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The Antigonadotropic Factor. Reversibility of the Prolan-Antiprolan Effect.

BERNHARD ZONDEK AND FELIX SULMAN.

From the Laboratory, Gynecological-Obstetrical Department, Rothschild-Hadassah Hospital, Jerusalem.

In order to explain the mechanism of the antigonadotropic function we thought it necessary to examine the following questions: Is prolan irrevocably destroyed by antiprolan or is the process reversible? While investigating the chemical properties[1] of antiprolan we found that prolan and antiprolan differ from each other as follows: Prolan is more sensitive to HCl than antiprolan but less sensitive to NaOH. n/10 up to n/15 HCl destroys prolan, but not antiprolan; n/10 up to n/15 NaOH destroys antiprolan, but not prolan. If the prolan-antiprolan effect is reversible it must be possible to destroy antiprolan by means of NaOH and thereby to release the prolan and render it biologically active once more. By using HCl we should be in a position to destroy the prolan, to liberate the antiprolan once more and to render it capable of again inactivating freshly added prolan. If the process is irreversible the prolan-antiprolan-complex cannot be changed in any way whatsoever either by NaOH or by HCl.

The following experiments make it evident that the prolan-antiprolan effect is reversible.

The reactivation experiments can be divided into two groups. I. Reactivation of the prolan out of the prolan-antiprolan complex by selective destruction of the antiprolan by means of NaOH. II. Reactivation of the antiprolan out of the prolan-antiprolan complex by selective destruction of prolan by means of HCl.

I. *Reactivation of Prolan.* Thirty mg. of antiprolan-acetone-dry powder (corresponding to 30 PAU*) was dissolved in 1.2 cc. of aqua dest. 0.8 cc. = 20 PAU of this solution was mixed with 20 RU of prolan (dissolved in 0.4 cc. aqua dest.). This neutral prolan-antiprolan mixture (= 1.2 cc.) was now divided into 2 parts (0.6 cc. each) with which Experiments 1 and 2 were carried out. Experiments 3 and 4 served as controls.

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[1] Zondek, B., and Sulman, F., PROC. SOC. EXP. BIOL. AND MED., 1937, **37**, 198.

* 1 PAU = 1 prolan-anti-unit is the smallest amount of antiprolan required to annihilate the gonadotropic effect of 1 RU of prolan in a test rat. Ten PAU must be tested at least in 1 rat. *cf.* Zondek, B., and Sulman, F., PROC. SOC. EXP. BIOL. AND MED., 1937, **36**, 708.

1. One-half (0.6 cc. prolan-antiprolan mixture) was placed into the incubator for 2 hours to enable union to take place. Then 2.0 cc. n/10 NaOH was added in order to release and destroy the antiprolan.

2. The other half (0.6 cc. of the prolan-antiprolan mixture) was also placed into the incubator for 2 hours to enable union to take place. Then for analogous reasons 2 cc. of m/10 NaCl solution was added. This control should prove that the prolan-antiprolan mixture neutralizes itself to complete ineffectiveness.

3. 10 PAU of antiprolan (0.4 cc.) was diluted with 0.2 cc. m/10 NaCl solution, placed into the incubator for 2 hours, and then 2.0 cc. n/10 NaOH was added. This control should prove that the antiprolan is destroyed at this concentration of NaOH.

4. 10 RU of prolan (in 0.2 cc. of aqua dest.) was mixed with a solution of 10 mg. of acetone-dry-powder of normal rabbit serum (in 0.4 cc. of aqua dest.). This mixture was placed in the incubator for 2 hours, then 2 cc. of n/10 NaOH was added. This control should prove that the gonadotropic factor was not destroyed by the concentration of NaOH applied in the presence of 10 mg. of serum protein.

The 4 tubes were kept at room temperature for 20 hours. Then 2 drops of brom-thymol-blue-indicator solution were added to each, and tubes Nos. 1, 3, and 4 were neutralized with several drops of normal hydrochloric acid. Tube No. 2 remained neutral. To tube No. 3 we added once more 10 RU of prolan. Then all 4 tubes were placed again in the incubator for 2 hours and finally the contents of each tube was injected into an infantile female rat. (The technique of the experiments has been reported upon in our previous papers.[2]) The result was as follows:

Rat 1 showed the gonadotropic reaction HVR I-III, the antiprolan having been destroyed by NaOH and the prolan thus rendered effective.

Rat 2 showed no reaction whatsoever, prolan and antiprolan having neutralized each other.

Rat 3 showed the gonadotropic reaction HVR I-III, the antiprolan having been destroyed by NaOH, the subsequently added prolan now being able to take effect.

Rat 4 showed the gonadotropic reaction HVR I-III, the prolan not having been affected by NaOH.

