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WHO EXPERT COMMITTEE ON BIOLOGICAL STANDARDIZATION

Geneva, 26 November – 2 December 1974

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WHO EXPERT COMMITTEE ON
BIOLOGICAL STANDARDIZATION
Twenty-sixth Report

The WHO Expert Committee on Biological Standardization met in Geneva from 26 November to 2 December 1974. Dr A. S. Pavlov, Assistant Director-General, opened the Meeting on behalf of the Director-General. He welcomed the participants, including the Representative of the International Atomic Energy Agency, and thanked them for coming to Geneva for this Meeting. He drew attention to the long history of biological standardization and pointed out that this meeting was concerned exclusively with hormones. He was sure the report of the Committee would be useful in the standardization and control of hormones of importance in human health and in the improvement of hormone assays.

GENERAL

The Committee discussed policies concerning criteria for including or excluding apparently aberrant data (outlying results or outliers) obtained from collaborative assays, in the calculation of combined potency estimates on the basis of which an international unit was defined for international standards for various hormones. It was pointed out that such outliers among assay results were discussed with the participant responsible and excluded only when there were clear technical grounds for doing so. If there was no reasonable explanation, however, weighted and unweighted mean potencies were calculated in the same way for all estimates and recorded in the report of the collaborative assay. Thus heterogeneity of variance has not necessarily been a basis for excluding assay results. Furthermore in the overall combination of results, analysis of variance is made on both weighted and unweighted potency estimates.

The Committee agreed that it is desirable to include tests for homogeneity of variance in statistical analyses of bioassay results and accepted the current practice of not excluding results on the basis of heterogeneity of variance. An exception is made only for results of those methods that clearly did not measure the same property of the hormone, e.g., biological and immunological activities. Such distinctions are clear from the name of the international standard or international reference preparation when it is established (i.e., “for immunoassay” or “for bioassay”).
The Committee was of the opinion that inclusion of all such assay results has introduced only minimal differences in potencies assigned to international standards or international reference preparations in collaborative studies in which a large number of assays by different methods are generally performed; as a result, the 95% fiducial limits of the combined potency estimates are narrow.

Since both weighted and unweighted means derived from all data are recorded in the reports of collaborative studies, users of international standards and reference preparations should take these facts into consideration in the calibration of national and other standards by their own assay methods.

PART I. INTERNATIONAL STANDARDS AND INTERNATIONAL REFERENCE PREPARATIONS

1. Human Urinary Gonadotrophins

The Committee noted the results of the collaborative assay referred to in its twenty-third report of the material intended for the replacement of the second International Reference Preparation of Human Menopausal Gonadotrophins (FSH and ICSH), Urinary, for Bioassay (2nd IRP).

Since preparations of human urinary menopausal gonadotrophins are administered to man in many countries, it was desirable to have an international standard for the control of potency of such preparations. The collaborative assay showed that the preparation studied was suitable for the bioassay of both urinary FSH and urinary LH. The Committee therefore established this preparation as the International Standard for Human Urinary FSH and for Human Urinary LH (ICSH), for Bioassay, in replacement of the second International Reference Preparation of Human Menopausal Gonadotrophins (FSH and ICSH), Urinary, for Bioassay.

The Committee also noted a proposal for defining the international units on the basis of the results from the collaborative bioassay which would maintain continuity of the units of the preparation being replaced. It was informed that this was acceptable to the participants in the collaborative assay. On this basis, the Committee defined the International Unit for Human Urinary FSH, for Bioassay, as the activity contained in 0.11388 mg and the International Unit for Human Urinary LH (ICSH), for Bioassay, as the activity contained in 0.13369 mg of the International Standard.

1 Unpublished working document WHO/BS/74.1080.
For practical purposes each ampoule can be used as containing 54 IU of FSH activity and 46 IU of LH(ICSH) activity. It is to be noted that the preparation it replaced (2nd IRP) contained, by definition, 40 IU of FSH and 40 IU of LH per ampoule. The Committee stressed that, as for this new standard, future standards and preparations calibrated against it should have their separate activities (FSH and LH(ICSH)) individually assessed.

The Committee noted that among the various bioassay methods employed, some heterogeneity of results was obtained by two laboratories using the ovarian ascorbic acid depletion assay method for LH(ICSH) and using the 2nd IRP as a standard. This may have been due to certain toxic constituents in the 2nd IRP, which are known to have such an effect on intravenous injection.

The preparation established was made from material processed in three batches, each of approximately 3500 ampoules, of which one was intended to serve as the replacement of the international reference preparation while the other two were to be distributed internationally as working material. Although all three batches had been processed under identical conditions, the results of accelerated degradation studies showed certain small differences in their stability. The reasons for these differences have not been resolved and the Committee therefore considered that continued stability studies should be made.

The Committee was informed that there were still available some 500 ampoules of the 2nd IRP now replaced. It noted that immunoassays of urinary FSH and LH(ICSH) are of clinical value; and that the IRP has been widely used as a working standard for these assays. The Committee therefore recommended that the remaining stocks should continue to be made available for this purpose, labelled as working material and not as the 2nd IRP.

The Committee further requested the National Institute for Biological Standards and Control, London, to obtain more highly purified preparations of the individual urinary gonadotrophins and arrange collaborative studies of their suitability to serve as reference material for the appropriate immunoassays.

2. Insulin

The Committee noted¹ the results of stability studies on the fourth International Standard for Insulin, Bovine and Porcine, for Bioassay, which were requested in its twelfth report² because the material was sealed

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¹ Unpublished working document WHO/BS/74.1083.
in ampoules in air and had some 6% average moisture content. This
procedure differed from that customarily followed for the preparation of
international standards (drying to less than 1% moisture and sealing under
dry nitrogen). These studies had been made over the last 14 years and an
interim report \(^1\) was made in 1963.

The results showed that the preparation, which consists of insulin
in a crystalline form, retained 95.8% of the original biological activity
after 12 years storage in the dark at 20°C and 65.7% after 14 years in
the dark at 37°C. Estimates derived from these data indicate that the
preparation stored at -20°C for 20 years would have retained at least
99.93% \((P = 0.95)\) of its original activity. The Committee was informed
of similar studies in the State Research Institute of Standardization and
Control of Drugs, Moscow, the results of which were in substantial agree-
ment with the above. The present international standard could be expected
to serve as a satisfactory material for many years if the present rate of
distribution is maintained.

The Committee was informed that this batch of highly stable crystalline
insulin had been prepared with particular attention to the removal of
contaminating proteolytic enzymes. Comparable stability should not be
assumed for all crystalline insulin preparations that are intended to serve
as national standards.

3. Calcitonins

The Committee noted \(^8\) the information, collected in response to the
request in its nineteenth report,\(^9\) on the nature and usage of calcitonin
preparations in man. In therapy, preparations of porcine and salmon
calcitonins are used in the treatment of Paget’s disease and may also be
used in acute hypercalcemia.

The two calcitonins differ in their primary chemical structure, and
comparisons of their activities in various bioassays have given widely
differing estimates of relative potency. This is particularly relevant to
the dosages used therapeutically. An international reference preparation
for each calcitonin was therefore required for the assay of the respective
hormones.

The Committee also noted \(^8\) that preparations of these calcitonins
had been obtained and studied. A stable preparation of porcine calcitonin
was available and the Committee agreed that this was suitable to serve as

\(^1\) Unpublished working document WHO/BS/631.
\(^2\) Unpublished working document WHO/BS/74.1077.
an international reference preparation. It therefore established this material as the International Reference Preparation of Calcitonin, Porcine, for Bioassay.

Earlier preparations of porcine calcitonin had been used with an assigned potency, the continuity of which had been maintained by calibration of the successive batches. The Committee agreed that it would be useful to maintain continuity of the unit of potency that had been thus assigned and had been used clinically in prescribing dosage, and on this basis, defined the International Unit for Calcitonin, Porcine, for Bioassay, as the activity contained in 4.74 mg of the International Reference Preparation of Calcitonin, Porcine, for Bioassay.

Since a suitable stable preparation of salmon calcitonin was also available, the Committee established this material as the International Reference Preparation of Calcitonin, Salmon, for Bioassay.

The Committee agreed that it would be desirable to define the international unit for salmon calcitonin so that it would be of the same order as the international unit for porcine calcitonin and noted a proposal along these lines which was acceptable. On this basis the Committee therefore defined the International Unit for Calcitonin, Salmon, for Bioassay as the activity contained in 0.02525 mg of the International Reference Preparation of Calcitonin, Salmon, for Bioassay.

In view of the fact that the units of potency of these calcitonins, although similar, are not identical, the Committee emphasized that when quantities of calcitonin preparations are designated the species of origin of each should be stated, this being particularly important when therapeutic changes are made from one type of preparation to the other.

The Committee also noted that calcitonin assays of human plasma were useful for diagnosis of medullary carcinoma of the thyroid. Since human calcitonin has been isolated and a synthetic preparation has been made, the Committee asked the National Institute for Biological Standards and Control, London, to collect further information on a suitable biological or chemical reference material for such assays.

4. Parathyroid Hormone

The Committee noted that information collected in response to the request in its nineteenth report showed that there was a use for bovine parathyroid hormone for administration to man. Such preparations are

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1 Unpublished working document WHO/BS/74.1077.
2 Unpublished working document WHO/BS/74.1078.
given for the clinical diagnosis of hypoparathyroid states. The Committee agreed that there is now a need for a stable international reference preparation for control of potency of preparations of this hormone. A quantity of partially purified bovine parathyroid hormone had been prepared and a collaborative assay made. The results showed that the preparation was suitable to serve as an international reference preparation for most currently used methods of bioassay of the hormone, and that it had adequate stability. The Committee therefore established the material as the International Reference Preparation of Parathyroid Hormone, Bovine, for Bioassay.

This preparation (hitherto coded under number 67/342) has already been widely used with an assigned potency approximately numerically equivalent to that of a pharmacopeial preparation from one country, and this had been acceptable. On this basis the Committee defined the International Unit for Parathyroid Hormone, Bovine, for Bioassay as the activity contained in 0.02765 mg of the International Reference Preparation.

Because this material had been found to give anomalous results in certain bioassays (e.g., the kidney adenyl cyclase assay, the intravenous chick hypercalcaemia assay) the Committee recommended that a more highly purified working reference preparation, appropriately calibrated in terms of the international reference preparation, be used in such assays.

The Committee noted that immunoassays of human plasma parathyroid hormone were of value in various clinical situations, e.g., in the differential diagnosis of hypercalcaemia. It agreed that a need existed for purified human parathyroid hormone to be used as reference material for such assays, but that there was no immediate prospect of this need being met. A preparation of purified bovine parathyroid hormone (hitherto coded under number 71/324) was available and had proved useful for these assays. The Committee therefore established this preparation as the International Reference Preparation of Bovine Parathyroid Hormone, for Immunoassay, and emphasized that it can be used for the immunoassay of human parathyroid hormone only in suitable assay systems. The biological activity of this preparation has been determined with reference to the preparation now established as the International Reference Preparation of Parathyroid Hormone, Bovine, for Bioassay. On this basis, the Committee defined the International Unit for Parathyroid Hormone, Bovine, for Immunoassay as the activity contained in 0.6 mg of the International Reference Preparation of Bovine Parathyroid Hormone, for Immunoassay.

The Committee asked the National Institute for Biological Standards and Control, London, in collaboration with interested workers, to obtain material of human origin that could serve as an international reference preparation. Owing to the scarcity of such material the Committee partic-
ularly emphasized the desirability of international collaborative efforts to prepare and characterize the preparation.

5. Renin and Angiotensins

The Committee noted \(^1\) that the further information that had been collected since its twenty-second report \(^2\) showed that there was widespread use of renin assays, particularly in the screening and diagnosis of different types of hypertension. Such assays usually depended on determining the rate of generation of angiotensin by the material under test, sometimes determined without reference to a standard.

The Committee also noted \(^3\) the results of a collaborative study of renin assay in which a preparation of human renin had been included as reference material. These showed that there was markedly improved agreement in results for estimates of renin activity in a series of samples when a common reference preparation of renin was used. The Committee agreed that an international reference preparation of renin would serve a useful purpose and that the preparation referred to above was suitable for this purpose. The Committee therefore established it as the International Reference Preparation of Human Renin.

The preparation has already been used with an assigned potency based on numerical equivalence with an animal unit which had been in existence for some time. On this basis, the Committee defined the International Unit for Human Renin as the activity contained in 188.7 mg of the International Reference Preparation.

The Committee also agreed that there is a need for chemical reference angiotensins for the direct assays of angiotensins themselves, particularly angiotensins I and II.

6. Human Chorionic Gonadotrophin and its Subunits

The Committee noted \(^4\) that immunoassays for human chorionic gonadotrophin (HCG) in urine and body fluids are of clinical value in a number of circumstances, such as: the diagnosis of pregnancy as early as nine days following the mid-cycle peak of LH, with implications for fertility control; the differential diagnosis of lower abdominal pain in women in the reproductive age group in whom impending rupture of a tubal pregnancy

\(^1\) Unpublished working document WHO/BS/74.1089.


\(^3\) Unpublished working document WHO/BS/74.1081.
is a possibility: the diagnosis and monitoring of treatment of trophoblastic neoplasms for which effective therapy is now available; and the diagnosis and study of the natural history and monitoring of therapy of non-trophoblastic tumours which may secrete biologically inactive fragments of HCG or the native hormone itself.

In the light of such manifold clinical applications, the Committee agreed that there is a need for an international reference preparation for immunoassay of this hormone.

The existing International Standard for Human Chorionic Gonadotrophin, for Bioassay (which is of urinary origin) has been used as such a reference material but is unsuitable for use in all immunoassay systems because of its heterogeneity. The Committee noted the offer of a quantity of highly purified HCG of urinary origin and its alpha and beta subunits. The information given was sufficient to demonstrate the stability and suitability of these three preparations to serve as international reference preparations.

The Committee therefore established the preparation of human chorionic gonadotrophin as the International Reference Preparation of Human Chorionic Gonadotrophin (HCG), for Immunoassay, and authorized the National Institute for Biological Standards and Control, London, to define the international unit for immunoassay on the basis of bioassay comparison with the existing International Standard for Human Chorionic Gonadotrophin, for Bioassay. The Committee asked the National Institute for Biological Standards and Control to process and ampoule the α and β subunits. It authorized that Institute to establish the preparations as international reference preparations and to define the international unit in each case as the activity in one microgram of the pure subunit preparation.

The Committee asked the National Institute for Biological Standards and Control to obtain further information regarding the long-term stability of these preparations and their contamination with the complementary subunit.

The Committee noted that these preparations of the subunits would also be useful in assessing the specificity of subunit assay systems.

7. Human Pituitary Gonadotrophins: FSH and LH (ICSH), for Bioassay

It has been customary to use the second International Reference Preparation of Human Menopausal Gonadotrophins, a preparation of urinary origin, as a standard for the bioassay of human pituitary gonadotrophins.

