THE RETICULO-ENDOTHELIAL SYSTEM AND HORMONE REFRACTORINESS

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There is considerable significance attached to the finding that antihormone substances appear in the blood of animals treated for varying periods with different hormone preparations. Attempts to simulate the effects of partial or complete removal of an endocrine organ in an unoperated animal with the use of the appropriate antiserum have met with success in several cases (1–4). Experiments with a view of determining the practicability of employing such antihormone sera in clinical cases characterized by excess hormone production are also being conducted in several laboratories (5–7).

No complete explanation has as yet been given concerning the nature of these antihormone substances. Many experiments support the view that the production of these inhibitory principles is dependent upon immunological reactions which develop because of the association of the injected hormone with protein material (8, 9). Several of the results obtained by Collip and his associates (10–13), however, might be interpreted to mean that reactions other than those which are truly immunological are also at play in the development of refractoriness following the injection of hormone preparations. Collip, Selye, and Williamson (14) have found, for example, that the injection of a pig pituitary extract into female rats for a prolonged period of time results eventually in severe atrophy of the ovaries, accompanied by castration cells in the pituitary, which they interpret as an inhibition of the rat's own gonadotropic secretion due presumably to antisubstance developed to the injected extract. This result has been corroborated by Severinghaus and Thompson (15) who found that the hypophyses of dogs injected repeatedly with sheep pituitary extract also showed castration cells. Although experiments such as these would, at first sight, tend to argue against the antibody-like nature of antihormones, Parkes and Rowlands (16) prefer to explain such results by assuming that the essential

hormone secreted by the animal's own pituitary may function as a specific haptene, either free or combined with homologous protein. This complex, although having no true antigenic properties for its own species, might conceivably be neutralized by the antisubstance developed by the animal in response to injection of pituitary extract of another species.

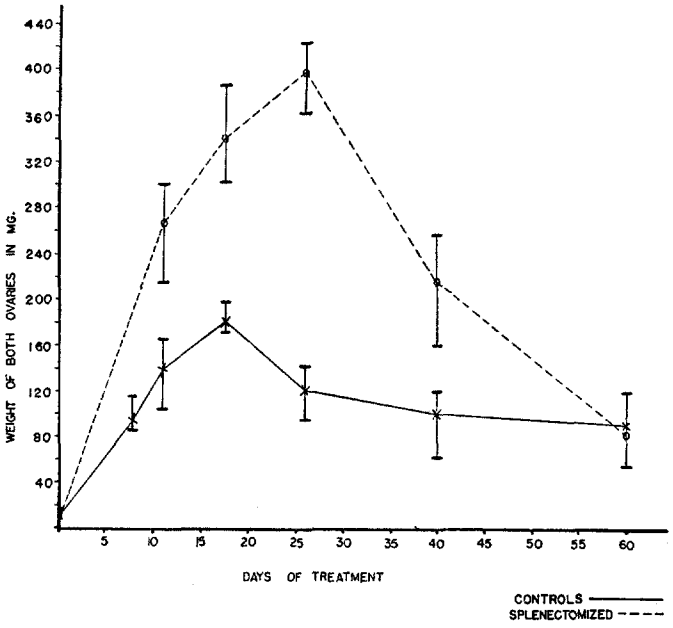
For many years, evidence has accumulated to show that the so called reticulo-endothelial system of cells participates actively in the production of various types of protective substances (17). Since the antagonistic substances which develop in response to injections of hormone materials may be considered protective in nature, we have investigated the possibility of some connection between the reticulo-endothelial system and the production of these inhibitory principles. Preliminary experiments in support of such a relation, using pregnancy urine extract in the female rat, have been reported elsewhere (8, 18). It is the purpose of this paper to give an account of more detailed experiments in which additional hormone preparations were used.

