LII. THE CHEMISTRY OF OESTRIN.

III. AN IMPROVED METHOD OF PREPARATION AND THE ISOLATION OF ACTIVE CRYSTALLINE MATERIAL.

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Introduction.

The preparation of oestrus-producing concentrates from any source may be said to consist essentially of two main processes: (*a*) the extraction of the hormone together with fatty substances from the raw material and the removal of the bulk of the saponifiable fat from the extract, and (*b*) the purification of the resulting concentrate.

In previous communications [Marrian, 1929, 1, 2] such methods have been described. The part of the process which may be said to fall under (*a*) was carried out by purely empirical methods and it was emphasised that the yields could not be considered to approach the maximum possible. The subsequent processes were studied in a quantitative manner and it was shown that the extracts could be considerably purified in several ways with no detectable loss of potency.

In this work the initial stages of the process have been modified in view of the now generally accepted belief that oestrin is acidic in character [Funk, 1929; Marrian, 1929, 2; Butenandt, 1929, 1, 2; Laqueur, Dingemanse and Kober, 1930], and of the author's finding that the hormone can be apparently displaced from its alkali salts by carbon dioxide [Marrian, 1929, 2]. The aim throughout has been to obtain greater yields of active material from the urine of pregnancy, and to concentrate the extracts with the minimum loss to a stage from which the final isolation of the hormone could be attempted.

For this purpose a series of small batches of urine have been extracted and purified separately; accurate assays of the potency being made on the initial extract and the product in each case. These concentrates were combined and an attempt was made to effect a further purification. This was successful in that highly active crystalline material appearing to be a single substance was isolated.

Since this work was commenced Butenandt [1929, 1, 2] and Laqueur, Dingemanse and Kober [1930] have reported the isolation of active crystalline materials from urine which, judging from the combustion results, appear to be identical. The latter workers, however, are doubtful if this substance is actually the pure hormone itself. In both cases the actual methods of isolation are not given in detail, and no mention is made of the yields obtained. According to these authors Doisy also described the preparation of an active crystalline substance at the Physiological Congress in Boston last summer. So far, however, no details of Doisy's work have reached the author. Wieland, Straub and Dorfmuller [1929] have also reported the isolation of an active crystalline substance which, however, does not appear to approach purity.

Experimental.

Method of assay. A standard technique for assaying the activity of the various fractions was employed throughout the work. The fraction to be tested was dried, weighed and made up to a volume of 500 cc. in a mixture of equal parts of alcohol and chloroform. 0·5 cc. of this solution was withdrawn and diluted to 10 cc. with alcohol. A small volume of such a testing sample, usually 0·1 cc. to 1·0 cc. depending upon the amount of active material expected to be present, was withdrawn and evaporated to dryness in a 10 cc. volumetric flask. The residue was dissolved in 0·5 cc. alcohol and made up to 10 cc. with water. This solution was then injected into seven ovariectomised mice in doses graded from $4 \times 0{\cdot}05$ cc. to $4 \times 0{\cdot}5$ cc., each dose being given in four injections at 12-hour intervals. From such preliminary tests an approximate idea could be formed of the activity of the fraction.

With this information a fresh aqueous dilution of an aliquot portion of the testing sample was made and injected into a group of twenty mice in four doses of 0·1 cc., the activity being determined in the manner described in previous papers [Marrian and Parkes, 1929; Marrian, 1929, 1, 2].

Provided that not more than 70 % and not less than 15 % of the mice showed an oestrous response, the results were satisfactorily accurate. When the response did not fall within these limits, as was sometimes the case with such rough preliminary tests, it was thought desirable to repeat the tests.

The use of a small amount of alcohol to obtain an "aqueous solution" has not been found to affect the accuracy of the results and no ill effects on the test-animals have been observed in consequence. In any case, this method seems preferable to that adopted by Butenandt [1929, 2], in which the active material is injected in a solution of sesame oil. The repeated injection of oily solutions into mice almost invariably leads to severe subcutaneous granulations, which may result in the incomplete absorption of subsequent injections.

Collection and extraction of urine. The urine was collected each day in vessels containing a small quantity of toluene, acidified with concentrated HCl in the proportion of 5 cc. to 1 litre, and extracted four times with ether.

