CLXI. THE GONADOTROPIC HORMONES (ρ -FACTORS).

III. FURTHER PURIFICATION AND PROPERTIES OF THE ACTIVE PRINCIPLES.

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SINCE the original observation by Zondek [1930] that the gonadotropic substances of pregnancy urine are precipitable by alcohol in sufficient concentration, numerous processes, based on this and other observations, have been devised by other workers with the object of obtaining a highly active substance in such a condition that it could be stored without loss of potency. Dickens [1930] made preparations by the alcohol precipitation process, and also by methods based on precipitation with ammonium sulphate or tannic acid. He obtained a product which, on many occasions, had a luteinising action in the immature mouse in doses of 0.015 mg., and in one instance only of 0.006 mg. Wiesner and Marshall [1931] by considerable modification of the alcohol precipitation process also obtained a product of comparable activity on many occasions, and in one instance by a process based on the precipitation of the active factors with phosphotungstic acid.

Fischer and Ertel [1931], in a comprehensive communication, describe various attempts at purification of commercial samples of the gonadotropic hormones which contained 4-5 m.u. per mg.; they obtained products containing, in some cases, as much as 130 m.u. per mg. by removal of phosphates with baryta or barium acetate, a method which was previously used by Wiesner and Marshall [1931], who, however, removed the barium more completely by the use of sulphuric acid. Some measure of purification was also obtained by Fischer and Ertel by adsorption of the active principles on kaolin or bolus alba at p_H 4.5 and elution with dilute ammonia. Electrodialysis at temperatures $< 10^\circ$ of a product containing 120 m.u. per mg. failed to effect further purification and even caused a destruction of active substances.

Marshall [1931] has shown that solutions of ρ -factor preparations can be selectively filtered through a series of "membran" and "ultrafein" filters supplied by the Membranfilter Gesellschaft, the coarser grades passing the active factors completely and the finer only to a small extent, if at all. In

the course of this work it was observed that even in those cases where the ρ -factors were almost quantitatively retained by the filter, the filtrate was quite strongly coloured. This observation suggested the possibility of removing a large part of the coloured organic impurities in this way, such impurities being, incidentally, the cause of the original hygroscopic nature of the products. The filters which seemed likely to be of use in this connection were "ultrafein mittel," "ultrafein fein," and "ultrafein Benzopurpurindicht." The former two were actually used in the experiments to be described.

The possibility of further purification *via* the phosphotungstates was also investigated but the results obtained were far inferior to those from the filtration technique.

Attempts at purification by combination with permutit in acid solution, with subsequent extraction with dilute ammonia were entirely unsuccessful.

Animal standardisation.

In order to determine the potency of different preparations, various dilutions were made in 0.85 % saline and injected in convenient amounts into several series of 5 mice each. The total volume injected into each animal was 0.25–0.50 cc. and this was divided into 5 doses given over a period of $2\frac{1}{2}$ days. With the highly active products the solution injected contained 0.01 mg. of solid substance per cc. After 2–3 days following the last injection the animals were killed, a positive result being regarded as one in which at least two animals of a series showed an ovarian response (*i.e.* corpora lutea or atretica). In some cases, as will be seen later, cornification of the vaginal epithelium was observed with a dose less than that required to produce an ovarian effect.

It should be borne in mind that accurate standardisation is out of the question where so many different factors are involved. This is particularly the case when one considers that, on present conceptions, there are two factors responsible for the effects upon the ovary, and that these factors are antagonistic to an extent which we are as yet unable to measure quantitatively. At the present time, although separation of ρ_1 and ρ_2 cannot be effected, we have no certainty that the relative proportions of the two are constant in different preparations. Although some such approximate standardisation is necessary in the absence of a more satisfactory method, its limitations must be recognised and an endeavour made to standardise at least the factors which can be controlled, such as the number of injections into which a dose is divided and which have been shown to exercise such a great effect in the "standardisation" of oestrin (α).

EXPERIMENTAL.