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1 Unpublished working document WHO/BS/74.1094.
This has frequently resulted in heterogeneous potency estimates, especially of human LH(ICSH), when different bioassay methods have been employed. The Committee therefore considered the question of making available a preparation of pituitary gonadotrophins of human origin, last referred to in the sixteenth and seventeenth reports, and agreed that there was a need for an international reference preparation of human pituitary FSH and LH(ICSH) for bioassay. Such a preparation would be used for the calibration of national standards for the control of preparations of human pituitary gonadotrophins for therapeutic use. The Committee noted the results of collaborative studies of a pituitary extract (hitherto coded under number 69/104) containing a number of pituitary hormones and considered it to be stable and suitable to serve as an international reference preparation of human pituitary FSH and of human pituitary LH(ICSH) for bioassay.

The Committee therefore established this preparation as the International Reference Preparation of Human Pituitary Gonadotrophins (FSH and LH(ICSH)), for Bioassay. In view of the fact that this preparation had already been widely used with an assigned potency for both gonadotrophins, which was acceptable to the participants in the collaborative assay and to a number of other investigators throughout the world, the Committee agreed that it would be useful to maintain the continuity of the respective units. The Committee therefore defined the International Unit for Human Pituitary FSH as the activity contained in 0.1670 mg and the International Unit for Human Pituitary LH(ICSH) as the activity contained in 0.0668 mg of the International Reference Preparation.

8. Human Pituitary Luteinizing Hormone (LH (ICSH)), for Immunoassay

The Committee noted the studies of the preparation of purified human luteinizing hormone referred to in its seventeenth report. This material (hitherto coded under number 68/40) was stable and of adequate purity and, used in many laboratories in several countries, had proved satisfactory for the assay of plasma LH(ICSH).

The Committee established this preparation as the International Reference Preparation of Human Pituitary Luteinizing Hormone (LH), for Immunoassay. It asked the National Institute for Biological Standards and Control, London, to arrange a limited collaborative assay to calibrate the preparation by bioassay in terms of the newly established International

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3 Unpublished working document WHO/BS/74.1086.
Reference Preparation of Human Pituitary Gonadotrophins (FSH and LH(ICSH)), for Bioassy, and authorized the National Institute for Biological Standards and Control to define the international unit for immunoassay on this basis with the agreement of the participants in the collaborative assay.

The Committee again drew attention to the problems that had arisen in the assignment of biological potencies when this pituitary material was used as a working standard for human pituitary LH(ICSH), resulting from the use of a urinary preparation (the second International Reference Preparation of Human Menopausal Gonadotrophins, for Bioassay), in attempts to calibrate the working material by various bioassay methods.

With the development of immunoassay of the subunits of LH(ICSH) and the possibility that the international reference preparation might be found to have some contamination with subunits, the Committee requested the National Institute for Biological Standards and Control, in collaboration with interested laboratories, to investigate this possibility and, if necessary, to obtain purer material.

9. Human Pituitary Follicle Stimulating Hormone (FSH), for Immunoassay

Imunoassays of FSH in human plasma have many important applications in clinical medicine and reproductive physiology. The Committee therefore agreed that a preparation of human pituitary FSH was required to serve as a reference preparation for immunoassay.

Various materials including international reference preparations of gonadotrophic hormones for bioassays have been used for this purpose, in particular the second International Reference Preparation of Human Menopausal Gonadotrophin, and the preparation (hitherto coded under the number 69/104) in view of the fact that there is currently no highly purified preparation of human pituitary FSH suitable for establishment as an international reference preparation, the Committee requested the National Institute for Biological Standards and Control, London, to obtain a preparation that was stable, free of subunits, and suitable for this purpose.

10. Human Thyroid Stimulating Hormone (TSH)

The immunoassay of thyroid stimulating hormone in blood has many clinical applications in disorders of the thyroid, pituitary, and hypothal-
It has been used particularly in the diagnosis of primary and secondary hypothyroidism and in conjunction with administration of thyrotrophin releasing hormone in the investigation of suspected hypothyroidism and hyperthyroidism. In view of this wide clinical usage, the Committee agreed that there was a need for a suitable reference preparation for immunoassay.

The Committee noted that studies had been made of the purified preparation of human thyrotrophin (TSH) referred to in its nineteenth report. This preparation of human TSH (hitherto coded under number 68/38) had already been extensively used as reference material for immunoassay and notwithstanding some content of LH, had been accepted. There was, however, evidence that the preparation, like other purified TSH preparations, lost some biological activity if it was kept for various periods at ambient temperatures.

Since no better material was currently available and because there was indeed an urgent need for a reference preparation for immunoassay, the Committee established the present preparation as the International Reference Preparation of Human Thyroid Stimulating Hormone (TSH), for Immunoassay.

On account of the marked heterogeneity of potency estimates in different assays, this preparation could not be calibrated by bioassay with the existing International Standard for Thyrotrophin, Bovine, for Bioassay, for the purpose of defining the international unit, but had been assigned by agreement a potency based on an approximate value in terms of the international standard. The Committee agreed that, while it would be useful to maintain continuity of the unit used, for convenience the previously assigned potency of 147 milli-units per ampoule should be rounded off to 150 milli-units per ampoule. On this basis the Committee defined the International Unit for Human Thyroid Stimulating Hormone (TSH), for Immunoassay, as the activity contained in 36.6 mg of the International Reference Preparation.

In the light of the reservations about the overall suitability of this reference preparation, the Committee asked the National Institute for Biological Standards and Control, London, to obtain a more suitable and highly purified preparation of human TSH to replace it as soon as possible. The Committee agreed that every effort should be made to ensure that the replacement material be stable and free of hormone subunits.

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1 Unpublished working document WHO/BS/74.1079.
11. Subunits of Human Glycoprotein Hormones, FSH, LH(ICSH), and TSH

The Committee noted \(^1\) that there was a need for reference material of the subunits of the human glycoprotein hormones, FSH, LH(ICSH), and TSH. This need applies particularly to reference materials of the beta subunits of these hormones, which confer their biological and immunological specificity. These preparations would serve for testing the specificity of the immunoassay systems used and also as reference materials in the immunoassay of the individual subunits themselves. Such immunoassays are becoming widely used in studies of the pituitary-thyroid and pituitary-gonadal axes.

The Committee requested the National Institute for Biological Standards and Control, London, in collaboration with interested workers, to obtain materials for the purpose of comparing the appropriate subunit reference preparations and to evaluate their suitability for the purposes envisaged. In view of the scarcity of the starting materials the Committee particularly emphasized the desirability of international collaborative efforts to prepare and characterize these glycoprotein hormone subunits.

The Committee decided that when hormone subunit preparations were established as international reference preparations for immunoassay, the international unit in each case should be defined as the activity in one microgram of the pure subunit preparation.

12. Human Placental Lactogen

The Committee noted \(^2\) that estimates by immunoassay of human placental lactogen in blood are widely used clinically, both in the differential diagnosis of threatened abortion and in the management of pregnancy, particularly for the assessment of fetoplacental function. For this purpose accuracy of measurement of actual concentrations of hormone (as distinct from changes in relative concentration) is critical. The Committee agreed that there was a need for an international reference preparation for immunoassay, particularly in view of the rapid proliferation of assay kits.

The Committee also noted \(^2\) that the National Institute for Biological Standards and Control, London, had obtained a quantity of material and had arranged a collaborative assay, of which only preliminary results were currently available.

\(^1\) Unpublished working document WHO/BS/74.1091.
\(^2\) Unpublished working document WHO/BS/74.1082.
In view of the urgent need for an international reference preparation for immunoassay, the Committee recommended that, with the agreement of the participants, the reference preparation be established and the international unit be defined as soon as possible, through the procedure available for this purpose.

13. Human Insulin

The Committee noted\(^1\) the results of the studies referred to in its twenty-first report\(^2\) of a preparation of human insulin suitable to serve as reference material for immunoassay of human insulin. This preparation consisted of pooled material obtained from different sources, had been characterized, and had already been widely used; it had proved satisfactory for use as reference material in immunoassay and in radioreceptor binding assay.

The Committee agreed that this preparation (hitherto coded under number 66/304) was suitable to serve as an international reference preparation and established it as the International Reference Preparation of Insulin, Human, for Immunoassay.

The potency of the preparation had been determined by bioassay with the 4th International Standard for Insulin, Bovine and Porcine, for Bioassay, and on the basis of the results obtained the Committee defined the International Unit for Human Insulin, for Immunoassay as the activity contained in 1.8233 mg of the International Reference Preparation. The Committee also noted\(^3\) that a preparation of homogeneous, single-component insulin had been offered, and asked the National Institute for Biological Standards and Control, London, to obtain the material with a view to using it to replace the present international reference preparation in due course.

Since insulin is derived from proinsulin by detachment of the C peptide, the assay of the latter in human plasma may become increasingly important for assessing the rate of secretion of endogenous insulin in diabetic subjects under treatment. The Committee therefore asked the National Institute for Biological Standards and Control, to collect further information on such usage so as to assess the need for reference material of human insulin C peptide.

\(^1\) Unpublished working document WHO/B8/74.1084.

14. Human Oxytocin and Human Vasopressin

The Committee noted ¹ that the International Standard for Oxytocin and Vasopressin, for Bioassay was available for the assay of preparations used therapeutically. In regard to the use of immunoassays of human oxytocin, vasopressin and their carrier proteins (the neurophysins) for clinical purposes, the Committee made the following observations:

(a) there was no evidence at present to suggest that the immunoassay of oxytocin was clinically useful;

(b) the immunoassay of human vasopressin could be of value in the differential diagnosis of diabetes insipidus and of hyponatraemic states in which inappropriate and excessive secretion of vasopressin was suspected;

(c) there was some evidence that the assay of the neurophysins may be of clinical value under circumstances similar to those noted for human vasopressin but the diagnostic value of such assays has yet to be firmly established.

The Committee therefore agreed that there was need for a reference preparation for immunoassay of human vasopressin and asked the National Institute for Biological Standards and Control, London, to obtain suitable material for this purpose, and to collect information regarding the possible usefulness of neurophysin assays. The Committee also noted ² that synthetic preparations of 8-Arg-vasopressin have been made and asked the National Institute for Biological Standards and Control to investigate the suitability of such a preparation to serve as reference material.

15. Gastrointestinal Hormones

The Committee noted ³ that assays of the hormones gastrin, secretin, glucagon, and cholecystokinin/pancreozymin are of interest in health and disease. For example, the immunoassay of serum gastrin is of diagnostic value in patients with suspected Zollinger-Ellison syndrome, whilst glucagon immunoassays have been useful in the investigation of certain pancreatic tumours. The Committee also noted ⁴ that there was considerable current interest in the simultaneous assay of various gastrointestinal hormones with a view to elucidating the nature of their physiology and interrelationships. The wide discrepancies in immunoassay results between laboratories

² Unpublished working document WHO/85/74.1090.
strongly suggest that there may be a need for international reference preparations for such assays.

The International Standard for Glucagon, Porcine, for Bioassay has been widely used as a reference material for immunoassay and has been found satisfactory for this purpose. The Committee therefore agreed that a proportion of the ampoules of this preparation should serve as an international reference preparation for immunoassay and established this material as the International Reference Preparation of Glucagon, Porcine, for Immunoassay. The International Unit of this preparation would remain as defined for the international standard for bioassay (1.49 IU per ampoule).

The Committee also noted that a preparation of human gastrin I (synthetic) had been obtained, and asked the National Institute for Biological Standards and Control, London, to arrange collaborative studies to determine its suitability to serve as biological or chemical reference material.

A preparation of secretin that might serve as reference material had been requested by the Committee in its seventh report but has hitherto been unobtainable. The Committee was informed that a synthetic preparation may be available shortly and requested the National Institute for Biological Standards and Control, London, to arrange the collaborative studies for establishing an international reference preparation.

The Committee asked the National Institute for Biological Standards and Control to obtain a preparation of cholecystokinin/pancreozymin and to arrange collaborative studies to determine its suitability to serve as reference material.


The Committee discussed the increasing value of assays of several peptide hormones that regulate anterior pituitary hormone synthesis and secretion. Examples are: thyrotrophin releasing hormone in the diagnosis of suspected hypothyroidism and hyperthyroidism; gonadotrophin releasing hormone in the functional evaluation of the hypothalamo-pituitary-gonadal axis and in the treatment of infertility; and growth hormone release-inhibiting hormone in the treatment of such disorders as acromegaly, diabetic retinopathy, and metastatic malignancy. These widespread uses indicate the urgent need for international reference material.

1 Unpublished working document WHO/BS/74.1092.
The Committee therefore requested the National Institute for Biological Standards and Control, London, in collaboration with the WHO Secretariat, to investigate the possibility of obtaining suitable material to serve internationally as reference preparations.

17. Other Hormones

The Committee was informed that assays of human prolactin in blood were in wide use, for example, in the investigation of patients with pituitary tumours and with inappropriate lactation. The Committee asked the National Institute for Biological Standards and Control, London, to obtain a purified preparation of human prolactin that could serve as an international reference preparation for the immunoassay of the human hormone.

The Committee also considered the use of immunoassays and certain in vitro bioassays of human corticotrophin (ACTH) in the diagnosis of Cushing’s syndrome and of primary and secondary adrenal deficiency as well as for the control of steroid replacement therapy and agreed that there is a need for an international reference preparation of ACTH for immunoassay. The Committee therefore asked the National Institute for Biological Standards and Control to obtain suitable material and to arrange collaborative studies.

PART II. QUALITY CONTROL OF MATERIALS USED IN ASSAYS

18. Recommendations for the Assessment of Assay Systems

The Committee studied the proposed “Recommendations for the Assessment of Binding Assay Systems (including Immunoassay and Receptor Assay Systems) for Human Hormones and their Binding Proteins (A guide to the formulation of requirements for reagents and assay kits for the above assays and notes on cytotoxic bioassay systems)”,¹ and a related document ² which had been prepared by the WHO Secretariat in collaboration with a number of experts. After making some modifications, the Committee agreed that the text was satisfactory and that it would be of value in developing procedures for the control of reagents and assay kits for these assays produced in different countries.

¹ Unpublished working document WHO/BS/74.1076.
² Unpublished working document WHO/BS/75.1095.
The Committee adopted these recommendations and agreed that they should be annexed to the present report (see Annex 1).

19. National Assay Service

The Committee was informed of some assay services for hormones and other substances that had been established or were being developed in certain countries and considered a document that described a model for the development of such national assay services: "Development of National Assay Services for Hormones and Other Substances in Community Health Care". This document had been prepared by the WHO Secretariat in collaboration with a number of experts. After making some modifications, the Committee agreed that the text was satisfactory and that it adequately embodied the principles for the development of national systems for assay services of health importance which could be implemented as a part of community health care services in different countries.

