CXC. A STUDY OF THE OESTRUS-PRODUCING HORMONE: ITS PREPARATION AND STANDARDISATION IN A WATER-SOLUBLE FORM.

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SINCE very full reviews of the literature concerning the oestrus-producing hormone have recently been published [Allen and Doisy, 1927; Parkes, 1928], it is not proposed to enter into a detailed historical summary here, and only those papers particularly concerning the aspect at present under consideration are briefly reviewed. It is proposed to describe experiments leading to a method for the preparation of the oestrus-producing hormone in a highly active, water-soluble form and its properties in this state. That such a product would have considerable advantages for clinical and experimental purposes over the existing oily material, requires but little comment. Whilst Ralls, Jordan and Doisy [1926] state that they can obtain from liquor folliculi an oily preparation containing 1 unit in 0.02 mg. of solid material, experiments here have not succeeded in reducing the unit by this method to below 0.5 mg., but this discrepancy may be due to differences in the technique of standardisation which are discussed later.

There have been many claims to the preparation of a water-soluble product. It will only be necessary to refer here to the main workers. Zondek and Brahn [1925] were among the first to describe a water-soluble extract, and in the same year Loewe [1925] obtained an active aqueous solution. Glimm and Wadehn [1926], and Frank *et al.* [1926] also describe similar preparations. By far the most important claim is that put forward by Laqueur and his co-workers [1925, 1926, 1927]. Although Prof. Laqueur has sent us water-soluble material of high activity, we have not succeeded in obtaining satisfactory results by means of the methods published by him and his associates. Whilst we have obtained active material by his method, the yields have been very small and inconstant. In 1927 [Dickens, Dodds and Brinkworth, 1927] a method was also published by us, but as has already been pointed out [Dodds, 1928], this has not proved satisfactory.

Since all these methods have been adversely criticised it was decided to attack the problem *de novo*. As the work progressed two separate problems arose; firstly the question of preparation, and secondly the method of standardisation.

In all these experiments placenta has been used as the starting material. The first attempts to produce a water-soluble preparation were made by acid

digestion of the placenta. Whilst an apparent aqueous solution containing the active principle could be obtained by this procedure, the crude material produced local abscesses in the animals. Although the final product had the appearance of an aqueous solution, the active principle could be extracted by means of ether or butyl alcohol and was associated with material of fatty nature; a colloidal suspension of fats had been produced. It was evident that a method had to be adopted involving the destruction of the fats. This was accomplished by the use of barium hydroxide in place of acid for the initial digestion of the placenta; after extraction of the unsaponified fat, this was resaponified with the barium hydroxide and a clear aqueous solution was obtained after removal of the soaps. The activity, however, had almost completely disappeared, and it was only possible to produce a pro-oestrus response in the animals by injecting this material according to the usual single dose method. It appeared from these experiments, therefore, that the potency was destroyed by this treatment.

Up to this point, except in the earliest experiments, standardisations had been carried out by the method of Coward and Burn [1927]: a number of ovariectomised animals were injected with a single dose of the material and the response was studied by vaginal smears, the results being calculated on the percentage of rats giving full oestrus. By this method the resaponified material gave no oestrus response. At this stage in the investigations an aqueous specimen of material was received from Prof. Laqueur which, when tested by the method of standardisation already referred to, was also found to be inactive, but when injected in a series of small doses according to the method described by Laqueur and his co-workers, proved to be highly potent. A re-standardisation on these lines of the aqueous material made by the barium hydroxide method resulted in a full oestrus response in a large percentage of animals, in contrast to an entirely negative result when injected in a single dose. With this knowledge it was possible to evolve a process for the preparation of water-soluble material of high potency.

EXPERIMENTAL.

Standardisation.

The following table gives the results of a single injection as contrasted with the response obtained when the quantity of the dose is divided into six equal portions.

Table I.

Batch No.	No. of rats	Dose	No. of rats showing oestrus	Result %
215	10	1 dose of 1 cc.	1	10
258	7	1 dose of 1 cc.	0	0
215	15	6 doses of 0.2 cc. (interval as under "method")	13	86
258	10	6 doses of 0.2 cc.	6	60
278	20	6 doses of 0.2 cc.	11	55
278	20	4 doses of 0.3 cc. in 12 hours	0	0

This demonstrates that the single injection method was not applicable to water-soluble material and, moreover, it fully confirms the claims of Laqueur and his co-workers as to the activity of water-soluble material when injected in multiple doses. In addition to dividing the dose, the time interval between the injections is very important. Thus, six doses distributed as described give a positive result, whereas the same total quantity, if given by the method described by Allen *et al.* [1924] is inactive. Results such as these led to the adoption of a method combining the multiple dose technique of Laqueur with the "statistical" method of Coward and Burn.

Method. All standardisations have been performed upon ovariectomised rats. When a weight of 140 g. is reached the ovaries are removed and vaginal smears are taken for 2 weeks. If no signs of cyclical activity are observed, the animals are then put into use. Six doses of 0.2 cc. each are injected into the animals, the preparations being diluted to contain approximately 1 unit in 1.2 cc. The injections are made as follows: first day, 9 a.m. and 6 p.m.; second day, 9 a.m., 3 p.m. and 9 p.m.; third day, 9 a.m. only. Smears are taken at 9 a.m. on the first and second days; at 9 a.m. and 6 p.m. on the third day; and again at 9 a.m. and 6 p.m. on the fourth day. It is rare for a rat to attain oestrus after the evening of the fourth day, and often signs of a return to the dioestrus condition are already present in the sixth smear. A minimum of 20 rats and, where possible, 100 rats are used for each batch. Smears have been stained with haematoxylin and eosin, since this enables a permanent record to be kept of the response elicited by various preparations.

Interpretation. One unit of activity is that quantity of material required to produce a full oestrus response in 50 % of the rats used (Coward and Burn). Oestrus has been taken to be present when cornified cells only are shown in the vaginal smear.

Table II.

