SUMMARY OF SOME LABORATORY METHODS IN ENDOCRINOLOGY

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In the diagnosis and treatment of endocrinological disorders, the clinician has had the assistance of the clinical laboratory for a number of years. For example, disorders of the parathyroid glands involve calcium metabolism, and diabetic conditions alter the level of blood and urinary glucose. All well-equipped hospital laboratories are prepared to investigate these problems. Certain studies, however, involve specialized technics which are not usually available to the physician who is faced with an endocrinological problem. Although these technics are specialized, they are not difficult and any of them can be mastered by the average technician. In this paper we summarize the methods employed at present in the endocrine laboratories of this institution. These laboratories are part of the clinical division of endocrinology which is under the direction of Dr. E. Perry McCullagh. The methods have been adapted or devised in the Research Division of the Cleveland Clinic Foundation. A considerable portion of this work was done in collaboration with Mr. W. Kenneth Cuyler who was particularly interested in studies relating to the endocrinology of the female.

Determination of Androgens in Urine

Several chemical methods[1,2,3] have been described for the determination of androgenic steroids. In our hands, none of these has been completely satisfactory. We therefore continue to use biological methods of assay.

The method is as follows: A 24 hour specimen of urine is collected without preservative. The urine is acidified, using 50 cc. of concentrated hydrochloric acid per liter, and hydrolyzed for 15 minutes by boiling over a free flame. It is then extracted by stirring violently for ½ hour with dibutyl ether, using 200 cc. of solvent per liter of urine. The ether is separated from the urine by decantation and the use of a separatory funnel. There is usually very little emulsification but any emulsion which exists is placed in a separatory funnel along with 15 cc. of a 10 per cent solution of sodium taurocholate. When this is thoroughly mixed, the emulsion breaks down and the solvent is separated carefully from the aqueous phase, being sure that the precipitate of taurocholate does not contaminate the dibutyl ether. A second extraction is done in the same manner and the combined extracts are washed twice with 25 per cent by volume of a saturated solution of sodium bicarbonate, and twice with warm tap water. The dibutyl ether is then evaporated to dryness by steam distillation in partial vacuum.

This is accomplished as is the usual vacuum distillation except that, instead of admitting small quantities of air into the distilling flask, steam is admitted from a source which has an independent vent into the sink. The amount of steam admitted is controlled by a small stopcock or thumbscrew. To assure complete dryness, the steam is shut off for a few minutes at the completion of the distillation. The residue is removed from the flask to a beaker using ethyl ether; 25 cc. of oil are added and the ether removed on a water bath.

The amount of hormone in the oil is determined by a method, the principles of which are based on the work of Gallagher and Koch[4]. One cc. of oil is injected into the pectoral muscles of each of five white leghorn capons daily for five days. On the sixth day the comb is measured and the size compared to the size before treatment. The growth is expressed as the increase in length plus increase in the height of the comb as measured in millimeters. Birds with small combs show less growth than birds with large combs. The average comb length is 57 mm. and the comb growth of all birds is corrected accordingly. The correction factor is 0.17 mm. If the original comb length is less than 57 mm., 0.17 mm. of growth is added for each millimeter of difference between 57 and the original comb length. If the comb is greater than 57 mm., a similar correction is made in the opposite direction.

The amount of androgenic material being excreted is expressed in terms of androsterone. The equation for calculation is $y^2 = .43x - 3$ where y is the growth of the capon's comb in millimeters (length plus height) and x is the amount of androsterone injected per bird per day. External conditions influence the response of the capon's comb to androsterone and if great accuracy is required, a group of control birds should be injected. Normally 0.1 milligram of androsterone (1 international unit) daily will cause a growth of 6.6 mm. If the control group varies from this, the comb growth of the birds used in the assay is corrected accordingly. Further details concerning the response of the capon's comb to androsterone can be found in a paper by McCullagh and Cuyler[5].

If capons are not available it is possible to use the growth of the chick's comb as a method of assay[6]. Statistical studies concerning the use of chicks will be published from this laboratory in the near future.