Experiments with Pregnant Mare's Serum on the Female Rat

1. Effect of Splenectomy on Ovarian Response.—It is known that in mammals the spleen is the largest single organ of macrophage tissue. Perla mentions that the rat, free of latent infection, is admirably fitted for experiments on reticulo-endothelial function, because of its high ratio of spleen weight to body weight as compared to other mammals; hence the rat was chosen as the experimental animal in this study. We were first concerned with noting how splenectomy would affect the ovarian weight response to injections of pregnant mare's serum. Groups of 25 to 30 day old normal and splenectomized female rats of a closely inbred strain were given daily subcutaneous injections of 5 r.u. gonadin[1] for periods as long as 90 days. Reference to Text-fig. 1 will show that the ovaries of the injected normal animals respond to the daily injections of hormone by increasing in size, reaching a maximal weight ranging from 172 to 200 mg. in about 17 days. This is then followed by regression, normal ovarian weights being attained in about 2 to 3 months despite continued treatment. It will be noted, however, that the ovaries of the injected splenectomized animals taken from the same litters show an increased growth over that of the injected controls, apparent as early as 10 days subsequent to spleen removal. The ovaries reach a maximal size in these splenectomized rats in

[1] We are indebted to Mr. Donald Wonder of the Cutter Laboratories, Berkeley, California, for generous supplies of gonadin, an untreated serum from pregnant mares.

about 25 days, their weights ranging, at this time, from 362 to 425 mg. as against 92 to 140 mg. in the similarly treated controls. The greater weight of the ovaries of the splenectomized animals is due to an increase in the numbers and sizes of the corpora lutea (see Figs. 1 and 2). With continued treatment the ovaries of the splenectomized animals begin to regress rapidly after 30 days and also reach normal size within 2 to 3 months.



TEXT-FIG. 1. Effects of the daily administration of 5 r.u. gonadin on the ovarian weights of young normal and splenectomized female rats. Each cross or circle indicates the value of the mean ovarian weight of from 5 to 8 animals. The vertical lines passing through the means represent the range in ovarian weight.

It is our assumption that the reticulo-endothelial cells are responsible for the production of the inhibitory substances following injection of antigenic hormone preparations. Consequently, an injected animal deprived of its spleen, an organ of high reticulo-endothelial tissue content, should produce less of the antagonistic principle than a similarly treated normal animal, as the above results show. As is well known, soon after removal of the spleen, compensatory reactions occur, involving an increase in the numbers and activity of the reticulo-endothelial elements in other organs such as the liver, lymph glands, and bone marrow (17). If our explanation is correct, a regression in ovarian size in the treated splenectomized animals might,

eventually, be expected to take place because of this reticulo-endothelial compensation.

2. "Blockade" Experiments with Trypan Blue.—Support of the explanation just given comes from certain of our experiments in which the ovarian regression was prevented, to some extent, by the administration of repeated doses of agents which cause a partial "blockage" of these compensating reticulo-endothelial elements. Beginning with the 15th day of gonadin treatment and every 2nd day thereafter, young splenectomized rats were given 1 cc. intraperitoneal injections of 1 per cent trypan blue[2] in physiological saline along with continued treatment with the hormone. At the end of 52 days, the ovaries of 8 such animals ranged in weight from 260 to 395 mg. as compared to a weight range of 91 to 264 mg. in 6 hormone-treated animals which had been splenectomized but not injected with dye. Examination of the livers, lymph glands, and bone marrows of these "dye-blocked" animals showed the hypertrophied elements to be greatly engorged with injected trypan blue particles.

It seems likely that this repeated treatment with the dye has interfered with a special phase of the metabolism of the reticulo-endothelial cells, specifically with their ability to produce a normal amount of the inhibitory or antihormone substance in response to injected hormone material. This would account for the maintenance of the large ovarian size in these dye-treated animals. It is difficult to continue dye injections beyond this time because of the gradual decrease in general resistance of the animals, resulting eventually in their death. We contemplate testing the efficacy of other blocking agents in future experiments. The ovaries of these dye-blocked animals are also heavily luteinized, the degree of luteinization being about the same at the end of 52 days of treatment as in the 25 day splenectomized animals not injected with trypan blue.

