LXXXIII. THE GONADOTROPIC HORMONES (ρ -FACTORS).

IV. THE PREPARATION OF EXTRACTS FOR CLINICAL USE, TOGETHER WITH OBSERVATIONS ON THE STABILITY IN SOLUTION AND STANDARDISATION PROCESSES.

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IN an earlier communication [Wiesner and Marshall, 1931] methods were described for the preparation of the gonadotropic principles of pregnancy urine in a solid form in which they could be stored indefinitely without loss of activity. One of these, that involving the use of phosphotungstic acid whereby the active principles were precipitated as phosphotungstates, has been shown to be satisfactory only for the preparation of extracts for use in certain laboratory animal experimentation; for this purpose the phosphotungstates were suspended in water and solution effected by the addition of alkali to p_H 7.0–7.5, such solutions being directly injected into mice which appear readily to tolerate relatively large amounts of phosphotungstates.

For clinical purposes, however, the phosphotungstic acid must be completely removed, a process which the author's recent work has proved to be difficult to accomplish satisfactorily (even as the barium salt) owing to the tendency of a part of the barium phosphotungstate to remain in colloidal solution. This process has therefore been abandoned.

The methods described by Dickens [1930] would no doubt be satisfactory for the preparation of clinical extracts, as would the benzoic adsorption method used by Funk and Zeffrow [1932] and Katzman and Doisy [1932], but in the preparation of such extracts considerations of time and cost are of considerable importance if they are to find any extensive use. The method to be described here is really a simplification of an earlier alcohol precipitation process; the solid preparation usually contains 5–10 mouse units (luteinising) per mg. and is of sufficient purity for use in the preparation of clinical extracts containing 50 units per cc., which is the strength used for experiments now in progress. The benzoic acid adsorption method has been repeated according to the details given by Katzman and Doisy [1932] without any attempt being made to explore the conditions for maximum yield; the result was satisfactory, but the method does not appear to the author to offer any definite advantage over that to be described here. An attempt was made to use picric acid in obtaining solid ρ -factor preparations from urine, and one specimen was isolated with a potency of 20 mouse units (luteinising) per mg., a total yield of over 50,000 units being obtained from 15 litres of urine; the specimen was, however, not free from protein, and, on deproteinisation, a considerable loss was sustained.

Various attempts to separate a further quantity of active material from these protein residues have been made, but have not so far yielded results which justify the labour involved, although the problem is being pursued.

In some experiments on the stability of ρ -factors in various solutions some interesting results were obtained, notably where it was shown that no loss of activity occurred in solution in concentrated ammonia (sp. gr. 0.880) at 0° during 24 hours, an observation which formed the basis of one attempt to recover some active material from the protein residues previously mentioned. The stability of ρ -factors at p_H 7.0 in 0.5 % phenol solution at room temperature has also been investigated with a view to determining for how long a clinical extract should be used after receipt. It was also found that a very dilute solution kept frozen for a week showed no loss of activity [*cf.* Dickens, 1930].

Attempts to effect further purification of the active principles by precipitation from protein-free solutions with uranium acetate were unsuccessful owing to the fact that such precipitation was by no means quantitative, although such a method was used by Reiss and Haurowitz [1929] for crude hormone preparations.

In view of Gulland and Macrae's observation [1932] that palladous chloride is an effective precipitating agent for a wide variety of compounds including the purine bases, this reagent was tried; however, whilst the active principles were precipitated from untreated urine, no precipitation occurred when protein-free solutions were used, and the method was of no value for further purification.

The method of Katzman and Doisy was also inapplicable to protein-free solutions, under the conditions described by them and found to be satisfactory for untreated urine.

The potency of all samples used in clinical experiments together with any others used in this work was determined exactly as described by the author in a previous communication [1932]. The relative merits of the different criteria of a "mouse unit" will be dealt with in the discussion of the experimental results.

EXPERIMENTAL.

Preparation of clinical product by alcohol method. Urine, in batches of 10–12 litres adjusted to p_H 7.0, is evaporated under reduced pressure at 40° to 1/10–1/20 of its original volume and treated with an equal volume of industrial spirit (95 %). The resulting precipitate is discarded and the clear filtrate treated with more alcohol to a concentration of 75–80 %; after standing for some time, usually overnight, at 0° the bulk of the supernatant fluid can be decanted, and the precipitate is collected in the centrifuge and washed once with 90 % alcohol. It is suspended in water (60 cc.), and 15 cc. of 20 % sulphosalicylic acid are added together with 70 cc. of 95 % alcohol. The whole is then centrifuged and alcohol added to the clear supernatant fluid to a concentration of 75–80 %. The precipitate which collects after standing for some hours at 0° is removed by centrifuging and washed successively with 95 % alcohol, absolute alcohol and finally ether, after which it is dried in a current of air at room temperature. The material obtained in this way is collected from several batches and dissolved (or suspended) in 10 times its weight of water and submitted to one further alcohol precipitation, any precipitate obtained in 50 % alcohol solution being again discarded; the product, which should be completely soluble in water and protein-free, is then ready for standardisation in the usual way.