Normal individuals excrete from 20 to 100 international units daily. Pituitary, testicular, and adrenal pathology may alter this output.

Assay of Androgens in Blood

Androgens may be measured in blood by the method of McCullagh and Osborn[7]. Thirty cc. of oxalated blood are laked with 270 cc.

of water and 15 cc. of concentrated sulfuric acid. This mixture is then hydrolyzed, extracted, and concentrated in the same manner as described for urine and is finally taken up in 5 cc. of sesame oil. The amount of hormone present is too small to permit of assay by the methods described above. It is possible to determine the amount of hormone in the oil by means of inunction methods[5]. Frank, Klempner and Hollander[8] describe a method of assay using the chick. The capon technic is briefly as follows: The comb of each bird is inuncted daily for 5 days with 0.2 cc. of sesame oil and the comb growth measured in the same fashion as when the injection method is employed. The growth response curve can be found in the literature[5]. The lower portion of the curve is almost linear, showing the relationship $x = 0.5\ y$ where y is the growth of the comb in millimeters and x the daily dose per bird of androsterone expressed as micrograms. The limits for normal individuals have not as yet been established but it seems probable that, expressed as androsterone, the level of blood androgens is similar to that of urinary androgens.

Determination of Estrogens in Urine

A 24 hour specimen of urine is acidified, using 5 per cent by volume of concentrated sulfuric acid. It is then hydrolyzed by boiling for 2 hours. The estrogens are extracted and concentrated by the technic described for the extraction of androgens. The extract is finally taken up in 30 cc. of sesame oil. This oil is then assayed by a modification of the method of Gustavson and D'Amour[9]. Spayed mature rats which have been observed for several weeks and are known not to have gone into estrus are used for the assay. One rat unit is that amount of hormone which will bring 50 per cent of the rats into estrus 72 hours following injection. The rat is considered to be in estrus when the predominating cell obtained in a vaginal smear is a non-nucleated cornified epithelial cell.

For most clinical purposes it is not necessary to know exactly how much estrogenic material is being excreted. It is therefore our custom to make a preliminary assay using 2 rats at each of three levels. One pair of rats receives 0.75 cc. of oil each, another pair 1.5 cc., and the third 3.0 cc. From the results obtained the approximate level of estrogenic excretion can be determined. If none or only one of the rats reacts with a positive vaginal smear, it is probable that the patient being studied has ovarian deficiency.

If more accurate results are required, sufficient material is still available to make possible an assay using 10 rats at a level indicated by the preliminary assay. Each of the 10 rats receives the same dose. The following table is taken from the data of Gustavson and

D'Amour and indicates the probable number of rat units which each rat receives as interpreted from the number of rats which came into estrus. If one rat comes into estrus the dose was 0.5 rat unit. If all 10 rats come into estrus the dose was at least 3 units. The corresponding values dependent upon the number of rats coming into estrus are shown in the following table:

Number of Rats Coming Into Estrus	*Number of Rat Units Each Rat Receives*
2	0.73
3	0.83
4	0.92
5	1.00
6	1.1
7	1.2
8	1.4
9	1.8

Obviously, then, the following formula can be used to calculate the amount of hormone an individual is excreting:

$$\frac{\text{No. of units per rat} \times 30}{\text{Dose per rat}} = \text{rat units per 24 hours.}$$

Normal women excrete from 20 to more than 40 rat units in 24 hours.

Chemical methods for the determination of estrogens have been available for some time. Most of these, however, have not been sufficiently sensitive to be satisfactory for the study of normal women or those with hypogonadism. A recent method published by Kober[10] has much merit and may obviate the necessity of the cumbersome and expensive bio-assay procedure.