Batch No.	No. of rats	No. showing oestrus	Calculated unit cc.	Difference between duplicates cc.
260	20	9	1.33	0.21
	30	16	1.12	
279	20	13	0.92	0.06
	20	14	0.86	
281	19	14	0.81	0.52
	20	9	1.33	
290	50	26	1.15	0.07
	52	29	1.08	
290 <i>a</i>	40	17	1.40	0.76
	36	10	2.16	
292	20	7	1.71	0.81
	21	14	0.90	

In view of the striking differences between the action of the hormone in oily and water-soluble form, we decided to construct a curve connecting the percentage of oestrus response and the number of rats used, similar to that recorded by Coward and Burn. Owing to the large number of determinations necessary in order to establish such a curve, our experiments upon this point

are at present incomplete. That the method described above is far from satisfactory is shown by a series of duplicate standardisations taken at random from our results.

Table II shows that even with the precautions of the method quoted, the unit may vary by as much as 5 to 50 %. Although Coward and Burn pointed out a year ago that there is a considerable possible error in the use of 25 rats, and the above experiments fully bear out their views, some writers do not even mention the number of animals used for a single test, and assume for their standardisation what must now be regarded as an entirely fictitious accuracy. The statistical method is much superior to the diminishing dose technique, and until further experiments have produced a better definition we have adopted the 50 % response as a unit.

As previously shown [Dickens, Dodds and Wright, 1925] the crude material produced by alcoholic extraction from ovaries considerably depressed the blood-pressure. The water-soluble material obtained by the method described below is, however, entirely without action on the blood-pressure, even in doses containing 50 units, when injected into a cat under chloralose and ether anaesthesia.

The effect on the uterus has also been examined by the method of Dale and Burn, and it was found that the addition of the extract in doses of 50 units to the uterine bath (125 cc.) produced a series of very small and slow contractions and relaxations. Further work is being done on this point.

Preparation.

Acid digestion of the placenta. Digestion of the placenta with acid [Dickens, Dodds and Brinkworth, 1927] results in nearly all the placental material entering into solution. The great stability of the hormone was shown by digesting minced placenta (1300 g.) with 650 cc. of 5 % hydrochloric acid in an autoclave at 150° with constant stirring for 7½ hours. The resulting suspension, when cold, was centrifuged, and the reaction of the clear, dark-coloured fluid (1770 cc., precipitate discarded) was adjusted to p_H 4, when an isoelectric precipitate appeared. This was dissolved in a minimum of 0.1 *N* alkali and was made up to 1000 cc. and 1 cc. of this slightly turbid solution produced oestrus.

This extract was very crude and the adsorption of activity on to the precipitate was not constant. Extraction of the activity from the acid digest was attempted with butyl alcohol [Dickens, Dodds and Brinkworth, 1927]. 2.5 kg. of minced placenta and enough concentrated hydrochloric acid to bring the p_H to 1.8 were heated in an autoclave at 150° with stirring for 1½ hours, and were allowed to cool. The mixture was filtered (filtrate 2430 cc., p_H 3.0) and was extracted with butyl alcohol for 10 hours in a constant-extraction apparatus. The dark-coloured extract deposited a colourless crystalline precipitate (amino-acids?) which was removed, and a coagulum separating in the aqueous part was extracted with butyl alcohol. The combined butyl alcohol extracts were evaporated *in vacuo* to a dark-coloured

residue completely soluble in hot water. The reaction was strongly acid, and on neutralising a turbidity appeared. The volume of this crude extract was 800 cc., and 0.5 cc. caused a definite oestrus response. The active material was still associated with fatty substance as was shown by ether extraction, evaporation of the ether extract yielding a suspension (A) of fat globules in water (480 cc.) of which 0.5 cc. produced a definite oestrus response. Centrifuging separated the fat droplets and standardisation showed that this layer carried with it the activity. It was therefore concluded that in order to obtain an aqueous preparation, preliminary destruction or removal of the fats was necessary. The simplest method of bringing this about appeared to be saponification.

Saponification. It was proved that the hormone in the fat-containing preparations is stable to the action of boiling alkali. Thus the suspension (A) (see above), was divided into four specimens. To the first 0.1 N sodium hydroxide was added until the p_H was between 8 and 9, to the second an equal volume of normal sodium hydroxide solution was added, and to the third an equal volume of 20 % potassium hydroxide. The fourth specimen served as a control. All the four specimens were heated in a boiling water-bath for 3 hours with vigorous shaking at intervals. Each was cooled, acidified with hydrochloric acid, and extracted with ether; the ethereal extracts were washed with a little water and evaporated. The residues, containing fatty acids, were emulsified in dilute sodium carbonate solution (volume of each specimen equal to half the initial volume of (A) used), and doses of 1 cc. were given (*i.e.* four times the dose of (A)). Each of the four specimens produced oestrus, showing that the active material had survived this treatment.

In order to determine the best conditions for saponification with minimum loss of activity, a quantity of fatty material was prepared from placenta by a modification of Allen and Doisy's method [Dickens, Dodds and Wright, 1925]. After freezing out most of the fats from the acetone solution of the final product, and precipitating the cholesterol by digitonin, 9.39 kg. of placenta gave 2.59 g. of clear oily substance (No. 217), of which a solution in olive oil containing 25 mg. per cc. was prepared for injection, the results of which are tabulated below for comparison with those obtained after saponification¹.

Table III.

Dose in mg.	No. of rats	Results
2.5	20	2 E, 5 E-, 11 P
5.0	10	5 E, 3 E-, 1 P
10.0	6	2 E, 2 E-, 2 P
30.0	6	5 E, 1 E-

Note. E denotes complete cornification; E- denotes a similar smear but containing a few nucleated epithelial cells; and P denotes a definite pro-oestrus smear.

For the following experiments a solution of 2.086 g. of No. 217 in 50 cc. absolute alcohol (41.7 mg. per cc.) was prepared.

¹ The experiments described here were performed before the results of Coward and Burn's work on the standardisation of the hormone were known to us.