Precipitation of ρ -factors by alcohol at varying p_H . This was originally undertaken with the idea of obtaining a more active precipitate at some definite reaction or possibly of effecting a separation of the principles.

50 cc. of a stock solution of ρ -factors was prepared in 0.85 % saline containing 200–250 m.u. per cc. (p_H 5.55). Four groups were taken. (1) Original + 10 % H_2SO_4 : p_H 0.94. (2) Original + 10 % acetic acid: p_H 3.5. (3) Original alone: p_H 5.55. (4) Original + NaOH: p_H 7.16. To each were added 4 volumes of absolute alcohol and the precipitates removed by centrifuging after standing for 1–2 hours at 0°. The supernatant fluid, after neutralisation and evaporation of the alcohol, was injected into immature mice in doses corresponding to 5.0 mg. (100–120 m.u.) of the original solid. Entirely negative results were obtained, showing that quantitative precipitation takes place within the range p_H 0.94–7.16 with 80 % alcohol; the precipitates were strongly active. At the same time it was observed that the precipitate formed at p_H 0.94 was much lighter in colour and entirely devoid of the stickiness which characterised the precipitates obtained at the other p_H values. This is of great value in facilitating manipulation in the final stages, and advantage has been taken of this fact in many of our recent preparations.

15 g. of crude material were suspended in 125 cc. of water (or preferably 0.85 % saline), and 10 % H_2SO_4 was added to p_H 0.9–0.95, about 25 cc. being required by an average sample but this quantity being subject to considerable variation. The precipitate formed on addition of an equal volume of alcohol was removed and the final product isolated as described earlier in the form of a very pale buff powder. There is an additional advantage in that the precipitate settles more readily and the bulk of the supernatant fluid can be decanted in a relatively short time without loss of active material. In this method all p_H values were determined by the use of the quinhydrone electrode.

Effect of ammonia (sp. gr. 0.880) on ρ -factors. A solution of 0.5 g. of ρ -factors (25 units per mg.) in 10 cc. of ammonia (sp. gr. 0.880) was allowed to stand at 0° for 18 hours. A small amount of insoluble matter was then removed in the centrifuge and the solution aerated (with addition of a drop of capryl alcohol) to remove as much ammonia as possible. The further precipitate produced on neutralisation with acetic acid to p_H 6.5 was removed by centrifuging, and the active principles were precipitated as usual by addition of alcohol. The yield was 0.4 g. of material, which was active in a dose of less than 0.05 mg., since 3 animals receiving this dose all showed many blood-points and corpora lutea (atretica). The above treatment therefore does not appear to have injured the active principles to any appreciable extent.

Action of ammonia (sp. gr. 0.880) on protein residues. 5.0 g. of protein residues (from ρ -factor preparations) in 50 cc. of ammonia were kept at 0° for 24 hours with occasional shaking. After addition of 150 cc. of water the mixture was kept cool whilst neutralising to p_H 6.5 with acetic acid. A further 25 cc. of 20 % sulphosalicylic acid was then added to remove unchanged protein, followed by an equal volume of alcohol. After centrifuging, the supernatant fluid was treated with alcohol to 80 % concentration, and the product was isolated as usual. A yield of 0.6 g. was obtained with an approximate luteinising unit of 1.0 mg. The results of the method do not therefore justify the labour involved.

A similar attempt to obtain active material from the protein residue by the use of 50 % alcohol containing 0.5 % of concentrated HCl at 0° was even less successful.

Loss of activity of ρ -factors in neutral solution. A phial containing a sterile solution of ρ -factors made up to contain about 50 m.u. per cc. (as used in clinical experiments) was kept at room temperature for 26 days. After this time doses equivalent to $\frac{1}{2}$ –3 units of the original gave no blood-points or corpora lutea in

the test animals, but the vaginae were open and the uteri enlarged. A similar test after keeping 17 days showed that the solution had lost approximately 50 % of its activity. In one instance it was also observed that a dilute solution lost practically none of its activity when kept frozen for 7–10 days.

Adsorption of ρ -factors from urine by benzoic acid. 4 litres of urine were adjusted to p_H 4–5 with acetic acid and treated with a saturated solution of benzoic acid in acetone exactly as described by Katzman and Doisy [1932]. The precipitate, after dissolution of the contained benzoic acid in acetone, was extracted with 90 cc. of water and centrifuged, the supernatant fluid again being treated with $2\frac{1}{2}$ volumes of acetone. The precipitate was collected as usual after standing overnight at 0° .