The Committee adopted the model describing the development of a national assay service and the recommendations made for implementing such a service, and agreed that the text should be annexed to the present report (see Annex 2).

20. Recurring Problems in the Standardization of Hormones for Bioassays and Binding Assays

The Committee discussed a number of problems that occurred in the standardization of hormones in various types of assay, particularly in binding assays, including immunoassays and receptor assays.

1. Principle of comparative assays—comparison of like with like

For each substance that it is not yet practicable to define completely by chemical and physical means, a common standard is needed for the assay of potency. This criterion is deceptively simple, and binding assays, like any other assays require that it be observed rigorously.

As for all comparative assays one fundamental assumption is that the substance assayed in the test sample and in the standard are identical, or at least similar, to the extent that the substance in the test sample behaves in the assay system as a dilution of the standard.

Binding assays depend upon the avidity of union between the binding site on the binding protein (antibody or other receptor) and one or more

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1 Unpublished working document WHO/Bf/74.1088.
2 Unpublished working document WHO/Bf/74.1085.
specific configurations on the surface of the substance bound. The energy of the bonds involved depends on the spatial closeness of fit and the nature of the binding forces between the bound substance and the binding protein.

Any difference between the standard and test sample in the constitution of the binding-sites on the substance in relation to the complementary site on the binding protein may give different values for their affinity constants. Such different affinities for the substance to be bound in the test sample and for the substance to be bound in the standard are likely to give disparate assay results with different reagents and under different conditions.

In radioimmunassay, in particular, other characteristics of the whole assay system may be important determinants affecting relative potency estimates, e.g., the completeness or otherwise of separation of the bound and free forms of the labelled antigen and the qualities of the antigenic determinants recognized by the antibody.

The nature and suitability of the reference preparation used in a particular assay must be determined not only by the basic principles of comparison of like with like but also by the nature of the particular biological question to which an answer is sought. Thus, even a material consisting of a crude mixture of substances could serve as a suitable standard for assay provided it was found in practice that its use led to clinically valuable information and provided that it was recognized that more highly purified preparations should replace such crude reference materials when they became available. The nature of the material used as a reference preparation and the type and degree of its characterization are greatly influenced by the nature of the assays (bio-, immuno-, or cytochemical) in which they are to be used.

2. Discontinuity of results due to dissimilarity between test sample and standard and to heterogeneity of a substance

2.1 Forms of each type of hormone

Until there is sustained general agreement on its chemical structure, a “hormone” is tacitly defined by one (or more) of its characteristic biological activities. A biological standard is the preparation used to quantitate that activity, i.e., it is the particular preparation in terms of which the potencies determined by assay are expressed. It is for convenience, and to enable such assay results to be expressed in universal terms, that international units are defined for international biological standards.

It is now generally realized that for many hormones currently assayed each type of hormone can exist in several different forms in biological fluids; one such may occur alone or in a mixture with other forms. Because they are structurally related they may react similarly, but not necessarily identically
or to the same extent, in a given immunoassay system. The forms may be
natural precursors, metabolites, allelic and genetically determined variants,
or variants derived from tumours; or they may be artefacts of the hormone
produced in vitro, during storage or by extraction procedures.

2.2 Form to be assayed

In setting up a standard for binding assay of a substance, there is thus a
need to identify the form(s) of the substance to be assayed. This, however,
is not always possible, e.g., in the case of the gonadotrophic hormones.
For many years, certain hormones in body fluids have been assayed solely
by their biological activity; but many of them are present in such small
amounts that it is not practicable to assay them in this way.

The new assay methods are often sensitive enough to measure very small
amounts of hormones but the limitations of immunoassays for evaluating
biological activity must be realized. On the other hand, for some substances
information that might be more useful for clinical purposes may be obtained
from immunoassays of one or more biologically inactive form(s) (e.g.,
proinsulin or C peptide) than of the active one, or of the total amount as
represented by the combined effect of all the forms.

2.3 Specificity

When heterogeneous or slightly dissimilar materials are compared, the
specificity of the whole assay system must be defined. If samples to be
assayed contain several different forms of a hormone, accurate quantitation
of one form necessitates making the assay specific for that form. Assay
specificity may be improved in different ways, but must be assessed on the
performance of the final whole assay system. Commonly agreed criteria
and reference materials are essential for the uniform quantitative evaluation
of specificity.

It is not, however, the purity of a standard that confers specificity on an
assay system used to estimate test samples consisting of a heterogeneous
mixture of substances that react with that system. The specificity of the
antisera used is also of importance, and the specificity of the assay is deter-
mined by the interaction of all the reagents within the assay system.

2.4 Antisera

Similar considerations apply in the case of changes in antisera used in
the assay system. For example, experiments have been performed in a
single laboratory in which assay conditions differed only in the specific
antisera used for determining potencies in aliquots of identical serum

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samples. Potency estimates differing at least twofold were observed under these conditions. In contrast, when all reagents including antisera were identical, variance in potency estimates between laboratories did not exceed the within-laboratory between-assay variance in potency estimates.

Thus some discontinuity in potency estimates can be expected to result from changes in antisera.

3. Discontinuity of assay results due to changes in the standard

In practice, because of the necessity for using assay systems of various degrees of non-specificity there is inevitably a likelihood of some discontinuity of assay results for estimates of substances. When a sample is assayed in different systems continuity of estimates would depend upon the systems having similar characteristics.

Discontinuous assay results are liable to occur with any one particular immunoassay system following a change from one standard to another of different quality because of the likelihood of dissimilarities (2.1 above) occurring between sample and standard. Consistently accurate estimates can be obtained only when assay systems are specific. With highly specific assay systems, accuracy of estimations is much less influenced by a change in purity of a standard.

On the other hand, non-specific assay systems (a category that includes many of those now available) are all liable to give erroneous estimates and may give different estimates when a standard is replaced by another of different purity.

A working standard, i.e., one routinely used in assays, must often be calibrated in terms of a common standard, e.g., a national standard, which itself might have been calibrated in terms of an international reference preparation. Such calibration must be done in relation to the particular assay system used, i.e., using the same reagents and under the same conditions. The potency assigned to a working standard obtained by calibration in a nonspecific assay system may not be correct or valid in another assay system. This could lead to a situation where, for example, a national standard, calibrated in terms of the international reference preparation using two different assay systems (sets of reagents and conditions), could be assigned a different potency with each system. Subsequently if that national standard (considering the case of one of those assigned potencies) is used to calibrate a single working standard that may be distributed with two different kits, the same working material would be assigned a different potency with each such kit. If the other of the two assigned potencies of the national standard were used, then two different potencies might be assigned.
to the working material with the two kits. Moreover, with any one of the kits, the results of assays of hormone in body fluids would depend on the particular potency assigned to the standard in it. It would be preferable therefore if each working standard, e.g., in a kit, could be calibrated directly with the international reference preparation. Further, with any change of any component of the whole assay system, each working standard should be recalibrated with the primary standard.

For this reason the aim has always been that any international standard or reference preparation established should be a preparation of the highest purity and homogeneity available. This is also desirable for any national standard, whether used to calibrate working standards or distributed as working material for routine use in all assays of the hormone.

4. Some problems of making standards for binding assay

4.1 Selection of material

Ideally a standard for immunoassay should consist of a homogeneous preparation of the substance in the same structural form in which it occurs in the sample to be assayed. In practice it may not be possible, because of scarcity, instability, difficulty of purification, or heterogeneity, to obtain enough of a homogeneous material. In certain instances, moreover, if a hormone exists in a large number of different forms, it may be impracticable, even if possible, to have a separate standard for each and every form, and a single standard is used to cater for all the forms of the hormone. Such a standard may consist of a heterogeneous mixture of forms, relying on the specificity of the assay system to measure the particular form desired, or a single form of the hormone is selected to serve for the measurement of the mixtures found in biological fluids. Selection of one from a number of different forms of a hormone poses the question “is it the right one for the purpose?”. In practice, dissimilarity and heterogeneity occur for most of the hormones at present assayed and discrepancies between assay results are in many instances unavoidable. Much can be done, however, to lessen the confusion that prevails for certain hormones.

4.2 Logistics

For certain substances (e.g., certain complex hormones like glycoproteins) for which the conformation normally found in plasma is not known, discontinuity of assay results due to dissimilarities between test and standard may be reduced if plasma or serum rather than extracted materials
is used as a working standard. This may, however, require large quantities of human plasma. Since discontinuity of assay results is also likely to occur with each change of standard, it would be reduced in magnitude and frequency if the number of different working standards used was kept to a minimum, and if enough of both primary (e.g., national standard or international reference preparation) and working standards were made to last for as long as possible. This could be achieved either by making very large numbers of ampoules (as in the case of the third International Standard for Corticotrophin for Bioassay) or, if the substance is sufficiently stable, by putting sufficient material for many assays into each ampoule.

The scale on which international standards and reference preparations of hormones are currently prepared is probably adequate to meet the needs of a large number of assay kit manufacturers in all countries only if national standards are established, or at least a working standard for each hormone is made available on a centralized national basis in each country.

4.3 Preparation of international biological standards and reference preparations

The general procedures still used to prepare WHO reference materials have been described in some detail. In essence a WHO standard for a hormone generally consists of some 2000–4000 ampoules; at each stage of processing the ampoules are handled (i.e., filled, freeze-dried, or sealed) as a single batch on one day. If the material is available in sufficient amount and in homogeneous form (e.g., crystalline insulin) it may be filled as a powder in quantities of 20–100 mg per ampoule. If smaller quantities are available, however, it is preferably filled as a solution containing inert carrier (e.g., serum albumin, lactose, mannitol) to provide bulk to the freeze-dried plug and to prevent adsorption to glass surfaces. The solution is filled into ampoules in accurately reproduced volumes (varying between ampoules often by as little as ± 0.2%) and freeze-dried under strictly controlled conditions. After secondary desiccation to constant weight the ampoule, containing dry nitrogen, is sealed by glass fusion. This procedure has proved satisfactory for preparing standards for a wide variety of biological substances, although a small (about 5%) irreversible loss of biological activity appears to occur with some glycoprotein hormones. It is usual when defining the international unit to determine the mean dry weight of the contents of a number of ampoules since the unit is defined as a particular weight of the standard. It would be inadvisable, when using the standard, to try to weigh out all (or even part) of the contents of each ampoule to...

Determine how many units of activity are contained therein. The entire contents of an ampoule should be reconstituted and, for practical use the number of units per ampoule (which is always stated) would be contained in the total volume after reconstitution.

Another procedure has been developed whereby microgram quantities of a substance may be filled into neutral glass capillaries, freeze-dried, and sealed under dry nitrogen. The precision of filling into such capillaries is a little less than that used for filling 1-ml volumes into ampoules. This procedure can be useful for ampouling small amounts (1–20 μg) of material with only small amounts of carrier or even with none. In these cases it would be impossible accurately to weigh the total dry contents of a number of ampoules and the international unit is then defined on an "assumed" mean dry weight of contents, taking into consideration the amount filled. As it would be equally impossible for users of the standards to attempt to weigh out the contents of the ampoules, the same procedure should be followed for reconstitution of the total contents of an ampoule and use of the stated number of units. It is the accuracy of fill and the precision (very small variation between ampoules) that make this a satisfactory procedure for an international standard or reference preparation consisting of freeze-dried material.

5. Expression of assay results

It is unlikely that there will be general agreement as to how results should be universally expressed, e.g., in terms of mass or molarity, or in terms of a particular reference preparation and/or in arbitrary units, even if any choice is possible. When the substance to be measured is easily obtainable as a "pure" chemical (e.g., digoxin), then mass has been the usual choice. Molarity has seldom been used in this context, but it has the advantage that the interpretation of comparative assays of hormone precursors or fragments becomes more meaningful. Moreover, the physiological significance of changes in the concentrations of various hormones would be more easily interrelated as molar than as mass concentrations. Unit notation systems (such as the international units of biological activity of insulin and HCG) have proved of value in their application to bioassay estimates of materials not fully characterized by physical and chemical means. Immunoassays do not necessarily (and probably only rarely) estimate the same characteristic of a molecule that is responsible for biological activity (indeed the substance being measured may not have any known assayable biological activity). There is a need for an unambiguous distinct means for the expression of estimates made by binding assays (immunoassays and receptor
assays). In conformity with the recommendations of the twenty-first Expert Committee on Biological Standardization it is desirable that reports of assay results should always state explicitly the method (chemical, bioassay, radioimmunoassay, or other binding assay systems) and also identify the standard, if applicable, its unitage, as defined or as determined by calibration, whatever the case may be.

It should be anticipated that potencies relative to a standard will eventually be replaced by mass or molar forms of expression for the result of assays of characterized materials in well defined systems.

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Annex 1

RECOMMENDATIONS FOR THE ASSESSMENT OF BINDING-ASSAY SYSTEMS (INCLUDING IMMUNOASSAY AND RECEPTOR ASSAY SYSTEMS) FOR HUMAN HORMONES AND THEIR BINDING PROTEINS

(A guide to the formulation of requirements for reagents and assay kits for the above assays and notes on cytochemical bioassay systems) ²

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¹ Many of these recommendations are applicable also to other substances, e.g., drugs, specific proteins, viruses, and substances involved in blood coagulation and fibrinolysis.
² Successive draft versions of this document were prepared by the following members of the WHO Secretariat: Dr D. R. Baingham, Head, Division of Hormones and Blood Products, National Institute for Biological Standards and Control, London, England (Consultant); Dr P. Mary Cotes, Division of Hormones and Blood Products, National Institute for Biological Standards and Control, London, England (Consultant); Dr R. M. Lequin, Head, Radioimmunoassay Laboratory, Department of Obstetrics and Gynaecology, Radboudziekenhuis, Nijmegen, Netherlands (Consultant); Professor B. Lurenfeld, Director, Institute of Endocrinology, Tel-Hashomer Government Hospital, Tel-Hashomer, Israel (Consultant); Dr A. von zur Mühlen, Medizinische Universitätsklinik, Göttingen, Federal Republic of Germany (Consultant); Dr A. S. Oosthoorn, Chief Medical Officer, Biological Standardization, WHO, Geneva, Switzerland; and Dr C. Retup, Department of Pharmacology, The University, Lund, Sweden (Consultant).
Introduction

It is desirable that this document be studied in its entirety before using any part of it for the control of assay materials.

The development of new methods of assay of biological substances, which are many times more sensitive than hitherto existing conventional bioassays, has aroused great interest. The assay methods now available include immunoassays, particularly radioimmunoassays, which employ an antigen-antibody reaction, as well as other types of saturation analyses where the reaction is between the hormone and some other binding substance, e.g., receptor assays. These assays have many fields of application in measurement, including that of protein (peptide) and non-protein hormones, as well as a variety of non-hormonal substances, e.g., drugs, vitamins, cofactors, nucleotides, tumour- and virus-associated proteins. They permit the measurement of minute quantities of the substance in body fluids. Recently, sensitive cytochemical bioassays have been developed. These methods employ a specific reaction between the substance to be measured and appropriate cells of a target organ. It has been claimed that the field that has benefited most from the use of these newer assays is endocrinology.

