Today much is being written and said about the use of hormonal substances in commercial lamb and cattle feeding.

Their use has been cleared in implanting lamb with combination progesterone-estradiol pellets and incorporating diethylstilbestrol in cattle feeds.

With this widespread commercial use of these physiologically active substances it is necessary to determine residual hormonal substances remaining in meat from animals having been administered these hormonal substances.

An assay is a technique either biological or chemical which is used to: (1) determine if an unknown substance has hormonal characteristics, and of what type hormonal substance it is; (2) the relative potency of this hormonal substance, i.e., a quantitative assay.

Biological assays are based on known effects which these hormones have on certain body functions or tissues. It is based on the premise that certain concentrations of unknown substances given to sufficient numbers of similar laboratory animals will elicit responses in relationship to potency. This response is then compared to that of a known standard to determine relative potency.

Chemical methods are based on the ability of the chemist to identify a compound and then to determine quantitatively the amount of hormone present in an unknown.

Because of the sizeable task of reviewing all types of hormone assays, this paper will deal with those most likely to be encountered by one interested in livestock and meats.

There are a number of problems to be encountered in assaying a hormonal substance by a biological method.

1. The variation of laboratory animal not only between assays, but within assay groups constitutes a major problem. Most bioassay techniques have set forth type of animals to be used, size, age, degree of maturity and quite often, strain. Certain inbred strains of laboratory animals have been developed for assay purposes.

2. Environmental conditions must be kept constant. This is particularly important when assaying substances such as thyroxin where the assay is based on CO₂ output and O₂ intake. Feeds must be carefully studied so as not to introduce endocrine
stimulating substances in this way. Temperature and light has been shown to have an effect on thyroid activity. Since endocrine interrelationships are so markedly important this could cause variation in expected results.

3. Even with highly inbred strains of animals held under ideal conditions, we may get abnormal endocrine functions in certain individuals.

4. The endocrine balance in living organisms is a very delicate relationship and when disturbed, a number of changes usually occur in other endocrine glands in the same organism. A notable example of this is the thyroid anterior pituitary balance.

5. Unknowns may contain biologically active substances which are not identical with the true hormone substance. A crude extract will contain a mixture of estrogen and other substances and the potency it exhibits will depend on the method used and the nature of the extract.

6. According to Emmens, different estimates can be obtained by altering such variables as: solvent used, spacing of injections, number of injections, etc. "The influence of such variables is so great that (except under special conditions) it is impossible to attach any precise meaning to the results of an assay unless it is known in advance that the substance under test is in pure form and is identical with the standard preparation in chemical constitution.

At the present, the most sensitive and accurate assays available are of the biological type. Even with these, only a few are considered to be reliably accurate from a quantitative standpoint. There is a strong tendency for the most sensitive tests to be the least accurate. Combined sensitivity and accuracy is yet to be reached.

In order to understand better the characteristics of the various types of assays and to realize their limitations, specific assay methods should be examined.

**Estrogen - Characteristics**

Estrogens are steroids having the basic steroid structure shown below.
Estrogens induce estrus and stimulate secondary sex organs and sex characteristics in normal and ovariectomized females. They stimulate growth of the uterine muscle and endometrium, motility of the uterus and fallopian tubes, increase capillary permeability in the uterus and vagina, thickening of the vaginal epithelium and lowering of the pH of the vaginal secretion.

They are necessary for mammary gland development and prepare mammary tissues for prolactin stimulation.

There are two general types, natural and synthetic.

The most potent natural type estrogen is estradiol - which is the main ovarian estrogen. The estradiol form is found in pregnant mares urine and is much less potent.

Another natural estrogen, estrone is found in urine, ox adrenal glands, and placentae. It is 1/4 - 1/2 as active as estradiol and is formed in vivo from estradiol. An international unit of estrogen activity is that of .1 ug. of international standard preparation of estrone.

Estriol, a third natural estrogen is found in urine and is less active than estrone. "The potency varies enormously with the test method." Values of 1:1 to 250:1 have been obtained for estrone/estriol ratio.