Yield = 0.12 g. with an activity of approximately 15 luteinising units per mg., which corresponds to 450 units per litre. It was not quite protein-free.

Attempt to adsorb ρ -factors on benzoic acid from protein-free solutions. 1 g. of ρ -factors (30 units per mg.) was dissolved in 200 cc. of water (p_H 5) and 20 cc. of a saturated solution of benzoic acid in acetone added with stirring. After standing overnight the precipitate was removed and treated as in the case of original urine. The yield was 0.06 g. of material of which 0.05 mg. failed to produce any effect in a group of mice. The filtrate from the benzoic acid precipitate was treated with 2–3 cc. of saturated KCl and 4 volumes of alcohol. A yield of 0.78 g. was obtained of material which had an activity of 40 units per mg. The active principles therefore appear to pass quantitatively into the benzoic acid filtrate under these conditions.

Preparation of clinical extracts. The solid preparations used for this purpose have usually contained about 5 luteinising units per mg., and 0.1 g. has been dissolved for every 10 cc. of solution required. In the first instance the solution is almost invariably acid through the presence of traces of sulphosalicylic acid, which are quite harmless when neutralised; sodium hydroxide is therefore used to bring the solution to p_H 7 or thereabouts (B.D.H. universal indicator), after which 0.5 % of phenol is dissolved in it. It is then filtered through a Seitz bacterial filter into sterilised apparatus, whence it is run into sterile ampoules. A stringent sterility test, using bullock's heart medium, is always carried out by the courtesy of the Royal College of Physicians Laboratory, Edinburgh, before any extract is issued.

Controlled precipitation of ρ -factors by phosphotungstic acid. To 1 litre of urine (filtered, p_H 7.3) were added 50 cc. of 20 % phosphotungstic acid (adjusted to p_H 6.1 with NaOH). No precipitate occurred, but on adding 10 % H_2SO_4 gradually the solution became definitely opalescent at p_H 4.5, and a flocculent precipitate began to settle at p_H 3.5. After standing for 1 hour in the cold the bulk of the supernatant fluid was decanted, the precipitate collected and washed with 1 % phosphotungstic acid at p_H 3.5. The precipitate was suspended in water and adjusted to p_H 7.0–7.5 with sodium hydroxide, any insoluble matter being removed. The total volume was made up to 500 cc., and 0.5 cc. of this gave a very strong positive ovarian reaction when injected in the usual way, i.e. the equivalent of 1 cc. of original urine contained 1 luteinising unit. A further precipitate obtained by continued addition of 10 % H_2SO_4 to the filtrate proved very toxic to the animals, but from the results obtained with the precipitate at p_H 3.5 the precipitation at that reaction must be fairly quantitative. This method was repeated several times with similar results, but in each case the removal of the phosphotungstic acid from the active precipitate was attended with such a large loss that the method was abandoned for the preparation of clinical extracts. It is interesting to note that, although ρ -factors can be

precipitated from protein-free solutions by phosphotungstic acid, precipitation can only be effected at a p_H considerably lower than 3.5, the reaction at which precipitation is almost quantitative from untreated urine.

DISCUSSION.

One of the most striking points which one encounters in the precipitation reactions of the gonadotropic hormones is the difference in the results obtained according as one uses original untreated urine or a product already partially purified. In a previous communication [Marshall, 1932] it was shown that the precipitability by alcohol was dependent to a considerable extent on the inorganic salt content of the solution, and that where this was almost negligible the further addition of ether was required before precipitation could be effected, and Dickens [1930] recorded a case in which he was unable to precipitate the product even by this means, although in this latter instance the solution was probably relatively dilute. In the present paper it has been shown that the p_H of the solution has little effect on the quantitative precipitation of ρ -factors by 80 % alcohol provided that sufficient inorganic salts are present, except in so far as a less gummy precipitate is obtained at more acid reactions. In adsorption experiments with benzoic acid, the work of Katzman and Doisy on untreated urine has been confirmed, whilst no adsorption whatever occurred under similar conditions from protein-free extracts. It therefore appears that the presence of protein and protein-like substances other than the active principles exert profoundly modifying influences on the chemical and physical behaviour of ρ -factors. Whichever of the published methods for obtaining potent gonadotropic extracts be adopted, a very considerable loss occurs during the removal of protein material at some stage of the operations; this is evidently due to powerful adsorption under the conditions used, since alkali is necessary to extract any further quantity of active material, a process which unfortunately also dissolves some of the protein. In the near future it is hoped to make some attempt to obtain increased yields by lessening the waste at present entailed in deproteinisation.

