CLXVII. THE PREPARATION AND PROPERTIES OF THE GONAD-STIMULATING HORMONE FROM THE URINE OF PREGNANCY¹.

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A NUMBER of preparations have recently been described which, given by injection or in some cases by mouth to immature animals, stimulate the sex glands to produce a precocious maturity. The principal sources of this material are: (1) the anterior lobe of the pituitary gland [Evans, 1923; Zondek and Aschheim, 1927; Smith and Engle, 1927; Putman, Teel and Benedict, 1928; (literature) Teel and Cushing, 1930]; (2) the urine of pregnant women [Zondek and Aschheim, 1927, 1928; Zondek, 1929, 1, 1930; Biedl, 1928; Evans and Simpson, 1929; Reiss and Haurowitz, 1929], and (3) the placenta [Zondek, 1929; Philipp, 1929, 1930; Wiesner and Patel, 1929; Wiesner, 1930; Collip, 1930, 1, 2].

All these materials have the common property of showing their action only *via* the ovary or testis, and are therefore without action upon castrates. Whilst differences occur in the results of administration of the above substances or of extracts therefrom, the general result is in the female to stimulate the ovary to the production of oestrin, which causes the uterus to become enlarged and hyperaemic and, in rodents, the vaginal smear to become cornified. The follicular mechanism is greatly stimulated, resulting in follicular ripening, and luteinisation of the walls of the Graafian follicles, with the formation of new corpora lutea. In certain species, particularly in immature mice, haemorrhagic spots may appear in the ovary due to the extravasation of blood into the unruptured follicles. The corpora lutea appearing after administration of extracts of the above substances may be normal corpora lutea resulting from true ovulation [Evans and Simpson, 1929], though more usually they are atretic bodies consisting of unruptured follicles containing an undischarged ovum. In the male the most marked effect is an increase in the size of the seminal vesicles.

The present investigation was designed to concentrate and purify the active principle or principles contained in the urine of pregnant women, and responsible for the formation of corpora lutea and haemorrhagic follicles upon injection into the immature animal. In the following account (1) the method of testing the potency of extracts, (2) their preparation and (3) the chemical and

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physical properties of the purified material are described. The relation to other work in the field and the probable nature of the active substance are discussed after the experimental part.

EXPERIMENTAL.

Method of assay.

All preparations were tested by the same technique throughout. The sample was dissolved in water and was diluted to a suitable extent for injection. Six doses, each 0.3 cc., were given over a period of 45 hours to each of five immature female mice about 3 weeks of age. It is important that at the end of the test the age should not exceed 4 weeks. The times of injection were as follows: 1st day, noon and 6 p.m.; 2nd day, 9 a.m., 3 p.m. and 9 p.m.; 3rd day, 9 a.m.: a similar quantity and distribution of the dose was used in an investigation of the Aschheim and Zondek pregnancy diagnosis test [Allan and Dickens, 1930]. On the morning of the 5th day the animals were killed and their ovaries, uterus and vagina were examined, sections for histological examination being cut in some cases. Microscopical examination of the ovaries, however, was used to confirm the macroscopic findings and not as a criterion of activity. It was considered that if the extract did not produce sufficient change for the latter to be visible on naked-eye examination, it was preferable to regard the substance injected as being inactive in the dose given. The morphological changes produced, considered here from the point of view of their possible value as standards of activity, are as follows.

(1) *Precocious opening of the vagina.* This occurred in every case when an active preparation was given, usually on the evening of the 3rd or on the 4th day of injection. A similar change is produced by injection of oestrin, and control experiments showed that the amount of oestrin necessary is very small, and may not be sufficient to induce oestrus in spayed animals. It is, therefore, unsafe to attach any importance to these changes unless a very much larger amount of the extract has been tested on ovariectomised animals and has been found entirely inactive.

(2) *Precocious oestrus.* The above objection also applies to the use of this standard. In addition there is the possibility of inhibition of oestrus by other factors contained in or produced by the extracts (*e.g.* by formation of lutein tissue). In fact many extracts which caused copious luteinisation were found to bring about only very slight vaginal cornification. This, taken with the possibility of incomplete removal of oestrin, makes the use of oestrus as a standard of activity very unreliable.

(3) *Changes in the immature ovary.* On the other hand the changes in the ovary following the injection of an active extract are very well marked and cannot be due to traces of oestrin. The ovaries are much enlarged in size and the morphological changes produced are of three kinds. (a) Ripening of immature follicles, so that the ovary may consist of a mass of enlarged cysts

filled with clear liquor folliculi. (b) Luteinisation of follicles resulting in the production of many corpora lutea, some of which contain a degenerating ovum (corpora lutea atretica). These bodies are readily recognised either by naked-eye examination or with the aid of a lens if they are small. Frequently almost the whole of the ovary consists of a mass of lutein tissue. (c) In the immature mouse after injection of an active extract, the ovaries very frequently contain also haemorrhagic follicles; these follicles appear as very easily recognisable dark red spots on the ovary [Zondek and Aschheim, 1927, 1928]. The production of corpora lutea and haemorrhagic follicles is so unmistakable that the use of these changes as a criterion of activity has the advantage of being quite definite and at the same time the possible presence of oestrin does not prejudice the result of the test in any way. Active extracts to which large amounts of oestrin were added before injection showed the same results on injection as the unadulterated material. This criterion of activity has therefore been used exclusively in the experiments recorded in the present paper. It is frequently found that slightly smaller doses of extracts than are necessary to bring about luteinisation will lead to follicular ripening, usually accompanied by vaginal canalisation, uterine enlargement and an oestrus or pre-oestrus vaginal smear. In such cases a larger dose of the preparation is given in order to determine whether it is capable of causing luteinisation when given in sufficient amount.

