the nitrogen of the sediment by microkjeldahl as previously described.[5]

Increasing slightly the concentrations of nicotinic acid and β-alanine improves the growth somewhat, but one or more additional substances evidently must be supplied for maximal growth.

Conclusions. Minute amounts of nicotinic acid, β-alanine, and pimelic acid, present together, permit about two-thirds the maximal growth of the diphtheria bacillus obtained from whole tissue extracts when added to a suitable control medium. The pimelic acid appears to be the least essential of the three. Differences appear between strains, even of the Park 8 organism, which may well be multiplied when further strains are examined.

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The Antigonadotropic Factor. Origin and Preparation.

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One of us[1] pointed out the fact that in rodents the gonadotropic effect diminishes if prolan from human pregnancy urine is applied in a protracted manner. Since that time the phenomenon has been repeatedly studied. Collip, Selye, *et al.*,[2] explained this as being due to the effect of antihormones, and succeeded in proving in the test-tube experiment the antisubstances against the hormones.

Proof of the presence of an antigonadotropic factor can be furnished only by the impairment of the gonadotropic reaction in rodents. As shown by our experiments[3] proof of the antigonadotropic factor by serological methods is not obtainable, pure prolan not forming any precipitins or complement-fixing antibodies. The sera of experimental animals which had been "immunized" with pure prolan over a period of more than 2 months do not show any serological reaction; they evince, however, a highly antigonadotropic effect.

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5 Mueller, J. H., *J. Bact.*, 1935, **29**, 383.

1 Zondek, B., *Hormone d. Ovariums u. d. Hypophysenvorderlappens*, Berlin and Vienna, J. Springer, 1st ed., 1931, p. 159; 2nd ed., 1935, p. 272.

2 Collip, J. B., Selye, H., *et al.*, PROC. SOC. EXP. BIOL. AND MED., **1934, 31, 487,** 566, 1113; 1935, **32**, 544.

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

The titration of our antigonadotropic sera was performed exclusively by impairing the production of the anterior pituitary reactions (HVR) in rodents. 0.5 cc. of an antigonadotropic serum or the corresponding amount of an antigonadotropic dry powder solution (see below) was mixed with prolan (the latter in increasing amounts), filled up to a total volume of 4 cc., placed in the incubator for 2 hours and finally injected into the experimental animals, infantile female rats 3-4 weeks old, weighing 25 to 30 gm. They were injected 4 times (if there were more than 4 cc. of the injection fluid, 6 times) at equal intervals (12 hours), spread over 36 hours. Sixty hours after the first injection we began with the vaginal smears, at intervals of 12 hours. One hundred and twenty hours after the first injection the animals were killed and ovaries and uteri examined.

One prolan rat unit is known to be the smallest amount of prolan able to produce the anterior pituitary reactions (HVR) I, II or III, in infantile female rats (Zondek[1]). We define as one prolan anti-unit (PAU) the smallest amount of the antigonadotropic factor able to prevent the anterior pituitary reactions (HVR) I, II or III, according to the above experimental order.

For the quantitative titration of the prolan the anterior pituitary reaction (HVR) I, *i. e.*, the bringing about of oestrus, is especially fitted. We hold that this method for the quantitative determination of the units is much more exact than the record of the mere increase in weight of the ovary. According to our experiments the weight increase is not in proportion to the prolan units and is not such a precise indicator as the oestrus reaction (HVR I). We applied as a minimum per rat 10 RU of prolan, which, in our control-experiments, always produced HVR I, II or III; but if the substance was added to an effective antigonadotropic serum these reactions were not obtained in any case.*

In a few cases we performed the titration of the antigonadotropic serum in mice and rabbits as well. These animals, however, cannot be compared with the rat for exactitude of the reaction, the latter being an especially sensitive and exact indicator for prolan A.

The antigonadotropic factor has its origin in the blood and is found especially in the serum. We can obtain it by repeated treatment of the experimental animals, the application of high doses of

* The rat test can be performed proceeding with simultaneous or subsequent single or repeated applications of prolan and antiprolan. (Details will be given in a later report.) Antiprolan may be given a long time before or even after prolan, but, from reasons of principle, we kept to the 2 hours-incubator-contact-method *in vitro*.

hormone does not seem to be as important as the daily repetition of the treatment with gonadotropic hormone. For preparation of the antigonadotropic factor we usually used the blood of rabbits, less frequently that of rats. In the rat we could observe the formation of the antigonadotropic factor following a prolonged application (twice a week subcutaneously), however the titre did not become very high. If we treated rats in this manner for one year twice weekly with 100 RU of prolan respectively after close of the treatment we still could observe swollen follicles and fresh corpora lutea, which is not obtainable, as is well-known, by daily treatment with gonadotropic hormone (Evans, Zondek).

Rabbits which were injected with 250 RU prolan 4 times a week subcutaneously and twice a week intravenously showed 2 months later underweight, atrophic, diffusely luteinized ovaries. The curve of the blood titre was approximately as shown in Table I.

TABLE I.