The ethereal extracts were washed once with water, evaporated to dryness and stored in alcoholic solution at 0°.

Saponification of the crude extract. When 50 litres of urine had been extracted, the alcoholic solution of the crude ether-soluble material was evaporated to dryness and heated with 400 cc. of 5 % aqueous potassium hydroxide solution in a boiling water-bath for 30 minutes. Carbon dioxide was then passed into the mixture for 3 hours. After dilution with an equal volume of water, the mixture was extracted twelve times with successive portions of 100 cc. ether. The combined ethereal extracts were washed four times with 50 cc. lots of 0·2 *N* HCl, twice with the same amounts of water and then evaporated to dryness.

Extraction with cold acetone. In a previous paper [Marrian, 1929, 1] it was shown that the unsaponifiable matter of the urine of pregnancy contained small amounts of a substance melting at about 233–234° which was believed to be a dihydroxy-alcohol.

Although the removal of this substance is probably unnecessary at this stage of the purification process, more of the substance was required for further examination. It was shown that this alcohol could be removed from the unsaponifiable matter by extraction with ice-cold acetone or ether, in either of which it is fairly insoluble. Since oestrin, according to recent work, has a low solubility in ether, the use of acetone seemed preferable for the purpose.

The dry unsaponifiable matter was dissolved in 30 cc. of boiling acetone and left at 0° for 12 hours. The flask was then immersed in an ice-salt freezing mixture for 1 hour to complete the separation of the alcohol, and the mixture filtered through an ordinary filter. The flask and filter were washed with a further 10 cc. of cold acetone. The residue in the filter was then washed back into the original flask with a boiling mixture of chloroform and alcohol. This was evaporated to dryness and treated with 15 cc. of acetone in precisely the same manner to remove remaining traces of the hormone. The combined acetone extracts, on evaporation to dryness, yielded a reddish brown gum.

Extraction with 50 % alcohol. The acetone-soluble fraction was heated for 5–10 minutes to boiling with 30 cc. of 50 % alcohol, and then kept at 0°. The next day the mixture was filtered, the flask and filter being washed with another 10 cc. of ice-cold 50 % alcohol. The insoluble material on the filter was washed back into the original flask with a hot alcohol-chloroform mixture. This solution, after evaporation to dryness, was treated with 20 cc. of 50 % alcohol with another washing of 10 cc. in the same way. The latter process was repeated twice, making in all four extractions with 50 % alcohol.

The combined 50 % alcohol filtrates were evaporated to dryness.

Extraction from ethereal solution with aqueous alkali. It was shown in a previous paper [Marrian, 1929, 2] that, owing to the acidic properties of oestrin, a considerable degree of purification could be attained by extraction of an ethereal solution of the hormone with aqueous alkali. By acidification

and ether extraction the oestrin could be recovered quantitatively from the alkaline solution.

The fraction soluble in 50 % alcohol was dissolved in about 10 cc. of alcohol and then diluted with 700 cc. of ether. The low solubility of this material in ether did not permit direct solution. This solution was then extracted ten times with successive 50 cc. lots of 5 % aqueous potassium hydroxide. The combined alkali extracts were acidified with 110 cc. of 12 *N* HCl and extracted eight times with 100 cc. portions of ether. The combined ethereal extracts were washed three times with water and evaporated to dryness.

The final product varied considerably in colour and appearance from batch to batch, but in general it may be described as a brown gum containing varying amounts of what appeared to be crystalline material.

The results of the whole method of concentration are shown in Table I. These results clearly indicate that by these methods very large yields of active material can be obtained and that the initial extracts can be enormously reduced in bulk with only about 40 % loss of activity.

Each fraction of batch P.U. 7A was assayed accurately to determine at which stage of the purification this loss occurred. The results given in Table II definitely show that practically the whole of the loss occurs during the saponification. The extraordinary uniformity of the extent of this loss, with the exception of batch P.U. 7C, suggests that incomplete extraction by the ether is not the cause. This point is now being investigated.

Table I.