Interpretation of the animal tests.

Whilst this method of testing shows quite definitely whether any particular preparation is active or not, attempts at quantitative interpretation have been found to be difficult. As with other methods depending on the determination of the minimum active dose, it is now well recognised that large errors may result. In some preliminary experiments therefore an attempt was made to use a method such as that introduced by Coward and Burn [1927] for the assay of oestrin, which method recognises and makes use of the individual variation in the responsiveness of different animals. It appeared that this method might be specially suitable for the present case, since it was found to be impossible to eliminate very considerable differences in responsiveness of different immature mice, due probably to slight irregularities in age and also to the great variations in the age of onset of natural puberty, such as have been recorded by many workers [Engle and Rosasco, 1927]. Since the animals cannot be used again after a test, the expense in the use of this method made it necessary to reduce the number of animals to small groups and to give a series of graded doses to these. The results of two such experiments are recorded in Table I.

Whilst there is some regularity in the results obtained, the figures show that it is doubtful if such a method is of much practical value at present. The proportion of the total number of mice injected showing a positive response falls off only gradually with diminishing value of the dose, particularly with specimen P.U. 16, and errors in individual estimates of the number of units by this method are therefore correspondingly great. Moreover, apart from the

Table I. *Effect of varying dose in inducing luteinisation and haemorrhagic follicles in small groups of mice.*

Specimen number P.U. 16			Specimen number P.U. 20		
Total dose cc.	No. of mice	Number positive	Total dose cc.	No. of mice	Number positive
0.18	5	3	1.8	3	3
0.09	8	3	0.36	8	8
0.06	7	2	0.18	8	7
0.045	7	2	0.045	11	8
0.036	5	2	0.036	8	2
0.026	7	2	0.026	8	0
0.018	8	0	0.018	8	0

expense, such an experiment is very tedious, and the advantage over the approximate idea of activity obtained by determination of minimum dose was considered insufficient to justify its use. It is evident that the accurate assay of the luteinising principle by methods at present available is much more difficult than the assay of the oestrus-producing hormone. Nevertheless, it is almost impossible to describe the experimental results without the adoption of a unit of some kind, and in the present paper the unit is provisionally taken as the minimum total amount of active substance which, injected into a group of five immature female mice under the conditions already described, produces definite (macroscopic) evidence of luteinisation or of corpora haemorrhagica in the ovaries of at least one animal: larger doses must produce a correspondingly higher response. This definition, though obviously very imperfect, has been found to lead to results of sufficient consistency to justify its use.

As in the case of oestrin [Allan, Dickens and Dodds, 1930] a single dose of the hormone is much less effective than the same total quantity spread out over a period of 48 hours. Thus a single dose of 0.5 cc. of P.U. 20 given to each of a group of 8 mice gave only one positive result, whereas with a total dose of only 0.18 cc. spread out over 48 hours, 7 of a total number of 8 mice injected, showed a positive response (Table I). Similarly, a total amount of 0.045 cc. given as 6 doses over 48 hours showed a positive result in 8 out of 11 mice injected: given as three doses spread over the same period the same total amount caused a positive response in only one of 8 mice injected. This result is doubtless due to the rapid loss through excretion and destruction when given in a single large dose. The divided dose method has, therefore, been used in all experiments.

Administration by mouth. Since the hormone withstands to some extent the action of digestive enzymes over short periods, it appeared probable that it might prove active when given by the mouth. In order to test this point mice 3-4 weeks old were given the aqueous solution of the hormone by means of a blunt cannula inserted well into the mouth. Doses of 0.3 cc. were used. This amount of fluid is swallowed by the animals; larger volumes cannot easily be given in this way to immature mice without considerable loss. The results of oral administration are summarised in Table II. It will be seen that doses 20 or more times greater than those required to bring about luteinisation when

given by injection are often, though not invariably, effective when given by mouth. Even with such very large doses only a small proportion of the total number of treated mice gave a positive response. It therefore seems probable that either the hormone is destroyed in the alimentary tract or it is not absorbed. The former possibility would be in agreement with the great chemical sensitiveness of the active principle.

Table II.

Batch No.	Injected (6 × 0.3 cc.) Active in dilution	(6 × 0.3 cc.) dilution	By mouth		
			Approx. no. of units per mouse	No. of mice	Result on ovaries
102	× 75	× 25	3	5	Nil
"	"	× 50	1.5	10	Nil
88	× 100	× 10	10	5	Nil
"	"	× 10 emulsified in 50 % olive oil	"	5	Follicular ripening only
117	× 80	× 1.9	40	10	"
"	"	× 3.9	20	10	Several corpora lutea in only one animal
122A	× 20	Undiluted	20	5	Nil
122B	× 80	"	80	5	One animal only, several corpora lutea and haemorrhagic follicles
"	"	"	80	5 (repeated)	One animal only; corpora lutea and haemorrhagic follicles
"	"	× 2	40	5	Follicular ripening only
"	"	Undiluted with food	40	5	One animal only; several corpora lutea, very large uterus
121D	× 50	Undiluted	10	5	Nil
"	"	× 5	—	5	Nil
"	"	× 5 with food	—	5	Nil

In two experiments the administration of the hormone was tried after mixing it with the animal's food. A mixture of bran and oats with the hormone was rapidly eaten. To each cage of 5 mice 3 cc. of the hormone solution mixed to a stiff paste with equal parts of bran and oats was given twice daily at 9 a.m. and 6 p.m. for 3 days. On the 5th day the mice were killed and examined as usual. The experiments showed that luteinisation may result by this method also from about 40 times the dose necessary to produce a similar effect when given by injection. Administration to mice by mouth by either method is, therefore, not a practicable method of giving the hormone.

