CONCENTRATION OF THE GONADOTROPIC HORMONE IN PREGNANT MARE'S SERUM*

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In 1930, Cole and Hart (1) discovered the presence of a gonadotropic hormone in the blood stream of mares in early pregnancy. It was detected almost simultaneously by Zondek (2). Cole and Hart established the following facts. The hormone is present in the period beginning at 37 days after the fertilizing coitus and lasts until approximately the 175th day but the presence of very appreciable amounts of it is limited to the period between 43 and 80 days. The period of greatest concentration occurs apparently somewhere in the interval between the 50th and 65th day and the native unaltered serum drawn from the mare's jugular vein at this time is capable of exerting clear gonadotropic effects in immature (21–26 day old) female rats or mice within a hundred hours following six doses of 0.005 cc. These investigators and others associated with them have continued study of the very interesting hormonal and tissue changes in pregnant mares and in the horse fetus (3–7). They have shown that during the period of high concentration of gonadotropic hormone in the mare's blood stream, its ovaries exhibit the formation *de novo* of many corpora lutea, an unique phenomenon; and that in the period immediately subsequent thereto, the fetal gonads exhibit an astonishing hypertrophy caused by the massive appearance there of interstitial cells.

It is apparent that equine hormonal and tissue conditions are fully as remarkable as those which characterize primates (with the Aschheim-Zondek reaction of pregnancy blood and urine) and that further inquiry here will be well repaid. Evans, Meyer and Simpson (8) included the hormone from the mare's blood in their comparative study of gonad-stimulating substances from the hypophysis and other

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sources and showed the relatively great concentration of gonadotropic hormone in this source.

The chemical properties of the gonadotropic substance of pregnant mare's blood have been studied by Goss and Cole (5) and by Cole, Guilbert and Goss (6). They found it possible to concentrate the serum at 36°C. in partial vacuum without appreciable loss of potency. Indeed, heating the serum to its coagulation point and even to the temperature of the boiling water bath for several minutes failed to destroy the gonadotropic principle. The active material failed to dialyze through a collodion membrane and could not be detected in ultrafiltrates. No effect was produced when massive doses of serum were fed to rats.

In agreement with Cole, Guilbert and Goss (6) we have found the active principle to be much more stable to alkalies than to acids at ordinary temperatures. No loss of potency could be observed in samples which had been allowed to stand 15 hours at 22°C. in range pH 3.7 to pH 11.0. Below pH 3.7 the loss of potency was distinctly apparent and potency decreased with increasing acidity.

Pepsin had little effect on the gonadotropic substance when the digestions were carried out at pH 4–5 at 37°C. for 4 hours. At pH 1.8–2.0 the activity was destroyed but this may not have been due to the action of pepsin since controls in which no pepsin was used were also inactivated at this pH and temperature.[1] Trypsin at pH 8.5 inactivated the hormone after 4 hours at 37°C. The products of peptic and tryptic digestion did not increase the gonadotropic effect of pregnancy prolan.[2] Inactivation was also observed when solutions at pH 7.5 were heated for 4 hours at 60–80°C. and the resulting product showed no tendency to increase the gonadotropic effect of pregnancy prolan.

When the acetone-dried serum was dissolved in anhydrous formic acid for 2 hours at 25°C. and the serum proteins recovered by precipitation with acetone, it was found that the gonadotropic activity had been lost and no increase in the activity of pregnancy prolan was observed when the inactivated material was injected with it.

The crude serum of the pregnant mare shows a very considerable

[1] Cole, Guilbert and Goss (6) found partial inactivation of the hormone followed peptic digestion at pH 3.

[2] *Cf.* Evans, H. M., Simpson, M. E., and Austin, P. R., *J. Exp. Med.*, 1933, **57**, 897.

gonadotropic potency. Cole and Hart (1) found the maximum ovarian response in rats followed 6 injections of 0.2 cc. of serum from a mare 78 days pregnant, autopsy being performed 96 hours after the first injection. Increasing the dose to 0.5 cc. showed no further increase in effect, while as has been mentioned, as little as 6 injections of 0.005 cc. of crude serum produced ovarian effects which could be detected by histological section.