Like estrone, estriol is formed in vivo from estradiol.

Estriol is excreted in the urine as sulfates and glucuronides.

The most prominent synthetic estrogen at the present is diethylstilbestrol.

This substance is highly potent orally as well as by injection. The potency of diethylstilbestrol when injected, lies between that of estradiol and estrone. Other types of synthetic estrogens are hexestrol, and dienestrol, both of which have similar properties to diethylstilbestrol.
Many synthetic estrogens and their estrogenic androgens are not themselves estrogenic but exert their effect after metabolic transformation in the body.

**Administration and Utilization of Estrogens**

The mode of administration of estrogenic substances has lately been of considerable significance.

The most useful vehicle for injection of natural or synthetic estrogens is an oil. "When injected, even large excessive doses of most estrogens are rapidly absorbed and little prolongation of action is attainable by increasing the amount injected.

Estrogens are readily absorbed in the intestines but when ingested, they exert relatively little estrogenic activity, owing to oxidation and conjugation in the liver. The liver can reversibly oxidize estradiol to estrone and to other products and it secretes estrone into the bile.

The rate of uptake of an injected hormone substance is a vital characteristic in its assay. Estrogens soluble enough in water or saline to be injected in such are even more rapidly lost from the site of injection and excreted or destroyed.

The characteristics of the hormonal substance itself offset the rate of utilization. According to Meischer (1) the longer the aliphatic chain the more greatly prolonged was the effect.

The minimum dose required to produce an estrogenic effect rises with the length of the aliphatic chain or chains, as shown by Parkes (2). The mechanism by which esterification exerts its characteristic effects is by delaying absorption from the site of injection.

When an injection technique is used, the potencies of the natural estrogens most closely approach one another if absorption is slow, but not too slow. This occurs with hydrolyzed extracts when multiple injections of an oil solution are given or by the addition of palmitic or other fatty acids.

Substances similar to these fatty acids are present in some extracts and may so equalize the potencies of the natural estrogens that useful and reasonably consistent results may be obtained by a two injection technique.

**Estrogen Assay Procedures**

As previously stated, estrogen causes cornification in the vaginal cells. This principle is used in the Allen-Daisey Vaginal Smear Assay

**Dosage:** Solutions made up so that .05 to .2 ml. is injected each time.

**No. of injections:** For routine assays - 2 injections are employed.

**Smear:** Smears are then taken 3rd and 4th day. Three smears are taken each time. Test is considered
positive if any of 3 smears is positive. All
smears are taken from dorsal vaginal wall.

Staining: Smears are then stained with methylene blue.

**Interpretation and Discussion of Data**

Dosage expressed as logarithm should be plotted against per
cent positive slides.

Sample data tabulation and graph is shown below.

<table>
<thead>
<tr>
<th>Group</th>
<th>Number</th>
<th>Dosage</th>
<th>Per cent Positive Response</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td></td>
<td></td>
<td>12 hrs.</td>
</tr>
<tr>
<td>1</td>
<td>10</td>
<td>0.5 I.U.</td>
<td>0</td>
</tr>
<tr>
<td>2</td>
<td>10</td>
<td>1.0 I.U.</td>
<td>14</td>
</tr>
<tr>
<td>3</td>
<td>10</td>
<td>2.0 I.U.</td>
<td>14</td>
</tr>
<tr>
<td>4</td>
<td>10</td>
<td>--</td>
<td>0</td>
</tr>
</tbody>
</table>

24 hour response plotted

Log Total Dosage (expressed in gamma or I.U.)

Probably one of the most accurate and sensitive techniques for
estrogenic assay is that set forth by Rubin-Dorfman (3).

The assay is based on the increase in weight of the immature
mouse uterus and is conducted as follows:
1. Mice are obtained 23 to 25 days of age.

2. Animals are injected subcutaneously once daily for 3 days with material suspended in corn oil. Daily dose was contained .1 cc. oil.

