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ACETYLCHOLINE CONTENT OF UTERI BEFORE AND AFTER ADMINISTRATION OF OESTRIN TO OVARIECTOMIZED RABBITS

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It is well known that administration of oestrin to ovariectomized rabbits causes hyperaemia of the uterus beginning some time between a few minutes and an hour after injection [Markee, 1932; Pompen, 1933]. The cause of this response is not understood; it might be attributed to the effect of oestrin upon the metabolism of the uterus, were it not a fact that the initial hyperaemia reaches its peak before the level of oxygen consumption of the uterus has begun to be raised appreciably by the treatment [MacLeod & Reynolds, 1938; Reynolds, 1938*b*]. The vasodilatation is not therefore an indirect effect of the hormone attributable to an elevated uterine metabolism, and another cause must be sought.

Pertinent to this observation is the further fact, established by visual observation of the uterus through abdominal windows in unanaesthetized rabbits [Pompen, 1933], that the initial vascular effects of oestrin may be inhibited by injection of atropine sulphate. According to current concepts of the nature of the action of atropine, this means that the effect may be due to acetylcholine, since the action of this substance on certain tissues is characteristically blocked by atropine [Dale, 1938; Cannon & Rosenblueth, 1937]. For this reason, the present experiments were carried out to measure the acetylcholine content of uteri before and after administration of oestrin [Amniotin, Squibb, 100–400 I.U./kg. body wt.] to ovariectomized rabbits. The hormone was carried in oil. Since the quantity of extract obtainable from any one uterus is small, it was necessary to compare assays made on several groups of rabbits.

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Procedure

The rabbits used were of mixed stock, purchased on the open market. Each animal was isolated for a period of three or more weeks prior to the operation of bilateral ovariectomy. The rabbits were not used for the experiment until 17–32 or more days post-operatively, with the exception of one rabbit used on the 8th day and another used on the 14th day.

Some uteri were obtained for extraction after killing the rabbits by concussion, others after killing by intravenous injection of air, and a few uteri were obtained under nembutal anaesthesia. The manner of killing the rabbit did not appear to affect in any way the subsequent results. The uterus was removed quickly through a long abdominal incision. It was held by means of forceps clamped on the Fallopian tubes. As the uterus was elevated it was freed cleanly from the mesometrium down to and including the cervices; the vaginal tissues were trimmed away. After making certain that the uterus was empty of fluid, it was weighed and placed in 10 % trichloracetic acid, 1½–2 c.c./g. of fresh tissue. From this point on, the methods of extraction and testing were exactly those indicated by Chang & Gaddum [1933], with the exceptions noted below which were necessary because of the small amount of tissue available for each experiment.

The uterus was finely minced in the trichloracetic acid, and allowed to stand for 2 hr. with frequent mixing (further mincing). After this the tissues were pressed in a mortar (this step not indicated by Chang & Gaddum) and filtered, in the process of which the tissues were washed with 7 % trichloracetic acid. The filtrate was then washed with ether three or four times until it was faintly acid to congo red. In about half the experiments the filtrate was kept overnight in a refrigerator at this point, while in the remaining experiments the extractions were continued and completed the same day. No difference could be detected between the two groups in the subsequent tests. The next step was evaporation at 38–40° C. under reduced pressure, to a concentration of 2/10–5/10 c.c. of fluid per g. of the original tissue. Chang & Gaddum recommend evaporation to the point where 1–10 c.c. of fluid remain for each gram of tissue. With one or two exceptions it was found in the early work that negative results were usually obtained when the more dilute extracts were employed, but upon further evaporation, activity could be demonstrated as described below. After evaporation the extract was again made just acid to congo red and the volume of fluid ascertained by taking it up in a syringe. The ultimate concentrations used are shown in Tables I–III.