It is interesting to note that, as reported previously (8), when follutein, a pregnancy urine extract, is used, it is possible with trypan blue injections, not only to prevent the regression in ovarian size which also occurs rapidly after about 30 days in splenectomized animals treated with this hormone preparation, but also to cause a further considerable increase in size for at least 25 days beyond this time. When pregnant mare's serum is employed, however, as seen above, the dye treatment can, at best, maintain the peak in ovarian weight attained by the 25 day-treated splenectomized animals. A possible explanation for the difference in result obtained here is that the

[2] Saccharated iron oxide injections have also been tried but they are not as effective as trypan blue.

reticulo-endothelial system, blockaded to the same intense degree, still responds to the injected hormone preparation. The response, however, although feeble in both cases, might conceivably vary to some extent, depending on the type and amount of hormone injected.

3. Inhibitory Effects of Plasma from Normal, Splenectomized, and Dye-Blocked Rats.—We were next concerned with determining whether blood obtained from hormone-treated (*a*) unoperated, (*b*) splenectomized, and (*c*) splenectomized dye-blocked animals would show any differences in anti-hormone content.

The inhibitory powers of the blood plasmas of three groups of immature female rats, injected with gonadin for different periods, were tested.

In one series, 5 immature female rats were splenectomized and then injected for 19 days with 5 r.u. gonadin. At the end of this time the animals were subjected to light ether anesthesia and the blood, drawn by cardiac puncture, collected in sodium oxalate. It was immediately centrifuged and the plasma pipetted off. The plasma samples of these 5 animals were then pooled. Similarly 5 normal female rats of the same age, with spleens intact, were treated for 19 days with 5 r.u. gonadin, and their plasmas, obtained as above, were also collected. In the second series, 5 splenectomized and 5 normal immature female rats were injected with 5 r.u. gonadin for 26 days, and the plasmas obtained from each of these two groups separately pooled. In the third series 5 immature females were splenectomized and injected for 15 days with 5 r.u. gonadin. Beginning with the 15th day and every 2nd day thereafter they were injected with 1 cc. 1 per cent trypan blue in 1 per cent saline along with continued daily doses of the hormone until the 40th day. The plasmas from these animals also were combined. Control plasmas for this series were obtained from 5 normal animals injected for 40 days with 5 r.u. gonadin. Plasmas from normal untreated rats, of approximately the same age, were also collected for general control purposes.

To avoid possible deterioration of the plasmas, dry powder preparations, made according to the method of Zondek and Sulman (19) were used, since in this form, the inhibitory capacity remains undiminished for at least 6 months. The method consists in precipitating the plasma with four volumes of acetone and washing the precipitate on a Buchner filter with acetone drawn through by suction. This is followed by three washings with ether and the precipitate then dried in a vacuum desiccator. 1 cc. of plasma yields about 50 mg. dry powder. Such powders retain about 90 per cent of the potency of the whole plasma. They may be made up in any desired concentration by dissolving in physiological saline to which a small quantity of 0.1 N NaOH is added, with subsequent adjustment of the pH to 7.0 with 0.1 N HCl.

The antihormone content of these powders was tested in fifty-six 25 to 30 day normal female rats. The animals were divided into three groups and each rat was injected for 8 days with 3 r.u. gonadin plus 3 mg. of the plasma powder to be tested. The gonadin was injected on one side of the body, and the powder preparation on the other to avoid mixing. The animals were then sacrificed on the 9th day, the ovaries carefully dissected, freed of connective tissue, and weighed on an analytical balance. The results obtained in this experiment were subjected to a statistical analysis for the determination of the standard error. A summary of this analysis is given in Table I.

It will be seen from Table I that as early as 19 days after beginning injection a difference in the inhibitory capacity of the plasmas obtained from normal and splenectomized animals can be detected. In all three groups the ovaries of the test animals treated with gonadin and plasma from the injected splenectomized animals are larger than those receiving gonadin and plasma obtained from injected normal animals. These differences are statistically significant as indicated by the values of the standard error and

TABLE I

Antihormone Content of Plasmas Obtained from Gonadin-Injected Normal, Splenectomized, and Splenectomized Dye-Blocked Rats

All rats were injected daily with 3 r.u. gonadin plus 3 mg. plasma powder for 8 days