The twenty-first WHO Expert Committee on Biological Standardization 1 pointed out in relation to hormones that immunoassays are valuable in diagnosis, in pharmacology, and in endocrinological research. The applications include tests for diagnosis, assays for determining dosage, and estimations for monitoring therapy affecting human hormone levels. However, a limitation on the use of immunoassays for evaluating hormonal bioactivity is that the methods measure a composite of antigenic activity, which is not necessarily related to the bioactivity of the hormone (e.g., growth hormone). At a meeting convened in Geneva in 1967 2 it was recommended that emphasis be placed on the development of biological microassays, which should preferably have a sensitivity comparable with

2 Those participating were: Dr D. R. Bangham, Director, Division of Biological Standards, National Institute for Medical Research, London, England; Dr R. Borth, Chargé de Recherches, Clinique Universitaire de Gynécologie et Obstétrique, Geneva, Switzerland; Dr C. Hamburger, Chief, Hormone Department, Statens Serum Institut, Copenhagen, Denmark; Professor R. Moricard, Université, Directeur, Laboratoire d’Hormonologie, Hôpital Broca, Paris, France; Dr C. Renup, Fachlicher Institut, Universiteit, Lund, Sweden; Dr G. R. Zahn, Chargé de Recherches, Polyclinique Universitaire de Médecine, Geneva, Switzerland; Dr A. S. Outshoorn, Chief Medical Officer, Biological Standardization, WHO, Geneva, Switzerland; Dr J. Uri, Medical Officer, Biological Standardization, WHO, Geneva, Switzerland. The advice of Professor A. E. Wilhelm, Department of Biochemistry, Division of Basic Health Sciences, Emory University, Atlanta, Ga., USA, was also acknowledged.

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Radioimmunoassays, with which they should be run in parallel. Possible techniques mentioned were organ perfusion, incubation or culture of organs and tissues using measurements involving micro-techniques, local application of hormones to organs, and micro-injection direct into responding tissues. Major developments in this field include some receptor assays and certain methods of cytochemical bioassay of hormones, which are sometimes more sensitive than those of immunoassay. If adequately validated they widen the scope of application of measuring hormonal activity.

The twenty-first WHO Expert Committee also considered that the possible application of immunoassays by national authorities in the control of hormone preparations used in therapy could be of importance. Developments since that time have not provided evidence that immunoassays could be used, to the exclusion of conventional bioassays, in the control of such preparations. A role for these methods, however, for control purposes would be to assess the absorption, metabolism, and fate of hormones administered to man and ultimately to enable the formulation of adequate specifications that must be fulfilled by therapeutic hormone preparations. There is already some use of immunoassays for in-process control of sequential batches in manufacture (e.g., for insulin).

On account of the great interest in these newer types of assay, there are many manufacturing establishments, laboratories, and institutions that prepare individual and matched reagents or grouped reagents in sets, usually called kits, for performing them. Many such materials are widely distributed, in many cases between countries. The control of quality of these materials is as important as the quality control of drugs or biological products, since incorrect results of tests or wrongful interpretation can be a major health hazard. There is a need, therefore, for guidelines for national control authorities to enable appropriate requirements to be formulated and applied for the control both of materials used in these types of assay and of assay performance. Representations to this effect have been received by WHO from Member States and international scientific societies working in this field. Control of quality of the reagents, however, is complicated in many instances by a lack of agreed criteria and specifications for the necessary reagents, either individually or when taken together in kits. It is, therefore, not possible at the present time to formulate conventional requirements for these reagents in the pattern of the Requirements for Biological Substances published by WHO; this may become feasible at some future time. What can be done at present is to give certain recommendations that should be followed in applying control measures for these

reagents. For meaningful national control programmes in each country the prerequisites are not only the legal and administrative machinery of the national control authority, but also the necessary laboratory facilities to provide the technical services needed. The role of such a national control laboratory is described in a document adopted by the twenty-second WHO Expert Committee on Biological Standardization.¹

The following recommendations are a guide to the formulation of requirements, or to the principles to be applied for control purposes, for individual reagents and kits used for the measurement of human hormones.² In many instances, however, only suggestions can be made on particular aspects of the preparation and assessment of the materials. Descriptions of technical procedures for manufacture are mentioned only where it is necessary to make certain points clear. Further, it is not the purpose of these guidelines to provide details of experimental assay procedures. Such technical information is available in the published literature. In particular, attention is drawn to the report of a Panel of Experts convened by the International Atomic Energy Agency.³

Evaluation of assay performance involves not only the initial assessment of the assay system used but also the monitoring of laboratory performance. It is of public health importance for each country to ensure the highest possible quality and consistency of assay performance by its laboratories. Recommendations for the development of national assay services were adopted by the twenty-sixth WHO Expert Committee on Biological Standardization.⁴

In drafting these recommendations, account has been taken of the opinions of consultants, any requirements for the manufacture and control of these materials that have been formulated in some countries, as well as information from both published and unpublished reports. In addition comments and advice as well as additional data relevant to these guidelines have been received from a number of experts and institutions to whom grateful acknowledgement is made (see Appendix 2, page 60).

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² The term "hormone" in this document is taken to include also hormonal substances (e.g., precursors, subunits), whether naturally occurring or synthetic.
General Considerations

To make an adequate assessment of each system, kit, or separate reagent, the national control authority should request and consider the data indicated in these guidelines, in order to determine the extent to which the recommendations have been followed. The purpose of these recommendations is to help ensure that sufficient and accurate information is supplied to enable the user to decide whether or not a particular reagent is likely to be suitable. Even after adequate quality control and release of the product by the national control authority, the decision as to acceptability of an individual reagent or of matched reagents (such as an antiserum paired with a labelled antigen) is within the responsibility of the user, who should base a decision on actual performance of the material in his assay system. On the other hand, in the case of complete assay kits, a manufacturer has a much greater degree of responsibility for demonstrating conformity with the specified criteria. Manufacturers should therefore provide sufficient information about their kits to enable assessment of performance to be made, under the conditions recommended for their use for a particular hormone. This could also facilitate the comparison of different manufacturers' products. It is advisable also that the national control authority should ascertain the extent of routine quality control exercised by the manufacturer of batches of kits. In the case of both kits and individual reagents, which are often made in a series of consecutive batches, consistency of performance both within and between batches should receive attention.

Any assay system should be evaluated as a whole (and not on the basis of criteria for individual reagents). Since the properties of the reagents used for assay of a particular hormone should be related to each other, it is the interrelated quality of the reagents that should be such as to provide acceptable performance, in regard to sensitivity and specificity of that assay system, as well as the precision of the estimates obtained.

The specificity of the assay is the ability to estimate solely the type and kind of hormone it is intended to measure. It should therefore be determined for the particular form of the hormone and type of test material it is intended to assay and the extent of freedom from interferences by substances other than the one it is intended to measure. As far as possible, the results of these determinations should be expressed in quantitative terms, e.g., the relative binding activity of the hormone and of molecules of similar chemical composition that are likely to occur in the biological specimens. Consequently, for each assay system (and this is of particular

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1 The specificity of assays should be checked using compounds likely to interfere in the maximum concentrations likely to be encountered in any clinical situation. Since testosterone is present in female plasma in a concentration of about 40 ng/100 ml and
importance in immunoassays), depending on the hormone there is a need for certain characterized reference materials for use in checking the specificity of the system by testing for the absence of cross-reaction with related hormones that may be present in the test samples. For peptide hormones these may consist of pure samples of various intact hormones and perhaps also of pure preparations of various related materials, such as a hormone precursor and hormone subunits. For steroid hormones, pure samples of various other known related steroids would be used.

Similar considerations apply in determining the sensitivity of the assay system. This could be related to the detectable amount of the hormone to be estimated. The limit of detection is the smallest amount determined in a single assay that, with a specified probability, can be distinguished from a suitable blank, and is that amount which can just be measured. Other characteristics that may be determined are the effect of a range of doses, and the slope of the dose-response curve.

The precision of estimates is taken to mean a measure of the reproducibility obtainable in the assay estimates. This needs to be considered as reproducibility within and between assays as well as between successive batches of any reagent or assay kit that may be released. It can be evaluated by repeated estimation of the hormone concentration in quality control samples of appropriate test materials (e.g., pools of plasma collected from subjects known to have high, medium, and low plasma levels of the hormone).

An important consideration is the conditions and reagents used to separate bound from free hormone. An assay system made up from potentially satisfactory reagents could give unsatisfactory or even invalid results if the separation system was not adequate.

Preparation of assay kits and reagents for performance of assays on clinical samples may involve the handling of radioactive materials and of materials potentially infected with viruses. For these reasons, personnel should be given appropriate training.

Particular hazards to workers arise in the preparation of radioactively labelled compounds. All work with radioactive materials should be carried out in conformity with accepted codes of practice for protection against radiation hazards. Appropriate measures to minimize such hazards should

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be taken under the guidance of the radiation protection officers of individual institutions. Publications are available that will give guidance in regard to radioactivity hazards. For all radioactive materials, whether in the preparation of materials or in the performance of assays, handling, labelling, and packaging must always conform to national rules and regulations and nothing in this document should be applied that is contrary to such national provisions.

Some considerations relating to precautions in dealing with viral material are set out in the requirements for certain viral vaccines in the WHO series of Requirements for Biological Substances. However, since many laboratories and institutions handling potentially contaminated materials are not experienced in dealing with viruses and virus containing substances, it would be desirable for some guidelines to be drawn up for international circulation to laboratories on the precautions to be taken and the correct procedures to be adopted for safeguarding the health of workers. Such guidelines should also include directions for proper disposal of waste material that might constitute a public health hazard.

Many methods exist for statistical analysis of assay results. This diversity poses a problem only when significantly dissimilar estimates can be shown to depend upon the methods used for the statistical analysis. In any treatment of data, it is preferable that curve fitting be done by an objective statistical method and computerized data processing is preferable (when such facilities are available). Information to validate the assay should include the determined within-assay and between-assay variability and tests of parallelism of the reference and test materials. Certain immunoassays, which have been regarded as the most precise, may have a small (e.g., 1-3%) within-assay variation (i.e., between replicates), but may show great between-assay variation (18-25%) and even much greater variation between laboratories.

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3 In general, a least-squares curve-fitting procedure should be used. Objective criteria should be stated for the rejection of points as outliers. Either linear or non-linear regression techniques may be used, depending upon the model used for description of the dose-response curves (e.g., the "logit-log" method and its modifications). Dose-response curves may be based on equations derived from the first order mass action law, or on empirical curve-fitting methods, such as multiple polynomials or the "spline" methods.
Owing to these variations, if interpretations are based on the results of serial determinations, it is preferable for the entire series to be assayed in a single group. In certain cytochemical bioassays, the between-assay variation is claimed to be similar to that of most immunoassays. Both within-assay and between-assay variability are best assessed on pools of plasma, serum, or urine containing different levels (high and low) of the hormone. The national control authority should apply these considerations in the calibration of quantitative reference material (e.g., laboratory standards in terms of national reference preparations) and in the assessment of kit performance for comparison of kits (or different batches of a kit) both within and between laboratories. Collaboration on statistical methods used nationally and exchange of information would be useful to all countries.¹

Attention is also drawn to certain recommendations of the twenty-first Expert Committee on Biological Standardization concerning the manner of expressing the results of immunoassays of hormones.² When the results of such assays are given, there should be added the qualification "by immunoassay"; in addition, if the results are given in units, it is essential that the reference material on which the unit was based should also be stated. In all cases, if any preparation (e.g., an international or other reference preparation) has been used to calibrate the reference material in the assay system, this should be identified. Problems that may arise in the calibration of reference material in different assay systems are considered in these guidelines (section 1.4, pp. 45-48).

Reference materials used in relation to immunoassays are of several kinds. Some of these are materials necessary for the assessment of specificity and sensitivity of the assay system when it is initially set up (see above p. 33), such as preparations of hormones closely related to the hormone it is intended to measure, unrelated interfering substances that may be present in test samples, relevant hormoneprecursors, and subunits of the hormone.³

¹ Information about the statistical treatment of the results of these types of assay may be obtained from the WHO Secretariat on application.
³ Thus, an assay for any one of the group of glycoprotein hormones (TSH, FSH, LH(HCG) and HCG) or any one of the group of gastrointestinal hormones (secretin, gastrin, and cholecystokinin/pancreozymin) should, when appropriate, be assessed for the level of cross-reaction with each of the other hormones of the group. An assay of glycoprotein hormones should also be assessed for cross-reaction, depending on the origin of the hormone (e.g., pituitary with urinary and vice versa). An assay for a particular steroid hormone (e.g., progesterone) should be assessed for ability to recognize materials that are chemically related to the hormone itself (e.g., 17-hydroxyprogesterone) as well as other less closely related steroids likely to occur in test samples such as plasma (e.g., testosterone, corticosterone). Likewise, hormone precursor (e.g., pro-insulin) and hormone subunit (e.g., of HCG) preparations should be appropriately used in assay characterisation.
The national control authority should supply or approve such reference materials needed for assay characterization. For certain polypeptide hormones, the World Health Organization is currently endeavouring to provide samples of subunits and possibly of purified intact hormone suitable to be used internationally as reference reagents for comparison purposes with similar materials available nationally for assay characterization.

Another kind of reference material is that included in every assay for the purpose of quantitating the assay estimate in terms of which the results of the assay are given. These reference materials are widely referred to as "standards" for immunoassay. In these guidelines, the recommendations below (section 1.4) relate to such reference materials ("hormone standards", "local standards", "laboratory standards", "house standards", "working standards") to be included in each assay. The World Health Organization has established or has under consideration a number of International Standards for hormones for bioassay and of International Reference Preparations of hormones for bioassay and for immunoassay. These are intended for the calibration of the above reference materials and are dealt with in Appendix 1 to this document (see p. 57).

1. Individual Reagents for Hormone Assays

1.1 Antigens or other substances to be bound

In some cases, depending on the services available in the country, the substances supplied for use in preparing the final reagent may be from different sources, e.g., a laboratory may supply only the radionuclide or the antigen already labelled. In other cases, the user may prefer to obtain the substance unlabelled and the labelling may be done in the laboratory making the assays.

Substances to be bound after labelling include antigens, which would be bound to appropriate antisera, and substances that would be bound to receptors or binding sites in materials such as cell constituents or plasma proteins. The quality of the antigen, or other substance, to be bound, the nature of the radionuclide and the process for labelling, the properties of the labelled substance, and the specificity of the receptor or binding site should all be considered in determining the final suitability of material for use in the intended assay system. Some of these properties will be dependent upon the other reagents to be used in the assay system. Some (but not all) non-antibody binding agents require that the substance to be bound has retained biological activity after labelling. Retention of biological activity is not usually required for labelled antigen to be used in a radio-
immunoassay, but retention of the relevant antigenic configuration is essential.