Effect on the male.

Zondek and Aschheim [1927, 1928], Engle [1929] and Brouha, Hinglais and Simonnet [1930] have observed the enlargement of the male sex organs, and particularly of the seminal vesicles, following the injection of urine of pregnant women into male rats or mice. The following experiments demonstrate that the purified extract, prepared by the method described below, has similar properties.

Young male mice were used, aged about 3-4 weeks and weighing about 8-10 g. at the beginning of the experiment. Three groups were used: A, castrated 3 days before the beginning of the experiment; B, normal controls, not receiving injections; and C, normal males. Each of the animals in groups A and C received daily doses of 1 cc. of a preparation which was found to cause formation of definite corpora lutea in females of the same age when given in doses of 0.015 cc. Some of the animals were killed after 5 days and the majority after 12 doses of the hormone. In both cases there was very marked enlargement of the seminal vesicles and slight enlargement of the testes in all the animals in group C, as compared with the controls (group B). In the castrated animals the seminal vesicles showed definite atrophy and were smaller than in the normal untreated controls. Table III gives some quantitative observations on this point, and Plate VI is a photograph of four typical animals at the end of the experiment. A similar result is brought about by the injection of testicular hormone [Dodds, Greenwood, Gallimore and Allan, 1930], but in this case the castrates also are affected.

Table III. *Effect of 12 doses of 1 cc. of preparation P.U. 96 on young male mice.*

(B) Normal controls (not injected)		(C) Normal males (injections as above)		(A) Castrates (injections as above)	
Body wt. g.	Wt. of two seminal vesicles (mg.)	Body wt. g.	Wt. of two seminal vesicles (mg.)	Body wt. g.	Wt. of two seminal vesicles
8.7	3	16.5	198	12.0	All very small ca. 3-5 mg.
15.7	7	12.8	101	13.2	
16.8	20	17.0	212	13.3	
12.7	10	12.0	138	13.5	
				11.8	

Changes following the prolonged injection of the hormone into rats. Two groups (A and C) of 5 male and 2 (B and D) of 4 female rats of about the same age were used. The average weight at the beginning of the experiment was 57 g. in the case of the males and 46 g. for the females. Doses of 0.5 cc. of the same preparation of the hormone as was used in the preceding experiment were given twice daily, with only one dose on Sundays, for a period of 1 month. On the 4th day the vaginas of all the injected females opened, and the typical cornified smear indicated precocious oestrus: the controls were 10 days later. There was no detectable effect on growth of the injected animals during this period. The average weights of the males at the end of the experiment were 146 g. (injected) and 145 g. (untreated); and of the females, 146 g. (injected) and 127 g. (untreated). It may be concluded that the growth-promoting hormone of the pituitary is not present in the extract obtained from urine of pregnancy by ammonium sulphate precipitation and subsequent washing with alcohol.

Analyses were made of the blood of the above groups. Since changes in

I II

Two mice receiving injections.

III
Normal control
of same age
and weight.

IV
Castrated mouse
(injections as in
I and II).

blood cholesterol have been described [Zondek, 1929, 2] following injection of extracts having similar properties to the one under consideration, this substance was particularly studied.

The results given in Table IV show that there is no definite change in the amounts of any of the constituents analysed, following prolonged administration of the hormone.

Table IV. *Blood analysis of treated and untreated rats.*

		mg. per 100 cc.
<i>Group A. Males</i>		
Injected;	Cholesterol	118, 107, 131, 144, 145: <i>Mean</i> 129
	Non-protein N	37, 45
	Urea	40, 28
<i>Group B. Females</i>		
Injected;	Cholesterol	145, 141, 130, 146: <i>Mean</i> 140
	Non-protein N	41, 40
<i>Group C. Control males</i>		
	Cholesterol	129, 140, 136, 130, 156; <i>Mean</i> 138
	Non-protein N	39. Urea 32
<i>Group D. Control females</i>		
	Cholesterol	105, 134, 160, 144; <i>Mean</i> 136
	Non-protein N	42

Preparation of the hormone from urine of pregnancy.

Concentration of urine in vacuo. The urine was concentrated to one-tenth of its volume in a jacketed vacuum-still at 40–50°. The cooled concentrate was shaken 5 times with ether and was then centrifuged. The clear dark-coloured concentrate (P.U. 16) was used for some of the standardisation tests described above after removing dissolved ether and diluting to a suitable extent. When tested on mice in the usual way it was still definitely active after diluting 70 times.

Dialysis of concentrated urine. As a preliminary comparison of two types of membrane, the concentrate prepared as above was first dialysed in parchment thimbles (Schleicher and Schüll) against distilled water for 18 hours in the refrigerator. The volume inside the thimbles was then 75 cc. and outside 100 cc. The solid matter was approximately equally divided (65 mg. per cc. inside, 57 mg. per cc. outside), but whereas the contents of the thimbles were very strongly active in a dose of 0.35 cc. (higher dilutions were not tried) the undiluted dialysate (1.8 cc. dose) was inactive when injected into mice. With unhardened collodion membranes (period of drying about 10 minutes) the activity was not entirely retained. 50 cc. of batch P.U. 16 was dialysed in such a sac for 16 hours against two changes of distilled water. The total volume of the dialysate was 240 cc. and of the inner fluid 60 cc. The latter was intensely active when a dose of 0.2 cc. was injected into mice (higher dilutions were not tried), whilst of the dialysate 1.8 cc. was also active, but 0.9 cc. total dose was without activity. It may therefore be concluded that most of the active principle remained undialysed, whereas a large part of the impurities passed

into the dialysate, since this was found to contain about 90 % of the total solids originally present in P.U. 16. It is evident from these experiments that the hormone passes either very little or not at all through parchment, but more rapidly through collodion. A very convenient method for removal of rapidly dialysable impurities is thus available. This behaviour on dialysis appears to differ from that observed by Zondek and Aschheim [1928].