Turning to the question of standardisation of gonadotropic hormones, it is at once evident both that careful standardisation is necessary for extracts used in any clinical experiments, and also that no entirely satisfactory method of accomplishing this has yet been worked out. In the first place it is obvious that a complete standardisation is a matter of considerably greater difficulty than is the case with oestrin standardisation, in which, although one may be dealing, in any given preparation, with a mixture of trihydroxy- and ketohydroxy-oestrin, apparently only one effect need be considered, namely the production of cornification of the vaginal epithelium of the ovariectomised mouse or rat. With the gonadotropic hormones it is necessary to consider the growth and maturation of immature follicles, the production of haemorrhagic follicles, and the formation of corpora lutea (or atretica), together with obvious ovarian hypertrophy and other effects such as vaginal canalisation and cornification of the epithelium. It was shown in Part I of this series [Wiesner and Marshall, 1931] that opening of the vagina in the immature mouse along with cornification might occur with a small dose of a given preparation, whilst with a much larger dose frequently neither of these effects occurred, although in the latter case ovarian hypertrophy was many times greater than in the former. This observation was then given as further support for the existence of two distinct factors, a hypothesis advanced by several workers, although it was recognised that this

fact by itself could prove nothing since the same substance may have opposite effects in small and large doses. The extent of the antagonism of these two factors is still unknown and cannot possibly be elucidated until final proof is forthcoming of their separate existence. A further complicating factor is the observation by the author, in the course of many hundreds of experiments, that the number and size of blood-points is many times greater with the larger doses, and that corpora atretica may occur without the formation of any blood-points. The evidence is, as yet, not sufficiently correlated to warrant any suggestion of the existence of a third factor, which, however, is not impossible, although it has previously been assumed that ρ_1 (Prolan A) is responsible both for maturation of the follicles and for the formation of blood-points. Although Zondek [1930] has obtained a product from the urine of patients with genital carcinoma which apparently only gives an oestrogenic effect, and Claus [1931] reported the separation of the oestrogenic and luteinising factors from dried pituitary preparations, the existence of more than one factor is still without final proof since the latter work has not been repeated and no claim of a separation of the two from products of pregnancy urine has yet been made. It is necessary to direct further work to this very important aspect of the problem.

Katzman and Doisy [1932] have stated that they "believe that it is correct to use the characteristic reaction, *i.e.* stimulation of follicle ripening, as a measure of potency and that this reaction lends itself more easily to a quantitative determination." The author believes that this view is quite unjustified in the present state of our knowledge and particularly in relation to the clinical experiments now being carried out in Edinburgh. Only if one were dealing with a single factor could this statement be literally true. Until the quantitative antagonism of ρ_1 and ρ_2 has been worked out, we can have no absolutely accurate knowledge of the relationship between an "oestrogenic" and a "luteinising" unit, in fact it is highly probable that the ratio of the two is variable for different specimens.

For the purposes of approximate standardisation of the clinical extracts used it was considered better to determine the "luteinising" unit, since it was this effect which it was desired to produce in the treatment of dysmenorrhoea and certain other disorders. The actual procedure has been referred to previously.

The discovery that no loss of activity occurs in solution in ammonia (sp. gr. 0.880) in 24 hours at 0° is interesting as indicating a greater stability under these conditions than one might have been led to suppose.

It will be noticed that after removal of proteins the active principles are left for 18 hours at 0° in 75–80 % alcoholic solution containing approximately 1 % of sulphosalicylic acid. Dickens [1930] has suggested that the loss of activity which he observed in solutions kept at 0° for a month or thereabouts was due to the slight acidity of the solution; this may very well be the case although the author is of the opinion that, at least at room temperature, such inactivation would proceed slowly even at neutrality. Although the methods described have yielded satisfactory extracts for the purpose desired, it is proposed, and it seems desirable, to determine whether appreciable inactivation occurs by the use of sulphosalicylic acid under the conditions indicated above.

SUMMARY.

1. Details are given for obtaining a gonadotropic hormone preparation from pregnancy urine suitable for use in clinical extracts. Solution is effected in 0.5 % phenol.

2. The stability of such sterile solutions at room temperature has been examined, and they have been found to lose approximately 50 % of their activity in the course of 17 days and 75 % or more in 26 days. Little activity is lost when extracts are kept frozen for 7-10 days.

3. ρ -Factors can be kept at 0° in solution in ammonia (sp. gr. 0.880) for 24 hours without appreciable loss of activity. An attempt, based on this finding, to obtain further quantities of ρ -factors from the protein residues proved uneconomic.

4. ρ -Factors cannot be adsorbed on benzoic acid at p_H 5 from partially purified protein- and phosphate-free preparations.

5. Palladous chloride cannot be used for further purification of protein-free specimens.

6. Criteria of different methods of standardisation are discussed, and it is shown that a completely satisfactory method must of necessity be more difficult to obtain than in the case of oestrin.

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