The Friedman Test

The technic and interpretation of the Friedman[11] modification of the Aschheim-Zondek[12] test as employed in this institution was described in this journal by Mr. W. K. Cuyler[13]. As a test for pregnancy 12 cc. of the urine to be tested is injected into the marginal ear vein of a mature female rabbit which has been isolated for three weeks. Parous rabbits rather than virgins are preferred. Twenty-four hours later the ovaries are examined. The gonadotropic hormone which is found in the urine of pregnant women will bring about the formation of mature follicles, ruptured follicles, hemorrhagic follicles, and fresh corpora lutea. In a high percentage of cases the presence of ruptured or hemorrhagic follicles indicates pregnancy. Several mature follicles are usually seen in the normal ovary of a rabbit.

An excessive amount of gonadotropic hormone may occur in conditions other than pregnancy, i.e., pituitary or chorionic tumors, teratomata of the testes, and hypogonadal states such as occur at the female climacteric or in the male in testicular deficiency. In some instances of this sort there is insufficient hormone to cause a frankly positive Friedman test when the technic outlined previously is employed. If larger quantities of urine are used and the injections continued over a longer period, these slight excesses can be demonstrated in the rabbit. For example, if 4 cc. of urine are injected three times daily on two consecutive days and the rabbit is examined on the third day, positive reactions will be obtained from urines which do not give positive reactions when the simpler technic is employed. Occasionally one encounters urines which are so toxic that the rabbits do not survive the injection. Such urines should be chilled, filtered, and carefully neutralized before injection. The difficulty can sometimes be overcome by administering the urine in numerous small doses and if necessary by precipitating the hormone with alcohol as described in the method for the quantitative assay of prolans. In detecting small quantities of gonadotropic hormone by this method the experienced observer will notice congestion and swelling of the uterine horns which is indicative of ovarian stimulation. When the test is used as a test for pregnancy one should not expect a positive reaction less than three weeks after conception. The output of gonadotropic hormone in the urine of pregnant women increases rapidly subsequent to that time and is invariably quite high in the sixth week. A negative Friedman test is a very reliable indication that an individual is not pregnant. A positive Friedman test indicates an excessive excretion of gonadotropic hormone and must be interpreted in the light of the numerous possible causes.

Quantitative Determinations of Gonadotropic Hormone

In assaying urines for the determination of pituitary or pituitary-like hormones, the method employed depends somewhat on the concentration of the hormone in the urine. When the concentration is very high the urine can be assayed directly. If, however, the content is low, as for example in the normal individual, methods of concentration must be employed. Several such methods have been published[14,15,16,17]. At present we are employing ethyl alcohol as a precipitant. Four volumes of 95 per cent alcohol are added to one volume of urine. This mixture is placed in the refrigerator overnight and the supernatant liquid removed by means of a syphon. The residue is centrifugalized, desiccated, and extracted with water, and the assay is carried out on the aqueous extract. The method of Heller, Lauson and Sevringhaus[18] is a satisfactory method in that the animal supply houses are able to furnish imma-

ture rats on demand and it is therefore unnecessary to maintain an animal colony. This method involves the use of 21 day old female rats. Each rat is injected once on the first day, twice on each of four subsequent days, and is autopsied on the 26th day of life. The uterus and ovaries are removed and weighed after expelling all fluid from the uterus. The extent of increase in weight over the controls is a measure of the amount of gonadotropic hormone present. A 24 hour specimen of urine from a normal young man will usually yield sufficient hormone to cause a marked stimulation in one rat. Men over sixty who are apparently normal have been observed to excrete larger amounts of gonadotropic hormone.

Semen Examinations

Some endocrinological disorders as well as other causes will result in partial or complete aspermia. Moench[19] and Hotchkiss[20] have discussed these problems in considerable detail. The methods employed in this laboratory in the examination of semen are as follows: The patient is requested to collect all the semen produced on the first ejaculation following at least three days of continence. The semen should be collected in a glass container because the chemical substances contained in rubber are injurious to the spermatozoa. The semen should be brought to the laboratory as quickly as possible after collection and should be kept as close to body temperature as possible until examination. The specimen is first measured for volume and for pH. It is then examined grossly for activity, the approximate percentage of spermatozoa immotile, slightly motile, and actively motile being estimated. The time of receiving the specimen is noted and the specimen is stored at room temperature. A microscopic examination is made at once and repeated subsequent observations are then made to ascertain the viability. Live sperms will be found in the semen of normal individuals several hours after collection; viability is frequently maintained for more than 18 hours.