Determination of saponification value. The solution (5 cc.) was boiled under reflux for 2 hours with 0.0909 *N* alcoholic potassium hydroxide (25 cc.), cooled and titrated against 0.0988 *N* hydrochloric acid using phenolphthalein (required 15.3 cc.). The saponification value was, therefore, 205. The contents of the flask were evaporated to a clear, reddish-brown, aqueous solution (5 cc.), which was neutral to phenolphthalein, and was injected in single doses of 0.5 cc. into rats (no. of animals 7; result, 5 E, 2 P).

Saponification by the calculated amount of alkali. In the above experiment an excess of alcoholic potassium hydroxide was used. In a similar experiment using the calculated amount (8.4 cc. 0.0909 *N*) of potassium hydroxide in alcohol after boiling under reflux for 2 hours, the reaction was neutral to phenolphthalein, and the solution was evaporated on the water-bath to a very small volume and made up to 5 cc. with water. The solution was very faintly opalescent. A dose of 0.15 cc. was injected into rats (8 animals; result, 1 E, 3 E-, 4 P). Saponification of 5 cc. by boiling for 1 hour with sodium in alcohol (4.5 cc. of a solution of 0.40 g. sodium in 100 cc. absolute alcohol) followed by evaporation and neutralisation with 0.5 cc. 0.1 *N* hydrochloric acid, gave an aqueous preparation (5 cc.) which set to a jelly on allowing to stand for some days. Doses of 0.15 cc. each were injected into rats (7 animals; result, 1 E, 5 P).

Removal of soaps. These experiments show qualitatively that the fats can be saponified without complete destruction of the hormone. If alkalis are used, the resulting product contains the corresponding soaps in aqueous solution. Removal of these soaps by replacing the alkali metals by barium was attempted in two ways: (1) by direct saponification of the oily material by boiling with barium hydroxide, and (2) by addition of barium chloride to the solution of potassium soaps obtained from the oily material as above.

(1) The stock solution (No. 217, 5 cc.) was boiled for 2 hours under reflux with the addition of the calculated amount (3.8 cc. 0.2 *N*) of barium hydroxide solution. The neutral alcoholic solution was evaporated, a little water being added, and the precipitate of barium soaps (*B*) was filtered off. The filtrate was freed from barium by cautious precipitation with 1 % sulphuric acid solution and filtration from the precipitate of barium sulphate. The clear yellow filtrate was evaporated to 5 cc. and contained 5.4 mg. of solid matter (dried at 110°) per cc. Doses of 0.15 cc. proved to be inactive (2 P only). A similar experiment in which twice the calculated quantity of barium hydroxide was used gave a similar result (P only being obtained even with three times the dose given above). The barium soaps (*B*) were taken up in hot 50 % alcohol and 1 % sulphuric acid was added until the reaction was strongly acid. After half an hour on the boiling water-bath, barium hydroxide solution was added until the acidity was reduced to a weakly acid reaction, the precipitate of barium sulphate was filtered off, and the alcohol removed from the filtrate by distillation. The fatty acids which separated were again emulsified by addition of dilute sodium bicarbonate solution, the final volume being 5.5 cc. Doses of 0.3 cc. were inactive. Thus the activity of the extract

could not be recovered from the precipitate of barium soaps and was apparently destroyed.

(2) Attempts to trace the exact stage at which the loss occurred were made by converting the fats into the potassium soaps and precipitating by addition of barium chloride solution. 23.5 cc. of the stock solution in alcohol (No. 217) were saponified as before by boiling with 0.1 *N* alcoholic potassium hydroxide solution (40 cc.) for 2 hours, the reaction remaining alkaline throughout. The slight excess of alkali was neutralised by 0.1 *N* hydrochloric acid (1.5 cc.) and the alcohol was evaporated. The resulting aqueous solution (23.5 cc.) was standardised (10 rats; single dose of 0.2 cc.; result, 5 E, 3 P). 5 cc. of this solution were diluted to 10 cc. and heated on the water-bath, and a slight excess of barium chloride solution (9.4 cc. approximately 0.1 *N*) was added. The resulting precipitate of barium soaps was centrifuged, and the clear supernatant fluid freed from the excess of barium by sulphuric acid. The final volume was 5 cc.; when injected into 6 rats in single doses of 0.25 cc. it was completely inactive.

These results were obtained only with fairly pure fatty material. We found in similar experiments in which crude oily preparations made from placenta by the usual alcohol process, with removal of the lipins but without subsequent purification by freezing and by digitonin, were boiled with barium hydroxide, much of the activity passed into the aqueous filtrate. In one experiment the oily material (10.5 g., which in doses of 75 mg. to each of 10 rats gave 9 oestrus responses) was dissolved in hot aqueous alcohol (160 cc. of 70 % alcohol) containing 5 g. of crystalline barium hydroxide in solution and the mixture was boiled under reflux for 2 hours. The reaction not being markedly alkaline, a further 2 g. of barium hydroxide crystals dissolved in 10 cc. of water were added, and boiling was continued for another half hour. A precipitate had formed, and distillation of the alcohol caused a further amount to collect. The residue was cooled on ice and centrifuged; the clear aqueous supernatant fluid when heated, became turbid, and this precipitate was also removed by filtration. Removal of the barium by sulphuric acid gave a faintly opalescent filtrate (40 cc.) (10 rats; 1 cc. each; result, 5 E, 3 E-, 3 P). This result suggests that the hormone was carried into the final solution in this experiment adsorbed on to a colloid (? cholesterol). That the final solution contained lipid material was shown by treatment in a continuous extraction apparatus with ether, evaporation of the ether yielding a trace of oily material which could not be redissolved in water, but which, on emulsification with dilute sodium bicarbonate solution and injection into rats, evoked an oestrus response. This method of preparation from the fatty material we regarded, at the time of these experiments, as unsuitable for the preparation of aqueous solutions of the hormone, since it failed with purified fatty material. As already described under "Standardisation," material of this type was found to be active when given to rats by the multiple injection method. It seemed probable that in the experiments in which soaps remained present in the final material