Source of plasma	No. of rats	Mean ovarian weight ± S.E.	Range	Mean difference / S.E. of difference	Coefficient of variation
			mg.		
19 day injected splenectomized	9	73 ± 2.3	62–88	5.7	9.4
19 day injected normal	9	53 ± 2.6	42–65		14.5
26 day injected splenectomized	11	69 ± 2.8	54–84	6.7	13.3
26 day injected normal	11	47 ± 1.7	39–56		12.1
40 day injected splenectomized and dye-blocked	8	66 ± 3.0	58–85	3.5	13.0
40 day injected normal	8	49 ± 3.8	37–65		20.8
Normal untreated rats	14	88 ± 2.2	74–101	—	13.2

S.E. = standard error.

the fact that the value of the mean difference divided by the standard error of the difference exceeds 2. It will also be noted that the inhibitory powers of the two sets of plasmas remain practically unchanged between 19 and 40 days of treatment.

It would appear from these results that the blood of the injected splenectomized and splenectomized dye-blocked animals possesses a lesser ability to inhibit simultaneously injected hormone than the plasma of similarly injected controls. This would explain the greater size of the ovaries obtained in the former as compared to the latter group of animals.

4. Effects of Bartonella muris Infection on Response to Gonadin.—The results thus far described can be reproduced consistently only in animals free of latent infection, especially that of *Bartonella muris*, which is quite preva-

lent among laboratory rats. The importance of considering this factor in experiments designed to test reticulo-endothelial activity has already been stressed by Perla (17) and by us in several previous reports. We have found that the ovaries of gonadin-injected rats, heavily infected with *Bartonella muris* before 25 days of age, grow as large as those of the injected splenectomized uninfected animals. Twelve 25 to 28 day old heavily infected bartonella animals, with spleens intact, were injected daily with 5 r.u. gonadin for 40 days. At the end of this time, both ovaries and the spleen were removed under ether anesthesia. The weight of the ovaries taken from these animals ranged from 186 to 390 mg. 11 of these 12 animals died within 10 days subsequent to the operation, showing, during this time, all the typical signs of bartonella infection such as the presence of many bartonella organisms in the red cells, followed by the development of an intense anemia. The 12th animal survived the operation, but this was the one in the series which had developed the smallest ovaries (*i.e.*, 186 mg.). To serve as controls, 12 uninfected 25 to 28 day old rats were subjected to the same treatment. The ovarian weight range in these animals was 55 to 162 mg. All 12, moreover, survived the splenectomy. Perla has shown that the primary effect of the bartonella organism is to attack the red blood cell, eventually causing its destruction. The phagocytic reticulo-endothelial elements throughout the body then ingest these injured cells and fragments, become tremendously engorged and soon disintegrate. Areas of necrosis may be seen in the livers and spleens of such animals (Figs. 3 and 4). Because of this injury to the reticulo-endothelial cells, the infected animals may not be able to produce the inhibitory principle to as great an extent as the uninfected controls and thus they might be expected to show a greater response to injected hormone material. The results show this to be actually the case.

Experiments with Pregnancy Urine Extract on Male Rats

Having thus brought evidence that the reticulo-endothelial system is concerned with the development of refractoriness to hormone preparations in the female rat, it was of interest to see whether similar effects could be obtained in male rats. Groups of young 40 to 50 gm. normal and splenectomized male rats were given daily doses of 10 r.u. follutein.[3] At the end of 25 days of treatment, the splenectomized animals showed much more striking effects than the similarly treated controls. Although the testes of

[3] We wish to thank Dr. J. A. Morrell of E. R. Squibb and Sons for supplying us with the follutein used in these studies.

the splenectomized animals were not significantly heavier than those of the treated controls, the organs of the former group possessed a more highly hypertrophied and hyperplastic interstitial tissue (Figs. 5 and 6). The reproductive accessories, moreover, were heavier in the animals deprived of their spleens. Thus, for example, in 8 splenectomized animals injected for 25 days, the seminal vesicles with their contained fluid ranged in weight from 780 to 1590 mg. whereas in 8 normal animals the organ showed a weight range from 370 to 675 mg. The heavier seminal vesicles obtained in the splenectomized animals revealed, when sectioned, a greater epithelial height and signs of greater activity than in the similarly treated controls (Figs. 7 and 8).