Testing of the extracts was limited to the frog's rectus abdominis muscle because it appeared to be, in all but one or two experiments, sufficiently sensitive (when eserinized, responses to 0·06–0·1 μg. were obtained, the bath had a capacity of 18 c.c., the lever magnification was 1 : 10 and the lever load was 3 g.). Known dilutions of actylcholine were employed in decreasing amounts exactly as recommended by Chang & Gaddum, that is to say, the agent was left in contact with the muscle for 3 out of every 10 min. and the chamber washed twice with Ringer-Locke solution between times. When eserine was used, 2 c.c. of 0·1 mg./c.c. of solution were used. All solutions were made fresh daily. In some of

the experiments, a series of graded responses was obtained by graded dosages of acetylcholine, after which the activity of the unknown extract was tested (as in Fig. 2), while in other experiments, the series of responses to known quantities of acetylcholine was interspersed with tests of the unknown extracts (as in Figs. 1 and 3). In either case the amplitude and form of the response made it possible to estimate the acetylcholine equivalent of the extracts with a fair degree of accuracy. Only occasionally were aberrant responses obtained which were not classifiable as potassium, choline or acetylcholine types, as described by Chang & Gaddum. In only one instance, however, could a modified type of response be subjected to confirmatory tests specific for acetylcholine. In this case, potentiation of the response occurred with eserine, and the response could be prevented either by atropine or by inactivation with sodium hydroxide.

Results

Acetylcholine equivalent of the uterus of untreated rabbits. Of nineteen ovariectomized animals comprising this group, extracts from eight were tested upon the uneserinized rectus muscle. All tests except two were negative in dosages ranging from the equivalent of $\frac{2}{3}$ g. of fresh uterine tissue in 0·3 c.c. of extract, to the equivalent of 1·35 g. in 0·36 c.c. of solution. Of the two extracts which were active, one showed an acetylcholine equivalent of 0·2 μg./g. of fresh uterus, and one gave a value equivalent to 5 μg./g. of fresh uterus. The latter is clearly a technical error, however, since a larger amount of the extract was inactive on the eserinized preparation later. With this experiment counted as a probable negative one, therefore, all but one of the eight extracts in this group yielded no evidence of an acetylcholine-like substance.

On the eserinized preparations of the frog's rectus muscle, thirteen of eighteen extracts tested (see Table I) were completely devoid of activity, and of the remaining five, three showed negligible activity. In one there was a trace of activity and in the others there was an activity equivalent to 0·03 and 0·08 μg. of acetylcholine per g. of fresh tissue. The remaining three extracts gave evidence of activity equivalent to 0·13, 1·0 and 2·0 μg./g. of fresh uterus respectively. In no case in this group was there sufficient material for a single confirmatory test for acetylcholine, as there was in the following group.

Acetylcholine equivalent of uteri 1 hr. after oestrin. In this group of thirteen rabbits, eight of the uterine extracts were tested on the uneserinized rectus muscle, and seven of these eight were tested subsequently on the eserinized rectus muscle. In Table II it will be seen that six of the eight extracts showed an activity equivalent to 0·5–4·0 μg. of acetylcholine per g. of uterus. One of the two inactive extracts, however, was definitely active when tested later on the eserinized rectus muscle, while only one was negative throughout. Within 1 hr. of injection of

TABLE I. Acetylcholine equivalent content of uteri of nineteen ovariectomized rabbits. Extracts tested on rectus abdominis of frog

Days ovariec-tomized	Concen-tration, c.c./g. fresh uterus	Non-eserinized		Eserinized		Remarks
		Dose g. fresh uterus	Response μg./g.	Dose g. fresh uterus	Response μg./g.	
20	0·36	1·35	*ca.* 0·2	0·27	0	Sensitive preparation;
				0·27	0	eserinized, to 0·06 μg.
20	0·90	1·5	0	1·5	0	See Fig. 1
20	1·35	1·0	0	1·7	Trace	0·06 μg. see Fig. 1 (possibly choline)
20	0·90	1·0	0	1·5	0	See Fig. 1
19	0·30	—	—	1·0	0	
				2·0	0	
28	0·30	1·0	0	1·0	0	
28	0·30	0·66	5	1·0	0	Doubtless a technical error, since eserine test was negative
28	0·50	—	—	1·58	0·08	
22	0·42	—	—	1·07	1·0	
22	0·71	—	—	1·0	0	
				1·5	0	
22	0·35	—	—	2·0	0·13	
17	0·47	—	—	1·81	0·03	
17	0·33	—	—	0·90	0	
21	0·42	1·0	0	2·77	0	
50	0·35	1·0	0	2·4	0	
	0·37	—	—	1·47	0	1 hr. after injection of 2 c.c. sesame oil
19	0·22	—	—	3·15	2	1 hr. after 4 c.c. sesame oil Not a sustained response
68	0·33	—	—	1·21	0	1 hr. after 4 c.c. seame oil
23	0·27	—	—	2·0	0	1 hr. after 4 c.c. sesame oil
				3·0	0	