after saponification (those in which potassium hydroxide or sodium ethoxide was used) the presence of these substances caused the material to act like the original oily preparations; whereas the use of barium, and consequent removal of the insoluble barium soaps, produced a different type of substance, in aqueous solution, inactive in one dose, but giving a positive response when administered by multiple doses. That it is possible to reverse this effect is shown by taking some of the water-soluble product, the preparation of which is described later, and adding potassium oleate solution to it before injection. In one experiment, the material in a single dose of 0.15 cc. injected into 10 rats gave an entirely negative response; but injection into 7 rats of a single dose of 0.1 cc. mixed before injection with 0.1 cc. of a solution of potassium oleate, produced 1 oestrus and 6 pro-oestrus responses; whilst the same material without potassium oleate, given to 15 rats as 6 injections, the total amount of which for each animal was equal to 0.08 cc., caused 13 of the rats to show an oestrus response.

Alkaline digestion of the placenta. These experiments suggested the use of alkali instead of acid for the digestion of the placenta, the object being the early elimination of as much of the saponifiable fats as possible. In a typical experiment 500 g. of the minced placenta together with an equal volume of water and 200 g. of crystalline barium hydroxide were heated on the steam-bath, the temperature inside the flask being about 85°, for 9 hours. After cooling and filtering (precipitate rejected), the filtrate, which was clear and light in colour, was freed from barium by sulphuric acid (alcohol extraction of the precipitate of barium sulphate yielded no active material). The clear aqueous solution so obtained (300 cc., filtrate "C") was active in single doses of 1 cc., and the potency seemed to be in solution since it could be filtered with practically no loss of potency. Investigations on the concentrations of barium hydroxide, time and temperature, showed that periods of boiling, even up to 8 hours with an equal volume of 20 % barium hydroxide, did not destroy the activity; whilst digestion for half an hour at the boiling-point was shown to be insufficient, since an alcoholic extract of the precipitate filtered off after digestion, and also of the precipitate of barium sulphate, gave active material in these experiments, and the final product was without activity—possibly due to insufficient saponification. Very slow digestion at 70° for 18 hours presented no advantage over brisk boiling for 2 hours in a steam-jacketed still under reflux with an equal volume of 20 % barium hydroxide solution, and this was the method finally adopted. Digestion of the placenta with alcoholic barium hydroxide has also been found to be suitable—boiling for 1 hour with two volumes of alcohol and barium hydroxide crystals to 10 % of the total volume effects very complete digestion, and on filtering from the precipitate and evaporating the alcohol a concentrated solution remains containing the activity. We have used mainly aqueous digestion in the preparation of our extracts.

Purification by alcohol and ether. The crude product obtained in this way

contained a large quantity of protein fission products. It gave a copious precipitate with picric acid, a small precipitate on saturation with ammonium sulphate, no precipitate with trichloroacetic acid, and a pink biuret reaction. It was toxic, producing a severe local reaction. In order to purify it and to remove the protein digestion products, precipitation by alcohol and ether was tried: the filtrate "C" obtained in the experiment described above (300 cc.) was evaporated to 150 cc. (p_H 2.8) and 10 volumes of absolute alcohol were added gradually with stirring. The alcohol was filtered from the resultant sticky precipitate of peptone (rejected, being found inactive and toxic). Removal of the alcohol left a viscous residue which was dissolved in water (52.5 cc.), single doses of 0.25 to 0.5 cc. being found active. The solution still gave a pink biuret reaction and a precipitate with picric acid. These properties were largely lost on reprecipitation from 95 % alcohol. The white precipitate forming was found to contain little or no activity. The alcoholic solution was added to twice its volume of ether when a brownish sticky precipitate collected, and this also was found to be without activity. The ethereal solution was evaporated, giving a small trace of yellow oil which, when suspended in water (75 cc.), had an acid reaction and was, therefore, neutralised by a small amount of sodium hydroxide. Doses of 0.5 cc. showed definite activity. (The use of acetone instead of alcohol and ether led to a similar product.)

This preparation still contained a small amount of fatty substance which was removed by boiling again with barium hydroxide. 20 cc. were added to 10 cc. cold saturated aqueous solution of barium hydroxide and the mixture was boiled. Evil-smelling bases were evolved, and boiling was continued until their removal was complete ($\frac{3}{4}$ hour). A precipitate had then settled and was removed from the solution by filtration, the barium was quantitatively removed from the filtrate by sulphuric acid and the volume made up to 20 cc. (Standardisation on 8 rats; dose 1 cc.; result, 1 E-, 4 P.) Trichloroacetic acid, copper sulphate, acid and alkali caused no precipitation, picric acid a very faint one, but the biuret and other protein reactions were still positive and a precipitate was obtained with tungstic, tannic, and phosphotungstic acids, and also with mercuric chloride. These precipitants were not suitable for purification, the active material being apparently divided between the precipitate and the liquid. (That a response was obtained by the single injection method in these experiments is to be attributed to the relatively crude nature of the final product.)