Alcohol precipitation. Preliminary experiments showed that the precipitation from concentrated urine by concentrations of alcohol above about 75 % is almost quantitative. The preparation of a typical batch of material by this method is given in the form of the scheme below. The urine¹ was a collected batch from a number of women in the later stages of pregnancy. It was first concentrated *in vacuo* at 40° to about one-fifth of its volume and was then precipitated with successive concentrations of 50 % and 75 % alcohol. The second of these precipitates (C) contained nearly all the activity contaminated with much inorganic material.

Alcohol precipitation of urine.

Precipitate C was a nearly white, light, non-hygroscopic powder. Rubbed with water, 91 % by weight dissolved, giving a clear dark-yellow coloured solution. It contained 79 % of inorganic matter, of which 11 % was phosphate (as P_2O_5). A dose of 5 mg. of this powder dissolved in water gave a definite luteinising reaction when injected into mice as described. This amount is equivalent to 1.7 cc. of the original urine. The powder has the advantage that it may be kept at ordinary temperature for a considerable time without deterioration—it was not possible to detect any falling off in activity of this particular batch after 12 months under these conditions, but it was still very crude, and in addition to the high concentration of phosphate contained ammonia, calcium, traces of chlorides and urates, and a considerable amount of protein. Thus it was coagulated by heat, precipitated by salicylsulphonic acid and gave very definite biuret and Millon tests. Dialysis of this material was successful in removing most of the inorganic matter, but it still left a large quantity of undialysable impurity. Precipitation of the hormone was there-

¹ I wish to express my thanks to Mr G. F. Marrian, University College, London, for the urine used in this particular experiment, which had previously been extracted with ether in the course of preparation of oestrin.

fore attempted, and after a number of unsuccessful attempts with more complex precipitants, ammonium sulphate was found to be satisfactory for this purpose. Half-saturation with $(\text{NH}_4)_2\text{SO}_4$ caused only a faint opalescence, but on full saturation a white gelatinous precipitate separated, and, whilst its weight was only about $\frac{1}{300}$ th of the weight of precipitate C taken, little or no loss of activity was found on testing the precipitate. It was, however, still contaminated by a considerable amount of protein which remained after the removal by dialysis of the accompanying ammonium sulphate. In fact dialysis in parchment against 10 changes of distilled water over 24 hours removed little else, the solids other than ammonium sulphate remaining practically unchanged after dialysis. The dialysed solution (P.U. 76) was coagulated by heat and precipitated by trichloroacetic or picric acids, and gave intensely positive Millon and definite biuret (reddish violet) and ninhydrin reactions. Its activity was apparently unaffected by the above dialysis through parchment.

Since dialysis fails to purify the material further when the initial step is alcohol precipitation, whilst the above experiments show that the hormone is precipitated by saturation with ammonium sulphate, it appeared that the original alcohol precipitation might with advantage be omitted.

Precipitation from urine by ammonium sulphate.

Table V shows some typical results of precipitating a number of combined batches of urine of pregnancy by saturating with ammonium sulphate.

Table V.

Batch No. P.U.	Urine volume (litres)	Final solution volume (cc.)	Animal testing		Total wt. substance given (mg.)	Units per cc. equivalent to dose given	Approx. no. of units per litre urine
			Dose of undiluted solution (cc.)	Result			
7H	1.0	83	0.18 0.036	V. active "	0.12	30	2,300
44	5.0	200	0.18 0.036 0.024 0.018	" " " Inactive	0.11	40	1,700
44A*	—	—	1.8 0.36 0.18	Active " Inactive	—	2.7	2,700
64	24.3	73	0.072 0.018 0.009	V. active " "	0.036	110	>330
70	15	250	0.09 0.045	Active "	0.14	22	>370
72	18	450	0.09 0.045	Active "	0.30	22	>550
96	39	500	0.045 0.023 0.015 0.0075	V. active " " Active	—	130	1,700

* Untreated urine used for precipitation of P.U. 44 above.

Method. As an example of the method, the preparation of batch P.U. 64 will be described in detail. The urine (24.3 litres), previously clarified by passing through the Sharples centrifuge, was found to be neutral to litmus. It was stirred up with recrystallised (not "commercial") ammonium sulphate (18.2 kg.) when a fine white precipitate separated slowly. The mixture was allowed to stand at 0° overnight, and was then again run through the Sharples super-centrifuge. This is almost the only practicable method of collecting the precipitate without considerable loss. Usually two passages through the Sharples are sufficient to separate the precipitate from the nearly clear fluid, which is discarded. The precipitate was carefully collected and consisted of a darkish, nearly dry, clayey deposit which could be scraped off with ease. It was then thoroughly washed with alcohol and afterwards with ether by decantation and filtration on a Büchner funnel. The alcoholic filtrate was very dark coloured and contained oestrin and impurities. The oestrin is readily obtainable in water-soluble form by the method of Marrian [1930] and the yield of this hormone is on an average about 300 rat-units per litre. The residue on the Büchner funnel after drying *in vacuo* was a bulky, nearly white, non-hygroscopic powder containing a considerable amount of adventitious $(\text{NH}_4)_2\text{SO}_4$. For further purification it was extracted with 50 cc. of water and centrifuged, whereby the greater part of the ammonium sulphate is dissolved out together with a small amount of coloured impurity. (This washing is tested by adding solid ammonium sulphate to ensure that there is no loss of dissolved hormone; if there is no precipitation the washing is discarded.) The solid matter remaining in the centrifuge tubes was then thoroughly extracted with about 600 cc. of water at the centrifuge, the large insoluble residue being rejected. A few drops of acetic acid may be used if necessary to remove a mucin-like opalescence sometimes occurring at this stage. The active material was then re-precipitated from the clear, pale-yellow extract by saturation with the calculated quantity of ammonium sulphate. A white floating precipitate separated at once. The mixture was kept overnight at 0° when the precipitate could be collected in the ordinary centrifuge without difficulty. It was washed into a small tube with saturated ammonium sulphate solution and finally washed at the centrifuge with alcohol and ether and dried *in vacuo* over sulphuric acid. The resulting material (Batch No. P.U. 64) was a white, very light, homogeneous powder and weighed 1.820 g., of which 0.090 g. consisted of impurities insoluble in water and readily removed by centrifuging the aqueous solution, while 1.447 g. of the remainder was accounted for by unremoved ammonium sulphate. These analytical results were confirmed by removal of the ammonium sulphate by dialysis, as is described below. The volume of the aqueous solution of the above was 73 cc. ($1.820 - 0.090 = 1.730$ g. total solids; 0.283 g. non-ammonium sulphate solids; or 4 mg. non-ammonium sulphate solids per cc.). In a dilution of 1 in 200, this solution was highly active, and 1.8 cc. produced typical haemorrhagic follicles in the ovaries of the experimental animals. The total dose given was therefore equivalent to 0.036 mg. of solid matter other than ammonium sulphate.