1.1.1 Antigens or other substances suitable for labelling

The antigen or other substance should be highly purified and should preferably be homologous and identical with the hormone to be measured, unless hormone from another species (e.g., porcine insulin in an assay system for human insulin), a hormone subunit (e.g., the β subunit of LH in the assay of LH), or synthetic material such as β 1-24 corticotrophin has been shown to be suitable and preferable for other reasons.

The antigen or other substance used for labelling should be free from known likely contaminants that might interfere with the assay, such as a precursor form of a hormone (e.g., pro-insulin) and related or degradation products (e.g., C peptide of insulin, deamidated insulin) and structurally related hormones and subunits (e.g., glycoprotein hormones). A number of tests and procedures are now used for characterization and assessment of purity.3

Although some glycoprotein hormones occur naturally as a family of closely related molecules (e.g., FSH, LH, TSH), for assay purposes the antigen or other substance (after labelling) in the form in which it is to be used should as far as possible be homogeneous. It is important that any different molecular forms present in the preparation of labelled material should be indistinguishable, both in their reaction with the antibody or binding substance (with which the tracer is used) and by the system used to separate bound and free labelled hormone.

Whenever the assay system is intended to be used to measure the biologically active form of the hormone, the identity of the hormone should be shown by biological assay in comparison with a characterized preparation of the hormone; this may be dispensed with when the identity of the hormone is not in doubt, e.g., steroids, which may be completely identified by physical and chemical means. Starting material from natural sources that is of low purity or unstable should preferably not be used for preparing the antigen or other substance to be labelled. After purification the preparation should consist of intact hormone molecules. In some instances, a biologically active synthetic molecule may conveniently be substituted for the native hormone.

3 Tests may include some of the following:

(1) (a) molecular size, (b) surface charge, (c) N and C terminal amino acids;
(2) amino acid and carbohydrate composition; (3) reaction with specific antisera that distinguish between the intact hormone and likely contaminants, such as subunits in preparations of glycoprotein hormones.
It is preferable for hormones and other substances suitable for labelling to be prepared in large batches so that each can be more fully characterized. It is unlikely to be practicable to carry out all available tests together with estimates of biological activity upon all preparations. The national control authority should demand such information as it considers necessary to approve release and distribution of the material, e.g., evidence of its identity, declared purity, and declared potency (in units or by weight, whichever is applicable). This, however, may be insufficient to enable the user to determine the acceptability of the material for his purpose. The manufacturer may therefore wish to supply further information, e.g., the results of other tests that may have been made.

Whatever information the manufacturer may supply, the national control authority should note that only in some cases may the material retain all the required properties indefinitely. It is desirable, therefore, that an expiry date be given, or at least that there should be stated the date of making the preparation and the dates the various tests reported were made.

Every container should be accompanied by at least the following information:

(a) marked on the container:
   - the name of the hormone followed by the words "for labelling";
   - the mass or concentration of hormone preparation in the container;
   - batch number;
   - recommended conditions for storage;

(b) on the leaflet in the package, in addition to the above:
   - the nature and amount or concentration of any added substances including any diluent buffer, carrier and bacteriostat that may have been added;
   - instructions, when applicable, for reconstitution of dry preparations and thereafter recommended storage conditions and period within which it should be used.

1.1.2 Radioactively labelled antigens or other substances ("tracers")

If the substance is provided already labelled, for direct use in assay, information should be given at least on the following points:

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(1) Information as under antigens or other substances suitable for labelling (section 1.1.1).

(2) The approximate amount (mass) of hormone present and its specific radioactivity at a stated reference date.

(3) The purity of the radionuclide used for labelling and the radiochemical purity of the labelled preparation at the reference date and, where applicable, the form and position(s) in a molecule at which the radionuclide has been introduced, as well as, where applicable, the average number of atoms of radionuclide per molecule of hormone.

(4) The proportion of the total radioactivity that is bound after incubation with a defined antiserum specific for the hormone, added in excess, under specified conditions at the reference date.

(5) Storage conditions recommended and probable rate of hormonal decomposition under these conditions (to indicate rate of irradiation damage or intrinsic instability in contrast to radioactive decay). In some instances it may be possible to indicate an expiry date after which the labelled material is unlikely to be suitable for use.

(6) The labelled hormone will not necessarily need to be purified (or repurified) prior to use in assays. If such purification is necessary, sufficient labelled material should be included to enable this to be done and instructions should be given for a suitable procedure.

Each batch of hormone after labelling should be assigned and should carry a batch number. This will identify it and enable it to be related to the batch of antigen prior to labelling. It will also enable users to be notified if unpredicted instability has occurred after issue.

1.1.3 Non-radioactively labelled substances

Labelling has also been carried out with non-radioactive markers (such as an enzyme or a bacteriophage) or chemically (e.g., with a substance carrying a fluorescent group). Substances labelled in this way are of interest in certain cases, for example for countries in which radionuclides may not be easily available or where the use of radioactively labelled substances has not been introduced. In general the principles for the evaluation and control of radioactively labelled antigens or other substances (section 1.1.2) are applicable.

The following information, at least, should be provided:

(1) Information as under antigens or other substances suitable for labelling (section 1.1.1).
(2) The approximate amount (mass) of hormone present.

(3) The proportion of the total amount of marker that is bound after incubation with a defined antiserum specific for the hormone, added in excess, under specified conditions.

(4) Batch number and recommended storage conditions and probable rate of hormonal decomposition under these conditions. In some instances it may be possible to indicate an expiry date after which the labelled material is unlikely to be suitable for use in assays.

(5) The labelled substance will not necessarily need to be purified (or repurified) prior to use in assays. If such purification is necessary, instructions should be given for a suitable procedure.

1.2 Antisera and other binding substances

The characteristics demanded of an antiserum or other binding protein for use in assay of a hormone will depend upon a number of its properties, upon other constituents of the particular assay system in which it is used, upon the separation procedure, and upon the physicochemical conditions in the assay system, such as the buffer and the type of test sample that is assayed.

1.2.1 Preparations for use as immunogens in antiserum production

In the case of peptide hormones of large molecular weight to be used as immunogens, the hormone should preferably be a highly purified preparation in order to avoid immunization with other materials, such as closely related hormones, hormone degradation products, or subunits. This may be particularly important in certain immunoassays, e.g., for HCG in pregnancy tests. In some cases, however, a high degree of purification may not be feasible or economic, and reliance may have to be placed on antisera resulting from further purification, e.g., by absorption procedures, chromatography.

In the case of small molecule hormones (such as steroids, short-chain peptide hormones, and T₃ and T₄) initial purity is essential. In addition, in each such case, the hormone may have to be conjugated to another, usually larger, molecule in order to make it immunogenic. In such instances the specificity of the antibodies may be improved by certain measures, e.g., making the chemical conjugation so that it does not interfere with those parts of the hormone molecule that confer its characteristic activity, and if possible so that it also masks those parts of the hormone that are
common characteristics of the group of hormones. In one case T₄ present in thyroglobulin has been used as immunogen. There may be instances, however, where an antiserum is desired for recognition of a group of hormonal substances rather than a single hormone. Under certain circumstances, if the assay system has been shown to be valid even with the use of a heterologous antiserum, e.g., antiserum against bovine parathyroid hormone for assay of human parathyroid hormone, this may be permissible.

In all cases, if an antiserum has been shown to be suitable the nature of the immunogen used is irrelevant.

1.2.2 Antisera used in immunoassays

It is desirable that assay systems, particularly if intended for estimation of hormones in physiological fluids, should be of sufficient sensitivity and specificity to permit direct estimation of the hormone in test samples. It would be preferable if this could be done without the necessity for extraction, purification, or concentration. There are, however, several situations where these procedures are unavoidable. Whatever the situation may be, acceptable quality of an antiserum should be evaluated using the whole assay system in which it is to be used. An antiserum that is satisfactory when used with a certain labelled antigen preparation may not necessarily be equally suitable with another.

Every antiserum preparation should be free of bacterial and mycotic contamination; if a bacteriostat is added it should be shown not to affect the product adversely in the concentration used, and the substance and amount added should be stated.

In the case of an antiserum supplied for use in immunoassays, the following information at least should be provided:

1. The type of hormone for which the antiserum is intended to be used.

2. The final dilution at which a stated proportion of a known trace amount of labelled hormone is bound after incubation in a known volume under specified conditions.

3. The equilibrium constant (K value) at stated temperatures (including +4°C) and under stated conditions, calculated from a Scatchard plot,

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1 As an example, when progesterone was conjugated to bovine serum albumin (BSA) in position 6, the resulting antiserum cross-reacted equally well with 5α-pregnan-3,20-dione. However, when progesterone was coupled in position 11, the resulting antiserum cross-reacted with only 3% of the related steroid.
and the Scatchard plot, to show the degree of homogeneity of the antiserum.¹

(4) Evidence of the extent of cross-reactivity of the antiserum with known or likely contaminants or cross-reacting hormones. This information will be in the form of a list of the names and amounts of compounds that if present in the test samples, are found to compete with labelled antigen for binding sites on the antibody under various conditions of testing the antiserum (in some cases curves of cross-reaction may be supplied).

(5) The immunogen used for preparation of the antiserum (e.g., steroids like 11-hemisuccinate progesterone, estradiol 17-β-6-BSA); or for coupled small peptides the name of the peptide, the nature of the coupling reaction, and the carrier substance.

(6) The species of animal immunized.

(7) The batch number (which should distinguish sera obtained at different times from one immune animal unless a pool of serum has been used to provide the batch) should be marked on every container and the recommended storage conditions and expiry date of the preparation stated.

(8) If the specificity of the antiserum has been altered by purification, this should be stated.

(9) Instructions to avoid repeated freezing and thawing and to prevent aggregation and accelerated destruction of the antibody.

1.2.3 Labelled antibodies

Antibodies to be labelled for use in immunometric and in two-site assay systems are generally prepared by initial binding to a sample of the antigen (hormone) that it is intended they should be used to measure, subsequent labelling of the complex, and then dissociation by separation of the labelled antibody from the antigen; in some cases the labelling may be done after the dissociation. The more highly purified the antigen used in such binding the more homogeneous and specific will be the resulting labelled antibody.

¹ Antisera containing antibodies in which the K value is 10⁻⁶ litres per mole or greater are more suitable for estimation of small amounts of hormones (picomolar range) in plasma, but antisera with lower K values (10⁻⁸ or 10⁻⁹ litres per mole) may be suitable for the assay of substances present in high concentrations, e.g., human placental lactogen.

The Michaelis-Menten hyperbolic calculation of the equilibrium constant has been shown to be unsuitable for this purpose (over-estimation of the K value).
Data to be provided about labelled antibody preparations (which may be supplied bound to an insoluble carrier) should include such information as relates to all radioactively labelled materials and, where applicable, data relating to other antiserum preparations (sections 1.1.2 and 1.2.2, respectively).

1.2.4 Other binding substances

In the case of assays where other binding processes replace antigen-antibody reactions, the agents used to bind the hormone include hormone-binding proteins from plasma (e.g., for cortisol, testosterone, progesterone, thyroxine) or membrane preparations from cell constituents of target organs carrying hormone-binding sites (e.g., kidney receptors for parathyroid hormone, ovarian or testicular receptors for LH and HCG). In the case of membrane preparations, if such binding agents are to be effectively used, it is usually necessary that the labelled substance for use in the assay retain its specific biological activity.

Many of the criteria for antisera used in immunoassays (section 1.2.2) are applicable to other means of binding. The choice between an antiserum and another binding agent will depend on comparison of the characteristic of the two materials as well as on comparison of the assay systems. The hormone-binding proteins in plasma usually have a rather low equilibrium constant (K value $10^{-8}$-10$^{-9}$ litres per mole) and are not so specific as properly prepared and selected antibodies or other tissue receptors (K value $10^{-6}$ litres per mole or greater).

The following information, at least, should be supplied with the preparation:

(1) The types of hormone substance for which the binding agent is intended to be used.

(2) The final dilution at which a stated proportion of a known trace amount of labelled hormone is bound after incubation in a known volume under specified conditions.

(3) In the case of assay systems in which the binding is the endpoint, the equilibrium constant (K value) at stated temperatures (including +4°C) and under stated conditions, calculated from a Scatchard plot, and the Scatchard plot, to show the degree of homogeneity of the binding agent.

(4) Evidence of the extent to which other hormones, metabolites, or likely contaminants may be bound. This information may be in the form of a list of the names and amounts of compounds that, if present in the test samples, are found to compete with labelled hormone for binding
sites under various conditions of testing the binding agent (in some cases curves of cross-reaction may be supplied).

(5) The species and source tissue of the binding substance;

(6) The batch number, recommended storage conditions, and expiry date of the preparation.

1.3 Reagents for separation of bound and free antigen

A variety of techniques are used for separation of bound and free antigens. It would be desirable for a separation system to have a wide latitude in relation to influences of variations in temperature, time, and pH, or from the presence of plasma or serum or of urine, whichever is applicable. Further it should be relatively simple to operate. Any separation system should have been shown to work effectively in the assay system for the declared hormone.

Each batch of a separation reagent, even when prepared by the same method and by the same manufacturer, should carry an identification number. In the case of antisera or unstable reagents an expiry date should be given.

Information provided with reagents specifically intended for separation purposes in hormone assays should include details of conditions under which they may be satisfactorily used, preferably with some indication of the acceptable latitude in the conditions for valid separations.

1.4 Reference material

The reference material ("standard") included routinely in every comparative assay for the purpose of quantitating the assay estimate should be as nearly as possible identical to the substance to be measured, i.e., fulfilling the criterion of similarity. Ideally it should consist of the homologous hormone (i.e., human). In other cases, synthetic material may be suitable (e.g., angiotensins, gastrin).

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1 Current separation techniques include: double-antibody method, adsorption methods, fractional precipitation, solid-phase methods, differential migration of bound and free fractions, and gel filtration.

2 A separation method working in buffer may not be suitable for application in an identical fashion to serum or plasma; one working in serum may not be suitable for urine or cerebrospinal fluid or tissue extracts. It is advisable to test the reagents for the particular hormone sample and separation method for which they are provided.

* See General considerations, pp. 33-34.
All reference materials should be adequately stable and, where appropriate, biologically active. Thus it is mandatory that the reference material be assessed both by bioassay and by accelerated degradation studies.

In the case of polypeptide hormones, these should be characterized by biological assay as well as by chemical and physical methods, as in the case of antigens prior to labelling (section 1.1.1). In the case of steroid hormones, prostaglandins, and some small peptide hormones it is essential that identity should be established by chemical and physical means. In these instances, the material used for immunoassays should be pure in substance and form (free from interfering substances such as dimers and isomers), and for bioassays free from substances that may interfere with the biological activity being assayed. However, the degree of purity of the reference material does not define assay specificity.