Extraction with ether and with butyl alcohol. In all experiments of this type the final product contained nearly all the protein disintegration products extracted by butyl alcohol. Alcohol and ether precipitation following the butyl alcohol extraction, besides involving the use of large quantities of these solvents, did not yield a product of sufficient purity. Direct ether extraction of the placental digest was insufficient, as shown by the large yield of active material obtained by subsequent extraction with butyl alcohol. Thus the filtrate obtained after digestion with barium hydroxide in the usual way

(17.1 litres, concentrated to 4.7 litres, from 10.1 kg. of placenta) was extracted in a shaking apparatus with ether (two extractions, each with 1.5 litres ether). The ether was distilled off and the light-coloured, oily residue was dissolved in alcohol, a trace of barium was removed by precipitation with sulphuric acid and the alcohol was evaporated from the filtrate, which was then made slightly alkaline and injected as an emulsion (volume 22.5 cc.; dose 0.1 cc. to each of 6 rats; result, 1 E-, 2 P). The aqueous layer after ether extraction was then extracted under the same conditions with butyl alcohol (two extractions, each with 1500 cc.), the butyl alcohol was evaporated and the large residue dissolved in about 1 litre of hot water, and this was shaken with ether (two extractions, each with 500 cc. ether). The ether extract was filtered, concentrated, and all traces of butyl alcohol were distilled off, and an equal volume of alcohol was added to the aqueous residue. Barium hydroxide solution (10 cc., 5 %) was added and the mixture was boiled under reflux for 2 hours. The resulting precipitate was filtered from the cold solution, the barium was removed by sulphuric acid, and the filtrate (75 cc.), a clear yellow solution, contained 15.6 mg. of solid matter per cc. It was standardised first in a single dose of 0.15 cc. (6 rats; result, 5 P) and later, after diluting 15 times, by injections of 6 doses of 0.2 cc. to each animal (total amount equivalent to 0.08 cc. of the undiluted material: 15 rats; result, 13 E, 1 E-, 1 P). The material contained in the aqueous layer after ether extraction, when concentrated, did not produce oestrus. This method, omitting the preliminary ether extraction of the barium hydroxide digest, has been repeated many times, and active material has consistently been obtained. The following table summarises the results from some typical batches (the multiple dose method of standardisation is used in this and in all the subsequent experiments).

Table IV.

Batch No.	Weight of placenta kg.	Final volume cc.	No. of rats	Approx. unit in cc. (undiluted)	Units per kg.	Solids (detd. at 110°)	
						per cc. mg.	per unit mg.
215	10.1	75	45	0.05	150	15.6	0.78
241	4.35	90	11	0.14	145	2.18	0.13
237	3.1	70	10	0.17	135	—	—
242	3.12	65	10	0.15	290	7.64	1.14
247	3.09	55	20	0.1	180	—	—

The material, though now without toxic and irritant properties, was still crude, as may be seen from the last column in the above table. It also contained a high percentage of nitrogen (for example, No. 241 had 0.335 mg. nitrogen per cc., or 0.047 mg. per unit) and contained substances giving a pink biuret reaction, and a faint but definite Millon reaction. Nessler's solution caused the formation of a cream-coloured precipitate; phosphotungstic acid also gave a voluminous precipitate. A portion (5 cc.), evaporated to dryness and extracted with chloroform, gave negative reactions for cholesterol. The substance is not precipitated by trichloroacetic acid or tungstic acid, nor by

95 % alcohol. Addition of chloroform to the alcoholic solution caused a white precipitate to separate. The properties of the solution suggested that it contained bases.

Removal of basic impurities. The ether extract (500 cc.) obtained as above from 4.83 kg. of placenta, after washing with water, was washed successively with (1) 10 cc. 0.1 *N* HCl, (2) 5 cc. 0.1 *N* HCl and water, and (3) 20 cc. water in two portions. The combined extracts, of which (1) and (2) were strongly acid in reaction, were warmed to remove ether, and a dilute solution of sodium sulphate was added to precipitate the small quantity of barium, which was removed; the filtrate was neutralised with 1 cc. of *N* sodium hydroxide and was found to be inactive. The nitrogen content was 0.168 mg. per cc., the total amount extracted being 11 mg. The ether solution was washed twice with water (the second washing gave no precipitate with silver nitrate) and was then evaporated. The pale yellow residue was dissolved in 50 % alcohol (50 cc.), 5 cc. of 5 % barium hydroxide were added and the mixture was boiled under reflux for 2 hours. The alcohol was distilled off and the aqueous residue was allowed to stand in the ice-chest overnight, after which the precipitate (*A*) was removed by centrifuging and the barium removed from the clear supernatant fluid by sulphuric acid. The resulting clear, pale yellow, aqueous solution (40 cc.) was combined with that obtained in a similar way by repeating the digestion of the precipitate (*A*) in 25 cc. of 50 % alcohol with 2 cc. of 5 % barium hydroxide for 1½ hours, and removing the soaps and excess of barium hydroxide. The combined volume was 65 cc. and a portion was diluted 24 times for standardisation. Six doses of 0.2 cc. were given to each of 20 rats, 10 of which showed an oestrus response; hence the unit was taken to be 0.05 cc. of the undiluted product. The total yield was 1300 units, or 270 per kg. of placenta. The solution contained 1.0 mg. per cc. of solid residue (dried at 110°) and analysis by the micro-Kjeldahl method (Pregl) showed 0.050 mg. N per cc.; thus the unit was 0.05 mg. and contained 0.0025 mg. N, the total nitrogen in the 65 cc. being 3.25 mg. and, therefore, the shaking with hydrochloric acid had removed 75 % of the total nitrogen contained in the ether extract. Further extractions with hydrochloric acid remove only a negligible quantity. The product, after purification in this way, no longer gave the biuret test, the Millon reaction was faint but positive, whilst phosphotungstic acid produced a very slight, or no precipitate. The colour given with the Folin phenol reagent was, in this experiment, approximately equal to that given by a solution containing 0.1 mg. of tyrosine per cc. (equivalent to 0.0004 mg. tyrosine-N per unit). In Table V, Nos. 258 to 279, the properties and yields obtained by this method in five typical batches are given.

Remarks on the method. Fresh placenta has been used in nearly all batches, but in a few cases in which the material was slightly putrescent the yields were considerably diminished. In one experiment when definitely putrid material was used, we obtained only 25 units per kg. of placenta. Three extractions with butyl alcohol are usually sufficient, though, as experiment No. 324 in Table V

Table V.