3. Twenty-four hours after injection, uterine weights and body weights were determined.

4. The uterine weight/body weight ratio was determined.

5. Four equal sized groups of animals were injected, two with a standard, two with an unknown.

The concentration of standard and unknown was chosen so that the following relationship held:

\[
\begin{array}{c}
\text{High dose std.} = \text{High dose unknown} \\
\text{Low dose std.} = \text{Low dose unknown}
\end{array}
\]

With this experimental design it is possible to calculate:

1. Relative potency of an unknown preparation.

2. Standard error of potency ratio.

3. Mean slope ( )

4. Significance of difference between respective slopes.

5. Index of precision the smaller the value of , the more the precision.

Most calculations were based on uterine ratio vs. log dose. However, with estrone, calculations are based on the log of uterine ratio vs. log of dosage.

This is necessary when dosages range is wide (.04 to .32 milligrams). In general, it is better not to assay too near the minimum or maximum response levels, because chances of sensitivity threshold variation may cause skewed results.

The mouse uterine weight method has the advantage over vaginal smear methods of estrogen assay in that the results are objective.

**Sensitivity**

The sensitivity of the mouse uterus is such that .4 micrograms of estrin, .02 micrograms of estradiol, 2 micrograms estriol can be detected.

**Intravaginal Pellet Type Estrogen Assay**

An assay technique by Albrieux (3) has been established using a pelleted type hormonal substance.
Blood pellets were made using 10 per cent sesame oil as a "binder". Blood is placed in cellophane tubes and suspended in front of an electric fan at room temperature. After drying, it is mixed with sesame oil and hammered into pellets weighing 40-80 mg.

Separate pellets made from serum or cells can be prepared by centrifuging blood.

Pellets are then inserted into the vagina with a trocar and are completely dissolved in 24 hours. This technique, while not widely used, offers possibilities for certain phases of assaying where the nature of the unknown might make it difficult to administer in other ways.

**Progesterone**

*Characteristics of the Hormone*

This hormone is secreted by the corpus luteum and has been isolated only from this gland. It is the only known, naturally occurring compound, with full progestational activity. It can be produced in crystalline form. It has been produced synthetically from the vegetable sterol stigmasteryl. It is excreted as pregnandiol.

[Chemical structures of progesterone and pregnandiol钠糖酸酯]

Progesterone, Pregnandiol Sodium Glucuronidate. Pregnandiol has no known hormonal action. However, it is an anesthetic. Changes in excretion of pregnandiol follow closely changes in the production of progesterone. However, pregnandiol and the excretory form of other related compounds such as desoxycorticosterone. Thus, the detection of this material in the urine cannot be taken as an accurate measure of the amount of progesterone secreted.

There are synthetic substances which are of minor practical significance.

*Ethinyl testosterone* is the most potent synthetic substance. (1/10 the activity of progesterone). It is as active by mouth as by injection, but is weak both estrogenically and androgenically. *Ethinyl androstenediol* has about one-half the activity of ethinyl testosterone.

Since progesterone is not effective to any extent when given orally, it cannot be incorporated into feeds for laboratory animals. Thus, the assay of animal tissues for residual progesterone is quite difficult. In addition to the problem of administration, we do not have such relatively simple response mechanisms as uterine weights and vaginal smears in
assaying progesterone. Physiologically, progesterone is responsible for the
development of the estrogen primed uterus and the maintenance of pregnancy.
Within a few days after fertilization, or on artificial administration of
progesterone, there is a rapid increase in cell division of the surface
epithelial cells. This activity is accompanied by a downgrowth of glands
and an increase in stromal cell proliferation.

A very sensitive assay method for progesterone has been developed
by Hooker (4). This technique is based on the principle that the endometrium
of the mouse exhibits hypertrophy of the stromal nuclei when stimulated by
progesterone. This technique is currently being used on samples of meat from
implanted steers on test at Michigan State.