Dialysis of the ammonium sulphate precipitate. 1.30 g. of the crude precipitate (P.U. 64) containing excess of ammonium sulphate, dissolved in 60 cc. of distilled water, was placed in Schleicher and Schüll parchment diffusion thimbles and dialysed against three changes, each of 5 litres, of distilled water, for 36 hours. This dialysis was unavoidably carried out at room temperature. The final volume of the dialysis residue was 75 cc., and the analytical results are summarised below:

	Before dialysis (P.U. 64)	After dialysis (P.U. 78)
Total solids	1,300 mg.	82 mg.
Total Nessler-N	230 „	0.99 „

Activity of the product. Before dialysis a dose corresponding to 0.21 mg. total solids, of which nearly 0.18 mg. consisted of ammonium sulphate, was active when injected into mice in the usual way. After dialysis a total dose of the clear aqueous solution (P.U. 78) corresponding to 0.017 mg. total solid matter was equally active, and when tested on 5 mice caused the definite appearance of numbers of corpora lutea in the immature ovaries. A slightly smaller dose (0.012 mg.) tested on the same number of animals produced well-marked uterine growth, but had no definite luteinising action. On the basis of the discussion of standardisation given above, the unit of this preparation may be taken as 0.017 mg. It is remarkable that such a simple process of preparation as precipitation with ammonium sulphate should give almost directly a product with such a high degree of purity from a complex mixture like urine in which it is present only in minute traces. It is evident that many of the other constituents also precipitated from urine by saturation with ammonium sulphate are to a great extent left in the insoluble residue on extraction with water, probably as a result of the treatment with alcohol and ether in the case of constituents such as protein, whose solubility is readily affected by such treatment.

Chemical properties.

The aqueous solution of the hormone prepared as described above is perfectly clear and straw-yellow in colour when concentrated. Owing to the small amount of solid matter contained in one unit (for example, with preparation P.U. 78 about the same as in a rabbit unit of purified insulin) it is possible to obtain extracts which are active on injection into mice but in which, owing to the high dilution, many chemical tests appear to be negative. Statements of negative chemical reactions are therefore of little value unless it is certain that a sufficient amount of active substance is present in the specimen tested. Although this point is an obvious one, it has not apparently been taken into consideration sufficiently in some investigations of the hormone under consideration. The following tests were all carried out on solutions definitely active in bringing about luteinisation after diluting 60 times. With extracts of greater purity it is necessary to use still more concentrated solutions, since even with the sample under consideration the solid matter was only about

1 mg. per cc. With this solution there was no trace of coagulation on heating and the usual protein precipitants gave completely negative tests. The precipitants tried included trichloroacetic, salicylsulphonic and picric acids. With mercuric sulphate in 5 % sulphuric acid, a slight precipitate slowly formed; lead acetate gave a slightly more bulky, white precipitate, whilst fairly voluminous precipitates separated on adding ammonium sulphate to saturation, tannic acid, or colloidal ferric hydroxide. The recovery of the activity from some of these precipitates is described later.

Colour reactions. The above solution (P.U. 78) gives a typical positive test with Millon's reagent and a positive reaction for tryptophan with the glyoxylic acid reagent; the latter test is very well-marked in the solution containing 200 units per cc. or more, but is also shown, though less strongly, by the above solution. The Molisch reagents give an intense clear red colour with purified specimens; charring occurs with cruder samples. Concentrated sulphuric acid alone gives a very faint rose-red colour (Liebermann reaction). The biuret test is a faint but definite violet. The solution after re-precipitation with ammonium sulphate, as in the above batch (P.U. 78), gives a negative reaction with the Folin uric acid reagent, whereas extracts which had not been so precipitated without exception contained considerable quantities of uric acid, the effect of which is to mask the reaction with copper sulphate and alkali owing to the formation of copper urate, so that an apparently negative biuret test is frequently given by the cruder extracts. The solution does not reduce Fehling's or Benedict's solution. Ehrlich's *p*-dimethylaminobenzaldehyde reaction is negative, as is also Knoop's test with bromine water. This latter point is of some interest since it shows that the positive bromine test given by many specimens of urine of pregnant women [Voge, 1929] is not due to the gonad-stimulating hormone which is the basis of the Aschheim and Zondek test for pregnancy. This observation is in agreement with the fact that a much higher percentage of accuracy of diagnosis is given by the biological test than by testing with bromine.