	Batch No. ...	P.U. 7A	P.U. 7B	P.U. 7C	P.U. 7D	P.U. 7E	Average
	Vol. urine (litres)	50	50	50	50	50	50
Initial acid ether extract	Wt. (g.)	22	21	21	18	20	20
	No. m.u.	2,069,000	980,000	850,000	444,000	792,000	1,027,000
	Yield, m.u. per litre	41,400	19,600	17,000	8,880	15,800	20,500
Final product	Wt. (g.)	0·364	0·253	0·471	1·107	0·516	0·342
	No. m.u.	1,208,000	546,000	614,000	267,000	445,000	616,000
	Yield, m.u. per litre	24,160	10,920	12,280	5,340	8,900	12,300
	Wt. 1 m.u. (mg.)	0·000301	0·000463	0·000767	0·000399	0·00116	0·000618
Loss in purification (%)		42	44	28	38	44	40

Table II. *Batch P.U. 7A.*

	Wt. (g.)	No. m.u.	Loss in m.u. (%)
Initial ether extract	22	2,069,000	—
"CO_2" unsaponifiable matter ...	2·845	1,278,000	38
Acetone-soluble	2·456	1,236,000	40
50 % alcohol-soluble	0·742	1,236,000	40
Alkali extract from ether solution	0·364	1,208,000	42

Extraction by ether from alkaline solution. According to Funk [1929] the alkali metal salts of oestrin possess a definite solubility in ether and on this account the hormone is present in the unsaponifiable matter from active fatty material. If this is the case, it should be possible, by thorough extraction with ether from alkaline solution, to remove all the activity.

Since the use of carbon dioxide in the saponification of these batches results in the presence of a large amount of material that would not be present in the unsaponifiable matter if prepared by ordinary methods, it was thought that repeated extraction of an alkaline solution by ether, without the use of carbon dioxide, might prove to be an efficient method of purification.

The whole of batch B was used to try out this method. The material, weighing 0·253 g. and containing 546,000 mouse units, was treated with 400 cc. of 5 % aqueous potassium hydroxide in which it readily dissolved. This solution was diluted with 300 cc. of water and then extracted twenty times with 100 cc. lots of ether. The combined ethereal extracts were washed first with 0·5 *N* HCl and then three times with water, and on evaporation yielded 0·0588 g. of solid material. On assaying the activity of this fraction, it was found that only 82,400 mouse units had been extracted. This corresponds to an activity of 1 m.u. = 0·000704 mg. as against the activity of the original material of 1 m.u. = 0·000403 mg. This method therefore is entirely worthless for the further purification of these extracts, and it seems extremely doubtful if the alkali salt of the hormone is appreciably soluble in ether. It seems more probable that the presence of oestrin in unsaponifiable matter is due to adsorption on the ether-soluble material.

The active material was recovered from the extracted alkaline solution by acidification and ether extraction, the two fractions being combined and re-tested.

Preparation of water-soluble extract. With part of the material obtained by the recombination of the fractions from the last experiment, an attempt was made to prepare an aqueous solution of such potency as might be useful for clinical work.

The material containing 235,000 mouse units was treated with 25 cc. of hot 0·1 *N* NaOH. The main bulk dissolved, yielding a deep red-brown solution containing a small amount of finely divided material in suspension. This solution was then neutralised carefully with 0·1 *N* HCl, using phenolphthalein as external indicator. By careful adjustment a point was reached where the solution was just acid to phenolphthalein, but no precipitation occurred. 0·25 cc. of "tricresol" was added and after making up to a volume of 250 cc. with water, the solution was left standing in a tall cylinder to allow the few suspended particles to flocculate and settle.

On assaying a portion of the clear supernatant liquid, which had a light brown colour, it was found to contain 670 mouse units per cc., so that the total activity corresponded to 168,000 mouse units. The remaining 67,000 mouse units were presumably adsorbed on the small amount of insoluble material.

Isolation of active crystalline material.

Butenandt [1929, 2] observed that the active crystalline substance isolated by him had a low solubility in ether. This fact suggested the possibility of using ether or a mixture of ether and some other solvent for the separation of solid material from the urine concentrates.

In preliminary experiments direct crystallisation from ether was found to be impracticable, owing to the low solubility of the concentrates even in large volumes of boiling ether. By solution in a very small volume of alcohol and addition of an excess of ether, however, a large amount of solid material could be precipitated.