The stability of the reference material for immunoassays under the recommended storage conditions should be determined by immunoassay using each time the same assay system, the same conditions of assay, and the same antiserum and other reagents, with the exception of the labelled antigen, which should be of approximately the same quality. For biological reference material for use in receptor or cytochemical bioassays the stability should be determined by bioassay. The test of stability in any case should be an accelerated degradation test of suitable design.1

For each hormone of interest, the national control authority should preferably provide a national standard for use in national laboratories and even for inclusion in assay kits. If this is not possible the national standard may be used only for the calibration of laboratory reference material used routinely or of the reference material supplied with kits. In such cases, the laboratory or kit reference material for immunoassay should be calibrated in terms of the national standard in the particular assay system in which it is intended that it should be used. This potency (which will be the one relevant for its use) should be reported giving reference to both the particular immunoassay system and the national standard used; it should be expressed in international units of immunoassay activity, where available. Where, in addition, the biological activity of the material has also been estimated, because different methods, e.g., in vivo methods and in vitro methods, may give different estimates of biological activity, the method of bioassay and the standard used should be stated; this potency should be expressed in international units of biological activity, where

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1 Data on such tests may be found in reports of international collaborative assays for the establishment of international biological standards and reference preparations referred to in reports of the WHO Expert Committee on Biological Standardization published in the WHO Technical Report Series.
available. For reference material for immunoassay, any estimate of potency by bioassay is intended to provide evidence of the proper identity of the reference material and to ensure that it is indeed a sample of the hormone for the estimation of which it is intended to be used. Where reference material is for use in receptor or other bioassays, calibration by bioassay of its potency is obligatory, and any immunoassays that may have been done only provide some additional information that may, or may not, be relevant to the routine use of the calibrated reference material.

If every assay system were entirely specific there would be no problem in the calibration of reference materials in terms of national standards, or of national standards in terms of international reference preparations. However, this is not the case, since for any one hormone the different assay systems intended for it do not all show identical specificities and various preparations available for use as reference materials differ and are not always homogeneous. They are thus likely to contain, even if in small amounts, some constituents that will be recognized as hormone in one assay system but not in another. For this reason the potency of a national standard measured in terms of an international reference preparation or of laboratory or kit reference material in terms of a national standard will depend upon the particular assay system used. This anomaly will diminish only as assay systems that are more specific become available.

In the meantime, it is important that the national control authority should be aware that such problems may be expected to occur. Material for establishment as national standards should preferably be preparations of the highest purity necessary to give satisfactory results when used with the other reagents, the control of specificity being achieved by the use of characterized reference materials (see above) except in certain cases, e.g., where the national standard has to consist of pooled plasma or serum or a urinary extract.

If a series of batches of reference material of the same peptide hormone is made, the manufacturer should set aside a batch for use as a house standard. Successive batches should be compared with the house standard by using the same assay system, and as far as possible the same reagents. The effect of this would be to ensure consistency of behaviour of batches of that reference material, provided they are used only in the same system with the same reagents. In view, however, of the difficulties of making batches that are closely similar in behaviour, it is desirable that large batches be prepared.

Every container of reference material should be accompanied by at least the following information:

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(a) marked on the container:

"reference material for ..." (here insert type of assay system and name of hormone for which it is intended); ¹
the content of hormone preparation expressed as mass, unitage,
or concentration, as applicable;
batch number;
recommended conditions for storage;
expiry date;

(b) on the leaflet in the package, in addition to the above:
the source of the hormone preparation (e.g., species of animal and
tissue source, or synthetic);
nature and amount or concentration of any added substances
(including buffer salts or bacteriostat);
instructions for reconstitution, if applicable, e.g., diluent to be
used with details of recommended carrier protein and there-
after recommended conditions for storage of the reconstituted
material and period within which it should be used.

It is desirable that any additional information available pertaining
to the characterization of the preparation should also be provided.

If the reference material is intended for use with a variety of assay systems, information should be provided on the behaviour of the material in the different systems for which it is claimed suitable (chemical, physical, biological, and binding assays). If the preparation is used in an assay system for which it has not been intended, its suitability for use in that system should first be ascertained, under the responsibility of the user, or with the approval of the national control authority if the preparation is to be so used nationally.

1.5 Other substances and devices used in hormone assays

A number of ancillary reagents and materials other than those referred to in the preceding sections are needed and used in the various types of assay mentioned. These may include various chemicals, buffers, and scintillatants; even the containers in which the reagents are to be put may be critical for the assay. In some cases, however, certain materials may be prepared in the laboratory making the assays.

¹ Examples are "Reference material for immunoassay of HCG", "Reference material for receptor bioassay of parathyroid hormone". In some cases, an alternative formulation may be preferred, e.g., "Standard for immunoassay of hormone...".
Any of these substances may be of as great an importance for the effective performance of the assay as the substances referred to in the preceding sections. Criteria for such ancillary reagents and materials are not discussed in these guidelines, but any materials supplied for either binding assays or bioassays should have been shown to be suitable and any descriptions of them should clearly state the nature of each preparation and its purpose. If supplied in a kit they should also have been shown not to affect the product adversely before the expiry date.

1.6 Other varieties of immunoassay, including pregnancy tests

Techniques for other varieties of immunoassay include agglutination of latex particles, agglutination inhibition (latex particles or erythrocytes), complement fixation and various precipitin reactions (including quantitative immunodiffusion). The most widely applied of these techniques is agglutination inhibition, which is used for pregnancy testing of urine—many millions of such tests being made annually—and for measurement of relatively low levels of hormone (e.g., in intrauterine fetal death) or of high hormone levels (e.g., in choriocarcinoma or hydatidiform mole).

The pregnancy tests most in use relate to the measurement of human chorionic gonadotrophin (HCG) in the urine. The haemagglutination inhibition test using coated sheep erythrocytes is a tube test requiring about 2 hours to perform, but it can measure as little as a fraction of 1 IU of HCG per ml of sample. The latex tests may be made on slides in a few minutes. The direct agglutination test is as sensitive as the tube tests but the latex agglutination inhibition test can measure about 2 IU of HCG per ml of sample. The commonest way of making these tests is by using pregnancy test kits. The result of the assay by such a kit must indicate the presence or absence of a minimum quantity of chorionic gonadotrophin accepted as a criterion of pregnancy.

The human chorionic gonadotrophin used for coating latex particles or sheep erythrocytes should preferably be of high purity, but on account of costs and other considerations this is not always feasible and it may be necessary to use only partially purified hormone.

The antiserum to HCG used in the test should be highly specific, preferably reacting only with HCG. Most of the available antisera, however, show cross-reactions with LH (and sometimes with FSH as well), owing to structural similarities among these hormones. The quantities of LH present in the urine are usually insufficient to interfere with the test for chorionic gonadotrophin. When such antisera are used to detect HCG, the differentiation of early pregnancy from other states in which urinary gonadotrophin concentrations are elevated poses problems. Generally,
however, since the hormone used as immunogen is relatively impure the specificity of the resulting antiserum should be improved by absorption and purification techniques. The specificity of the antiserum may also be improved by using subunits of chorionic gonadotrophin as immunogen.

Since an international standard for HCG antiserum does not exist, the potency of an antiserum could be evaluated on the basis of amounts that will react with known amounts of HCG, i.e., in terms of equivalents of international units of the hormone. In practice, the dilution of the antiserum is adjusted to the maximum dilution that will cause agglutination in the absence of HCG but show no agglutination in the presence of a specified amount of HCG. This amount of chorionic gonadotrophin is that which is accepted as indicative of pregnancy. For any antiserum to be used, all such amounts should be designated by bioassay in International Units of the WHO Second International Standard for Chorionic Gonadotrophin, Human, for Bioassay (established in 1963) and in no other units; at the present time, in some countries, the minimum value is expressed as a concentration and set at 2-5 IU per ml of urine.1 Each lot of HCG antiserum should be tested using a calibrated reference preparation of HCG to show that not less than the stated amount of hormone will inhibit agglutination. Further, HCG antiserum should be free of bacterial and mycotic contamination; if a bacteriostat is added it should be shown not to affect the product adversely in the concentration used.

Under laboratory conditions, errors in these tests are of the order of 2%. When the tests are performed by untrained persons, however, errors are of the order of 5% or greater. High levels of HCG could give a false negative result (due to the prozone or antigenic excess zone). In making an assessment of performance it would be important not to be influenced by errors that are unrelated to the quality of the reagents concerned. These include inefficient mixing, incorrect measurement of drops from pipettes, and wrong conclusions drawn from the reaction seen, owing to turbidity, etc., of the urine and improper interpretation.

The national control authority should determine the acceptability of individual reagents and of kits on the basis of the recommendations in this document and the performance of the system as a whole in relation to the accepted clinical criteria. This is extremely important for pregnancy test kits, particularly those that have recently been developed for use at home by non-technical persons.

1 The limit is set at this high value in order to minimise the likelihood of false positive results. The International Reference Preparation of HCG, for Immunoassay (established in 1974) should not be used for this purpose.
Every pregnancy testing kit should carry at least the following information:

- the type of test it is intended for, i.e., pregnancy tests, or semi-quantitative assay of HCG;
- a statement on the label of the minimum amount of chorionic gonadotrophin that will give a positive reaction (expressed in IU's);
- storage conditions (if relevant) and expiry date;
- a list of components of the kit and detailed instructions for the performance of the test and for interpretation of the result;
- the source of the antiserum included in the kit and whether or not it is adsorbed; if adsorbed the nature of the carrier particles.

Final containers, droppers, etc., supplied with the kits should be sterile, colourless, and transparent.

In the case of kits intended for home use, detailed instructions should be given in simple and explicit terms understandable by non-technical persons; such instructions should be validated in the country and for the population by whom the kits would be used. They should also include recommendations for action to be taken on the result of the tests, whether positive or negative, i.e., positive results indicating pregnancy should be confirmed by appropriate technical services; if negative results occur, the test should be repeated but not less than six weeks after the first day of the last menstrual period, in the absence of factors that may render this timing invalid.

If HCG assay kits are supplied for uses additional to pregnancy testing, i.e., for quantitative measurements of the hormone in urine, adequate instructions should be supplied on the procedures to be followed and the precautions to be taken in making such assays.

2. Assay Kits

In the preceding sections, which refer to individual reagents that may or may not be supplied in bulk form, mention has been made of matched reagents, e.g., an antigen and an antiserum. The term kit, however, is in principle in this document applied to a complete group of reagents that comprises all the materials required to make a particular assay by a defined procedure. For the assay of each of several human hormones a variety of kits is now available. Not all of them, however, are supplied in the com-

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1 It is undesirable for the term "assay kit" to be used for a kit intended for only a part of the assay test procedure, e.g., a kit for labelling only.
ple form with all the reagents needed. The national control authority should decide which materials may be omitted from a particular type of kit based on the considerations below, provided that the suitability of the substitutes has been validated. Use of a kit with unsuitable substitutes may give discrepant results, which could constitute a health hazard.

(1) The reference material ("standard") for inclusion in every assay for quantitation may be omitted if an appropriate national standard is available. When the reference material is included, however, it may be a sample of such a national standard that the national control authority requires to be included in the kit.

(2) The antigen may be supplied in the kit for labelling by the user, or it may be included already labelled. It may be omitted, however, if the user has to obtain the labelled antigen from another source.

(3) The reagents for the separation may be omitted, at the discretion of the national control authority, if suitable materials for a satisfactory method of separation of bound from free hormone can be obtained elsewhere.\(^1\)

(4) Certain chemicals, buffers, etc., or any particular type of container, e.g., for taking the test samples, may be omitted at the discretion of the national control authority, if suitable materials can be obtained elsewhere.

All kits should be supplied with a clear statement of the reagents provided with them and with a list of all materials (and apparatus) that must be provided by the user (or national control authority) to carry out an assay. This will enable the user to check if he will be able to provide himself with the other materials needed.

In the case of a particular individual reagent it has been emphasized that its evaluation and suitability would depend on the other reagents and the particular assay system in which it would be used. It often happens, therefore, that a particular preparation may serve satisfactorily under certain circumstances but be quite unacceptable to a user under others. For this reason, many manufacturers prefer to supply their materials only in the form of kits. The acceptability of each kit for the stated purpose should be assessed from the performance of the kit as a whole and not on the basis of information relating to its individual constituents.

If certain materials have been omitted, the national control authority should determine its acceptability as a kit under precisely specified conditions for performing the particular assay. These may include the use of the

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\(^1\) In some cases the user may prefer a separation method in use in the laboratory making the assays, in which case it would be his own responsibility to validate the results.
particular national standard if the reference material is not supplied; if the antigen for labelling or the reagents for the separation systems are omitted, the performance should be assessed on those materials that may be supplied by the manufacturer, separately from the kit, for the purpose of kit evaluation, or those that may be recommended by him. An assay kit should perform reliably under the conditions recommended for its use, including any procedure recommended by the manufacturer for calculation of results.

Since kits are often made as a series of batches, consistency or performance should be achieved both within and between the batches released for use.

The following information at least, should be supplied with all kits:

1. the name and type of the hormone it is intended to measure and the type of sample (plasma, serum, urine, or extract) to be evaluated;
2. information that should be marked on each container, i.e., on each whole kit:
   - batch number;
   - recommended conditions for storage;
   - expiry date;
   - if applicable, the identity and amount of radioactivity (at a stated date) of any radionuclide present;
   - identification of the reference material ("standard") included in the kit.
3. a list of components of the kit and detailed instructions for the performance of the assay, including processing of the sample if this is necessary;
4. general information on the performance of the kit: e.g., level of sensitivity, specificity, accuracy and precision of estimates, obtainable within the dating period.\(^1\)

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\(^1\) The evidence for this general information could include some of the following, depending on the availability and feasibility of obtaining the data:

1. A representative dose-response curve, showing data points as well as the "best-fit" curve.
2. Summary statistics showing the mean, standard deviation, and range of \(\%(B/B_0)\), slope and intercept for dose-response curves for several repetitive assays.
3. An antibody dilution curve.
4. Cross-reactivity data (relative ED\(_{50}\)'s for material obtained from closely related species likely to cross-react).
5. Demonstration of parallelism of standard and unknown over two or more orders of magnitude.

(Continued on following page)
(5) precautions to be observed in the use of the kit, e.g., hazards to personnel, reagent instability, critical or difficult stages in the procedure, and, if important, details of taking the sample.

