CXXIX. OBSERVATIONS UPON THE PREPARATION AND STANDARDISATION OF THE OVARIAN HORMONE.

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ATTEMPTS to demonstrate the presence of an active principle in the ovary date back to the end of the last century, but the first real advance was made by Adler [1912], who published an account of the production of artificial oestrus in animals by the injection of aqueous extracts of whole ovaries. This investigation is entitled to special notice, since it was the first in which a definite attempt at standardisation was made. Adler checked his results by histological examination of the uterine mucosa of his test animals. This work was followed by a series of more elaborate chemical investigations. Iscovesco [1912] extracted whole ovaries with alcohol and obtained a fatty substance possessing activity. Fellner [1913] demonstrated that the active principle was thermostable and soluble in alcohol, ether and acetone.

Very similar results were obtained by Seitz, Wintz and Fingerhut [1914], and later by Herrmann and Fränkel [1915]. The method of the latter workers consists in extraction of the ovaries with alcohol. The solid material is filtered off, and the resulting solution is evaporated to dryness. The residue is extracted with ether, and acetone is added to precipitate the lipoid fraction. The active principle remains after the solvent has been removed by distillation. As much cholesterol as possible is separated from it by fractional crystallisation, after which it is stated that the material can be distilled *in vacuo* at 190°.

Recently the matter has been re-opened by Allen and Doisy [1923], who applied the vaginal smear method of ascertaining the onset of oestrus. Their method for the preparation of the active material is similar to that described by Herrmann and Fränkel. They were able to prepare active material from liquor folliculi, whole ovaries and placenta, but not from corpus luteum. They lay great stress upon the use of liquor folliculi, and appear to incline to the view that the active principle is located mainly in this fluid. In view of the importance of these observations, it was thought that an investigation of these earlier experiments would be of interest.

Method of preparation.

This consists of slight modifications of those employed by Herrmann and Fränkel, and Allen and Doisy. Pigs' ovaries were used as the main source of supply.

The minced tissues were extracted with twice their weight of alcohol, care being taken not to lose any fluid during mincing. After stirring, the mixture was pressed out in a hand press through jean, and the solid material was then extracted in a Soxhlet apparatus with alcohol for 6-9 hours. The alcoholic extracts were then combined and filtered through Chardin papers.

The filtrate was evaporated almost to dryness *in vacuo*, and the residue was taken up in a small volume of alcohol and filtered. On standing, a precipitate of fats and cholesterol separated which was removed by filtration. The solution was again taken down to dryness, and the residue was dissolved in a small quantity of ether, and twice its volume of acetone was added. The precipitated lipins were filtered off. The solid material was then redissolved in ether and again precipitated with acetone. This process was repeated several times. The filtrates were then combined, and evaporated to dryness. The residue was dissolved in a small quantity of alcohol, and some cholesterol was removed by freezing. By repeating this process, the greater part of the cholesterol can be removed. The final product is obtained by distilling off the alcohol *in vacuo*. Some experiments were tried with alternative methods of extraction. The following is an example.

Aqueous extraction.

A small yield of active substance was obtained by mincing 2.78 kg. ovaries (pig) and washing thoroughly with 2.8 l. water. This washing was repeated once more with the same volume of water and the combined washings were acidified until the reddish colour of the washings changed to grey. The washings were concentrated *in vacuo* in a bath at 65° until the volume was approximately 150 cc. Two volumes of alcohol were added and then dilute sodium hydroxide solution until the maximum precipitation occurred. The solution was filtered and the filtrate was once more concentrated to dryness. The solid matter was taken up in hot 95 % alcohol, the solution was filtered and lipins were precipitated by the addition of two volumes of acetone. The precipitate was filtered off and the liquid concentrated. The yield was 4.4 g., the rat-unit of which was 150 mg., corresponding to about nine rat-units from 1000 g. ovaries.

Yields.

The method of preparation already described has been applied to a series of tissues. The ovaries were not specially selected, and were therefore obtained from animals in various stages of the oestrous cycle.

In the following table the yields obtained can be seen.

Table I.

Source	No. of animals	Weight of tissue	Yield g.	Rat unit mg.	Rat unit per kg.	Notes
<i>Pig ovaries, whole ovaries including liquor folliculi</i>	148	1050 g.	1.05	75	14	Without Soxhlet extraction
	70	960	1.14	80	14	
With Soxhlet extraction:						
	92	720	3.69	15	340	
	218	2100	28.8	150	91	
	280	4650	33.9	100	73	
	240	2290	36.0	200	80	
	800	6960	30.35	50	87	Ovaries had very small follicles
			crude			
			15.85	30	70	
			purified			
	228	2140	7.6	50	70	
	290	5410	17.75	50	65	
<i>Liquor folliculi only (pig)</i>		250 cc.	0.4	16	100	
		720 cc.	3.7	25	200	
		120 cc.	1.20	50	200	Four ovaries only each with large follicles
<i>Tissue only from above</i>		50 g.	2.25	200	255	
<i>Human ovarian cyst</i>		1100 cc.	Trace	Inactive		
<i>Placenta (human)</i>		385 g.	3.1	200	50	
			crude			
			0.45	15	85	
			purified			
<i>Dried pig ovaries</i>		200 g.	13.0	350	150	Dried in vacuo at 40°