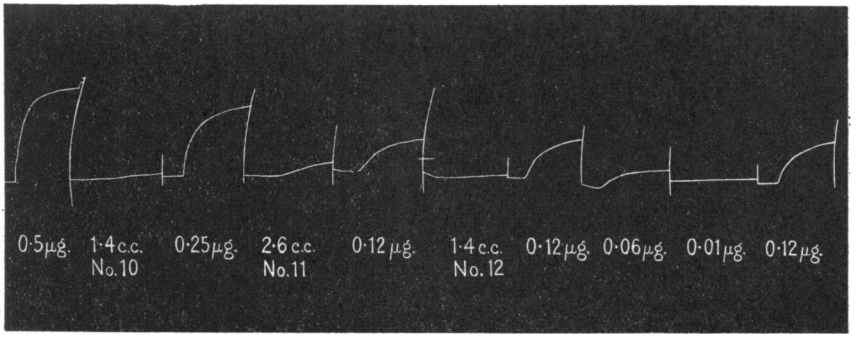

Fig. 1. Tests for an acetylcholine-like substance in extracts of uteri obtained from untreated, ovariectomized rabbits. Eserinized rectus abdominis muscle of frog used for the test. No. 10, 1·4 c.c. = 1·5 g. fresh tissue; no. 11, 2·6 c.c. = 1·7 g. fresh tissue; no. 12, 1·4 c.c. = 1·5 g. of fresh uterine tissue. See Table I and text for description.

TABLE II. Acetylcholine equivalent content of uteri of thirteen ovariectomized rabbits 1 hr. after injection of oestrin (100–400 I.U./kg. body wt.)

Days ovariectomized	Concentration, c.c./g. fresh uterus	Non-eserinized: Dose g. fresh uterus	Non-eserinized: Response μg./g.	Eserinized: Dose g. fresh uterus	Eserinized: Response μg./g.	Remarks
14	0·16	1·2	1·6			
26	0·55	0·55	4	0·4	1·0	1 g. inhibited by atropine (see Fig. 2)
27	0·50	1·0	1·5	0·8	0·5	
27	1·0	1·0	0·5	1·0	0·2	1 test, inhibition by atropine; 1 test, inhibition by NaOH. Eserine responses 3x non-eserine ones (see Fig. 3)
				0·8	0·5	
25	0·34	1·0	0	1·75	Trace	
				1·5	1·0	
25	0·52	1·0	0	1·2	0	Relatively insensitive eserinized frog rectus; small response to 0·5 μg. acetylcholine
25	0·66	—	—	2·0	0	
18	0·18	—	—	1·3	0·2	
18	0·20	—	—	0·66	0·5	Atropine inhibition, 1 test. Inactivation with blood, response diminished by two-thirds
24	0·50	—	—	0·5	1·0	1 test, inhibition by atropine
27	0·32	1·0	2	1·0	0·2	1 test, inhibition by atropine. 1 test, inactivation by NaOH. 1 test, complete inactivation by blood
27	0·50	1·0	2	0·6	0·25	
25	0·50	—	—	2·0	0·5	Uterus transplanted to anterior abdominal wall for 2 days. 1 test, atropine inhibition successful. 1 test, NaOH inactivation, successful
				1·0	0·5	

oestrin, therefore, the presence of an appreciable quantity of an acetylcholine-like substance may be detected in the uterus. This is not the result of the oil alone, since injection of 2–4 c.c. of sesame oil into ovariectomized rabbits is without effect upon the activity of extracts of the uterus prepared in the usual way (see last four tests in Table I). It was also ascertained that neither alcohol-soluble nor water-soluble oestrin had a detectable effect upon the eserinized rectus preparation when added directly to the bath.