Procedure:

The hormone is dissolved in sesame oil. The standard amount ad-
ministered was .0006 ml. Undesirable distension was produced if
more was injected. In order to administer such small amounts, a
micrometer syringe technique must be established.

Test animals used were adult female mice which had been ovarecto-
mized 16 days before test. The technique of administration is
quite detailed. The animals are anesthetized, and then the mid-
ventral section opened to expose the uterine horn.

The test substances are carefully injected into the uterus with
the section being tied off to prevent leakage of the material.
The animals are then killed 48 hours after injection. The uterine
segment is removed and fixed in Lavdowsky's fluid. Paraffin
sections 6 microns in thickness are stained with Harris'
hematoxylin and Eosin.

A positive test is described as follows: Stromal nuclei are
enlarged and appear smooth and oval in outline as compared to
shrunken nuclei and clumped chromatin granules, in non-treated
cells. This appearance in anyone of the stromal cells is
considered positive.

The authors feel that the response obtained is specific for
progesterone since 6 micrograms of desoxycorticosterone, 48 micrograms of
testosterone, .00075 micrograms of estradiol or .6 ug. of estrone did not
produce similar results, although there was some enlargement of nuclei.

Sensitivity:

The minimal effective dose (least amount which induces a positive
response in any stromal nuclei) is said to be consistently .0002 micrograms
of progesterone.

This method could be adapted to a quantal assay in which 100 per
cent positive responses could be expected with minimal effective dose.
Progesterone - Chemical Assay:

Edgar (5) has developed a method by which micro-quantities of progesterone can be estimated in blood from the ovarian vein of sheep and from follicular fluid of Graafian follicles and ovarian cysts. The method involves extraction and partition between organic solvents and the semi-quantitative estimation by chromatographic separation on filter paper. Amounts varying from 1.5 to 0.3 micrograms per ml. of fluid could be detected.

The best method according to Emmens is that of Venning (6). By this technique, butanol extracts the sodium pregnanadiol glucuronidate from the urine. After careful separation and filtration the Na P.G. residue is dissolved in hot alcohol and dessicated. The amount present is determined by weighing.

Testosterone:

This is a male sex hormone classified as an androgen. Testosterone is the most potent androgen, being six times more potent than androstosterone. Androgens affect the following biological activities:

1. Metabolism
2. muscle mass
3. electrolyte metabolism
4. enzyme concentrations of various muscles and glands.

Androgens of course, cause the development of secondary sex characteristics in males. There is evidence of some similarity in effects of androgens and female sex hormones.

Transandrostenediol, dehydroisoandrostenediol, androstenedione, and testosterone produce uterus growth in the immature rabbit. Transandrostenediol produces stratification and cornification of the vaginal epithelium in the castrate rat.

Most of the androgen is derived from the testes. However, it is possible that these are androgenic substances secreted by the adrenals.

Administration:

Androgens are ineffective when administered orally. Thus, making an assay of their potency or presence in carcasses more difficult. When crystalline androgen is implanted subcutaneously, slow absorption and prolonged effects are obtained.

Assay Methods:

There are a number of various assay techniques for androgens. The most successful are those using:

2. Seminar vesicle and prostate response in the rodent.
Capon Comb Growth

Gallagher and Koch (7) developed a method using as a response tool the comb growth of capons. Five daily injections of testosterone unknown in 1 ml. oil are used in a group of capons, while injections of testosterone standard in 1 ml. oil are made in another group of capons. From this a characteristic curve was developed, which used a dose-response relationship.

Bliss (8) elaborated on this technique by running live concentrations of unknown and two concentrations of standard and then determining potency ratio. The total concentration of testosterone propionate used for each animal should be in the range of 20 to 160 micrograms. At least 32 animals should be used on standard and unknown. A linear response is obtained when log dose plotted against log response.

Emmens (9) applied testosterone directly to combs. Obtained straight line for log dose response.

Chick Comb Method

Dorfman (10) injected chicks with hormone suspended in oil. Obtained a straight line log dose - response relationship.