Stability of the hormone.

Heat. As has been pointed out by Zondek and Aschheim the hormone is very readily destroyed by heating its solution to temperatures above about 70°. Boiling for 30 seconds was found to destroy the activity completely, in confirmation of the thermolabile nature of this hormone. In some cases during preparation, allowing the temperature to rise to 65° has been found to result in a diminished yield of active material, and even prolonged standing of the solution at room temperature is to be avoided. The solid substance however may be kept for long periods at room temperature without appreciable loss of activity.

Acids and alkalis. The hormone is very readily inactivated by treatment with acid or alkali. In the choice of methods of purification and for other purposes it is necessary to have some more accurate information on this point.

In the following experiments two preparations of varying degrees of purity were exposed to different concentrations of acid (HCl) or alkali (NaOH). In all cases the time during which the action was continued was 15 minutes at 40°. The solution was then cooled and carefully neutralised. It was then diluted to a suitable extent for injection and the activity estimated in the usual way by determination of the minimum active dose on groups of five mice. The results are given in Table VI.

Table VI.

15 minutes at 40°			
Normality of acid or alkali	Estimated percentage destruction of activity		Batch No. P.U.
	HCl	NaOH	
0.002 (p_H 8.5)	—	ca. 0	80
0.02	25	—	80
0.03	>75	>25	80
0.03	>75	50	121 D
0.18	>80	80	121 D
0.20	90	90	121 D

It will be seen that under these conditions almost complete inactivation occurs with 0.2 *N* acid or alkali. For example, the original specimen P.U. 121 D was still definitely active when diluted 50 times, but after this treatment a dilution equivalent to a fivefold dilution of P.U. 121 was only feebly active and a tenfold dilution entirely without effect on the ovaries. With more dilute solutions of NaOH or HCl there is still a marked loss of activity, particularly in the acid solutions. Biedl [1928] has remarked on the sensitiveness of the hormone to acid. From the above table it will be seen that at 40° incubation for even 15 minutes with *N*/30 HCl reduces the activity to less than a quarter of its previous value. In alkali of the same concentration about half the activity is lost. The buffering due to the very small weight of the hormone and associated impurities is so slight that it can be ignored in these experiments: for example, only 0.2 cc. of *N*/10 NaOH was required to be added to 30 cc. of specimen P.U. 80 to make the resulting solution definitely alkaline to phenolphthalein. No detectable loss of activity occurred on incubating this solution for 15 minutes at 40°, a fact which is in accordance with the use of very dilute alkali in extraction of the luteinising substance from the anterior pituitary [Putman, Teel and Benedict, 1928].

Trypsin and pepsin. The enzyme preparations used (Liquor Trypsini Co., Allen and Hanbury, diluted 1 to 25 and Pepsin scales B.D.H., 2 % solution) were first tested for their action on alkaline and acid caseinogen solutions in the usual way (Cole, "Practical Physiological Chemistry," 8th ed.). 10 cc. of acid caseinogen (0.5 % solution) were completely digested by 1 cc. of the pepsin solution in 10 minutes; 10 cc. of alkaline caseinogen (0.1 % solution) were completely digested by 0.1 cc. of the diluted trypsin preparation in 15 minutes.

For the digestion with pepsin two batches of hormone were used. The first was active after diluting 20 times and the second after diluting 120 times, in

the usual doses. Higher dilution of both preparations were found to be inactive. Both solutions were cautiously made definitely acid to Congo red by the addition of one-fifth of their volume of $N/10$ HCl: one-sixth of their volume of the pepsin solution was then added. After incubation for 15 minutes at 38° and subsequent careful neutralisation and dilution to an amount equivalent to a fivefold dilution of the original specimen, both preparations were found on injection into mice to be still definitely active; the more potent of the two preparations was very strongly so.

The same two preparations were used for the experiments with trypsin, and also the same quantities of hormone and enzyme solutions. The reaction was adjusted with $N/10$ NaOH so as to be faintly alkaline to phenolphthalein throughout. After 15 minutes the tubes were removed from the bath, cooled, and neutralised. The dilutions injected were the same as in the pepsin experiments; using groups of five mice no difference could be detected between the activity of the control specimens (alkali alone, and alkali and boiled trypsin together) and those digested with the unboiled enzyme. It is permissible to conclude that the hormone is not rapidly attacked by these enzymes, though evidence of inactivation has been obtained in both cases by the use of more concentrated enzyme solutions and longer periods of digestion, as in the experiments of Reiss and Haurowitz [1929].

Precipitation of the active fraction.

The experiments described above show clearly that the ammonium sulphate precipitate from urine or from aqueous solutions of the partially purified hormone carries down with it the activity. The small loss of activity in experiments in which several consecutive precipitations were made on the same batch of material shows that the precipitation is probably nearly quantitative. As the purity of the preparation and its dilution in the solution increase, the time taken for separation of the ammonium sulphate precipitate increases, and it is therefore necessary to leave the mixture at 0° for some days if maximum precipitation of activity is required.

The use of ammonium sulphate in the initial stages of the purification provides a valuable method of removing the greater part of the inactive constituents but has the disadvantage that it is then necessary to remove the excess of the precipitant by dialysis. Although active material was obtained after cautious precipitation with $N/10$ barium hydroxide in the cold, this method of removing the sulphate was found to be accompanied by a much greater loss of activity than the very small loss occurring in dialysis, and was probably caused by adsorption of the active fraction on to the bulky precipitate of barium sulphate. Biedl [1928] reports the ease of adsorption of the hormone from its aqueous solution.