Again, to obtain the above results it is essential to employ bartonella-free rats. When young heavily infected male rats, with spleens intact, were injected with 10 r.u. pregnancy urine extract daily for 25 days, effects of about the same intensity as those observed in treated bartonella-free splenectomized animals were obtained. In 6 heavily infected rats, treated in this way, the seminal vesicles with contained fluid ranged in weight from 890 to 1680 mg. We interpret these results as due to injury of the reticulo-endothelial system caused by the consequences of the disease.

Experiments with Thyrotropic Hormone on the Guinea Pig

Experiments were now conducted to determine whether this relation between the reticulo-endothelial system and the production of antihormone substances is also true for thyrotropic hormone extract, using in this case another type of experimental animal, the guinea pig.

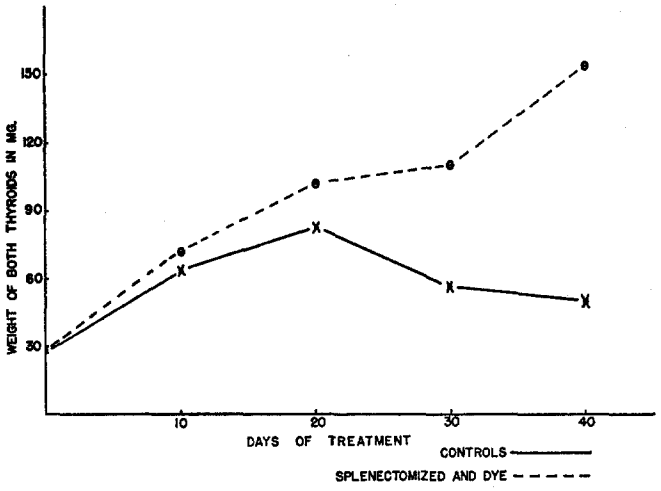
Groups of young normal and splenectomized male guinea pigs (weight 150 to 200 gm.) were given daily subcutaneous injections of either 3 mg. Collip (10) or 5 mg. Rowlands and Parkes (20) ox thyrotropic extract. In another group, young splenectomized guinea pigs received daily subcutaneous injections of the Rowlands and Parkes extract, and then beginning with the 15th day of treatment and every 2nd day thereafter were injected intraperitoneally with 1.0 to 1.5 cc. of 1 per cent trypan blue dissolved in physiological saline along with continued daily doses of the hormone extract.

Twice weekly, during the course of the experimental treatment, metabolic rate determinations were made for each animal in an apparatus similar to the one described by Tainter and Rytand (21). The time necessary for a consumption of 20 cc. of O_2 was determined. Each value recorded represented the average of at least 5 consecutive readings which rarely showed a range greater than 10 per cent after the animals had become accustomed to the chamber. The chamber was thermostatically controlled at 31°C. which is the critical temperature for the guinea pig. The animals were fasted for approximately 12 hours before each determination was made. The experimental values shown in Text-fig. 3 are given as the percentage increases over normal. The normal

metabolic readings were obtained from control groups of untreated animals of approximately the same age and weight as the treated animals.

Groups of animals were sacrificed at various periods during the experiment. The thyroids were then dissected, weighed on an analytical balance, fixed immediately in Bouin's solution, and prepared for histological examination.

In the normal animals receiving daily injections of the thyrotropic hormone extract, the thyroid weights increased and reached maximal values in about 20 days. At this time also the follicular epithelium in the gland assumed its greatest height and the scanty colloid was highly vacuolated.