The combined concentrates from batches P.U. A, C, D and E, weighing 1·457 g. and containing 2,534,000 mouse units, were treated in this way. The dried material was warmed for a short time with 0·75 cc. of alcohol, when it softened to a mobile oily consistency. To this were added 10 cc. of ether, when a heavy granular precipitate was thrown down. The flask was placed in an ice-salt freezing mixture for 45 minutes, and then filtered rapidly on a small Büchner funnel. The filtrate was evaporated to dryness and again treated with alcohol and ether in the same manner, when a further small amount of solid was separated. The combined solid precipitates were washed with a few cc. of alcohol-ether mixture to remove as much of the more soluble gummy material as possible.

This solid material was light brown in colour and weighed 0·1442 g. On assaying, it was found to contain 1,233,000 mouse units, or about half of the total, corresponding to an activity of 1 m.u. = 0·000116 mg. This is slightly more potent than the concentrate described by the author in a previous paper [Marrian, 1929, 2].

The material was still too highly pigmented for purification by means of recrystallisation to be effected. An attempt was therefore made to clean it by treatment with charcoal. The solid matter was dissolved in 20 cc. of alcohol and boiled under a reflux condenser with a small amount of "norite" for 30 minutes. The mixture was filtered hot, the flask and filter being washed with four successive 10 cc. portions of boiling alcohol. The alcoholic filtrate was evaporated to dryness and the process repeated a second time.

The product was now nearly white in colour. The weight was 0·1439 g., showing that the pigment removed formed only a small fraction of the total bulk. It contained 1,050,000 mouse units, corresponding to an activity of 1 m.u. = 0·000137 mg. It is doubtful if this apparent slight decrease in potency is significant. The difference of about 13 % is only slightly outside what is believed to be the probable error in the method of assay. Preliminary experiments showed that this material, which was amorphous, could be crystallised from aqueous methyl alcohol. It was therefore dissolved in 10 cc. of boiling 57 % methyl alcohol and left overnight at 0°. 0·0767 g. of crystalline material were obtained in this way. The crystals were still slightly

brown in colour, but the bulk of the pigment appeared to be in the mother liquor. The recrystallisation was repeated with 5 cc. 57 % methyl alcohol. The twice recrystallised material weighed 0·0613 g. Two tests on this gave totals of 498,000 and 556,000 mouse units, corresponding to an average activity of 1 m.u. = 0·000116 mg. The potency is not considered to be significantly different from that of the original non-crystalline solid.

The mother liquors from the two crystallisations were evaporated to dryness and treated with 12 cc. of boiling 42 % methyl alcohol. On cooling to 0° a further crop of crystals was obtained, which were rather more pigmented than those of the first batch. This material weighed 0·0275 g. and contained 256,000 m.u., corresponding to an activity of 1 m.u. = 0·000108 mg.

The mother liquor from this recrystallisation contained less than 125,000 mouse units.

Properties of the crystalline substance.

The first batch of crystals were very slightly pigmented. On microscopic examination two types of crystals were observed (Plate II A). It was shown, however, that these two types were merely different forms of the same substance. On recrystallisation from 57 % methyl alcohol at 0°, the crystals were all of the type shown in Plate II B. On recrystallisation at ordinary temperatures, the crystalline form was of the second type shown in Plate II A.

The substance appeared to have no definite melting point. From 222° onwards a slight darkening and shrinkage were observed. At 256–260° the substance melted completely and simultaneously decomposed.

On treating a small quantity of the substance in chloroform solution with H_2SO_4 and acetic anhydride (Liebermann-Burchardt reaction) a pale orange-yellow colour with a marked green fluorescence resulted. This is very similar to the colour reaction observed by Wieland, Straub and Dorfmuller [1929] with their preparations. Butenandt [1929, 1], on the other hand, observed no characteristic colour reaction with these reagents.

A preliminary micro-combustion and molecular weight determination showed that the substance contained three oxygen atoms to about eighteen carbon atoms. The percentage of carbon was somewhat lower than that in the substances isolated by Butenandt [1929, 1, 2] and Laqueur, Dingemanse and Kober [1930]. In view of the fact that there is at present no satisfactory criterion of purity, these results are probably only of value in so far as they indicate the approximate molecular dimensions. The close agreement between the observed value for the molecular weight and the figure corresponding to the formula suggested by the analysis, is probably fortuitous.