3. Cytochemical Bioassays

3.1 General

The procedures developed in cytochemistry offer special advantages for hormone assays. Sensitive cytochemical bioassays have recently been developed and considerable interest has been aroused in their application to the assay of several hormones at levels of detectability hitherto attainable only by radioimmunoassay. In fact, in several instances they are considerably more sensitive than their equivalent immunoassay and so require much smaller samples of plasma. Cytochemical bioassays thus fulfill the need not only for more sensitive assays but also for methods that measure only bioactive hormones and other substances. This could be relevant, particularly to hormones that may be biologically active in vitro, but may have lost some activity in vivo, e.g., desalinated HCG, decomposed human growth hormone. The procedure followed in each case is the microscopic inspection of a selected cell-type and the microdensitometric analysis of a characteristic biochemical activity, e.g., cytochemical chromogenic reaction. The technology allows the measurement of the biochemical changes in specific target cells of an organized tissue, which may be induced by disease processes, by damaging agents (such as histamine), and by drugs and poisons. Quantitative evaluation of amounts of hormone is made by appropriate statistical design of the assay method and use of a standard for the hormone in terms of which the relative potency of test materials is given. The sensitivity and precision of these assays depend partly on their being...

(6) Recovery experiments, showing ability to obtain 100% recovery over the working range of the assay.
(7) A measure of within- and between-assay variability, for at least one and preferably three dose levels.
(8) Scatchard plots and equilibrium constants.
(9) Data related to the time course of the RIA reactions for one or more temperatures.
(10) Comparison with other methods of assay (e.g., gas chromatography, double isotope methods, colorimetric methods, and bioassay).
(11) Detailed information on all parameters likely to affect assay performance (e.g., time, temperature, pH, ionic strength, serum effects, "blanks", reagent concentrations, special additives, methods for separating bound from free antigen, etc.).
(12) Data to substantiate the fact that the assay system is working satisfactorily in the intended application (e.g., physiological studies, clinical studies, etc.).
(13) Appropriate references to the scientific literature.
(14) The range of normal (and pathological) values, if given, should be for the population and particular environment for which the kit is to be used.
designed as within-animal assays. Conventional use of standards in this way enables the results of cytochemical bioassays to be expressed in international units for the hormone, where these have been defined.

Many of the considerations that apply to the immunoassay of hormones, including the principle of similarity, the form of each hormone to be assayed, the purity, homogeneity, and identity of the standard used in the assay, apply also to these assays.

3.2 Requirements

The biological materials required for making cytochemical bioassays include the animals and tissues or cells providing the target. The responsibility for selection of these materials rests with the laboratory making the assays.

The requirements for these assays are:

1. The target organ must be obtained from a suitable animal. The species may be critical and the ideal weight of the animals will vary with the assay. For some assays, the animals require hormonal pretreatment (as in the assay of LH).

2. Segments of the target tissue need to be maintained in vitro for a specified short period of time. They are then subjected to the hormone diluted with culture medium. For section assays, the culture medium includes a colloid stabilizer to protect the sections. Not all available culture media and colloid stabilizers are intended for maintaining in vitro the pieces of tissue or the sections for these particular assays, and some may be unsuitable for this purpose. Any medium or stabilizer supplied for use in these assays should have been shown to be satisfactory for the stated purpose.

3. The segments, after rapid chilling, must be sectioned at low temperature. It is essential that cryostats for this purpose should be capable

(a) of maintaining the correct temperature both of the cabinet and of the knife;

(b) of cutting sections of equal thickness to an accuracy of ±5%.

4. Microdensitometric measurement of the cytochemical chromogenic reaction must be performed.

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1 Because thickness varies according to the speed of cutting, cryostats should be equipped with an automatic cutting device, so that the speed can be regulated but remains constant for any given setting.

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Methods of measuring the cytochemical response are fundamental to the performance of these assay systems.\textsuperscript{1} For most of these assays it is essential that the diameter of the microdensitometer scanning-spot should be 0.25 μm (or at the most 0.3 μm).

3.3 Validation of results, precision, and sensitivity

Validation of results requires the determination of within-assay variation (variation between replicates or serial sections should not exceed \( \pm 5\% \)) and between-assay variation, and tests of parallelism of the reference and test materials. It is advisable to make the measurements on two different dilutions of the test plasma. The potencies calculated from the two dilutions of each sample should give results that agree to within \( \pm 15\% \).

\textsuperscript{1} The World Health Organization is currently in the process of developing plans that may include the formulation of specifications for the control of apparatus relevant to particular tests in accordance with resolution WHA27.62 adopted by the Twenty-seventh World Health Assembly.
WHO BIOLOGICAL STANDARDS

International standards and reference preparations of hormones are held and distributed by the WHO International Laboratory for Biological Standards at the National Institute for Biological Standards and Control, Loudon, England. Samples are distributed to national laboratories on request and free of charge. They are intended for calibration of national or laboratory standards or reference preparations of hormones for use in bioassay or immunoassay (whichever is applicable). They are not available in sufficient quantity to be used routinely in laboratories for such assays.

The international standards and international reference preparations of hormones that have been established by the World Health Organization include the following:

<table>
<thead>
<tr>
<th>International standards for bioassay</th>
<th>Form in which dispensed and definition of international unit</th>
</tr>
</thead>
<tbody>
<tr>
<td>Oxytocin and vasopressin (antidiuretic hormone), bovine (established 1957)</td>
<td>Acetone-dried powder of whole posterior pituitary gland of the ox (2 oxytocic IU, 2 vasopressor IU, and 2 antidiuretic IU per mg)</td>
</tr>
<tr>
<td>Insulin, bovine and porcine (established 1958)</td>
<td>Insulin recrystallized from a mixture of 52% bovine and 48% porcine pancreas (24 IU per mg)</td>
</tr>
<tr>
<td>Chorionic gonadotrophin, human (established 1963)</td>
<td>Active principle from human urine of pregnancy, with lactose, freeze-dried (5300 IU per ampoule)</td>
</tr>
<tr>
<td>Serum gonadotrophin, equine (established 1966)</td>
<td>Active material from the serum of pregnant mares, with lactose, freeze-dried (1600 IU per ampoule)</td>
</tr>
<tr>
<td>Prolactin, ovine (established 1962)</td>
<td>Freeze-dried purified prolactin from anterior pituitary gland of sheep (22 IU per mg)</td>
</tr>
<tr>
<td>Corticotrophin, porcine (established 1962)</td>
<td>Purified corticotrophin from anterior pituitary gland of the pig, with lactose, freeze-dried (5 IU per ampoule)</td>
</tr>
<tr>
<td>Thyrotrophin, bovine (established 1954)</td>
<td>Tablets of a blend of purified thyrotrophin from anterior pituitary gland of the ox, and lactose (approximately 1.48 IU per tablet)</td>
</tr>
<tr>
<td>Growth hormone, bovine (established 1955)</td>
<td>Dried growth hormone from anterior pituitary gland of the ox (1 IU per mg)</td>
</tr>
<tr>
<td>Glucagon, porcine (established 1973)</td>
<td>Freeze-dried material from a solution of porcine glucagon, with lactose and sodium chloride (1.49 IU per ampoule)</td>
</tr>
</tbody>
</table>
Human urinary FSH and Human urinary LH(ICS)H
(established 1974)

*International reference preparations for bioassay*

Erythropoietin, human, urinary
(established 1970)

Calcitonin, porcine (established 1974)

Calcitonin, salmon (established 1974)

Parathyroid hormone, bovine
(established 1974)

Human pituitary gonadotrophins FSH
and LH(ICS)H (established 1974)

Renin (established 1974)

*International reference preparations for immunoassay*

Growth hormone, human
(established 1968)

Parathyroid hormone, bovine
(established 1974)

Human insulin (established 1974)

Porcine glucagon (established 1974)

Human chorionic gonadotrophin
(established 1974)

Human chorionic gonadotrophin alpha subunit (established 1974)

Freeze-dried residue of extract of urine from postmenopausal women, with lactose (54 IU FSH and 46 IU LH(ICS)H per ampoule)

Freeze-dried residue from an extract of human urine, with sodium chloride (10 IU per ampoule)

Freeze-dried residue of purified porcine calcitonin with mannitol (1 IU per ampoule)

Freeze-dried residue of purified synthetic preparation of salmon calcitonin with mannitol (80 IU per ampoule)

Freeze-dried residue of trichloroacetic acid extract of bovine parathyroid gland with lactose (200 IU per ampoule)

Freeze-dried residue of extract of human pituitary glands, with lactose (10 IU FSH and 25 IU LH(ICS)H per ampoule)

Freeze-dried residue of purified extract of renin from human kidneys, with lactose and phosphate buffer (0.1 IU per ampoule)

Purified growth hormone from human anterior pituitary gland, with sucrose and buffersalts, freeze-dried (0.350 IU per ampoule)

Freeze-dried residue of purified hormone from bovine parathyroid glands, with human plasma albumin and lactose (2 IU per ampoule)

Freeze-dried residue of recrystallised human insulin, with sucrose (3 IU per ampoule)

Freeze-dried residue of purified porcine glucagon, with lactose and sodium chloride (1.49 IU per ampoule)

In preparation

In preparation
Human chorionic gonadotrophin beta subunit (established 1974)

In preparation

Human pituitary LH (CSH) (established 1974)

Freeze-dried residue of extract of luteinizing hormone from human pituitaries, with human albumin and lactose.¹

Human TSH (established 1974)

Freeze-dried residue of extract of thyroid stimulating hormone from human pituitaries with human albumin and lactose (150 IUU per ampoule)

International Units of Hormone Preparations for Immunoassay

Some explanation is appropriate here of the definition of international units for international reference preparations of hormones for immunoassay. The twenty-first WHO Expert Committee on Biological Standardization ² decided that when defining an international unit for an international reference preparation of a human hormone for immunoassay, it was desirable to define this as nearly as possible equal in biological activity to an existing international unit for bioassay (i.e., by comparing the two preparations by bioassay), even when the latter unit is for a standard originating from another species. When a reference preparation for immunoassay is replaced, however, the international unit should be defined by comparing the new reference preparation with the previous reference preparation by immunoassay.

It is desirable that each hormone for all reference materials ("standards") to be included in each immunoassay for quantitation should be directly compared with the international reference preparation if it exists. If a calibrated national standard is available in sufficient quantity, it can be used routinely in all immunoassays of the particular hormone (supplied as an individual reagent and included in all kits). If a national standard does not exist, manufacturing establishments or laboratories making immunoassays should each calibrate their own reference material directly with the international reference preparation. If the national standard for the particular hormone exists but is insufficient to be used throughout the country routinely in all assays, it may be possible for only the national standard to be calibrated directly with the international reference preparation so that it may be made available to manufacturers for the calibration of their own reference material. It is preferable, however, for the national control authority to provide in adequate quantities the necessary national standards, particularly of human hormones, for distribution within the country to laboratories that carry out these assays.

Where international units (IU) have been defined for the international materials established by WHO, calibration of laboratory or national reference materials would enable the results of assay of human hormones to be expressed in IU; this practice would carry many advantages apart from those for national authorities concerned with the control of hormone preparations administered therapeutically to man, the specific purpose for which IU for biological substances are defined. Problems in the calibration of reference materials are dealt with in section 1.4, page 45.

¹ The unitage per ampoule will be assigned when the study has been completed (see p. 14).
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DEVELOPMENT OF NATIONAL ASSAY SERVICES FOR HORMONES AND OTHER SUBSTANCES IN COMMUNITY HEALTH CARE

One of the major developments in modern medicine is a shift in emphasis away from the diagnosis and clinical care of established diseases in an advanced state, which may be impossible to cure or difficult to alleviate. The present tendency is towards a public health approach, i.e., the early diagnosis and detection of prodromal and preclinical states that may be more amenable to treatment and in which treatment can be life-saving. This approach will depend to an increasing degree on sensitive, specific, and accurate analytical techniques. Such techniques are of particular importance in fertility regulation, nutritional growth problems, and the assessment of the well-being of the mother and the unborn child.

With the recent rapid development of newer in vitro methods for the assay of hormones, including immunoassays, it has become increasingly evident that these procedures have wide application in relation to human medicine. It must be emphasized that these assays are just as important in microbiology, medical oncology, veterinary medicine, and clinical pharmacology as they are in endocrinology. At present, in many countries the use of these assays is pioneered by research workers, by hospital laboratories (using kits and reagents available commercially), and by services provided by independent laboratories on a commercial basis. The availability of these assays, however, is of major public health importance. In developing countries, the possibility of performing these assays at all has to rest heavily on the availability of kits for particular assays of interest.

In view of the expected rapid expansion of demand for these assays in all countries, a case can be made for providing such services on a national basis. The primary argument in favour of such a course is that it would result in considerable cost saving, but other benefits, such as improved reliability of results and more effective use of health resources, would

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1 The draft of this document was prepared by: Dr D. R. Bangham, Head, Division of Hormones and Blood Products, National Institute for Biological Standards and Control, London, England; Professor J. Landon, Professor of Chemical Pathology, St Bartholomew's Hospital, London, England; Professor B. Lunenfeld, Director, Institute of Endocrinology, Tel-Hashomer Government Hospital, Tel-Hashomer, Israel; Dr R. M. Leguin, Head, Radioimmunoassay Laboratory, Department of Obstetrics and Gynaecology, Radiobouwziekenhuis, Nijmegen, Netherlands; Dr A. S. Outschoor, Chief Medical Officer, Biological Standardization, WHO, Geneva, Switzerland; Dr C. Rerup, Department of Pharmacology, The University, Lund, Sweden.
acquire. It would also make available a source of better, specialized advice on the interpretation of results of assays and their application. Further, a national service could also help to prevent unnecessary duplication of facilities in several places in a country where these assays are needed. The cost of developing it within community health care would be very much less than if laboratories develop their own assay facilities individually.

A national assay service could perform important functions in collaboration with the national authority responsible for the control of preparations used therapeutically, and depending on the degree of extension and coverage of the national service other aspects of national control of relevant biological products could be included among its functions.

A number of functions must be coordinated in order to achieve a satisfactory national assay service. It should not be envisaged, however, that such a service needs necessarily to be established as a completely separate system, independent of other services in medicine and public health. On the contrary, it should be developed so as to make use of existing services and facilities wherever possible, drawing on expertise and resources that are already available. The scope of the national service here outlined is concerned only with in vitro assays; it does not require the presence of the patients themselves, or involve the administration of radioactive substances to the patient in the laboratory. It relates specifically to assays of human hormones, but a service of the kind described could be extended to other substances, including specific proteins (carcino-embryonic antigen, alpha-fetoprotein), drugs (digoxin, gentamicin), viruses (hepatitis B antigen), and proteins involved in blood coagulation and fibrinolysis.

Since national assay services of the kind envisaged are not yet developed in many countries, the recommendations herein would, in many instances, be based on the development of potential resources and estimated needs. Once such services have been developed to a greater or smaller degree in several countries, the expertise gained and the additional information available would be most valuable and enable the present recommendations to be improved.