Among the other precipitants whose action has already been mentioned, it was found that apart from solvents like alcohol and acetone only two caused nearly quantitative precipitation of the activity. These reagents were "colloidal

iron" and tannic acid. The following experiments were made with the solution of the alcohol- and ether-washed ammonium sulphate precipitate obtained from 39 litres of urine of pregnancy in the manner already described. This precipitate (P.U. 96) was extracted with 500 cc. of water, and the extract was slightly acidified with acetic acid and centrifuged. The perfectly clear solution was found to be active in dilutions of 1 to 120 and feebly active in dilutions of 1 to 240 (total dose given equivalent to 0.6 cc. of original urine). It contained 70.0 mg. per cc. total solids, dried at 100°, of which the greater part was ammonium sulphate.

Precipitation by "colloidal iron." To 50 cc. of the above solution, 2 cc. of dialysed iron B.D.H. (previously diluted five times with distilled water) were added drop by drop. The bulky precipitate, much of which was due to the ammonium sulphate present, centrifuged readily and was drained from the clear supernatant fluid. This together with the water washings of the precipitate had a volume of 75 cc. and was again precipitated with "colloidal iron"; this second precipitate was quite inactive when suspended in 50 cc. of water and injected into mice in the usual way. The original precipitate on shaking with 50 cc. of water formed a uniform fine suspension, and after diluting 80 times was intensely active, producing many corpora lutea and haemorrhagic follicles in the ovaries of the injected mice. In a dilution of 1 in 160 it was also active, though less strongly, and resulted in luteinisation in the ovaries of four of five mice injected. There was therefore probably a nearly quantitative precipitation of the activity by "colloidal iron," even in the presence of ammonium sulphate. This method appears to provide a possible basis for further purification, but up to the present no satisfactory means has been found for removing the iron from the precipitate without losing the activity.

Precipitation by tannic acid. Satisfactory results in this respect were found when tannic acid was used as a precipitant. To 50 cc. of the above solution, previously acidified with acetic acid, 20 cc. of a 20 % solution of tannic acid (Kahlbaum) was added. This was the minimum amount to coagulate the bulky precipitate which was greyish-white in colour and centrifuged very compactly. It was washed at the centrifuge with water (40 cc.); the washings formed a negligibly small precipitate with more tannic acid. The whitish precipitate was suspended in water (20 cc.) and cooled to 0°. $N/10$ $Ba(OH)_2$ (35 cc.) was run in cautiously with brisk stirring until the precipitate turned green. The suspension of barium tannate was filtered quickly at the pump, and the filtrate was titrated at 0° with $N/10$ H_2SO_4 (15 cc.). After standing overnight at 0° the precipitate of $BaSO_4$ was removed by filtration and the filtrate and washings (total volume 150 cc.) tested upon mice (P.U. 104). Diluted 1 : 20 it was strongly active, 1 : 40 it was also definitely active, but 1 : 100 no macroscopic evidence of luteinising activity was observed. The solid residue from 5 cc. dried at 100° was 4.0 mg. or 0.80 mg. per cc. and the ammonia content determined by direct nesslerisation was equivalent to 0.075 mg. N (or 0.36 mg. $(NH_4)_2SO_4$) per cc. The above active dose (total 1.8 cc.; dilution $\times$ 40)

is, therefore, equivalent to 0.036 mg. total solids of which nearly half appears to be ammonium sulphate. Probably the latter was imperfectly removed by the washing of the original tannic acid precipitate, the experiment was therefore repeated with more thorough washing; see below. The solution so obtained (P.U. 104) was perfectly clear and nearly colourless. After concentrating *in vacuo* at 30–35° to 10 cc. it was pale yellow in colour and still perfectly clear. On adding absolute alcohol (140 cc.) and ether (100 cc.) there was a slight opalescence which did not coagulate even on standing overnight at 0°. Attempts to bring about precipitation by addition of acetic acid or by acetone were unsuccessful. The solution was therefore again concentrated to 3.5 cc. *in vacuo* at 30–35°. This very concentrated clear solution was precipitated by addition of acetone (70 cc.), the white precipitate was collected at the centrifuge and dissolved with a slight trace of opalescence in 30 cc. of water. For the animal tests this solution was diluted before injection 1 : 90 and 1 : 180. Both dilutions were strongly active. The acetone supernatant fluid was concentrated to dryness *in vacuo* and the residue was dissolved in 40 cc. of water; the undiluted solution produced no changes in the ovaries. It appears therefore that the precipitation was quantitative, though some loss of activity probably occurred on repeated evaporation of the faintly acid solution of the hormone even at the low temperatures used. The solution of the acetone precipitate contained 2.06 mg. per cc. total solids, dried at 110°, and 0.16 mg. ammonia-N by direct nesslerisation. Since a total dose of 1.8 cc. of a 1 : 180 dilution was active, this dose was equivalent to 0.02 mg. of total solid matter of which about one third was due to the trace of ammonium sulphate present.

In order to remove the last traces of ammonium sulphate and other soluble impurities from the hormone, the tannic acid precipitate prepared as in the previous experiment was thoroughly washed with water at the centrifuge. 290 cc. of the same batch (P.U. 96) as that previously used was cooled to 0° and treated with 29 cc. of glacial acetic acid and 45 cc. of a 20 % solution of tannic acid (Kahlbaum). The precipitate was then washed with two successive quantities of 75 cc. of 2 % tannic acid and finally with two further quantities, each 75 cc., of distilled water. The whole precipitate was then drained from the last washing and suspended in 100 cc. of water. One cc. of a 1 in 350 dilution of this suspension was strongly active and on injection produced corpora lutea and haemorrhagic follicles. The remainder (99 cc.) was chilled to 0° and Ba(OH)₂ (27 cc.) was run in to precipitate the tannate. The precipitation of BaSO₄ by sulphuric acid (1.5 cc. N/10) was very slow and required 48 hours at 0° before a clear filtrate could be obtained. The filtrate and washings of the precipitate were free from excess barium (total volume 145 cc.) but still gave a faint colour with the Folin type of Nessler reagent, though the colour appeared to be a slightly different shade of yellow from that given by ammonia. Compared in the colorimeter with a standard (NH₄)₂SO₄ solution the colour given by the hormone solution approximately matched that given by a solution containing 0.005 mg. ammonia-N per cc. 10 cc. of the solution were