The yields are similar to those obtained by Allen, Doisy and their co-workers. We have been unable, however, to secure the large yields from liquor folliculi, and from human placenta, described by these workers. The yields obtained by us from liquor folliculi and from the residual ovarian tissue are of the same order. Our findings, therefore, do not support the contention that the active principle is mainly confined to the liquor folliculi. Of a series of commercial preparations tested all proved inactive. One specimen of whole ovaries, dried at 40°, was found to yield 150 rat-units per kg. by the above method of extraction. No active principle could be obtained from hen's eggs. As a negative control, 1 kg. pancreas was worked up by the process. No active material resulted.

Chemical properties.

The active principle consists of a clear, light brown oil, readily soluble in alcohol, ether, acetone, and olive oil. It can be heated to 200° without loss of activity. It withstands saponification only to a limited extent, as can be seen from the following experiments.

1 g. sodium was dissolved in 20 cc. absolute alcohol, and the solution added to 10 cc. of an alcoholic solution of the active principle containing 6.28 g., the rat-unit being 40 mg. The mixture was heated on a boiling water-bath, in an atmosphere of hydrogen for 40 minutes. After cooling, the contents of the flask were poured on to powdered ice, and the resulting liquid was thoroughly extracted with ether. The extracts yielded 0.4 g., which was found to give a positive reaction in rats in doses of 40 mg. The fatty acids obtained

by acidification of the aqueous layer were found to be inactive physiologically, even in doses of 0.5 g. In another experiment, where the reaction was allowed to proceed for 3 hours, the product was quite inactive. It appears therefore that the activity is slowly destroyed under these conditions. Attempts at distillation have up to the present been unsuccessful, owing to excessive frothing.

Purification by digitonin.

The cholesterol-containing substance obtained by the usual alcoholic extraction process (3 g. = 37 rat-units) was dissolved in 60 cc. hot 95 % alcohol. A solution of digitonin (1 g.) in hot 90 % alcohol (100 cc.) was added, and after 1 hour the fine crystalline precipitate was filtered off, and the filtrate was concentrated to dryness on the water-bath. The residue was taken up by ether, the extract filtered and the ether removed. The clear, pale yellow, oily residue (0.75 g.) no longer gave the Liebermann-Burchardt reaction for cholesterol. By this treatment with digitonin the rat-unit was reduced from 80 mg. to 25 mg. The above yield therefore represents approximately 30 rat-units, showing that there is little loss in this method of purification.

Method of testing extracts.

This depends on the work of Stockard and Papanicolaou [1917] with the guinea-pig, which was extended to the rat by Long and Evans [1922]. It was demonstrated that a definite oestrous cycle exists in these animals, and that the various stages of the cycle can be accurately determined by examining smears from the vagina. Four stages of the oestrous cycle in the rat can be recognised: pro-oestrus, oestrus, post-oestrus and post-ovulation. The total duration of the cycle is 96 hours. The changes found in the smears depend on proliferation of the vaginal epithelium and keratinisation of these cells, proliferation of the uterine mucous membrane and migration of leucocytes. In cases of doubt, the animal may be killed and the genital organs examined both with the naked eye and microscopically.

Ovarian extracts were standardised by the method of Allen and Doisy, *i.e.* by determining the minimum amount which will bring about artificial oestrus in ovariectomised adult animals. Rats were used almost exclusively. The extracts were injected subcutaneously, dissolved in olive oil or suspended in weak bicarbonate solution. If the extract was active, oestrus appeared sometimes after 24 hours and usually within 36 hours of the time of injection.

The extracts containing much cholesterol were not easily absorbed and large "granulomata" appeared at the site of injection. When extracts freed from cholesterol were employed, practically no nodules resulted or they were much less marked. The total dose of extract to be given can be administered at one injection as absorption is quite slow. The same results were obtained from one dose as from a spread-out series of small doses which amounted to the same total. The responsiveness of the animals was found to wear off with

the lapse of time after ovariectomy and larger doses became necessary for the production of oestrus. Thus 12 mg. of a liquor folliculi extract produced definite oestrus in a freshly spayed rat, while 50 mg. produced only slight pro-oestrus in a rat spayed 3 months previously. It must be remembered that 3 months corresponds to 30 oestrous cycles in the rat, which is equivalent to $2\frac{1}{2}$ years in the human female. After 3 months the genital organs of ovariectomised rats are very thin and resemble fibrous cords. The effect of the preparation was also tested upon a female monkey: 100 mg. were injected subcutaneously, and within 3 days typical menstruation commenced. That this response was not the onset of a normal period was excluded by previous observations upon the animal. In a series of experiments in which the extract was administered by the mouth to ovariectomised rats, oestrus could not be induced even when very large doses were given.

Depressor effect.