Batch No.	Wt. of placenta kg.	Final vol. cc.	Standn. no. of rats	Approx. unit cc.	Units per kg.	Solids (detd. at 110°)		Nitrogen	
						per cc. mg.	per unit mg.	per cc. mg.	per unit mg.
258	4.83	65	20	0.05	270	1.0	0.050	0.05	0.0025
260	8.01	82	50	0.050	205	0.52	0.026	0.016	0.0008
274	6.2	200	20	0.20	160	0.50	0.10	0.036	0.007
278	7.0	162	20	0.18	130	—	—	—	—
279	5.35	375	40	0.46	150	0.20	0.092	—	—
280	11.35	320	20	0.15	180	0.37	0.06	—	—
281	10.5	345	110	0.25	190	0.14	0.035	0.007	0.0017
282	11.05	120	20	0.071	210	1.18	0.084	—	—
284	9.65	100	20	0.05	200	—	—	—	—
285	15.98	135	20	0.067	125	0.56	0.069	0.04	0.0047
288	14.2	120	20	0.083	105	0.148	0.0123	—	—
295	33.9	227	20	0.052	125	0.28	0.018	0.0147	0.00094
304	32.4	225	19	0.08	90	0.30	0.025	0.013	0.0011
305	17.7	105	20	0.055	110	—	—	—	—
309	31.0	310	20	0.09	110	0.20	0.018	0.006	0.0006
321	6.45	90	20	0.066	210	1.12	0.07	0.11	0.007
324 a)	14.0	150	20	0.07	156	0.48	0.034	0.043	0.003
324 b)		90	20	0.2	33	0.64	0.12	0.060	0.012
328		130	20	0.06	150	0.40	0.024	0.026	0.0015
331	1.78	100	20	0.35	160	0.16	0.05	0.012	0.004
332	1.70	160	20	0.9	100	0.18	0.16	0.016	0.014
345	61.5	550	20	0.07	125	0.18	0.013	0.028	0.002
292	13.15	130	41	0.11	90	0.09	0.010	0.010	0.001
310	5.0	60	20	0.13	92	0.18	0.023	0.012	0.0016
311)	5.0	100	20	0.38	52	0.68	0.26	0.085	0.032
312)		120	20	0.30	80	0.36	0.108	0.033	0.010
313	16.0	160	20	0.15	65	0.18	0.027	0.015	0.0023

Notes.

No. 279. Alcoholic barium hydroxide used for digestion of placenta.

No. 321. Placenta had been frozen at -5° for 15 days before use.

No. 324 a. First three extractions with butyl alcohol, of barium hydroxide filtrate, combined and worked up as usual.

No. 324 b. Second three extractions from above (making 6 in all) worked up separately from 324 a by the usual process.

Nos. 331 and 332. The same batch of placenta was divided into two portions, No. 331 was used at once, and No. 332 was stored for 5 days in an equal volume of 0.5 % solution of tricresol in water at room temperature before digestion with barium hydroxide as usual.

No. 292. Three ether extractions.

shows, the extraction is then not quite complete. Direct extraction with ether is much less convenient owing to the formation of emulsions and is also less efficient as in the following example: the placenta (10 kg.) was boiled in the usual way with barium hydroxide and the resulting filtrate was concentrated and exactly half was extracted with ether (three extractions separated in the centrifuge). The combined ethereal extract after evaporation was treated with barium hydroxide as usual and the final aqueous product was No. 311, containing about 260 units. The filtrate, which had been extracted as above with ether, was afterwards extracted with butyl alcohol and the extract treated in a similar way (No. 312, a further 400 units). The other half of the original barium hydroxide digest was extracted directly with butyl alcohol as usual and gave about 460 units when treated in the usual manner. These results are summarised at the end of Table V where the result of an experiment (No. 313)

is included in which a mixture of butyl alcohol (30 %) and ether (70 %) was used for extraction; this, however, was not so complete as when butyl alcohol alone was used. The use of specially purified butyl alcohol of very low nitrogen content does not increase the yield or degree of purity of the final product.

Whilst the washing with hydrochloric acid of the ether solution obtained from the evaporated butyl alcohol extract is not accompanied by any loss of potency, the use of alkali for this purpose results in a partition of the activity. In one experiment on this point, the ether extract resulting from 1.55 kg. of placenta (140 cc.) was shaken mechanically for $\frac{1}{4}$ hour with 0.1 *N* sodium hydroxide in two portions of 100 cc. each. The ether was then washed with water and the combined washings and alkaline extracts were made fairly strongly acid with *N* sulphuric acid. This solution was then shaken with ether, and, after evaporation of the ether, the residue was taken up in 50 % alcohol, 10 cc. of 5 % barium hydroxide were added and the mixture was boiled under reflux for 2 hours. After removal of alcohol, barium soaps, and then excess of barium, the filtrate (107 cc.) contained 125 units (20 rats; 6 doses of 0.2 cc. each; result, 14 E; approximate unit 0.85 cc., containing 0.29 mg. solids and 0.0082 mg. N per unit). The ether extract, after the above washing with sodium hydroxide, was evaporated and put through the usual process. The final volume was 174 cc. containing 85 units (20 rats; 6 doses of 0.2 cc. each; result, 6 E; approximate unit 2.0 cc., containing 0.17 mg. solids and 0.0017 mg. N per unit). This is an example of the activity being carried with the sodium soaps, and appears to be an absorption phenomenon, numerous examples of which have occurred in connection with the hormone.

The next point requiring quantitative investigation was the saponification with barium hydroxide of the oily residue obtained on evaporation of the ether. In certain of the earlier experiments a considerable loss of activity occurred at this stage, and we have found that this was due, not to inactivation, but to adsorption of the active material on the precipitate of barium soaps, and was probably associated with incomplete saponification. If the precipitate of barium soaps was filtered off and again boiled with barium hydroxide solution under reflux, a further yield of active material was obtained in these cases. In our later experiments we have also preferred to use an aqueous medium for the saponification rather than an alcoholic one, since the danger of loss from this cause appears to be less in an aqueous suspension. Under these conditions this operation is accompanied by a small loss only, as the following experiment shows.

