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Influence of Age of Animal and Nature of Injected Hormone Preparation on Antihormone Production.

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In the course of experiments dealing with the injection of various hormone substances, it became clear that the amount of antihormone produced was related to the age of the animal employed. In general, it appeared that adult rats, in response to injections of anterior pituitary-like gonadotropic hormones, produced greater quantities of antihormone substance and became refractory sooner than immature animals. This report is concerned with a description of results obtained recently in a detailed study of this phenomenon, along with some additional observations on the relative amounts of antihormone produced in response to 2 different anterior pituitary-like gonadotropic preparations.

Follutein,* a purified human pregnancy urine extract and Gonadin,* an untreated serum from pregnant mares, were the substances used in these experiments. Thirty-two immature Bartonella-free female rats (25-30 days of age) were divided into 4 groups. Daily subcutaneous injections of 5 R.U. Follutein were given to Group 1 for 35 days and to Group 2 for 95 days. Group 3 received daily injections of 5 R.U. Gonadin for 35 days and Group 4 for 95 days. Similarly, 28 Bartonella-free mature female rats (4-5 months of age) were separated into 4 groups and injected, as the immature animals, for 35 and 95 days with comparable amounts of the 2 hormone preparations. At the termination of the 35- and 95-day periods of treatment, the animals in each of the groups were subjected to light ether anesthesia, and the blood, drawn by cardiac puncture, was collected in tubes. The sera of the animals in each of the 8 groups were pooled and stored in a refrigerator during the course of the experiment.

These sera were tested for their antihormone content in the following way. Groups of immature female rats (25-28 days of age) were injected with 2.5 R.U. of either Follutein or Gonadin plus 0.2 cc of the appropriate serum daily for 5 days. The serum was

* We are indebted to Dr. J. A. Morrell of Squibb and Sons for generous supplies of Follutein. We wish also to thank Mr. Donald Wonder of the Cutter Laboratories for supplying us with the Gonadin.

TABLE I.

	Serum tested	No. of test animals	Mean ovarian wt ± S.E. (mg)	Range in ovarian wt mg
Follutein Groups				
A.	35 day injected, immature	15	30.8 ± 2.0	19-47
B.	95 " " "	11	18.2 ± 1.0	13-25
C.	35 " " mature	12	14.9 ± 1.1	11-22
D.	95 " " "	13	12.5 ± 1.1	7-18
E.	Controls	18	39.7 ± 1.5	27-50
Gonadin Groups				
F.	35 day injected, immature	13	31.3 ± 2.1	26-49
G.	95 " " "	16	27.2 ± 1.6	18-35
H.	35 " " mature	12	25.5 ± 1.2	18-35
I.	95 " " "	12	20.1 ± 2.1	12-32
J.	Controls	22	42.3 ± 2.7	26-69

S.E.—Standard error.

injected on one side of the body and the hormone on the other to prevent mixing. To serve as controls, one group of immature rats was injected with 2.5 R.U. Follutein and another group with 2.5 R.U. Gonadin daily for 5 days. On the 6th day, the animals were killed, the ovaries carefully dissected, freed of connective tissue, weighed immediately on a torsion balance and then fixed in Bouin's. The ovarian weights are given in Table I.

A number of conclusions are to be drawn from these data. (1) The serum of the injected adult rats possesses a greater inhibitory capacity than that of similarly treated rats which were immature when treatment was begun. This holds for both hormone preparations employed, the results being more pronounced with Follutein than with Gonadin. The serum of the young rats injected for 95 days shows greater protective power than that obtained from 35-day injected young animals, this result being more definite in the Follutein-treated animals than in those injected with Gonadin. The greater inhibitory effect of the serum from the 95-day injected young animals is probably due not only to the longer period of injection but also to a greater production of the antagonistic principle as the animal becomes older. (2) Although Follutein and Gonadin, in the dosage employed, produced approximately the same increase in ovarian weight in the various serum donor animals, the results indicate that more antihormone substance is formed for the pregnancy urine extract than for the pregnant mare serum preparation. As can be seen, this is true for both the immature and mature injected animals.

Histological examination of the ovaries from the animals employed to test the antihormone capacity of the anti-Follutein and anti-

Gonadin sera showed the same general picture after the 5 days of treatment. Both mature follicles and corpora lutea were seen.