Experiments with permutit. A solution of 1.0 g. ρ -factors (20 units per mg.) in 10 cc. water was acidified to p_H 4.5 with sulphosalicylic acid, shaken vigorously at intervals with permutit and allowed to stand for half an hour. The permutit was removed by centrifugation, washed with water acidified with

sulphosalicylic acid, and extracted with 10 cc. of 1 % ammonia (sp. gr. 0.880, 1 : 100) by shaking. After centrifugation the supernatant fluid was neutralised with 10 % acetic acid and 4 volumes of absolute alcohol and 1 volume of ether were added. The precipitated product was washed with alcohol, then with ether, and dried. A dose of 0.025 mg. failed to produce any ovarian response in the immature mouse. No attempt was made to determine whether the product was active in larger doses, since the sole object of the experiment was to investigate the possibility of utilising the method as a basis for further purification.

Experiments with phosphotungstic acid. A solution of 1.0 g. ρ -factors (20 units per mg.) in 9 cc. of water and 1 cc. of 10 % sulphuric acid was centrifuged to remove a small amount of insoluble matter, and fractional precipitation resorted to. Fraction *a* was produced by addition of 2 cc. of 20 % phosphotungstic acid; fraction *b* by addition of 3 cc. of phosphotungstic acid to the supernatant fluid from *a*; fraction *c* by further addition of 5 cc. of the acid to the supernatant fluid from *b*. All three fractions were washed with absolute alcohol and dried. They were then suspended in water; solution was effected by cautious addition of 10 % ammonia (sp. gr. 0.880; 1 : 10), and phosphotungstic acid removed with barium hydroxide. The excess of barium was quantitatively removed with sulphuric acid and the active ρ -factors were precipitated by addition of 4–5 volumes of alcohol. Washing and dehydration were effected by means of alcohol and ether as previously described. Fractions *a* and *b*, tested in immature mice, were found to contain 40 units per mg. Fraction *c* was almost inactive.

Filtration experiments. The products used in the filtration experiments were all entirely free from protein and usually had a potency of about 20 units per mg.; they were prepared by the alcohol precipitation method, involving the use of barium hydroxide, previously described by Wiesner and Marshall [1931] for the preparation of their *E* 198. The following is a résumé of the technique which has been employed in some dozen instances. A solution of 2 g. of ρ -factors in 100 cc. of distilled water (adjusted to p_H 7.2) was filtered at the water-pump on two filters ("ultrafein mittel" or "fein"), the effective filtering area of which was about 7 cm. in diameter. When the volume of residual liquid on each filter was reduced to 5–10 cc., distilled water was added and the filtration allowed to proceed until the residual volume was not more than 5 cc. This washing process was repeated three times, by which time the filtrate was colourless. The residual liquid was thoroughly washed off the surface of the filters and the solution centrifuged until quite clear. Addition of 5 volumes of absolute alcohol gave merely an opalescent solution, but the purified ρ -factors were precipitated by the further addition of an equal volume of ether. The operation up to this point took about 7–8 hours. The precipitate was removed by centrifugation, washed with alcohol and ether, and dried. The yield was usually 0.15–0.2 g. The potency of the product, in every case in which filtration was as complete as described above, was 200 units

(luteinising) per mg. It is interesting to note that in two experiments 60 % of the animals receiving 0.0025 mg. of active substance showed complete cornification of the vaginal epithelium, the vaginae being well open in all cases 3 days after the cessation of injections. This means that the oestrogenic potency of these samples is 400 units per mg. and it also confirms Wiesner and Marshall's findings with regard to the differential effects of small and large doses with cruder preparations, although in this series of experiments, which deals with liminal doses as regards luteinising activity, the vaginae were open without exhibiting cornification in the larger doses. In the course of this work it has been noticed that the filtrates frequently possessed a certain degree of activity. This may be due to two causes, (1) the relatively concentrated solutions used for filtration (400 units per cc.) whereby activity in the filtrate was more readily observed in the doses given, and (2) the slightly increased permeability of the membranes after continued use. A product was obtained from these filtrates which was compared for its nitrogen content with the highly active substance remaining on the filter.