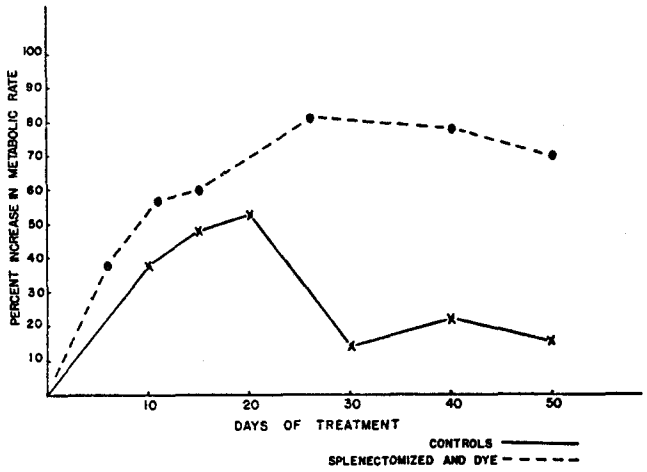


TEXT-FIG. 2. Effects of daily injections of 5 mg. ox pituitary thyrotropic extract (Rowlands and Parkes) on the thyroid weights of young normal and splenectomized dye-blocked guinea pigs. Each cross or circle represents the mean thyroid weight of from 4 to 8 animals.

This was soon followed by regression, so that after about 40 days of treatment, the thyroid weights and histological appearance had returned almost to normal. The changes in metabolic rate closely followed the alterations in thyroid weight (Text-figs. 2 and 3). In the splenectomized animals, accentuated effects were maintained for at least 35 days, but this, too, was soon followed by regression, due presumably to reticulo-endothelial compensation. More significant results were obtained in the splenectomized guinea pigs, which had received, in addition, the treatment with trypan blue. In these animals, marked effects such as increased thyroid weights which averaged 150 mg., metabolic rates which ranged 50 to 80 per cent above normal, a heightened follicular epithelium, hypertrophied interstitial

tissue, and vacuolated colloid in the gland were still obtained after 90 days of treatment (Text-figs. 2 and 3; Figs. 9 and 10). The effects of the plasmas obtained from the injected normal and splenectomized dye-blocked animals, as tested in tadpoles and guinea pigs, have already been described in a preliminary report (22) and will form the subject of another paper.

It is of interest to note that results similar to these have been obtained recently by Murphy, Lowther, and Pagniello (23) in the rat. Although the rat is not very sensitive to thyrotropic hormone, the animals which had



TEXT-FIG. 3. Effects of daily injections of 5 mg. ox pituitary thyrotropic extract (Rowlands and Parkes) on the metabolic rates of young normal and splenectomized dye-blocked guinea pigs. Each circle or cross represents the value of the mean metabolic rate of from 4 to 8 animals.

been dye-blocked with trypan blue and given simultaneous injections of thyrotropic hormone showed significantly greater increases in thyroid weight than found in control animals injected with higher doses of the hormone alone.

DISCUSSION

The results described seem to show a definite relation between the so called reticulo-endothelial system of cells and the formation of antagonistic substances following the injection of different types of hormone preparations. Since removal of the spleen and dye-blocking in animals result in the production of a smaller amount of inhibitory substance, it would appear that this sytem of cells is the actual site of formation of antihormone material. With regard to the blockade experiments mentioned above, it

is not to be concluded necessarily that the injections of the dye completely arrest reticulo-endothelial function. It is well known, for example, that ingestion of particles of one type of dye by the phagocytic reticulo-endothelial elements does not necessarily interfere with the ability of these cells to take up particles of another dye. The evidence, however, is almost unequivocal in support of the finding that continued and prolonged injections of dye, as carried out in our experiments, do eventually interfere with the ability of these histiocytic elements to form their various types of protective substances.

Since the reticulo-endothelial system has been shown to be concerned with the production of such substances as antibodies, agglutinins, antitoxins, etc., the results obtained would tend to indicate that the antihormone substances are antibody-like in nature. In support of this, Twombly (24) has reported that prolan, partially inactivated by heat or completely inactivated by aging, still is capable of evoking as much antihormone as active preparations, and that the extent of the precipitin reactions to prolan is directly proportional to the antihormone content of the sera. Werner (9) has found that it is the method of preparation of the extract which determines whether or not refractoriness to injected thyrotropic material will develop, and that guinea pigs rendered refractory to one type of extract still respond to another. Along the same lines, Rowlands and Young (25) have recently demonstrated that more antithyrotropic principle is evoked in response to injections of a crude thyrotropic extract than to a highly purified extract prepared from it. On the other hand, Zondek, Sulman, and Hochman (26) claim that prolan boiled for 1 hour still retains 0.3 to 1.0 per cent of its gonadotropic activity and that this is sufficient to evoke antihormone production. If, however, urinary prolan is completely inactivated by heat, it is no longer capable of inducing antihormone formation. Gordon, Levenstein, and Charipper (27) have recently found that injection of a purified pregnancy urine extract (follutein) which contains considerably less antigenic material than a crude pregnant mare serum preparation (gonadin) evoked the production of more antihormone than gonadin, even though the latter induced greater precipitin reactions than the former. Sulman (28), in addition, has performed experiments which indicate that prolan, free from human urinary antigen, injected into rabbits evokes considerable antihormone but no antibody formation.