Duration of treatment weeks	Titre per cc. of serum PAU
4	10†
6	20
8	80
12	100
20	120
26	150

† 1 PAU = 1 prolan anti-unit (see above).

N.B. At least 10 units must be assayed in a test rat.

It is necessary to have an exactly titrated antigonadotropic factor always in stock and to be in a position to preserve and concentrate it. For that reason we worked out the following method: The serum to be examined is poured into 4 times its volume of acetone, whereat a finely flaked precipitation forms. The precipitate is instantly freed from the supernatant fluid on a Buchner or Seitz filter; after that it is washed 3 times with acetone, while being constantly stirred and withdrawn by suction, and finally washed again 3 times with ether. For 5 minutes air is drawn through the filter, until the precipitate is absolutely dry. The resulting fine rose-colored powder is preserved under vacuum in the exsiccator, and there it is preservable at least for 6 months. The loss of titre along with the precipitation of the serum is relatively small, about 10%. In general, one cc. of the serum produced 50-70 mg. of dry powder; (one mg. = 1-2 PAU). The latter is easily soluble in distilled water, in slightly acid and slightly alkaline buffers as well as in diluted acetone and alcohol. It is of importance that the precipitate results as a

very fine-grained one, coarse-grained serum powder being very slowly and not completely soluble.

The antigonadotropic factor was further concentrated by salting out procedures with saturated ammonium sulphate solution (SAS). Using serum or adequate solutions of acetone dry powder in distilled water (60 mg. in one cc.) we were able to precipitate the antigonadotropic factor almost quantitatively at 48% SAS (pseudoglobulin fraction). The fractions below this concentration were found to contain only slight amounts of the antigonadotropic factor. Using a more concentrated solution of the antigonadotropic factor (60 mg. dry powder in 0.5 cc.) we were able to precipitate it already in the globulin fraction at 28.6% SAS.

In analogy to the preparation from the blood we used the following method to determine the content of the antigonadotropic factor in the tissues: The tissues were cut into very small pieces, pounded in a mortar with sea-sand, and finally extracted in the shaking-machine for 4 hours (after addition of 10 times the amount of distilled water). After being centrifuged, the dim rose-colored viscid extract was filtered and then precipitated with 4 times the volume of acetone. The precipitate is washed 6 times with acetone, while constantly being stirred on a Buchner or Seitz filter, then dried 3 times with ether, and finally air is drawn through for 5 minutes. The resulting fine white powder is placed into the exsiccator over night and is once more extracted on the following day. The extraction takes half an hour respectively in the shaking-machine; at first 4 times with distilled water, then twice with an alkaline glycocoll-buffer (pH = 9.6) and finally twice with an acid glycocoll-buffer (pH = 3.5). After having combined these extracts we have a clear brownish solution, which may be reprecipitated into a fine-grained acetone dry powder. As mentioned above in connection with the serum, this powder is easily and completely soluble in any required amount of distilled water.

Urine was assayed as well by means of this concentration method for its content of antigonadotropic factor.

We studied the following organs as to their capacity of forming and storing antihormone: ovaries, testes, liver, spleen, musculature, urine. The results obtained were as follows:

The serum of a rabbit treated daily with 250 RU prolan for 26 weeks had a maximal titre of 150 PAU per cc.

Liver, spleen and musculature of the experimental animal contained nothing of the antigonadotropic factor.

The urine of the experimental animal contained nothing of the antigonadotropic factor.

Castrated male and female rabbits formed the antigonadotropic factor in their blood in the same manner as normal animals.

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The Antigonadotropic Factor. Species Specificity and Organ Specificity.

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There are contradictions to be found in literature as to the specificity of the antigonadotropic factor. Bachmann, Collip and Selye,[1] Fluhmann,[2] Meyer and Gustus,[3] Brandt and Goldhammer,[4] Twombly,[5] Sulman,[6] Thompson,[7] hold that the species specificity of the antigonadotropic factor is proved. Gegerson, Clark and Kurzrok,[8] Rowlands,[9] Parkes and Rowlands,[10] however, presented evidence against the species specificity. Recently Collip[11] reported similarly. However, the latter workers had other test objects (inhibition of ovulation in the mated rabbit or of the oestral cycle in the normal rat) than the former,[1-7] and than we did. Fluhmann,[2] Brandt and Goldhammer,[4] Gegerson, Clark and Kurzrok,[8] Parkes and Rowlands[10] are opposed to the presence of an organ specificity. Selye, Collip and Thompson,[12] however, favor it. To investigate this we performed the following experiments. We used the technique described in our first report,[13] that of the exact titration of the gonadotropic hormone against adequate amounts of the antigonadotropic factor in infantile female rats and mice.

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11 Collip, *Canad. Med. Assoc. J.*, 1937, **36**, 199.

12 Selye, Collip and Thompson, PROC. SOC. EXP. BIOL. AND MED., 1934, **31**, 487, 566.

13 Zondek and Sulman, PROC. SOC. EXP. BIOL. AND MED., 1937, **36**, 708.