Nitrogen could not be detected in the substance.

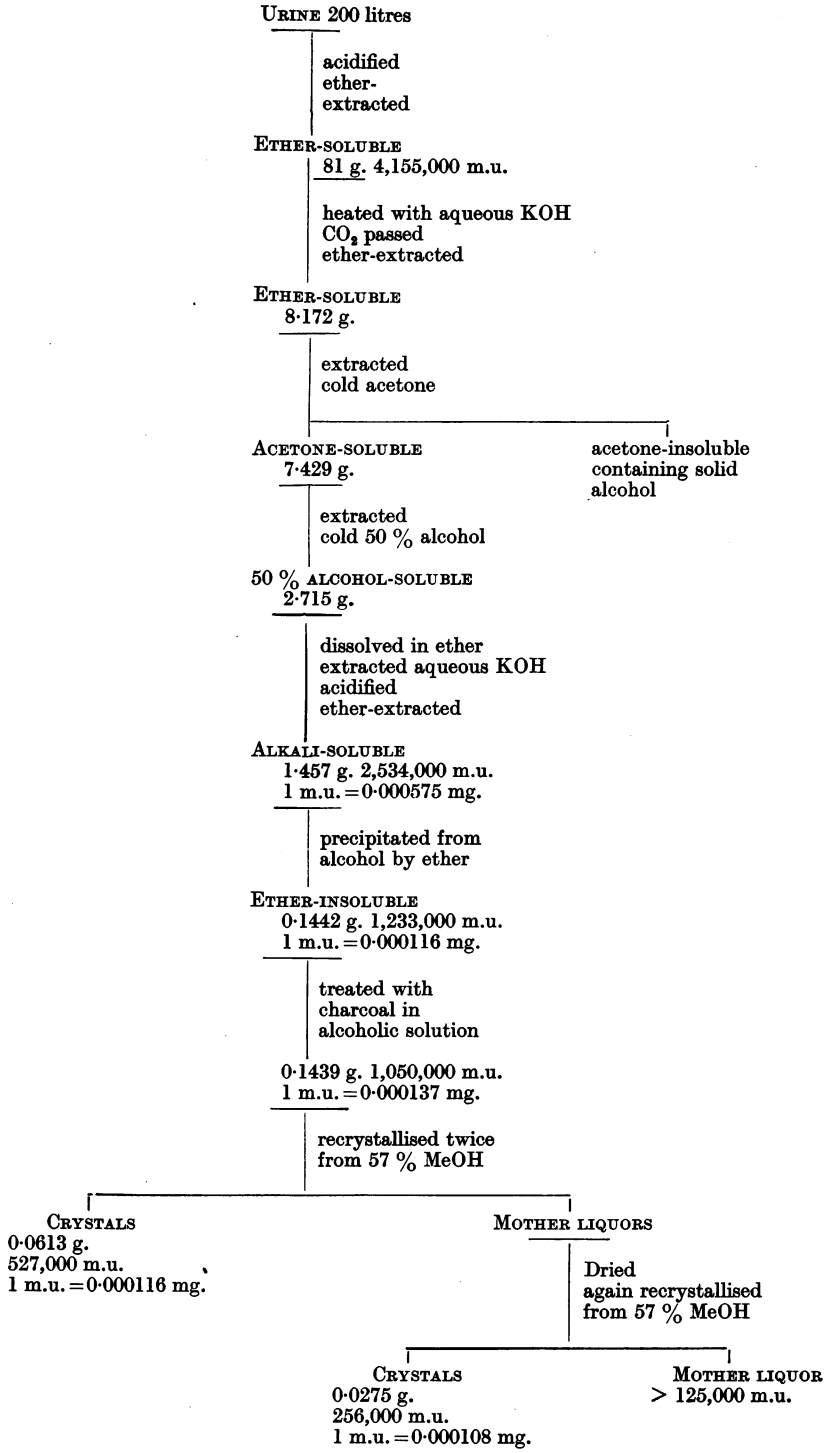
4·541 mg. gave 12·415 mg. CO_2 and 3·50 mg. H_2O.