This experiment, as well as that following, can be reproduced at any time, if the dilutions indicated are strictly adhered to. If either

2 Zondek, B., and Sulman, F., PROC. SOC. EXP. BIOL. AND MED., 1937, **37**, 198.

the acid or the alkali is too concentrated, both prolan and antiprolan will be destroyed. If too dilute one or the other will not be completely destroyed. In order to be certain that the prolan was rendered completely inactive we used in some of the experiments an excess of antiprolan; we neutralized 10 RU of prolan with 20 or 30 RU of antiprolan. In this case too we succeeded in reactivating the prolan by selective destruction of the antiprolan by NaOH.

II. *Reactivation of Antiprolan.* Thirty mg. of acetone-dry-powder (corresponding to 30 PAU of antiprolan) was dissolved in 1.5 cc. of aqua dest. and neutralized with 30 RU of prolan (dissolved in 0.3 cc. of aqua dest.). The neutral mixture (totalling 1.8 cc.) was placed in the incubator for 2 hours to enable a complete union to take place. Then the mixture was divided into 3 parts (0.6 cc. each) and Experiments 1-3 were carried out.

1. To 0.6 cc. of the mixture 3 cc. n/10 HCl was added in order to release the 10 RU of prolan from the complex and to destroy it. Twenty hours later 10 RU of prolan was added in order to combine with the released antiprolan (see below).

2. To 0.6 cc. of the mixture (for reasons of control) 3 cc. m/10 NaCl was added. This experiment should prove that the prolan is completely inactivated by the adequate amount of antiprolan.

3. For reasons of control as well 3 cc. m/10 NaCl was also added to 0.6 cc. of the mixture and 20 hours later 10 RU of prolan was added in order to demonstrate that there was no excess of antiprolan in the mixture (see below). As a further control Experiments 4 and 5 were carried out.

4. Ten PAU of antiprolan (dissolved in 0.6 cc. of aqua dest.) was added to 3 cc. of n/10 HCl, kept at room temperature for 20 hours and subsequently neutralized with some drops of n/NaOH. Then 10 RU of prolan was added to demonstrate that the antiprolan had not been destroyed by the produced concentration of hydrochloric acid and, therefore, was able to inactivate the 10 RU of prolan (see below).

5. Ten RU of prolan (dissolved in 0.6 cc. of aqua dest.) was mixed with 10 mg. acetone-dry-powder from normal rabbit serum (in 0.5 cc. of aqua dest.). To this mixture we added 3 cc. n/10 HCl and after 20 hours' contact at room temperature neutralized with normal NaOH. This control should demonstrate that the produced concentration of hydrochloric acid destroys the prolan if serum protein is present. The 5 tubes were allowed to stand at room temperature, as described above, in order to enable the hydrochloric acid to take effect. Then 2 drops of brom-thymol-blue-indi-

cator solution were added to every tube. Tubes Nos. 2 and 3 were neutral. Tubes Nos. 1, 4, and 5 were neutralized with some drops of NaOH. Then—as already mentioned above—10 RU of prolan was added to tubes 1, 3, and 4. Finally all 5 tubes were placed once more in the incubator for 2 hours, and the contents of each tube was injected into an infantile female rat. The result was as follows:

Rat 1 showed no gonadotropic reaction whatsoever, for the prolan which had been added at first had been destroyed by the HCl. That is why the subsequently added 10 RU of prolan could be inactivated once more by the released reactivated antigonadotropic factor.

Rat 2 showed no gonadotropic reaction whatsoever, for the 10 PAU of antiprolan and the 10 RU of prolan had annihilated each other completely.

Rat 3 showed the gonadotropic reaction HVR I-III, for by the *second* addition of 10 RU of prolan an excess of gonadotropic factor had been produced which could no longer be bound. The 10 antiprolan units present had been quantitatively bound by the first 10 RU of prolan.

Rat 4 showed no gonadotropic reaction whatsoever; the previously used 10 PAU of antiprolan not having been affected by the hydrochloric acid was thus still able to neutralize the subsequently added 10 RU of prolan.

Rat 5 showed no gonadotropic reaction whatsoever, for the 10 RU of prolan contained here had been destroyed by the hydrochloric acid.

Basing upon our previous investigations we arrive at the following conclusions regarding the mechanism of the antigonadotropic function:

Antiprolan is not a hormone *strictu sensu.* It is to be expected from an (anti)hormone that it can be demonstrated in the blood under normal conditions. We have not yet succeeded in doing so with antiprolan.

We demonstrated[2] that antiprolan is still effective if injected into animals 8 days previously to prolan. Consequently antiprolan maintains its effectiveness in the blood for 7 days. Hitherto no hormone is known which remains active in the blood for such a lengthy period, if applied in aqueous solution subcutaneously. Furthermore, our investigations[3] demonstrate that there is a high degree of species and organ specificity (99.5 and 93%) which also speaks against the hormonal character of antiprolan.