Seminal Vesicle - Prostate Response Method

Principle: - Testosterone will repair the damages to seminal vesicles and prostate caused by castration.

Procedure:

Testosterone standard and unknown are dissolved in corn oil of which .1 to .2 ml. are injected daily (each animal receiving equal amounts of oil.)

Total dosage with testosterone should fall in range of 0-5 mg. Androsterone has been administered up to 30 mg. Variation in length of treatment ranges from 8-10 days. Rats are killed, body weights taken and seminal vesicles and prostate removed.

The seminal vesicles are then weighed (with prostate off). The log of the dose is then plotted against the gland weights.

Thyroxin

This hormone secreted by the thyroid gland is of vital importance in regulating the rate of energy exchange in the body. It can be observed by its effect on basal metabolic rate and in oxygen uptake of tissue slices.

Among the physiological characteristics with which thyroxin is associated are:
1. amount of adipose tissue
2. rate of glucogenesis
3. rate of excretion of urinary nitrogen
4. rate of intestinal absorption of oxygen and intestinal peristalsis.
5. respiratory quotient
6. levels of blood lipids
7. store of body proteins
8. storage of liver glycogen
9. level of blood sugar
10. rate of secretion of urinary H₂O
11. rate of excretion of urinary calcium and phosphorus.

On the endocrine system, thyroxin affects:
1. secretion of thyrotropic hormone from the anterior pituitary
2. secretion by adrenal cortex by ACTH stimulation
3. conditioning of reactive parts of body epinephrine
4. appears necessary for adequate secretion of prolactin and consequent milk secretion.

There are a number of assay methods available which apply some of the above characteristics in response characteristics.

Goiter Prevention Method

Principle - when a goitrogenic substance (thiouracil) which inhibits thyroxin production is given, we get increased thyrotropic hormone secretion and resultant hypertrophy of the thyroid.

This thyroid hypertrophy can be prevented by administering thyroxin to restore normal balance.

Procedure:

100-200 gram male rats are grouped (8-10). Five groups are usually sufficient to establish a response curve. All groups are given .1 per cent thiouracil in feed during treatment period. (Dosage should be given to fall in middle of response curve if possible.) All but one group of thiouracil treated rats are then injected with 1-4 micrograms/100 g. body weight of thyroxin.
After 14 days, thyroids are removed and weighed. Response is reported as micrograms thyroxin/100 grams body weight. An illustration of graphic presentation is as follows:

![Graph showing the relationship between thyroid weight and body weight.](image)

Note: when curve intercepts normal weight line - can be taken as measure of thyroid secretion rate since it represents the amount of DL-thyroxin required to maintain normal thyroid pituitary balance.

**Metabolic Rate Method**

Dressler and Halling (11) reported a method of assaying thyroxin by its effect on the metabolic rate of guinea pigs.

**Procedure:**

Feed and water are withheld from guinea pigs for 18 hours before determination. Oxygen consumption and CO₂ output is then determined with metabolism being expressed as ml. O₂ or CO₂ exchanged per 100 g. body weight per hour. These control readings are taken on 3 separate days.

Thyroid preparation is administered orally for 4 consecutive days. One hundred hours after the first dosage B.M.R. determination is made and percent increase noted for each animal over control value.

A reference curve is estimated by graded dosage with standard thyroid powder. The potency of the test preparation is then computed in terms of the standard.

The thyroid equivalent can be established by reference to standard slopes.

The percent increase in oxygen consumption is substituted for $\gamma$ in the equation. Then the equation is solved for $X$ - to get logarithm of thyroxin dosage that must be given orally to give the same response.
In the use of this type of assay, the reference curve should be established for a standard substance similar to those being tested.

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12. Emmens, "Hormone Assay".
MR. AUWAN: We sure enjoyed the topic that you presented, and certainly when you came to some of the chemical compounds it was more enjoyable.

At this time we will ask H. D. Naumann to lead the discussion.