The evidence thus would seem to indicate that true immunological reactions play a rôle in the production of antihormones following injections of hormone preparations containing antigenic material. Other data, however,

cannot be explained entirely on such a basis. In any event, the results reported in this paper show that the antihormone substance formed in response to injections of hormone preparations containing considerable protein or practically devoid of antigenic material, supplies another example of a protective substance produced, at least to some extent, by the versatile reticulo-endothelial system.

SUMMARY

1. Young splenectomized female rats, free of latent infection, show greater increases in ovarian weight in response to injections of pregnant mare's serum than do young normal rats with spleens intact.

2. The regression in ovarian weight which occurs after about a month in such injected splenectomized animals may be prevented by repeated injections of an agent like trypan blue which causes blockage of the compensating reticulo-endothelial elements.

3. The plasmas obtained from these splenectomized and splenectomized dye-blocked animals possess less antihormone substance than plasmas from similarly treated normal animals.

4. Young female rats, heavily infected with *Bartonella muris*, and therefore possessing an injured reticulo-endothelial system, develop heavier ovaries in response to injections of pregnant mare's serum than do normal uninfected rats.

5. Similar differences in effect on the testes and seminal vesicles of young normal, splenectomized, and bartonella-infected male rats have been obtained using pregnancy urine extract.

6. Young splenectomized, and splenectomized dye-blocked guinea pigs injected with thyrotropic extract, show heavier and more highly active thyroids than normal hormone-injected animals.

7. These results are explained on the basis that the reticulo-endothelial system participates in the production of antihormone substances.

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EXPLANATION OF PLATE 34

FIG. 1. Ovary of bartonella-free young normal rat injected daily for 25 days with 5 r.u. pregnant mare's serum. ×9.

FIG. 2. Ovary of bartonella-free young splenectomized rat injected daily for 25 days with 5 r.u. pregnant mare's serum. Note increase in numbers of corpora lutea as compared with Fig. 1. ×9.

FIG. 3. Liver of bartonella-infected young rat injected daily for 40 days with 5 r.u. pregnant mare's serum. Note area of necrosis. Ovaries from this animal at the end of this time weighed 247 mg. ×400.

FIG. 4. Liver of bartonella-free young rat injected daily for 40 days with 5 r.u. pregnant mare's serum. No necrotic areas present. Ovaries from this animal at the end of this time weighed 92 mg. ×400.

FIG. 5. Testis of bartonella-free young splenectomized rat injected daily for 25 days with 10 r.u. pregnancy urine extract. Note greater amount of interstitial tissue as compared with that shown in Fig. 6. ×240.

FIG. 6. Testis of bartonella-free young normal rat injected daily for 25 days with 10 r.u. pregnancy urine extract. Refractoriness has already developed. Note relatively scanty interstitial tissue. ×240.