The technic employed in determining the number of sperms is similar to that used in counting blood cells. About 500,000,000 sperms are found in the ejaculate of a normal individual. If the number of sperms in the specimen seems high a red count pipette is employed for dilution and the areas designed for counting white cells are used. One may multiply by 500 to get the number of sperms per cubic millimeter and then by 1000 to get the number per cubic centimeter. If the count is low a white count pipette should be used and the areas designed for white cell counts utilized. One should multiply by 50 to get the number of spermatozoa per cubic millimeter and then by 1000 to get the number per

cubic centimeter. The diluting fluid employed is 1 per cent formaldehyde and 5 per cent sodium bicarbonate.

In studying the morphology of the sperms, a smear is stained by the technic followed by Moench[21]. Slides are air-dried, fixed by heat, and then treated with a 10 per cent solution of chlorazene for ½ to 2 minutes. They are then washed with water and 95 per cent alcohol and stained for 1½ to 5 minutes with a modified Williams stain which is made in the following manner:

Ziehl-Neehaon's carbolfuchsin — 50 parts
Saturated alcoholic sol. bluish eosin — 25 parts
95% alcohol
These materials are mixed and filtered.

After staining, the slide is washed with distilled water and counter stained for 1 to 5 seconds with methylene blue (saturated alcoholic solution of methylene blue diluted to one third of strength with distilled water). It is finally washed with distilled water.

Examination of Vaginal Smears

The epithelium of the vagina gives an indication of the activity of the ovaries. This has been studied by Papanicolaou[22] and Papanicolaou and Shorr[23]. The smear is made from fluid removed from the vagina with a glass pipette, using a heavy rubber bulb to furnish the suction. The tip of the pipette should be placed 5 to 6 cm. within the vaginal orifice. The fluid is spread as a heavy film on the slide and is immediately fixed in equal parts of 95 per cent alcohol and ether. After fixation for at least one half hour it is passed successively through 80 per cent alcohol, 70 per cent alcohol, 50 per cent alcohol, and distilled water. It is then stained for five minutes with Ehrlich's hematoxylin, washed in water for 15 minutes, and stained for 4 minutes with 0.5 per cent eosin. It is washed with water, stained with 0.5 per cent water blue for 5 minutes and again rinsed. The slide is then carried through alcohol water solutions until it is finally placed in absolute alcohol and xylol and mounted in balsam or gum damar.

In most instances in order to determine the exact type of smear in a given individual, repeated examinations must be made. During the cycle the smear changes in a fashion similar to that in lower mammals. In the normal individual during the so-called copulatory period which probably corresponds to the time of ovulation, the predominant cell is a cornified epithelial cell. The test is a valuable aid in determining whether or not a dose of estrogen is adequate for the treatment of a patient whose symptoms are atypical and a poor guide.

Determination of Iodine

The iodine to be measured is converted to iodide by digestion in an alkaline solution and separated from interfering substances by distillation from acid solution. It is then oxidized to iodate, which in turn is used to liberate iodine from an excess of added iodide in acid solution. The iodine is titrated with sodium thiosulfate. In the analysis of tissues or materials containing only a small proportion of organic matter, the alcoholic extraction may be omitted. In foods where it may be necessary to use large samples to obtain the 2 or 3 micrograms necessary for accurate estimation, the fusion may be conducted in several crucibles and the alcoholic extracts combined.

Fusion: 10 cc. of blood (or an amount of material containing about 2 to 3 micrograms of iodine) are boiled with 12 cc. of saturated potassium hydroxide solution in a 300 cc. nickel crucible until foaming ceases. The Bunsen flame must be carefully manipulated at the beginning to control excessive foaming; goggles should be worn as a protection against possible spatter due to careless manipulation. Organic material may be washed down the sides of the crucibles with a little water. The crucible is placed in the muffle furnace at 250° for 30 minutes (or less) to drive off gases. The temperature is then raised to 360° over a period of 30 minutes, and allowed to remain at this temperature another 10 minutes, after which the crucible is removed.