It was now important to see whether the degree of antihormone activity of the serum could be related to any of its immunological properties such as its ability to form precipitins. Precipitin tests on the sera obtained from the 95-day injected animals were carried out as follows: 1-2, 1-5, and 1-10 dilutions† of the Gonadin preparation (1 cc contains 50 R.U.) and the original concentrated Follutein (1 cc contains 500 R.U.) were prepared, using 1% saline as the diluting agent; 0.1 cc of each of these dilutions was placed on a slide and alongside each, 0.05 cc of the different undiluted sera was added in such a way as to just allow the 2 drops to make contact. Macroscopic and microscopic examinations of the different preparations for precipitin formation were made after they had been allowed to stand for one hour. The relative amounts of precipitin formed were recorded and the results are shown in Table II.

Considering first the sera obtained from the Follutein-injected animals, it is seen that serum D which possessed greater antihormone capacity than serum B, showed a greater precipitin reaction. Similarly, for the Gonadin-injected animals, serum I which showed greater protective power than serum G, at the same time yielded more precipitins. Thus, it would appear that for either the Follutein or Gonadin-injected animals' sera, the greater the antihormone con-

TABLE II.
Dilution of Hormone Preparation.

Serum used	Full strength	1-2	1-5	1-10
1. Follutein				
B	++	++	+	+
B^1	0	0	0	0
D	+++	+++	++	+
D^1	0	0	0	0
Control*	0	0	0	0
2. Gonadin				
G	++++	++++	+++	++
G^1	++	+	+	0
I	+++++	+++++	++++	+++
I^1	++	++	+	0
Control*	+	0	0	0

Letters B, D, G and I refer to the untreated sera obtained from the experimental groups of animals mentioned in Table I. B^1, D^1, G^1 and I^1 refer to the same sera after precipitation with the non-specific protein in normal serum and urine.

0, +, ++, +++, ++++, +++++, indicate increasing degrees of precipitin formation.

*Control serum was obtained from untreated normal, mature female rats.

† It was necessary to use these low dilutions of hormone because of the relatively low precipitin titers of the different sera.

tent, the greater the precipitin reaction. On the other hand, it is also to be noted that the sera obtained from the Gonadin-injected animals which possessed smaller antihormone content than those of the Follutein-injected rats, revealed significantly greater precipitin reactions.

It remained now to investigate to what extent non-specific factors participate in these precipitin reactions. Quantities of the sera obtained from the 95-day Gonadin-injected animals were precipitated with optimal proportions of normal horse serum, as determined by the method of Dean and Webb.[1] Similarly, portions of the sera from 95-day Follutein-injected rats were treated with optimal amounts of normal human urine extract made in the same way and in the same concentration as the pregnancy urine extract. After precipitation was complete, the mixtures were centrifuged, and precipitin reactions with the supernatant fluids again carried out as above. Account was taken of the extent to which the sera had been diluted in this process. The results, also indicated in Table II, show that the ability of the serum from the Follutein-treated animals to form precipitins is completely lost after absorption with normal human urine extract. Similarly, after precipitation with normal horse serum, the precipitin-forming power of the Gonadin-injected animals' sera is considerably reduced; they still, however, retain some capacity to form precipitins to Gonadin.

Despite a considerable amount of work on the subject, the question as to whether the protective substances which appear in the blood stream of animals treated with hormone preparations are immunological in nature or whether they are true antihormone substances in the sense used originally by Collip, has still not been definitely settled. In support of the former contention, Twombly[2] and Brandt and Goldhammer[3] have reported that pregnancy urine extracts, which had lost their gonadotropic activity through ageing or boiling, still were capable of inducing antihormone formation. Rowlands and Young[4] have shown that more antihormone is evoked in response to injections of a crude pituitary thyrotropic preparation than to a more potent purified thyrotropic extract prepared from the former. On the other hand, Sulman[5] has found that purified prolan, free from

1 Dean, H. R., and Webb, R. A., *J. Path. and Bact.*, 1926, **29**, 473.

2 Twombly, G. H., *Endoc.*, 1936, **20**, 311.

3 Brandt, R., and Goldhammer, H., *Klin. Woch.*, 1938, **17**, 236.

4 Rowlands, I. W., and Young, F. G., *J. Physiol.*, 1939, **95**, 410.

5 Sulman, F., *J. Exp. Med.*, 1937, **65**, 1; *Arch. Int. Pharm. et Thér.*, 1939, **61**, 319.

human urinary antigen, still induces antihormone but no antibody formation.