C 74·53 %, H 8·56 %.

Mol. wt. by Rast's camphor method: 288, 289.

Calculated for $C_{18}H_{24}O_3$: C 75·00 %, H 8·33 %, molecular weight 288.

Table III. *The purification of batches P.U. 7 A, C, D and E.*



Discussion.

By the methods described in the earlier part of the paper, very large yields of oestrin can be obtained in a concentrated form from the urine of pregnancy with a relatively small loss of total activity. Such concentrates make a suitable starting point for the preparation of aqueous solutions suitable for clinical or physiological use. The advantage of the method of obtaining aqueous solutions of the hormone, as described in this paper, over methods previously in general use, is that far higher concentrations of the active substance can easily be obtained. The only limit appears to be the solubility of the alkali salts of the hormone and of the substances associated with the hormone. In the older methods, the very much lower solubility of the free acidic substances in water was the limiting factor. The importance of such highly concentrated aqueous solutions for physiological and clinical use is made clear by recent work carried out in collaboration with Dr A. S. Parkes [Marrian and Parkes, 1930].

Two points in connection with the crystalline substance are at present not clear. In the first place, are the active crystalline compounds isolated by Butenandt [1929, 1, 2], Laqueur, Dingemanse and Kober [1930] and the author identical? In the second place, are any of these substances the pure hormone itself, or are they merely inactive substances on which is adsorbed a small amount of the active hormone?

Butenandt [1929, 2] observed that his active substance melted with decomposition at 240°, Laqueur, Dingemanse and Kober [1930] do not record a melting point for their substance[1], while the author's preparation melted very indefinitely with decomposition at 256–261°. The decomposition which occurs makes it obvious that the melting points cannot be accepted as satisfactory criteria of purity.

The close agreement between the combustion figures of the first two preparations suggests that these two substances may be identical (Butenandt, C 78·31 %; H 8·13 %; Laqueur, Dingemanse and Kober, C 78·61 %; H 8·25 %). A somewhat lower figure for the carbon content was, however, obtained for the author's preparation.

Butenandt reports an activity of 8 million mouse units per g., Laqueur, Dingemanse and Kober 8–10 million per g., while the author's preparation has also an activity of 8 million mouse units per g. These figures, however, are not so uniform as they might at first appear, as the methods of testing are different in each case. Laqueur and de Jongh [1929] test their materials in six doses spread over a period of 48 hours. A similar technique has been adopted by Allan, Dickens and Dodds [1930]. The latter show that this method gives approximately a 25 % higher response than that of giving four doses over 36 hours. Experiments confirming this relationship between the two methods have been carried out by the author.

[1] Shortly after going to press, a more detailed paper appeared in *Deutsch. med. Woch.* from Laqueur's laboratory, in which the melting point of crystalline "Menformon" is given as 240°: decomposition is not mentioned.

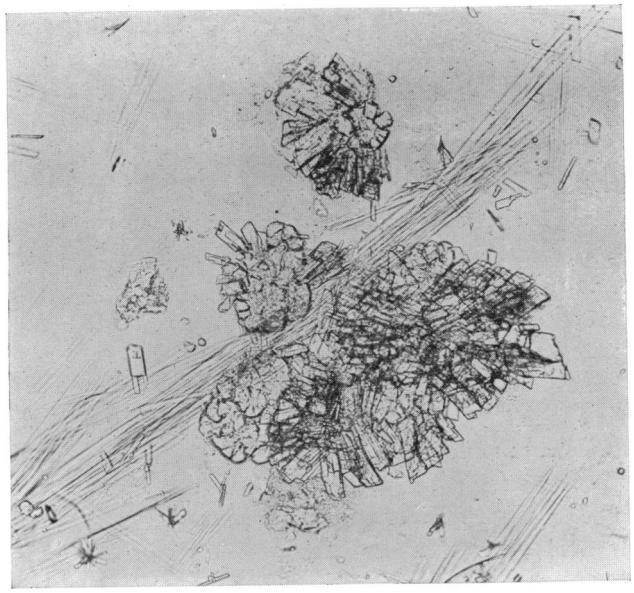
Butenandt [1929, 2], on the other hand, administered his preparations as a single dose in sesame oil solution. In later work [Butenandt and Ziegner, 1930], he shows that when four and six injections are administered over 36 and 40 hours respectively, the apparent activity of the preparation is raised from 8 to 30 and 40 million mouse units per g. respectively. These results, while providing further data on the relationship between the four and six dose methods, are of little value for deciding the point at issue, namely, the relationship between the activity of a preparation given in a single dose in oil solution and that of a multiple dose in what is virtually an aqueous solution. Allan, Dickens and Dodds [1930] show the relationship between single and multiple doses in aqueous solution and also single doses in an oil-water emulsion. The latter is, however, hardly the same as a true oil solution. Rapid absorption of the hormone must occur directly from the aqueous phase.