MR. NAUMANN: Thank you, Mr. Chairman.

The recent popularity of the use of hormones has caused quite a good deal of concern in the place I am working. The physiologists and the endocrinologists are pretty much up in the air about it because from the standpoint of public health it could be a real problem if something got out of control. They are particularly questioning in their own minds the validity of many of the assay techniques that are being used today from the standpoint that a lot of them are biological assays and as a result they are used on test animals and there isn't definite proof that the response of the test animal is analogous to the response of the human with whom we are ultimately concerned. The other question is perhaps just as important in that in many of these products the hormones specifically seem to have a cumulative effect or at least that is a strong possibility. So the reviews of hormone assay methods in my opinion are pertinent at the moment.

Do you have any questions to direct to the speaker?

MR. KASTELIC: I should like to make a comment about the point of the amount of a substance, whatever it may be, that may be present in tissue. I have seen reference in several reports, as you all have recently, concerning how little there may be of a given substance in meat. I think we should make it absolutely clear that the quantitative amount is not the most important thing. The most important thing is what amount will produce physiological effects.

I should like to drive home this point by pointing out that it matters little if it takes a fraction of a gram of a biologic substance to kill a person and it takes several pounds of sodium chloride. If the end result is fatal, it matters not how much it took. That is one point I should like to put across.

I think, too, there has been a dangerous point in the conclusions drawn in some of these experiments. That concerns the effect that one observes when he deals with a short-term experiment or an acute toxicity test. Personally I am far more concerned about chronic effects than I am about acute, because if we can produce effects immediately that are symptomatically obvious, we are warned, but there are instances where it may take years, indeed, before the results of the ingestion or the use of some material might come about and be recognizable as such. It is in this latter category that I think we should express the most concern; yet I hear people with practically no temerity whatsoever saying, as the result of their assays, that there are not likely to be any effects. I think that is naive, and I am glad that in the remarks Mr. Deans made he brought forward some of these things.
Another thing that I think we should be concerned about (this is my third point) is that if by one criterion you produce no evidence of effect that does not preclude the possibility that there are no effects. In the case of the stilbestrol assay the uterine weight of the mouse is frequently used to establish whether or not the material may be present. That is one thing, but the animal organism is extraordinarily complex and there may be other effects.

I am not an alarmist. I want to make that clear, but let's be a little thoughtful about some of these assays before we write some of the things that have been written.

MR. BULL: Mr. Chairman, is it possible to hook a hot carbon on these estrogens that are fed the animal? If so, has it ever been done?

MR. BUTLER: The question?

MR. NAUMANN: Can you use a tracer technic on these hormones? Who would care to answer that question?

MR. KASTELIC: There are some papers concerned with the tagging of carbon in diethylstilbestrol. The carbon tag in these instances has been the ethyl groups. We also have two-ring structures, and while it has been indicated from such studies that the material is not metabolized I would not be satisfied with such information until the remaining part of the molecule had been tagged and until the degradation products, if any, are carefully mapped out. Tagging one piece of a molecule is not sufficient.

MR. BULL: In connection with Dr. Kastelic's remarks about long-time effects, there is rather prevailing sentiment among medical men that the use of estrogen by the ladies in face creams, bust developers, etc., causes cancer. The same is true about the use of testosterone among you old fellows.

MR. NAUMANN: Do we have other comments or questions?

Well, I want to take the liberty, since Deans has mentioned this in his paper, of directing a question to him, although perhaps it isn't really within the scope of the title. What were the results of these progesterones on meat tissues?

MR. DEANS: On our last bunch we did not get any at all. We have different hormone treatments at this time. The thing we are comparing here is an implant as well. Of course, we have estradiol. We have different hormone dosage. So we don't want to go out on a limb and predict what we are going to get. We didn't get any the last time.

MR. NAUMANN: Thank you.

Any other questions? Then I will turn it back to the Chairman.

MR. AUNAN: At this time we will hear from Dr. Breidenstein of the University of Illinois.

# # # # #