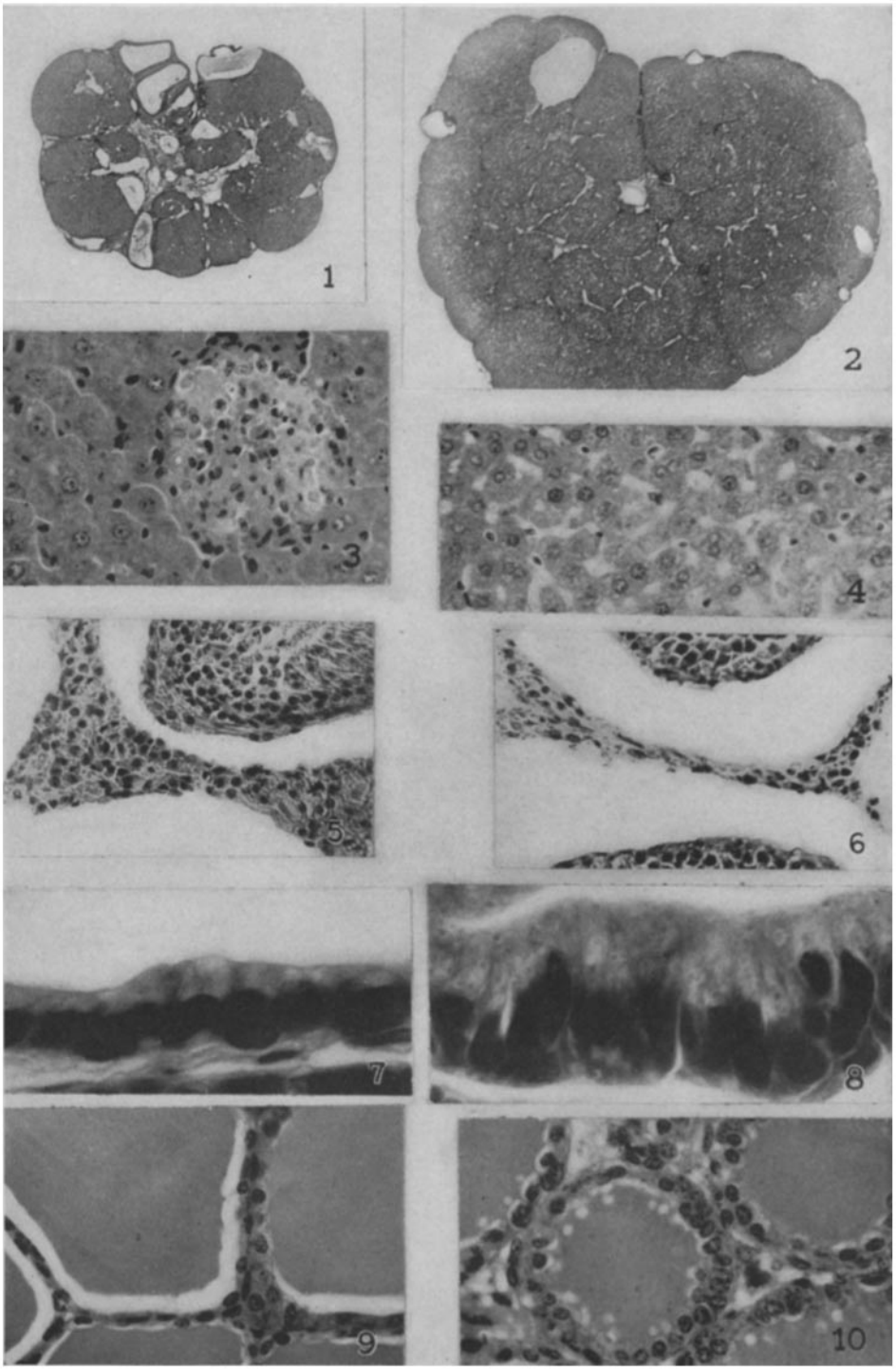
FIG. 7. Epithelium of seminal vesicle from animal mentioned in Fig. 6. Note the relatively low and inactive cells. ×1350.

FIG. 8. Epithelium of seminal vesicle from animal mentioned in Fig. 5. Note higher and more active cells as compared with those shown in Fig. 7. ×1350.

FIG. 9. Thyroid of normal guinea pig injected daily for 90 days with 5 mg. Rowlands and Parkes thyrotropic extract. Refractoriness is complete. Note the low epithelium and abundant non-vacuolated colloid. ×400.

FIG. 10. Thyroid of splenectomized dye-blocked guinea pig injected daily for 90 days with 5 mg. Rowlands and Parkes thyrotropic extract. Note the higher epithelium; also the less abundant and vacuolated colloid. Compare with Fig. 9. ×400.

All figures represent sections of organs fixed in Bouin's solution and stained with hematoxylin-eosin.



(Gordon *et al.*: Reticulo-endothelial system)