Extraction: Sufficient water is added to the fused mass to form a fluid paste on cooling. This is extracted by thorough stirring with 25 cc. of 95 per cent ethyl alcohol, which is decanted from the sludge into another 300 cc. nickel crucible. The extraction is then repeated four more times with 10 cc. portions of alcohol. To the combined extracts 0.5 cc. of saturated potassium hydroxide is added. The alcohol is evaporated on a steam bath and the crucible placed in the muffle furnace at 385° for 15 minutes.

Distillation: The ash is dissolved in water and transferred quantitatively to a modified 50 cc. Claissen flask which has the auxiliary tubulation sealed, thus eliminating the use of a rubber stopper and reducing the condensing surface. The end of the tube leading from the condenser goes to the bottom of a 50 cc. extraction flask which contains water just sufficient to cover the end of the condenser tube, 0.2 cc. of a 3 per cent solution of sulfuric acid, and 0.2 cc. of a 10 per cent solution of sodium bisulfite. To the contents of the Claissen flask are added quickly 2 cc. of 50 per cent solution of sulfuric acid, 1 drop of a 10 per cent solution of ferric sulfate, and 2 cc. of a 3 per cent solution of hydrogen peroxide (superoxol diluted to make a 3 per cent solution). More acid is added if necessary to make the solution definitely acid, as

indicated by the presence or absence of ferric hydroxide. A glass bead is added to prevent bumping. The flask is immediately closed with the stopper and dropping funnel, the outlet of which has been drawn to a point. The contents of the flask are then boiled vigorously over a microburner, care being taken, however, to avoid flooding of the side arm of the Claissen flask. One or two additional 2 cc. portions of 3 per cent hydrogen peroxide are added through the dropping funnel during the distillation. Heating is discontinued during these additions to avoid carrying hydrogen peroxide over with the distillate. The distillation is discontinued when the volume in the Claissen flask is reduced to about 5 cc. or when sulfates begin to crystallize on the side of the flask.

The extraction flask is placed on a wire gauze with asbestos center and the contents are boiled gently for 2 minutes to expel carbon dioxide and sulfur dioxide, a glass bead being used to prevent bumping. The solution is immediately made alkaline to litmus paper with 10 per cent potassium hydroxide; this should not require more than 3 drops. The solution is then carefully boiled down to a volume of 5 or 6 cc.; 1 drop of methyl orange is added, and the solution is neutralized by the addition of 3 per cent sulfuric acid. Two drops of acid in excess are then added along with 5 drops of bromine water which should cause the solution upon shaking to turn yellow immediately. It is then boiled down very cautiously to about 2 cc. and cooled on ice. This should require from 3 to 5 minutes in order completely to remove excess bromine with a minimum loss of iodine.

Titration: One drop of a 1 per cent solution of starch and 2 drops of a 1 per cent solution of potassium iodide are added and titration is carried out with a 0.001 normal solution of sodium thiosulfate which is delivered from a microburette or a 0.2 cc. pipette graduated to 0.001 cc. A blank (usually 0 and never more than 0.02 cc.) should be run on all reagents.

Calculation: One cc. of 0.001 normal sodium bisulfite is equivalent to 21.2 (micrograms) of iodine. When 10 cc. of blood are used as described:

(Titration - Blank) x 212 = micrograms iodine per 100 cc. blood.

Interpretation: Normal blood contains usually from 7 to 14 micrograms of iodine per 100 cc., of which one half or less is alcohol insoluble. This fraction is increased in hyperthyroidism and may represent active thyroid substance. Eighteen per cent or less of the total iodine in normal blood is probably in an inorganic form. This is increased by the administration of iodine. In cretinism the total amount of iodine decreases. In hyperthyroidism it is usually increased.

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