Intravenous injection of active ovarian extract dissolved in olive oil was carried out on anaesthetised animals. As controls, solutions of cholesterol in olive oil were employed. As will be seen from Fig. 1 A the control injections produced a slight irregularity of the blood pressure in doses from 1–4 cc. The ovarian extract had a marked depressor effect; a fall of 24 mm. Hg resulting from injection of 0.1 cc. (Fig. 1 B) and a fall of 46 mm. Hg from a dose of 0.2 cc. (Fig. 1 C). With a dose of 1 cc., the depressor effect was more prolonged (Fig. 1 D). No marked effect on respiration was noted.

Fig. 1. Cat. Ether. Blood pressure. At A, 4 cc. of emulsion of cholesterol in olive oil injected. At B, ovarian extract in olive oil 0.1 cc. At C 0.2 cc. At D 1 cc. Time in seconds.

One experiment was carried out in which daily injections of ovarian extract were administered to a rat over a period of 14 days. One rat-unit was contained in each 1 cc. of the solution used and the injections were given daily in doses of 1 cc.

Smears from the vagina were studied. Oestrus appeared normally after 36 hours. Typical cornified cells were present till the sixth day of the experiment. No leucocytes or fibrin were found. On the sixth day a characteristic pro-oestrus smear appeared followed by oestrus cells on the seventh day.

Further pro-oestrus smears were obtained on the tenth day and succeeded by oestrus cells. On the fourteenth day the animal was killed. The uterine horns and body were distended and considerably congested. The vagina was widely dilated, *i.e.* typical oestrus conditions were present. Apparently the effect of continuous injection of the ovarian principle was greatly to prolong the period of oestrus. The post-ovulatory stage (appearance of leucocytes) was not noted and there was no resting stage; but oestrus lasting as long as 5 days was followed by a new pro-oestrus and then further oestrus. Throughout the period of the experiment the vaginal fluid had the characteristic watery appearance with small opaque granules like small rice grains as is seen in natural oestrus.

Interaction between insulin and the active principle.

That the onset of menstruation disturbs the carbohydrate metabolism to some extent has been pointed out by many workers.

Thus it is known that the advent of the period in a diabetic patient is often accompanied by an increase in the blood and urine sugar content, although the diet remains constant.

This suggested that there might be some interaction between insulin and the active principle of the ovary. Since the oestrous cycle has been fully worked out in mice, and their response to insulin is also well known, it was decided to employ these animals to test the point.

In the following tables can be seen the effect of varying doses of insulin upon mice not in oestrus, mice in natural oestrus, and mice in which artificial oestrus has been induced by injection of the ovarian principle.

A similar experiment was performed upon rabbits, the results of which appear in Table V.

Effect of insulin on female mice.

The mice were maintained at a temperature of 28°.

Table II. *Normal mice, not in oestrus.*

Insulin solution: a clinical solution diluted 1 in 300.	
Dose cc.	Result
0.5	Completely comatose 1 hr. 25 mins. after injection. Revived by glucose
0.4	Comatose 2 hrs. after injection. Revived by glucose
0.3	Slightly sleepy condition 2 hrs. after injection
0.2	Nil
0.1	Nil

Table III. *Normal mice in natural oestrus.*

The same solution of insulin was injected as in Table II.

Dose cc.	Result
0.5	No effect visible up to 5 hrs later, when the mice were fed
0.4	
0.2	
0.1	

Table IV. *Mice in artificial oestrus induced by injection of ovarian extract (from pig's ovaries).*

Insulin solution was a 1 in 100 dilution of a clinical solution.

Dose cc.	Mice in artificial oestrus	Non-oestrous controls
0.4	No apparent action	Comatose in under 2 hrs. and recovered on administering glucose
0.2		

Table V. *Effect of ovarian extract on the reaction to insulin of normal rabbits.*

Rabbit no.	Insulin weight mg.	Blood-sugar		
		Resting	1 hour	2 hours
Before injection of ovarian extract:				
9	0.5	110	70	40
12	1.0	98	40	29
14	1.5	120	33	19
After five injections 0.1 g. of ovarian extract at six hourly intervals:				
9	0.5	110	97	89
12	1.0	125	79	54
14	1.5	105	50	41

These experiments demonstrate the fact that the active principle of the ovary possesses the property of inhibiting the action of insulin. It is interesting to note that animals in natural oestrus respond in the same manner as those in which artificial oestrus has been induced.

SUMMARY.

1. A physiologically active principle can always be extracted from the ovary by means of alcohol, as a number of earlier workers have found.
2. The substance is an oil, soluble in alcohol, ether and acetone, and can be obtained free from cholesterol. Experiments are described showing its behaviour towards sodium ethoxide on saponification.
3. The method of standardisation is discussed, together with the general physiological properties.
4. By means of a series of injections an ovariectomised rat was kept in a state of continuous oestrus for a period of 14 days.
5. Administration by the mouth proved to be ineffective.
6. The extract was found to have a marked depressor action.
7. It was found that the substance inhibited the blood-sugar reducing action of insulin.

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