On the eserinized frog rectus muscle, all but two of the twelve extracts obtained from the thirteen rabbits in this group were active, being equivalent in activity to 0·2–1·0 μg. of acetylcholine per g. of fresh tissue. A striking and rather constant feature of these results is the fact that the

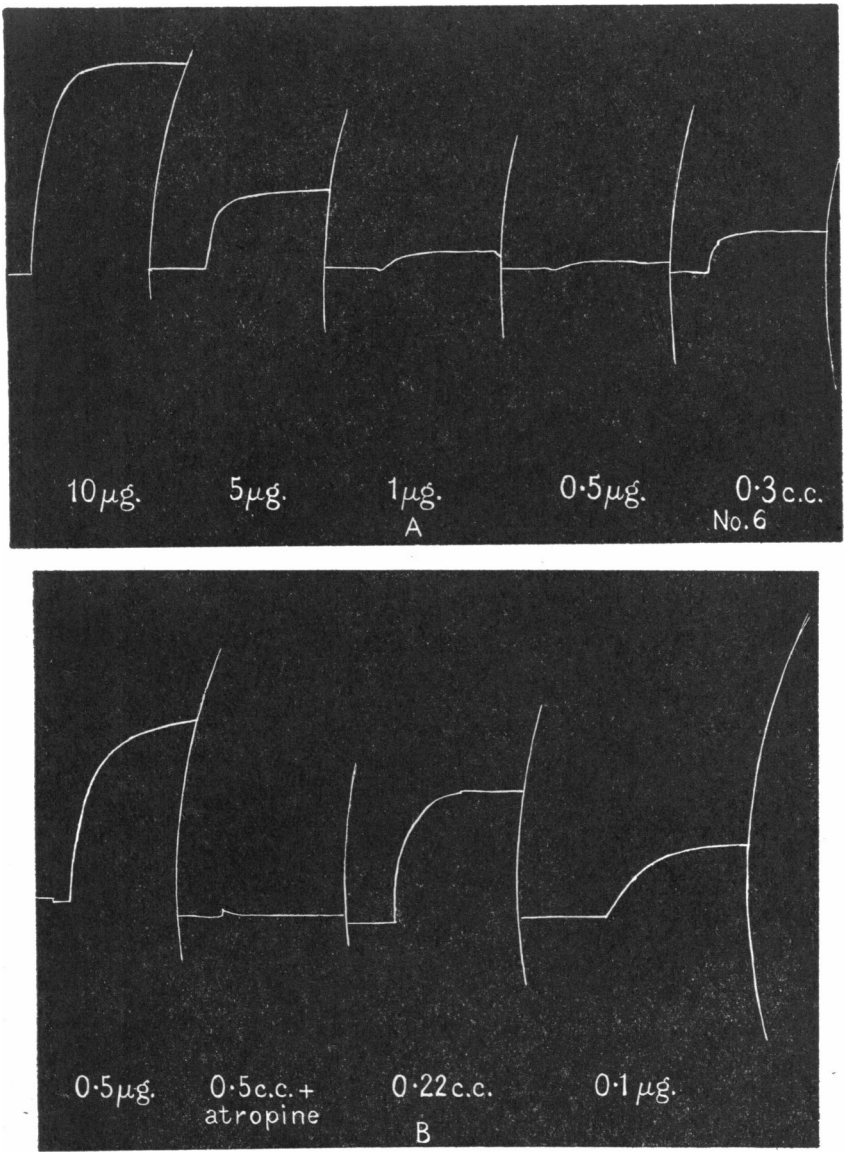

Fig. 2. Top, uneserinized rectus preparation. Extract prepared from uterus of a rabbit injected for 1 hr. with 100 I.U. of oestrin per kg. of body weight. 0·3 c.c. = 0·55 g. of fresh tissue. Bottom, same preparation eserinized. Atropine inhibition of the equivalent of 0·9 g. of fresh tissue. 0·22 c.c. = 0·4 g. fresh tissue demonstrating eserine potentiation of the response. See Table II and text for description.

concentration of the acetylcholine-like substance of these extracts diminishes on standing, during the period of half an hour or more when the frog muscle is eserinized. This may be attributable to the use of solutions which were a bit too alkaline (*p*H 4·5) and so unfavourable for the maintenance of acetylcholine. Even so, potentiation was clearly observed in each of the seven cases tested. This is exemplified in Fig. 2. Thus, on the uneserinized muscle, 0·3 c.c. of extract (equivalent to 0·55 g. of fresh tissue) gave a response of small amplitude (Fig. 2A, 0·3 c.c.), whereas after eserinization (and inhibition of a response by atropine) 0·22 c.c. of extract (equivalent to 0·40 g. of tissue) gave a response about three times as great as the first one (Fig. 2B, 0·22 c.c.). This potentiation was characteristic of these experiments and is typical of acetylcholine. In a control experiment performed to test this loss of potency in the extracts it was observed that acetylcholine was partially destroyed on standing in the concentrated extract. Ostensibly, therefore, the loss of potency in the seven unknown extracts tested on the eserinized preparations was attributable to destruction of some of the active material, presumably acetylcholine, in the extract.