evaporated to dryness and the residue, dried at 100° , weighed 1.70 mg.: the total solids therefore were 0.170 mg. per cc. For the animal tests the above solution was diluted 1 : 60 and 1 : 240. With the former dose, of 5 mice injected the ovaries of three animals showed macroscopically the definite formation of corpora lutea. The substance in these doses had little effect on the uterus, though some slight enlargement was noted. In the higher dilution the microscopic findings showed slight luteinisation of enlarged follicles, but, in accordance with the provisional definition of activity adopted, this finding was merely regarded as confirming the unit contained in the 1 : 60 dilution. The total dose given in this dilution (1.8 cc.) was, therefore, 0.006 mg.; this represents the smallest weight of substance definitely active in bringing about luteinisation obtained in any of these experiments up to the present. Unfortunately, slow formation of a white precipitate, accompanied by inactivation of the material, occurred on standing for a few weeks in the refrigerator. Injection of suspensions of the precipitate or of the supernatant clear colourless solution was found to yield negative results even in doses equivalent to 10–20 times the amount given above. It is evident that the highly purified hormone should not be kept in solution even at 0° for any appreciable time, but should be preserved in the solid state, as in the preceding experiment. Further work is in progress to attempt to determine the cause of the inactivation; at present it seems possible that the acidity of the solution (very faintly acid to methyl orange) remaining after precipitation of the barium may have been responsible for the loss in this particular experiment.

DISCUSSION.

From the above results it is evident that the material obtained by the method of precipitation used is identical with that described by Zondek and Aschheim. These authors now believe that two thermolabile factors are concerned, referred to by Zondek and Aschheim as prolan A and prolan B, and by Wiesner [1930] as rho 1 and rho 2. One factor (prolan A, rho 1) causes follicular ripening and the other (prolan B, rho 2) is the luteinising or "kyogenic" principle. Prolan A is obtained almost free from prolan B by precipitation with alcohol from the urine of non-pregnant women, *e.g.* from a proportion of cases of genital carcinoma [Zondek, 1930], but prolan B has not yet been prepared free from prolan A. In the experiments described above there is no evidence of any separation of these effects occurring during purification, hence it may be concluded that if there are these two distinct substances in the urine during pregnancy they are both precipitable not only by alcohol, but also by ammonium sulphate, "colloidal iron" and tannic acid under exactly the same conditions.

A point of great importance is the relation of this material to the active maturity-producing principle recently obtained in a microcrystalline form by Collip [1930, 1, 2] from human placenta. Is the substance described in this paper a relatively crude form of Collip's "emmenin"? This is definitely

not so, since the latter withstands boiling in aqueous solution, is soluble in all concentrations of alcohol and is almost as active when given by mouth as by injection. Also Collip now states [1930, 2] that corpora lutea "have seldom been found" in the ovaries of immature rats which have been treated with either a fraction soluble in 85 % alcohol or with the recrystallised final product. It appears that the luteinising factor present in Collip's earlier extracts [1930, 1] and removed during the process of purification used in the later extracts is closely related to the substance or mixture of substances the preparation of which is described in this paper. This is made very probable by the fact [Collip, 1930, 2, addendum] that a substance with similar physiological properties, inactive when given by mouth, precipitable by alcohol, and injured by boiling, occurs together with the follicle-ripening principle in some of his placental extracts.

The chemical properties of the concentrate prepared by ammonium sulphate from the urine as described above show that it contains degradation products of protein, though it is quite free from protein itself. As yet, however, it is not clear how far the chemical tests are due to the active principle itself and not to associated impurities precipitated with it from the urine.

SUMMARY.

1. The gonad-stimulating hormone is precipitated from the urine of pregnant women by saturation with ammonium sulphate.

2. Injection of the hormone into immature animals (mice and rats) leads to precocious sexual maturity. In the female the formation of corpora lutea and of haemorrhagic follicles in the ovary is observed, and in the male great enlargement of the seminal vesicles. The hormone is active by mouth only when very large doses (40 units) are given, and is without action on castrated animals.

3. The extract is without effect on the growth-rate of normal rats. Injection over a long period does not cause any detectable change in the blood cholesterol or urea contents.

4. By the method of preparation described, the yield of active principle is satisfactory. Aqueous solutions containing up to 1000 mouse-units per cc. have been obtained, and the injection of 0.02–0.006 mg. of the material causes the above changes in female mice.

5. On dialysis the hormone passes only very slowly through parchment, more rapidly through collodion.

6. It is inactivated by boiling, and by the action of acids and alkalis; the activity is reduced to below 50 % by treatment for 15 minutes at 40° with $N/30$ NaOH or HCl. It is not rapidly destroyed by trypsin or pepsin, though prolonged treatment causes inactivation.

7. In the purified solution containing 200 units per cc. the biuret reaction is faint but definite; the Millon, glyoxylic and Molisch tests are positive. No

precipitate is given with trichloroacetic, salicylsulphonic or picric acid, and the solution remains clear on boiling.

8. The hormone is precipitated without loss of activity from its aqueous solution by complete saturation with ammonium sulphate, by alcohol or acetone, by "colloidal iron" or tannic acid.

9. A method of purification based on the use of tannic acid is described.

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