In view of the fact that pregnant mare serum contains considerably more protein than the pregnancy urine extract employed, it would appear from our results that the amount of antihormone produced is not dependent solely on the total amount of antigenic material in the hormone preparation. This conclusion is to be reached from our finding that pregnant mare serum evokes the formation of less antihormone substance but more precipitins than a highly purified pregnancy urine extract. In fact, after absorption of the antiserum to Follutein with the non-specific antigen in normal human urine, its ability to form precipitins with the pregnancy urine extract is lost completely. In view of Sulman's experiments, this latter result indicates that the ability of pregnancy urine extract to induce precipitin formation is due to non-specific materials of the carrier substance. This non-specific factor must also operate in the case of the pregnant mare serum, although its capacity to form precipitins with the antiserum precipitated with normal horse serum is still slightly retained. In this connection, de Fremery and Scheygrond[6] have found that rabbits treated with serum of a non-pregnant mare failed to develop any inhibitory substance for the gonadotropic action of pregnant mare serum.

The reason why Follutein should evoke the production of more antihormone substance than Gonadin is not clear. It may mean that the gonadotropic complex in the former is a more powerful antihormone evocator than that present in the latter. The difference in antihormone production may also, in some way, be related to the relative proportions of gonadotropic factors (*e.g.*, FSH and LH) in the two preparations, since pregnant mare serum most likely contains more FSH than human pregnancy urine. It would be of interest to compare the amounts of antihormone principle formed in response to injections of relatively pure preparations of FSH and LH.

The results obtained in this study show that although true immunological reactions probably play a rôle in the formation of antihormone substances after injection of hormone preparations containing carrier antigenic material, other reactions not of the orthodox immunological type also may participate to some extent. Zondek and his coworkers[7] have also concluded that reactions not of the usual immune type operate in the production of antiprolan.

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[6] de Fremery, P., and Scheygrond, B., *Nature*, 1937, **139**, 1016.

[7] Zondek, B., Sulman, F., and Hochman, A., PROC. SOC. EXP. BIOL. AND MED., 1939, **40**, 96.

Summary. (1) Mature rats produce more antihormone principle in response to injections of anterior pituitary-like gonadotropic preparations than rats which are immature when treatment is begun. (2) Of 2 gonadotropic preparations tested, the one containing a large amount of protein evoked less antihormone production but more precipitins than the one containing less antigenic material.

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Pathology of B_6 Deficiency in the Rat and Response to Treatment with 2-Methyl-3-Hydroxy 4, 5-Dihydroxymethyl-Pyridine (Vitamin B_6).

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Vitamin B_6 deficiency in rats was first described by György.[1] From the macroscopic appearance of the skin lesions he coined the term "rat acrodynia". Many investigators have since confirmed György's description of the symptoms. This investigation was initiated in order to study the histopathology of this deficiency and the histogenesis of the changes effected by synthetic vitamin B_6.

Twenty-one-day-old rats were placed on the following diet:

	%
Cornstarch	68
Purified casein	18
Crisco	8
Salt mixture	4
Cod liver oil	2

supplemented with 40 micrograms each of thiamin and riboflavin and 0.5 mg of nicotinic acid. No attempt was made to supply factor II in this series of experiments. The characteristic dermatitis became evident after 6 to 9 weeks. The animals were maintained on the deficient diet for 72 to 86 days. By this time the symptoms were very severe: The paws were denuded, edematous and moist, the ears thickened and scaly, the snout swollen and some ulcers present under the tongue. Many of the rats showed roughness and redness of the surface of the lower abdomen.

Histological findings: At this stage the epithelial cells of the *ears* were somewhat larger than normal. The stratum granulosum was wider and instead of the usual 1 to 2 layers in thickness, extended

[1] György, P., *Biochem. J.*, 1935, **29**, 741.