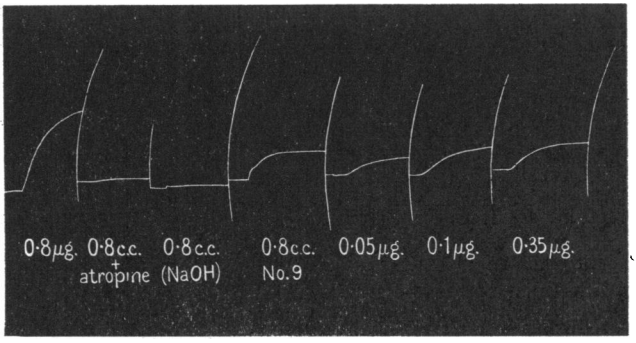

Fig. 3. Records from tests on an eserinized frog rectus preparation. Demonstration of inactivation of a potent extract by neutralization with NaOH and almost complete inhibition of a response by means of atropine. 0·8 c.c. = 0·8 g. of fresh uterine tissue. See Table II and text.

Identity of the active substance. The identity of the active material with acetylcholine is proved by the four confirmatory tests carried out whenever sufficient extract was available. First, potentiation of the responses occurred in all seven attempts. Secondly, atropine inhibition (20–40 mg. of atropine sulphate added to the bath), as demonstrated in Figs. 2 and 3, was obtained five times in five attempts. The use of this drug on the rectus abdominis is evident only when large doses are used, and it is

not entirely specific for acetylcholine; it includes in addition the effects due to salts. Thirdly, three out of three attempts to inactivate active extracts by neutralization with NaOH and standing for a period in excess of 20 min. at room temperature were successful. Finally, two attempts to inactivate extracts with blood were successful. One, with human blood, was completely successful, while in the second with rabbit blood the response was diminished by two-thirds. Taken together, these facts clearly indicate that the active agent in the uterine extracts prepared in this work is acetylcholine. Furthermore, since this substance is not present in significant amounts prior to the administration of the hormone, one is forced to conclude that its presence is attributable to the action of the hormone, oestrin, upon the uterus *in situ*.

Independence of connexion with the central nervous system. In one of the foregoing experiments noted in Table II (last expt.) the uterus, minus the cervical region with the uterine cervical ganglia, had been transplanted to the anterior abdominal wall in a series of three operations [for technique, see Kaminester & Reynolds, 1935]. The last of these operations was performed only 2 days before the uterus had to be taken for study, since the rabbit developed a severe middle ear infection. The uterus, however, was healthy in every respect; it was well vascularized and it yielded sufficient extract for two tests which were positive, and in addition, confirmatory tests, namely, inhibition by atropine and inactivation by NaOH, were successfully carried out.

Two other transplants, not noted in the tables, yielded negative results. These tissues had been transplanted for a period of 3 weeks, commencing 1 week after ovariectomy. On gross examination the tissues were not normal in appearance. They were translucent, all but avascular, and had a water content (dried to constant weight at 85° C.) of 92·7 and 95·7 % respectively. The range of water contents in the other uteri of this series ranged between 76 and 89 %, averaging between 82 and 83 %. It is therefore clear that with normal transplanted uterine tissue (without time, however, for nerve degeneration to be complete) the acetylcholine content of the uterus increases as a result of the injection of oestrin. Since this is a peripherally mediated effect of the hormone it is essentially a "cholinergic" action of the hormone [Reynolds, 1938*a*].