A few experiments have been carried out by the author bearing on this question. Graded doses of the active crystalline material dissolved in sesame oil were administered to a few mice by the method in which four injections are given. Too much reliance cannot be placed on the results, since groups of twenty were not used on account of the adverse effects of the oil injections. The results indicated, however, that the material had at least twice the apparent activity displayed when injected in aqueous solution. Thus, tested by this method, the crystalline material has an activity of about 16 million units per g. This, although lower than Butenandt's figure of 30 millions, is more nearly of the same order.

The remaining discrepancy might be accounted for by a difference in the total volume of oil injected. Marrian and Parkes [1929] showed that, with aqueous solutions, the volume of the dose had no appreciable effect upon the apparent activity. With oil solutions this cannot be the case. Within certain limits the activity of a preparation varies inversely with its rate of absorption when administered subcutaneously. Absorption from a large volume of oil must necessarily be slower than from a small volume, and an increased apparent activity may result with the larger volume. With still larger volumes of oil, the rate of absorption must be still further slowed and then presumably a decrease in apparent activity would be observed. With aqueous solutions the rate of absorption is so rapid that these considerations do not apply.

It is considered, therefore, that the apparent difference between the activity of Butenandt's material and the one described here is not so great as might at first appear.

Whether these crystalline substances are actually the hormone itself is a point that can only be decided by future work. Laqueur, Dingemanse and Kober [1930] are doubtful on this point, since some of their preparations showed an activity of as much as 14 million mouse units per g. Until the activity has been shown to remain constant after repeated recrystallisations from different solvents, no conclusions should be drawn.



A

B

Active crystalline substance isolated from the urine of pregnancy.

SUMMARY.

1. By acidification and ether-extraction of urine from pregnant women, yields of oestrin varying from 8000 to 40,000 mouse units per litre can be obtained.

2. Methods are described by which these extracts can be greatly purified with a loss of only 40 % of the activity throughout the process. This loss occurs entirely in the initial stage of saponification. The mouse unit of these purified preparations is of the order of 0·0006 mg.

3. From such purified preparations an active crystalline substance has been isolated. The mouse unit of this substance is about 0·00011 mg.

4. The possible identity of this substance with the active substances recently isolated by Butenandt and by Laqueur, Dingemanse and Kober is discussed.

5. There is no proof at present that this substance is actually the pure hormone.

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REFERENCES.

Allan, Dickens and Dodds (1930). *J. Physiol.* **68**, 348.
Butenandt (1929, 1). *Naturwiss.* **45**, 879.
—— (1929, 2). *Deutsch. med. Woch.* **55**, 2171.
—— and Ziegner (1930). *Z. physiol. Chem.* **188**, 1.
Funk (1929). *Proc. Soc. Exp. Biol. Med.* **26**, 568.
Laqueur, Dingemanse and Kober (1930). *Nature*, **125**, 90.
—— and de Jongh (1929). *J. Pharm.* **36**, 1.
Marrian (1929, 1). *Biochem. J.* **23**, 1090.
—— (1929, 2). *Biochem. J.* **23**, 1233.
—— and Parkes (1929). *J. Physiol.* **67**, 389.
—— —— (1930). *J. Physiol.* (In press.)
Wieland, Straub and Dorfmuller (1929). *Z. physiol. Chem.* **186**, 97.