A national assay service could comprise a multi-tier system of central and peripheral (rural) laboratories, as well as a national centre. The number of such laboratories would depend on factors such as the demand for assays, national resources and expertise, the number and density of population, and administrative independence of states or provinces within a country. Thus, a small country might have a more compact establishment of one or two peripheral laboratories and one central one, which might even serve as the national centre. A larger country might have many peripheral laboratories and several central laboratories as well as national centres. It should be emphasized, however, that the system should be flexible and
also be developed in conjunction with the most knowledgeable persons or institutions in the country for each particular hormone assay. It could be envisaged, therefore, that one laboratory with expertise for a particular assay could function as the central laboratory for that assay as well as serve as a peripheral laboratory for a number of other assays.

Since the main purpose of the assay service is in relation to human medicine, a system should be established whereby there are frequent consultations with clinicians, workers from laboratories, the national control authority, representatives of any national control laboratory, radioisotope laboratories, pharmacologists, statisticians, governmental authorities, and commercial establishments, so as to ensure the continued maintenance of an efficient service. Consultations of this kind would also be of importance for discussions on the interpretation of results, their significance and their implications for diagnosis and therapy, and for improved specifications and criteria for preparations used in man, as well as for reagents and materials used in assays.

In the case of certain substances (e.g., human pituitary hormones) source materials for the preparation of assay reagents are scarce and difficult to obtain. The development of centralized national services for collecting and processing such materials (e.g., national pituitary collections) is therefore of considerable importance for national assay services.

A. Peripheral (rural) laboratory

In each country at least one and perhaps several peripheral laboratories would be needed. Each would generally be sited in a suitable department of a hospital or other institution and serve a particular geographical region or group of the population.

The laboratory would perform (a) assays that are needed frequently (e.g., human placental lactogen assays for assessment of placental function, some steroid assays in fertility regulation, T4 assays in goitre endemic areas, and screening assays for cancer); (b) assays that are simple to perform (e.g., pregnancy tests), and (c) assays for which a quick result is often urgently needed (e.g., digoxin). If equipped to do so, it would also perform certain more complex assays, especially those (e.g., on inpatients) for which results are desirable within a few days (one week) and for which blood samples have to be assayed soon after collection, or where samples cannot conveniently be sent to a central laboratory under suitable conditions.

The facilities of a peripheral laboratory could be built around existing expertise, either in a governmental or non-governmental institution or in a commercial establishment. Expertise in assay of a particular hormone
may also already exist, for example, in a university department in the biomedical faculty. In such cases, the assay service for that hormone could be provided by such a laboratory even though a peripheral laboratory might be developed elsewhere in the same area for assays of other hormones.

The personnel and facilities needed for a peripheral laboratory would include the minimum of a chemist/biochemist and a technician; some secretarial services would also be needed. Equipment must include a radioactivity counting device and a refrigerator for storage at +4°C (if possible a deep freeze for −20°C would also be useful). A refrigerated centrifuge or reasonable access to one is also essential. If most of the assays in a peripheral laboratory are made using disposable kits, a minimum of other laboratory equipment, such as glassware, is all that is needed. With such minimum staff and equipment, perhaps 100 assay samples a week could be dealt with. This number could be increased greatly if automated equipment, e.g., diluting devices, were provided, and if more technicians and a larger capacity counter were available. Statistical services could be obtained from the central laboratory by suitable means. It would be essential to provide continuous maintenance of equipment.

Since handling and disposal of radioisotopes would be involved for many of the hormone assays, national regulations and control measures for institutions and personnel working with radioactive isotopes would be applicable. A peripheral laboratory would only rarely do its own labelling of antigens; if it does then it should be suitably equipped and appropriate safety measures applied.

B. Central laboratory

A central laboratory would generally be sited in, or adjacent to, the clinical pathology or clinical diagnostic department of a large hospital and serve a large group of the population, i.e., in a geographical area, or in certain cases even the whole country.

A central laboratory would perform, in addition to the range of assays carried out by peripheral laboratories, those assays that were more complex and technically difficult (e.g., TSH in thyroid disease, FSH and LH and certain steroids in fertility control, human growth hormone for assessment of abnormalities in the growth and development of children, alpha-feto-protein in neural crest deficiency, and tumour-associated antigens); and certain assays for which a delay of some weeks in the return of results was acceptable. A central laboratory may be designated to deal only with partic-

1 Simple manual instruments, inexpensive but accurate, are available. In rural areas where servicing may be difficult, consideration should be given to having two identical counters with interchangeable components.
ular assays based on the available expertise. In a certain area or country, therefore, more than one central laboratory may be required for the coverage of all the kinds of hormone assay needed. Thus, it could be envisaged that a particular laboratory could function as a central laboratory for the assay of certain hormones and as a peripheral laboratory for assaying others.

The central laboratory would be expected to provide certain reagents, e.g., labelled antigens and certain quality control samples, for its own use and for the peripheral laboratories within its region. It would also, when called upon, provide expert advice on the interpretation of results. It would participate in work on the assessment of reagents and kits and collaborate in assays to calibrate national standards (for use in the country) and in quality control and other collaborative studies in conjunction with the national centre.

Training of technicians on methodology would be provided and education of academic and higher technical staff on interpretation of results would be planned so as to build up expertise within peripheral laboratories for the area served by the central laboratory. In addition a central laboratory would train staff on precautions for handling radioactive materials and human materials potentially contaminated with viruses; such instruction would also include the proper disposal of waste materials.

Another important consideration is that kits for hormone assay now available are deceptively simple to operate so that unqualified persons who are unaware of or unable to understand their pitfalls think they can, and often do, make these assays. Erroneous results and uninformed interpretation under these circumstances could become a definite health hazard, unless they are critically evaluated.

Personnel for a central laboratory should include at least one senior officer, qualified in medicine or the biological sciences and with special training in the assay methodology (including labelling with radioisotopes) and interpretation, and several technicians, at least one of whom should have been specially trained in such methodology and handling of equipment. The personnel may also comprise an additional chemist or biologist. A clerk/secretary and typist would be needed.

Equipment in the central laboratory should include: at least one automated radioactivity counter for γ- and/or β-emitting isotopes; refrigerators at +4°C and –40°C or below; refrigerated centrifuges; fume cupboard; automated pipetting device(s); access to a fast data-handling facility.

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1 The laboratory should be equipped to comply with national specifications for handling radioactive isotopes.
which would serve the central laboratory and one or more peripheral laboratories.

The central laboratory would have access to at least an animal house or animal house facilities for the preparation of the reagents required for these assays.

C. National centre(s)

A national centre could provide the services of a central laboratory if this is required, at least for the assay of certain hormones. In any event, the national centre would act as the main coordinating laboratory of a national assay service. It could be sited in one of the central laboratories of the service, or in another appropriate biomedical institute. The duties of a national centre would include:

(a) The organization of a supply of working reagents, including:
   (i) pooled antisera or other binding proteins and certain labelled antigens the preparation of which is technically difficult in quantities adequate to serve routinely for assays in the country;
   (ii) suitable quality control serum samples, reference reagents, etc.;
   (iii) scarce reagents, e.g., human pituitary hormones.

(b) The organization and coordination of a national scheme to monitor laboratory performance in central and peripheral laboratories. This would include the supervision and maintenance of quality control charts for each hormone assay system in each laboratory, central or peripheral.

(c) The coordination of statistical analyses of assay results.

(d) The organization of training and education programmes for medical, scientific, and technical staff, including formulation of the syllabus and the provision of training in theoretical and practical methodology. In many instances, this should be carried out in the peripheral (rural) laboratories themselves.

(e) Improvements of assay methods and new applications in specific fields in which the laboratory is particularly experienced.

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1 It would be very valuable if such quality control samples that had previously been analyzed by a number of laboratories could be obtained for low, normal, and elevated levels of various hormones. It would be best if these were true physiological or pathological samples, as the interfering material present in made-up samples is often very different from that in clinical samples.
If the laboratory is not itself the designated national control laboratory for reagents and materials, collaboration with the national control laboratory in:

(i) provision of national standards for immunoassay, calibrated in terms of international reference preparations where they exist;
(ii) national control of manufactured reagents and kits and putting into effect a batch release system;
(iii) formulation and application of national requirements for the control of reagents and materials used in immunoassays;
(iv) collaboration with international organizations and scientific bodies concerned with standardization and control of relevant materials and methods.

D. Quality control of reagents and assessment of laboratory performance

It is desirable that the requirements recommended by WHO or other suitable requirements for the control of reagents and materials for immunoassays should be adopted and implemented. A national quality control scheme should include validation of new reagents, and assay systems.

If circumstances permit it would be desirable to adopt a national scheme for licensing of all reagents used by a national service; this would include the registration of manufacturers of kits and reagents and release of batches only on the recommendation of the national centre in conjunction with the national control laboratory.

The monitoring of laboratory performance would be achieved on coded samples, e.g., pooled plasma or urine, blanks, samples with added known amounts of hormone, distributed from the national and central laboratories. Analysis of the results would enable an assessment to be made of the general pattern of values obtained and the relative position of each laboratory’s results within that pattern. It could also lead to the collection of up-to-date information on average estimated “normal” and “abnormal” ranges for the populations served.

E. Consultative and supporting information

Since the provision of assay services with a minimum of cost to the country is a prime objective, it would be advisable to arrange for the supply of kits that are capable of being used not only for the purpose of a single test, as is frequently the case, but with sufficient reagents for the purpose of many assays on the same occasion in a single laboratory.
Certain operational problems would require development to suit the particular resources and requirements of each country. Thus, a communication system, perhaps in the form of an information booklet continuously brought up to date in the light of new evidence, should provide information on: the range of assays available; suitable preparation of the patient for each type of assay (e.g., with respect to diet, drugs); the method and precautions necessary for obtaining and handling samples of body fluids for testing; current opinion on the useful application of these assays and the interpretation of results.

Suitable forms for requesting assays should be provided so that requisite information regarding the patient, the handling of the sample, and interpretation of the results can be entered on it. It would facilitate the analysis of quality control data on laboratory performance, as well as of data for the calculation of estimated average “normal ranges”, if such forms were machine readable.

**F. International services**

The development of assay services has been helped by the activities of international organizations, such as the World Health Organization and the International Atomic Energy Agency, and by the activities of international scientific societies. These activities include the provision of international specifications and reference reagents and the training of workers and scientific meetings. Much help has also been given by various governmental and non-governmental bodies in the form of the supply of characterized scarce reagents (e.g., human pituitary hormones) and by holding discussion groups on technical scientific problems.

It is envisaged that such activities should be extended at the international level. For example, it may be desirable to develop the interchange of personnel for training, and to designate one or more centres to coordinate information concerning developments in this technology, to provide scarce reagents, and to help governmental authorities to develop their national control of assay kits and of reagents used in assays.
Annex 3

REQUIREMENTS FOR BIOLOGICAL SUBSTANCES
AND OTHER SETS OF RECOMMENDATIONS

The specification of requirements to be fulfilled by preparations of biological substances is necessary in order to ensure that these products are safe, reliable, and potent prophylactic or therapeutic agents. International recommendations on requirements are intended to facilitate the exchange of biological substances between different countries and to provide guidance to workers responsible for the production of these substances as well as to others who may have to decide upon appropriate methods of assay and control.

Recommended requirements and sets of recommendations concerned with biological substances formulated by international groups of experts and published in the Technical Report Series of the World Health Organization are listed hereunder:

<table>
<thead>
<tr>
<th>No.</th>
<th>Year</th>
<th>Requirements for Biological Substances:</th>
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| 178 | 1959 | 1. General Requirements for Manufacturing Establishments and Control Laboratories  
     |      | 2. Requirements for Poliomyelitis Vaccine (Inactivated) |
| 179 | 1959 | Requirements for Biological Substances:  
     |      | 3. Requirements for Yellow Fever Vaccine  
     |      | 4. Requirements for Cholera Vaccine |
| 180 | 1959 | Requirements for Biological Substances:  
     |      | 5. Requirements for Smallpox Vaccine |
| 200 | 1960 | Requirements for Biological Substances:  
     |      | 6. General Requirements for the Sterility of Biological Substances |
| 237 | 1962 | Requirements for Biological Substances:  
     |      | 7. Requirements for Poliomyelitis Vaccine (Oral) |
| 274 | 1964 | WHO Expert Committee on Biological Standardization:  
     |      | 8. Requirements for Pertussis Vaccine  
     |      | 9. Requirements for Procaine Benzylpenicillia in Oil with Aluminium Monostearate |
| 70  |      |                                         |
293 1964 WHO Expert Committee on Biological Standardization:
10. Requirements for Diphtheria Toxoid and Tetanus Toxoid

323 1966 WHO Expert Group:
Requirements for Biological Substances (Revised 1965)
1. General Requirements for Manufacturing Establishments and Control Laboratories
2. Requirements for Poliomyelitis Vaccine (Inactivated)
7. Requirements for Poliomyelitis Vaccine (Oral)
5. Requirements for Smallpox Vaccine

329 1966 WHO Expert Committee on Biological Standardization:
11. Requirements for Dried BCG Vaccine
12. Requirements for Measles Vaccine (Live) and Measles Vaccine (Inactivated)

361 1967 WHO Expert Committee on Biological Standardization:
13. Requirements for Anthrax Spore Vaccine (Live — for Veterinary Use)
14. Requirements for Human Immunoglobulin
15. Requirements for Typhoid Vaccine
9. Requirements of Procaine Benzylpenicillin in Oil with Aluminium Monostearate (Revisions adopted 1966)

384 1968 WHO Expert Committee on Biological Standardization:
16. Requirements for Tuberculins
17. Requirements for Inactivated Influenza Vaccine

413 1969 WHO Expert Committee on Biological Standardization:
4. Requirements for Cholera Vaccine (Revised 1968)
18. Requirements for Immune Sera of Animal Origin

444 1970 WHO Expert Committee on Biological Standardization:
19. Requirements for Rinderpest Cell Culture Vaccine (Live) and Rinderpest Vaccine (Live)
20. Requirements for Brucella abortus Strain 19 Vaccine (Live — for Veterinary Use)

444 1970 WHO Expert Committee on Biological Standardization:
Development of a National Control Laboratory for Biological Substances (A guide to the provision of technical facilities)
WHO Expert Committee on Biological Standardization:

21. Requirements for Snake Antivenins

WHO Expert Committee on Biological Standardization:

7. Requirements for Poliomyelitis Vaccine (Oral) (Revised 1971)

WHO Expert Committee on Biological Standardization:

4. Requirements for Cholera Vaccine (Revised 1968) (Addendum 1973)

6. General Requirements for the Sterility of Biological Substances (Revised 1973)

17. Requirements for Inactivated Influenza Vaccine (Addendum 1973)

22. Requirements for Rabies Vaccine for Human Use

WHO Expert Committee on Biological Standardization:

Recommendations for the Assessment of Binding-Assay Systems (including Immunoassay and Receptor Assay Systems) for Human Hormones and their Binding Proteins (A guide to the formulation of requirements for reagents and assay kits for the above assays and notes on cytochemical bioassay systems)

Development of national assay services for hormones and other substances in community health care