Diminution of the acetylcholine content of the uterus six and more hours after oestrin. Using eserinized frog rectus preparations for the most part, the activity of uterine extracts prepared from seven rabbits at the end of 6 hr. after the injection of oestrin have been examined and, similarly, extracts obtained from seven other rabbits at the end of 12 hr. after

administration of the hormone have been tested. The data from these experiments are summarized in Table III. Here, it will be seen, extracts from four of seven rabbits at the 6 hr. period failed to have any acetylcholine potency, while the remaining three showed potencies of 0·1, 0·1 and 0·08 μg./g. of fresh tissue, respectively. At 12 hr. only two showed

TABLE III. Acetylcholine equivalent content of uteri of ovariectomized rabbits, 6 and 12 hr. after administration of oestrin

Days ovariectomized	Concentration, c.c./g. fresh uterus	Eserinized frog rectus: Dose g. fresh uterus	Eserinized frog rectus: Response μg./g.	Remarks
		6 hr. after oestrin		
32	0·50	1·65	0	
18	0·27	2·5	0	
		2·5	0	
18	0·30	2·9	0·08	
20	0·50	3·0	0·1	
8	0·14	2·4	0	An atypical response, an initial peak with gradual relaxation subsequently
		0·6	0	
25	0·58	2·39	0·1	
25	0·13	1·6	0	
		2·9	0	
		12 hr. after oestrin		
27	0·43	1·75	0	1 g. on uneserinized rectus was ineffective
34	0·66	2·0	0	2 g. on uneserinized rectus were ineffective
		5·3	0	
33	0·38	2·6	0	
53	0·33	1·85	0	
49	0·30	3·5	0	
43	0·23	2·84	0·36	
43	0·30	1·75	Trace	

any activity, and in one of these it was immeasurable. The other extract had an acetylcholine content of 0·36 μg./g. of uterus. These results show that by the sixth hour there is appreciable waning in the acetylcholine content of the uterus, and by the twelfth hour this substance has virtually disappeared.

These results clearly show that the cholinergic action of oestrin given by injection upon the uterus is of a transient nature. This conclusion is significant when one considers the physiological changes which take place in the uterus following injection of oestrin and the order in which they occur. This is briefly discussed below.

DISCUSSION

When oestrin is injected into ovariectomized rabbits, the initial hyperaemia of the uterus noted at the outset coincides with an increase in its acetylcholine content. In consequence of the vasodilatation, profound

changes take place in the distribution of fluid in the uterus. Thus in the rat, as the vessels enlarge they become increasingly permeable and an accumulation of water takes place in the tissues in the course of the next 6 hr. [Astwood, 1938]. As a result of such changes in the uterus of the rabbit, the density of the vessels comprising the vascular bed of the endometrium diminishes from about 200 vessels per sq. mm. of average cross-section to about 100 per sq. mm. [Fagin & Reynolds, 1936]. These vascular changes precede the onset of intermittent, oestrous motility which commences some 10 or more hours after the injection of the hormone, and they coincide with the onset of a rapid elevation of metabolism (oxygen consumption) of the uterine tissues [MacLeod & Reynolds, 1938].

From the foregoing considerations it is evident that the rise and fall in the acetylcholine content of the uterus precede the changes in metabolism and muscular activity in the uterus which, like the vascular changes, are equally attributable to the specific action of the oestrogenic hormone. It thus appears that acetylcholine is a vasodilator upon a tissue which is quiescent, which possesses a low level of metabolism, and in which the blood vessels are of small calibre, offering considerable resistance to the rapid flow of blood through the tissue. By means of the initial vasodilatation in the uterus, even in bits of transplanted uterine tissues in which demonstrable nervous elements are not present [Markee, 1932], a ready access of oestrogen to the tissues is assured. Subsequently the other, anabolic effects of the hormone are effectively exerted upon the uterus.

Summary

1. The acetylcholine content of the uterus in four groups of ovariectomized rabbits was investigated. These were (1), untreated rabbits, (2) rabbits 1 hr. after treatment with oestrin, (3) rabbits 6 hr. after treatment with oestrin, and (4) rabbits 12 hr. after treatment with oestrin.

2. The acetylcholine content of uteri from untreated rabbits was at best found to be low, and in seventeen out of nineteen animals no evidence of the presence of this substance could be detected.

3. One hour after the injection of oestrin, eleven out of thirteen rabbits were found to have an appreciable quantity of acetylcholine present in the uterine tissues.

4. By the 6th hour after the injection, the acetylcholine content of the uterus was not measurable in four out of seven rabbits, and in the remaining three it was not present in significant amounts. By the 12th

hour acetylcholine was observed in only two of seven rabbits, and in one of these it was present only as a trace.

5. Oestrin caused a transient increase in the amount of acetylcholine extractable from the uterus. This substance was obtained equally well independently of the connexion of the uterus with the central nervous system.

6. The physiological significance of these results is discussed.

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