The ether extract obtained in the usual way from 14 kg. of placenta was divided into two equal portions and both were evaporated to small oily residues; one of these was dissolved in a very little alcohol and poured into water and evaporated to drive off most of the alcohol, giving a fine, stable suspension in water (126 cc.). Injection of this material (diluted 8 times for standardisation; 6 doses of 0.2 cc. each to 20 rats; unit 0.14 cc. (undiluted) containing 0.56 mg. of solids and 0.0035 mg. N per unit) showed that it

contained in all 880 units. The oil from the other half of the evaporated ether extract was also suspended in a similar volume of water, 10 cc. of 5 % barium hydroxide were added to the aqueous suspension, and the mixture was boiled under reflux for 1 hour. The slight precipitate which separated during boiling was removed by filtration and barium was precipitated from the clear filtrate by heating on the water-bath and cautiously adding 0.1 *N* sulphuric acid until the reaction was very slightly acid. The precipitate of barium sulphate was filtered off and was washed with a little hot water. The filtrate (145 cc., diluted 7 times for standardisation; 6 doses of 0.2 cc. to each of 19 rats; unit 0.19 cc. (undiluted); 0.026 mg. of solid material and 0.0017 mg. N per unit) contained in all 760 units. The loss in this experiment was within the experimental error of standardisation.

Great care is necessary in the removal of barium from the final product. In the above example the addition of an excess of 0.5 cc. 0.1 *N* sulphuric acid to the total volume increased the "solid" content (weight of the residue at 110°) by nearly 50 %. If, however, care is taken to determine the reaction, either electrometrically or by the use of an external indicator, the sulphuric acid neutralisation is satisfactory. To avoid this operation, precipitation by carbon dioxide after making alkaline with ammonia may be used (precipitation with carbon dioxide from the solution without addition of ammonia is incomplete). The filtrate from the final boiling with barium hydroxide, representing 10.7 kg. placenta, was divided into two equal portions. The first (No. 336 *a*) was freed from barium in the usual way by sulphuric acid (volume 110 cc., diluted 12 times for standardisation; 6 doses of 0.2 cc. to each of 19 rats; estimated unit 0.19 cc. approx.; solids 0.38 mg., 0.032 mg. N per cc.). To the second (No. 336 *b*) a dilute solution of ammonium hydroxide was added until the solution smelt definitely of ammonia. Carbon dioxide was then passed through the solution until precipitation of the barium was complete, when the precipitate of barium carbonate was filtered off and the ammonia quantitatively removed from the filtrate by distillation in a current of steam. The residual solution gave no precipitate with sulphuric acid. (Volume 80 cc.; for standardisation, diluted 18 times; 6 doses of 0.2 cc. each to 20 rats; estimated unit 0.1 cc. undiluted; solids 0.56 mg., 0.021 mg. N per cc.) This experiment shows that the latter method of removing barium is also satisfactory.

The method finally adopted. This is based on the experiments described above, and the procedure is described in detail, since the exact conditions are of importance, for a quantity of 10 kg. of dissected and minced placenta; this is added to 10 litres of hot water containing in solution 200 g. of crystalline barium hydroxide. The mixture is boiled for 2 hours in a steam-heated, enamelled iron vessel, furnished with a reflux condenser, and is afterwards poured on to filters made of "jean" (filter paper may be used for small quantities of material). The liquid filters readily, and the filtrate is concentrated in a shallow, steam-jacketed, enamelled iron pan to a volume of about 4 litres. This, together with the slight precipitate which may have separated, is extracted by mechanical

shaking for periods of $\frac{1}{2}$ hour with three or four successive quantities, each of 1 litre, of butyl alcohol. The combined extracts are distilled under diminished pressure until nearly all the butyl alcohol is removed and the residue is dissolved in 2 litres of hot water and is filtered through a folded paper. The precipitate is washed with ether and the ethereal extract is added to that obtained from the aqueous portion (by shaking in a separating funnel with three or four successive quantities, each 0.5 litre, of ether). The combined ether extract is washed with water¹ (two washings of about 500 cc. each) and is then concentrated (transferring to a small flask at the end of the evaporation) to a slight, oily residue which is freed from the remaining trace of butyl alcohol by distilling *in vacuo* with a little water. The residue is dissolved in a minimum amount of alcohol (about 5 cc.) and is transferred to a volume of about 200 cc. water. The suspension thus obtained is shaken with three successive quantities of about 300 cc. ether, and the combined extracts are then washed three times with about 50 cc. of approximately 0.1 *N* hydrochloric acid, and finally with distilled water until the washings are free from chloride. The ethereal solution is then evaporated and the very small residue is dissolved in a minimum quantity of alcohol (2 or 3 cc.) and the alcoholic solution is poured into about 100 cc. of distilled water, and at this stage the extraction with ether may be repeated a third time if desired. This is not generally necessary, however, and after removing nearly all the alcohol by evaporation from an open dish on the water-bath, 10 cc. of a 5 % solution of crystalline barium hydroxide in water are added to the aqueous suspension, and the mixture is heated under reflux in a boiling water-bath or is gently boiled on a sand-bath for a period of 1 to 2 hours. The contents of the flask are then cooled to room temperature, and the clear solution is filtered from the slight precipitate which has formed. The filtrate is set on one side and the precipitate is thoroughly extracted with successive small quantities of hot alcohol. The alcoholic extract is evaporated to small volume and is then added to about 50 cc. of distilled water; 5 cc. of 5 % barium hydroxide are added and the mixture is boiled as before for 1 hour, when it is cooled and filtered. The combined filtrates from the first and second boilings with barium hydroxide are next heated on the boiling water-bath and the barium is removed by either of the methods described. The final product should be quite free from traces of barium or of sulphuric acid, and is usually faintly acid in reaction (about p_H 5). The results obtained from 16 typical batches are given in Table V, Nos. 280 to 292.