Chemical investigation of the products obtained.

Nitrogen content. Determinations of nitrogen were made on a considerable number of specimens using a micro-Kjeldahl method. Merck's perhydrol (0.5 cc.) was used to assist the oxidation as it was found to be very effective for this purpose, whilst containing only minute traces of nitrogen. Table I gives the values obtained with the original product, the product from the filtrate and the highly active residue on the filter.

Table I.

Substance	N content %	Activity (units per mg.)
Original product (102)	5.2 5.3	ca. 20
Filtrate (102 b)	4.6 4.6	> 2
Filtrate (102 e)	4.4 4.4	< 2
Active residue (102 f)	8.4	200
Active residue (102 a)	8.3 8.5	200
Active residue (102 d)	7.9 8.1	145

The figures given are consistent with the hypothesis that the active substance is a N-containing compound, the highest percentage being found in the most active product, and the lowest in the relatively inactive product from the filtrate. Specimen 102 d was submitted to less efficient washing in the process of filtration and has therefore a somewhat lower activity than 102 a and f. Many other specimens with an activity of 200 units per mg. gave similar results. No allowance has been made in the above figures for ash content of the specimens. No. 102 f had an ash content of 7.16 %, the residue

containing no calcium or barium and only a minute trace of iron; the N content on an ash-free basis is therefore 9.09 %. Fischer and Ertel [1931] give N values of 10.0–11.10 % on an ash-free basis for a product with an activity of 120 units per mg.

Qualitative tests (on the highly active product).

- (1) No halogens, phosphorus, or sulphur are present.
- (2) No pentoses (phloroglucinol and Bial's tests) are present.
- (3) Millon's test is definitely positive.
- (4) Molisch's test gives a colour which is much redder than that given by glucose.
- (5) Phosphotungstic acid, in presence of sulphuric acid, gives a flocculent white precipitate which is biologically definitely active.
- (6) The glyoxylic acid reaction is very doubtful.
- (7) Addition of 5 volumes of alcohol alone to a relatively concentrated solution (2000 m.u. per cc.) does not precipitate the active factors. Further addition of ether or a small volume of saturated sodium chloride solution is required; in actual practice ether was always used.
- (8) The ninhydrin reaction for α -amino-acids is negative alone, but following brief boiling with dilute sulphuric acid and subsequent neutralisation the reaction is obtained.
- (9) Knoop's bromine test for histidine is negative.

Tyrosine and tryptophan content. An approximate tyrosine and tryptophan determination was made on the highly active product by Folin and Marenzi's method [1929]; results: tyrosine, 1.25 %; tryptophan, 0.6 %. Owing to the small amount of material which remained after the completion of other experiments, it was not possible to repeat these analyses with extraction of the alkaline hydrolysates with toluene, which procedure was found by Macfarlane and Fulmer [1930] to be necessary for obtaining accurate tryptophan values with certain proteins. The figure recorded above is, therefore, almost certainly too high, and the rather doubtful qualitative glyoxylic acid reaction obtained is explained.

Attempt to demonstrate the presence of carbohydrate. A partially purified specimen (20 units per mg.) was first used and the following technique adopted [cf. Osborne and Campbell, 1900]. 1 g. of the product (20,000 m.u.) was boiled under reflux with 20 cc. of 10 % sulphuric acid. After hydrolysis the sulphuric acid was removed quantitatively with barium hydroxide and the precipitate of barium sulphate and humin centrifuged off. The precipitate was washed once with 10 cc. of distilled water. The clear supernatant fluid (p_H 7.0–7.5) was evaporated to 10 cc. and 1.0 g. of sodium acetate and 0.5 g. of phenylhydrazine hydrochloride were added. The hot solution was immediately filtered from some tarry material and then further heated at 100° for 1 hour and allowed to cool overnight. The precipitate was collected and washed with water and a little 95 % alcohol. To the solution in boiling alcohol

1–2 parts of hot water were added and the solution was allowed to cool slowly. The yellow-brown product which separated had the typical crystalline form of glucosazone. After a further crystallisation the product had M.P. 183–185°; yield 10 mg.