Goss and Cole (5) attempted to concentrate the active principle by fractional precipitation of the serum with salts. They found that the fraction precipitable between the concentrations 20 and 27 per cent sodium sulfate contained the greater part of the activity while only 11 per cent of the serum protein was found in this fraction. Evans, Meyer and Simpson (8) prepared acetone-ammonia extracts from the acetone-dried serum and precipitated solutions of these acetone-ammonia extracts with flavianic acid. These procedures gave potent materials still contaminated by inactive serum protein.

With the hope of finding a better method of attack on the problem of the isolation of the active agent we have made a comprehensive study of the behavior of the hormone towards a number of adsorbents following the technique which has yielded conspicuous success in the purification of enzymes by Willstätter and his school. This study has shown that aluminum hydroxide preparations are excellent adsorbents for the gonadotropic substance found in the serum of the pregnant mare.

When aqueous solutions of the acetone-dried proteins of pregnant mare's serum were adjusted to pH 3.5 and treated with suspensions of aluminum hydroxide (Willstätter Type A and Type B) (9) it was found that the active substance was readily adsorbed on the surface of the aluminum hydroxide. In this way it has been possible to separate the active agent from a large part of the inactive protein material. Subsequent washing of the aluminum hydroxide on which the hormone had been adsorbed, using acetate buffer at pH 3.5 or water, removed practically none of the active material. The elution of the active substance was, however, easily accomplished by dilute ammonia or by converting the aluminum hydroxide into the basic phosphate with disodium phosphate solution in the usual manner. The active solutions

obtained by elution were freed from salts by dialysis in Visking[3] membranes and their potency and content of solid material determined.

In this way preparations were obtained which gave, in a total dose of 0.006 mg., an ovarian reaction in mice comparable to the maximum effect observed with pregnancy prolan. A description of typical experiments will make the procedure clear.

EXPERIMENTAL

Chemical Procedures

30 gm. of acetone-dried serum was dissolved in 6000 cc. of 0.005 N NaOH and the solution adjusted with dilute HCI to pH 3.5 using the glass electrode (10, 11). 300 cc. of a suspension of freshly prepared aluminum hydroxide Type B, containing 3 gm. of air-dried solid, was added and the solution vigorously stirred for 30 minutes. The aluminum hydroxide on which the hormone had become adsorbed was now collected by running the solution through a Sharples supercentrifuge until it came through perfectly clear. The cake of adsorbent was removed from the steel cylinder and shaken up with 100 cc. of molar acetic acid-sodium acetate buffer of pH 3.5 for 30 minutes. The suspension was centrifuged and the supernatant discarded. The precipitate was shaken up with 200 cc. of 0.5 per cent aqueous ammonia and the ammoniacal suspension allowed to stand at 5°C. overnight. The suspension was centrifuged and the supernatant decanted, chilled, brought to about pH 8.0 with dilute HCl, and dialyzed. The elution could be equally readily accomplished by the use of disodium phosphate solution instead of ammonia.

The superiority of the aluminum hydroxide suspensions as selective adsorbents for the hormone became apparent to us as a result of a careful study of a group of adsorbent materials and their applicability to the problem. Kaolin, kieselguhr, alundum, tricalcium phosphate, Lloyd's reagent, permutit, aluminum hydroxides Types A, B and C and talc were systematically studied in regard to their ability to adsorb the hormone. The aluminum hydroxides Types A and B proved by far the best adsorbents of the group. Aluminum hydroxide Type C was considerably less efficient. In very acid solutions Lloyd's reagent adsorbed an appreciable amount of the active material.