Physical properties. The clear liquid obtained as described above is colourless, or very pale yellow in colour, often showing a strong blue fluorescence. It may be evaporated in an open dish on the steam-bath giving a residue which retains the activity of the original preparation, is freely soluble in absolute alcohol, but does not redissolve readily in a small volume of water. The final product appears to be in the form of a solution (true or colloidal) since it passes through filter paper

¹ It may with advantage be washed with hydrochloric acid and water at this stage also.

with little or no diminution of activity, and may be filtered through a Berkefeld candle without great loss. Thus 560 cc. of material prepared as above, containing 7.0 units per cc. were filtered through a previously moistened Berkefeld candle, which had been tested to retain bacteria. The total volume thus obtained was 558 cc., and this, standardised on 52 rats, was shown to contain 5.0 units per cc. The loss was, therefore, about 35 %. This test was performed with an impure product containing 0.08 mg. of solids per unit. Further evidence as to solubility is given by dialysis. Some of the material, after filtration through the Berkefeld filter (20 cc.; 5.0 units per cc.) was placed within a Schleicher and Schüll parchment diffusion thimble (tested after the experiment and found not to allow egg-albumin to pass through) so that, after inserting the manometer tube, a slight pressure was indicated; this did not diminish during the experiment. The thimble was allowed to stand for 24 hours in a beaker containing 75 cc. of distilled water. At the end of this time the thimble was removed and its contents (the change in volume being negligibly small) were diluted three times, after which 6 doses of 0.2 cc. were given to each of 19 rats, three of which showed an oestrus response (before dialysis in double this dilution a similar injection into 52 rats caused a 50 % response). The outside solution (75 cc.) was injected without dilution as 6 doses of 0.2 cc. each into 18 rats, when 6 showed a definite oestrus response. The material is thus dialysable with some loss of activity. This is in agreement with the findings of Laqueur *et al.* [1926, 3; 1927]. The preparation is stable to boiling, and we have used this method, as well as heating in the autoclave at 120° for 15 minutes, for sterilising the product. Tricresol may be added without obvious effect on the activity. After filtration through the Berkefeld candle, the material described above was divided into two portions to one of which 0.1 % of tricresol had been added, and both were sealed in sterile glass vessels and placed in an incubator at 37° for 3 months. At the end of this time they were removed and after diluting the contents six times with water, 6 doses of 0.2 cc. were given to each rat. The specimen without tricresol (injected into 40 rats) produced a 58 % response; that with the tricresol (36 rats) gave a 44 % response, whereas standardisation before incubation (52 rats) showed oestrus in 50 % of the animals. These variations, however, are within the limits of error of the method of standardisation.

Ether extraction. The active material is soluble in ether and may be completely removed from the aqueous solution of the hormone by continuous extraction with ether. The ethereal extract on evaporation yields a slight colourless residue which dissolves completely in warm water and the solution contains active material. This has not yet been fully investigated quantitatively; there appears, however, to be a considerable loss of activity, but this may possibly be accounted for by peroxides which were present in the samples of ether used.

The nitrogen content. As will be seen from the last two columns of Table V the product made in this way always contains nitrogen, as a rule to the extent of 5 or 10 % of the total solids. This is, of course, no indication that the pure

hormone contains nitrogen, indeed a contrary result has been observed by Laqueur *et al* [1925] and by Allen and Doisy [1927], and as our analytical figures show, there is no relationship between the nitrogen content and the activity. The nitrogenous constituents appear to be non-volatile in steam, non-basic, or, if basic, to give a hydrochloride and sulphate soluble in ether, and about 15 % only of this total nitrogen is liberated by distillation with concentrated sodium or potassium hydroxides, the remainder being apparently stable to alkali. With the purest products the Millon reaction is exceedingly faint, whether performed in the usual way or with the modification of Weiss. The biuret reaction is negative. All batches have given a faint colour with Nessler's solution, the depth being of the same order as that given by a solution of ammonium sulphate containing 0.001 mg. nitrogen per cc. There does not appear to be any quantitative relationship between this coloration and the activity of the product. The main part of the nitrogenous constituent is extractable with ether from the aqueous solution. Investigation of these points is being continued.

Experiments upon the further purification of this material are in progress. Fractional precipitation with alcohol and ether shows that the potency can be greatly increased.

It may be noted that active material has been obtained from the urine of pregnant women by butyl alcohol extraction, followed by the process described earlier. Two litres of urine, when so treated, gave 240 cc. of aqueous solution of which 6 doses of 0.2 cc. given to 20 rats produced 12 oestrus responses. This corresponds to 120 units per litre, and hence confirms the work of Zondek [1928].

The preparation has been used clinically in several centres. Whilst it is as yet too early to state the results, experiments have proved that large doses may be given daily to women without any untoward results. The clinical reports up to date indicate that the preparation will have a definite application in the treatment of various disorders such as amenorrhoea and premature menopause. This aspect will be dealt with elsewhere.

SUMMARY.

1. A method is described whereby the oestrus-producing hormone is constantly obtainable in good yield from placenta in the form of an aqueous solution.
2. This material is inactive in a single dose but produces oestrus when given as a series of small injections. Standardisation of this product is discussed and the use of a method combining the multiple dose technique with the statistical method is described.
3. The weight of one unit as calculated on the basis of this method of standardisation is reduced to 0.01 mg.

4. The physical and chemical properties of this preparation are described, and it is shown that active material will pass through a porcelain filter and that it may be dialysed through parchment.

5. The material is stable to prolonged incubation and to short exposures to high temperatures.

6. The substance is without effect on blood-pressure or respiration and no ill effects have been observed when it has been injected daily for some months into patients.

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ADDENDUM.

When this paper was completed, the communication of Thayer, Jordan and Doisy [1928] came to our notice. These authors convert their very active, oily material into water-soluble form, by a method which presents many similarities to that used by Laqueur and his co-workers [1927]. The experiments described above have shown that our material, the unit of which is 0.01 mg. or more, is inactive when given in one dose or by the method described by Allen *et al.* [1924]. Unfortunately Thayer, Jordan and Doisy give no details of standardisation in the paper referred to and, therefore, it is not possible for us to compare the potency of their material with that of our own.

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