A similar technique using 0.4 g. (80,000 m.u.) of the highly purified product failed to give any trace of osazone. Fischer and Ertel [1931] were also unable to obtain an osazone from a product with an activity of 100–120 m.u. per mg.

Attempt at crystallisation. 2 cc. of saturated ammonium sulphate were added to 0.1 g. of highly active ρ -factors in 1 cc. of water (p_H 7.0). After standing for 2 days at 0° some amorphous product had separated but no crystalline material could be detected.

Biological experiments.

In addition to the standardisation of preparations already referred to, a number of preparations were tested for their efficacy in inducing ovulation in the rabbit. With samples of which 1 m.u. = 0.005 mg. it was found that 0.1 mg. would produce rupture of one or more follicles with probable ovulation although ova were not isolated. It is proposed later to determine whether any relationship exists between the mouse unit (luteinising) and the rabbit unit (ovulation) with a view to obtaining evidence of the identity, or otherwise, of the factors. This point is of particular interest in view of Leonard's [1931] suggested differentiation.

DISCUSSION.

The filtration technique described above has been found extremely useful in the preparation of small quantities of a highly active, almost salt-free product. The use of the ultrafiltration method also enabled the author to remove some coloured resinous impurities which could not be removed by ordinary dialysis through collodion or cellophane membranes. As a result of this work it has been made clear that the precipitability of ρ -factors from protein-free solutions by alcohol is dependent on at least a small inorganic salt content, since sodium chloride can replace ether as a precipitating agent for the opalescent solutions obtained by the addition of alcohol alone. The fact that the ninhydrin reaction was only obtained after a brief hydrolysis with mineral acid is important as support for the view that the product is polypeptide in nature. When, in previous work, a qualitatively very definite positive Millon's reaction was obtained, it seemed possible that tyrosine formed an intrinsic part of the active molecule, as was also suggested by Fischer and Ertel. The roughly quantitative determinations reported here would seem to cast some doubt on this hypothesis, although the presence of a polypeptide has been more or less definitely established. Further, that such polypeptide, if it may be assumed to be the active principle (or principles), does not possess its activity merely by virtue of the presence of the fundamental —CO—NH— linkages which it contains, is obvious from the finding that a solution of

purified ρ -factors loses its entire activity on heating at 100° for half an hour, whereas such heating does not decompose the polypeptide sufficiently to give the ninhydrin reaction. If, therefore, our assumption that the active principles are polypeptide in nature is correct, we must of necessity postulate some other rearrangement of the molecule or splitting off of some side-chain in a key position without effect on the polypeptide linkage. The only other alternative would be that the active principles of unknown nature were adsorbed on the polypeptide.

It seems hardly likely that any polysaccharide forms an essential part of the molecule, as suggested by Fischer and Ertel, in view of their own failure to obtain any osazone following either brief or prolonged hydrolysis, but also by reason of the author's demonstration of the presence of a sugar in a partially purified sample (containing 20,000 m.u.) coupled with his inability to do so in a highly purified sample containing 80,000 m.u.

SUMMARY.

1. A further purification of ρ -factor preparations by an ultrafiltration method is described. "Ultrafein mittel" and "fein" filters of the Membranfilter Gesellschaft are used. Specimens containing 200 m.u. per mg. have been obtained.

2. Neither the use of phosphotungstic acid nor that of permutit yielded sufficiently satisfactory results.

3. The presence of a polypeptide in the highly purified product is established by the application of the ninhydrin reaction before and after hydrolysis.

4. The presence of a sugar complex in the active substance is doubtful.

5. Not more than 1.25 % of tyrosine is present in the purified product which also contains about 8.4 % of nitrogen and 7 % of ash. The presence of tryptophan is very doubtful.

6. 0.1 mg. of the product will induce rupture of the follicles with probable ovulation in the rabbit within 24 hours.

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