It was noticed that the effectiveness of the aluminum hydroxide preparations depended in an unusual degree on the pH of the hormone solution. The table illustrates this point very clearly. Solutions of the acetone-dried serum of pregnant mare's blood were adjusted to definite pH's, using the glass electrode, and were treated with the

[3] Regenerated cellulose tubes manufactured by the Visking Corporation, Chicago.

same amounts of aluminum hydroxide Type B. After adsorption the supernatants were tested for the presence of the hormone.

Average Weight of Ovaries of Mice Injected with Supernatants Left after Adsorption with Aluminum Hydroxide Type B

Dose	Hydrogen ion concentration						Controls
	pH 9.8	pH 8.5	pH 7.3	pH 4.7	pH 3.5	pH 2.2	
	mg.	*mg.*	*mg.*	*mg.*	*mg.*	*mg.*	*mg.*
Equivalent to 14 mg. dried serum	15	14	15	11	3	19	11
Equivalent to 7 mg. dried serum	10	13	11	10	3	15	12
Equivalent to 1.4 mg. dried serum	5	3	3	3	2	3	4

Biological Procedures

In testing the potency of chemical fractions groups of 3 mice 6–10 gm. in weight and 21–25 days of age were injected on 3 successive days. Autopsy was performed 96 hours after onset of treatment. Ovaries were observed under a binocular microscope, dissected and weighed. The ovarian development taken as representative of any fraction was the average weight of pairs of ovaries in the group of 3 mice. All preparations were tested in a series of dilutions in order to determine maximal and minimal doses. The mouse gave sufficient ovarian response to this powerful gonadotropic hormone to make it a thoroughly satisfactory animal form for following the purification of the hormone from pregnant mare's serum. When giving the maximal response the ovaries weighed 12–15 mg. (After injection of the hormone found in the urine of pregnant women (prolan) the maximum ovary weight attained is only about 6–8 mg.) The infantile ovaries would weigh from 1–2 mg. Ovaries giving the minimal observable response weighed about 3–4 mg. The most potent product obtained by absorption gave the minimal ovarian response when given in a total dose per mouse of 0.003–0.002 mg. At the 0.006 mg. dosage the ovaries weighed 6–8 mg. (*i.e.* were as large as could be produced by any dose of prolan), and at a 0.012 mg. dose the maximum ovarian weight (12–15 mg.) attainable with this product was reached.

Cole, Guilbert and Goss observed the gonadotropic effects of pregnant mare's serum when injected into immature male rats (6). Accordingly, we have similarly studied the effect of the concentrated material. The preparation was injected for 10 and 20 day periods giving 5.5 mg. per rat per day. (This particular preparation gave the minimal response in female mice following injection of a total dose of

0.012 mg.) At the end of 10 days the testes weighed 1516 mg. and the seminal vesicles 200 mg. (the control organs weighed respectively 495 mg. and 8 mg.). After 30 daily injections the testes weighed 2620 mg. and the seminal vesicles weighed 979 mg. (control organs weighed 1155 mg. and 13 mg. respectively). Histologically it was found that after 10 daily injections the testicular tubules were already noticeably increased in diameter and that there was a marked increase in interstitial tissue. After 30 days the tubules were even larger, the spaces between tubules were densely packed with interstitial tissue and spermatozoa were found in the lumen of the tubules.

SUMMARY

The gonadotropic hormone of the blood of the pregnant mare has been greatly concentrated by adsorption on active aluminum hydroxide followed by elution. The preparations so obtained gave demonstrable gonadotropic effects within 100 hours in 21 day old female mice following three subcutaneous injections of 0.001 mg. in 1 cc. of physiological saline.

As is well known, other gonadotropic substances do not cause conspicuous development of the male gonads but injections of comparatively large doses of these preparations into immature male rats caused marked development of the testes, which in 10 days were trebled in weight. An astonishing increase in the weight of the seminal vesicles resulted, for these organs were approximately 75 times heavier than in controls.

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