Is antiprolan a ferment? For neutralizing prolan a constant

[3] Zondek, B., and Sulman, F., PROC. SOC. EXP. BIOL. AND MED., 1937, **36**, 713.

amount of antiprolan is necessary; consequently prolan and antiprolan quantitatively neutralize each other. The time of the reaction of prolan with antiprolan does not affect the result. This fact suggests the assumption that antiprolan is not a ferment, as does the fact that prolan and antiprolan can be reactivated out of the prolan-antiprolan complex.

Is antiprolan an antibody? The serological investigations performed hitherto do not favor the assumption of an antibody, for complement fixation, precipitation and skin reactions cannot be performed with a serum rich in antiprolan, provided that the animals had been "immunized" with a pure prolan.[4] The antigonadotropic reaction certainly does not belong to that group of immune-reactions which are unmistakably provable by serological methods.[4-9]

At any rate we find especially among the toxins functions similar to those of antibodies but without the presence of serologically recognizable antibodies. It was not until the application of Ramon's flocculation method (which allows the toxins and antitoxins to flocculate—under optimal quantitative conditions—in the water-bath at 45°C.) that we knew that there were substances similar to the antibodies, the strong activity *in vivo* being easily provable but of which the serological proof *in vitro* may only be achieved under especially controlled conditions. We did not succeed with the help of Ramon's method in bringing about flocculation in the antigonadotropic serum after having added purified prolan.

While hitherto all serological reactions of antiprolan were negative we succeeded, however, by protracted treatment of rabbits with gonadotropic hormone from mare's serum (prosylan) in demonstrating spasmodically occurring evanescent antibodies which gave the complement fixation. One of us (S.) will report in detail upon this elsewhere. We mention it here in order to demonstrate that although by the serological methods used hitherto the antiprolan cannot be identified as an antibody and prolan as an antigen, this negative finding does not prove the antiprolan reaction not to belong to the group of the immune-biological reactions. In any case the following findings favor the assumption of an immune-biological reaction:

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4 Sulman, F., *J. Exp. Med.*, 1937, **65**, 1.

5 Twombly, *Endocrinology*, 1936, **20**, 311.

6 Brandt, R., a. Goldhammer, H., *Z. Immunitatsf.*, 1936, **88**, 79.

7 Bachmann, C., PROC. SOC. EXP. BIOL. AND MED., 1935, **32**, 851.

8 Gustus, E. L., Meyer, R. K., a. Dingle, J. H., PROC. SOC. EXP. BIOL. AND MED., 1935, **33**, 257.

9 Eichbaum, F., a. Kindermann, V., *Z. Immunitatsf.*, 1935, **86**, 284.

1. The injection of antiprolan protects a rat for 8 days against the effect of prolan. This process especially points to a passive immunization.

2. Antiprolan is not to be found in the albumin but in the globulin of the serum, namely in certain fractions of the globulin (pseudoglobulins)[2] thus resembling some of the antibodies.

3. The reversibility of the antiprolan effect also speaks in favor of an immune-biological reaction.†

While our experiments suggest that antiprolan is neither a hormone-like nor a ferment-like body, yet on the other hand we find some, if not all, properties corresponding to those of an immune-biological body. It is possible that here we have to deal with a new kind of body approaching very closely the immune-bodies without producing the vitro-reactions typical of those bodies.

Summary. The inactivation of prolan by antiprolan is a reversible process, since prolan and antiprolan may be released from a neutral prolan-antiprolan mixture and thereby reactivated.

The assumption is made that antiprolan is neither an (anti)hormone *strictu sensu* nor a ferment, but is possibly a new kind of factor approaching very closely the immune-bodies to which it is in some respects quite similar.

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Effect of Vitamin D on Growth of Tubercle Bacilli.*

WM. STEENKEN, JR., AND E. R. BALDWIN. (Introduced by L. U. Gardner.)

From the Research and Clinical Laboratory, Trudeau Sanatorium, Trudeau, N. Y.

It was shown[1, 2] that feeding irradiated cream or oil concentrates moderately high in vitamin D to tuberculous guinea pigs produced no change in the course of the disease.

To control this observation further it was decided to observe the

† In a previous report[3] we expressed the supposition that antiprolan destroys prolan. The experiments reported here, however, show that this supposition was wrong for now we can prove the reversibility of the reaction.

* This study was made possible through a grant from the Wisconsin Alumni Research Foundation.

1 Loewen, David F., and Oatway, Wm. H., *Am. Rev. Tuberc.*, 1936, **33**, 733.

2 Steenken, Wm., Jr., and Baldwin, E. R., *Am. Rev. Tuberc.*, 1937, **35**, 656.