CCXLIII. THE PREPARATION OF CONCENTRATED ANTIGONADOTROPIC FACTOR (ANTIPROLAN)

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THOMPSON [1937] showed that when antigonadotropic sera are salted out with 33–50 % sat. $(\text{NH}_4)_2\text{SO}_4$, the antigonadotropic factor may be quantitatively recovered from the pseudoglobulin fraction. Zondek & Sulman [1937] described independently several methods of preparation of the antigonadotropic factor, viz. precipitation with acetone and salting out with $(\text{NH}_4)_2\text{SO}_4$ directly from the serum or from a solution of an acetone-powder prepared therefrom. Harington & Rowlands [1937] confirmed and extended these results in an extensive series of experiments.

The following methods of preparation have now been studied by us:

(1) Direct precipitation, utilizing acetone, alcohol, benzoic acid, flavianic acid and tannic acid, both alone and combined.

(2) Fractional precipitation, utilizing $(\text{NH}_4)_2\text{SO}_4$, KI plus NaCl, precipitation at the isoelectric point, precipitation by CO_2 , precipitation by dialysis and combinations of these methods.

(3) Adsorption and elution, utilizing kaolin, Lloyd's adsorbent, aluminium hydroxides A and B prepared as described by Willstätter, "Fasertonerde", franconite and benzoic acid.

(4) Coagulation and elution, utilizing acetone, alcohol and heat.

(1) Direct precipitation

(a) *Precipitation by acetone.* The method of precipitation by acetone was described by Zondek & Sulman [1937]; 1 mg. of the acetone-powder contains 1–3 P.A.U.¹), the concentration depending on the titre of the original serum. The loss by precipitation amounts to 10 % at the most. This method of preparation renders storage of antigonadotropic factor in the desiccator without loss in titre for more than a year possible. Rabbit antisera treated by this method yield an acetone-powder which dissolves readily in water and normal saline giving concentrations of active substance 3–4 times greater than in blood. The acetone-powder from goat antisera, on the other hand, is difficultly soluble even to the original blood concentration, and its solutions always retain a slight opalescence.

(b) *Precipitation by alcohol.* The technique of the alcohol precipitation is the same as that of the acetone method. The results with alcohol are not as good,

¹ 1 P.A.U. = 1 prolan anti-unit is the smallest amount of antiprolan able to neutralize the gonadotropic effect of 1 R.U. of prolan. At least 10 units must be assayed in a test rat [cf. Zondek & Sulman, 1937].

however, since the alcohol-powder is only incompletely soluble and the solutions are more opalescent than are those obtained with the acetone-powder.

(c) *Precipitation by benzoic acid.* On addition of a concentrated solution of benzoic acid in acetone to antiserum a precipitate is formed. This precipitate can be freed from benzoic acid by rinsing in acetone and the residue dried with ether. The dry powder thus obtained is difficultly soluble in water or normal saline, and is no richer in antiprolan than the material obtained in the simpler direct precipitation with acetone.

(d) *Precipitation by flavianic acid.* With a few drops of 10% aqueous flavianic acid maximum precipitation of a 1:10 dilution of prolan antiserum is obtained. After 48 hr. in the ice chest, such a precipitate was centrifuged and freed from flavianic acid by washing with 0.5% ammonia in 96% alcohol. The residue was dried by washing first with acetone and then with ether. The dry powder obtained was partly soluble in distilled water. The water-soluble portion (A) was rich in protein but poor in antiprolan, and could be refractionated by salting out with 50% sat. $(\text{NH}_4)_2\text{SO}_4$ into two components both of which contained but little antiprolan. The water-insoluble portion (B) dissolved in Locke solution (as well as in *N*/50 ammonia and buffers of pH 9.5) giving a solution which was poor in protein, could not be further fractionated with $(\text{NH}_4)_2\text{SO}_4$ and was no richer than A in antiprolan. Quantitative analysis showed that the flavianic acid precipitated only 50% of the total antiprolan and that this percentage was distributed equally between fractions A and B of the precipitate. The method described is not therefore promising.

(e) *Precipitation with tannic acid.* The addition of a few drops of a 10% aqueous tannic acid to prolan antiserum brought about maximal precipitation. The sedimented material proved to be rich in protein as well as in antiprolan, but attempts to separate the two were unsuccessful.

Of the five methods of antiprolan precipitation tested, therefore, the acetone method alone gives satisfactory results.

(2) Preparation by fractional precipitation

(a) *Fractional precipitation with sat. $(\text{NH}_4)_2\text{SO}_4$.* The results obtained with this method were described by Zondek & Sulman [1937]. Rabbit serum fibroglobulin (25% sat. $(\text{NH}_4)_2\text{SO}_4$) and euglobulin (32% sat. $(\text{NH}_4)_2\text{SO}_4$) fractions were found to be free of antiprolan, the whole of which was contained in the pseudoglobulin (48% sat. $(\text{NH}_4)_2\text{SO}_4$) fraction; transition fractions (32–48% sat. $(\text{NH}_4)_2\text{SO}_4$) contained antiprolan only in proportion to their contents of pseudoglobulin.

The same experiments when carried out with goat antiserum gave different results in that with this serum about 50% of the antiprolan was brought down with the euglobulin, and only 50% with the pseudoglobulin fractions [cf. Harington & Rowlands, 1937]; as in the rabbit the fibroglobulin and albumin fractions contained no antiprolan. Our results with salting out in diluted antisera were not promising, but with acetone-powder from rabbit antisera salting out was successful in antiprolan solutions of twice the physiological concentration: at 9.1% sat. $(\text{NH}_4)_2\text{SO}_4$ a very light precipitate containing no antiprolan separated; at 18.2% no further precipitate was obtained but at 23.1% saturation a heavy inactive precipitate formed; at 28.6% saturation a further light precipitate containing the entire antiprolan content of the antiserum was obtained; further inactive precipitates were obtained at 33.3% saturation. The fractional

(NH_4) $_2\text{SO}_4$ precipitation method in acetone-powder solution is effective therefore for concentrating the antigonadotropic factor in rabbit antiserum. Experiments with goat antisera indicated that this method may not give the same favourable results with antisera from other species.

(b) *Fractional precipitation with KI plus NaCl.* In 1 ml. prolant antiserum diluted with 2 ml. distilled water was dissolved 1 g. KI; the resulting solution was treated with 1 g. NaCl which did not entirely dissolve. On standing in the ice chest for 24 hr., a light precipitate containing about 50 % of the serum antiprolan separated out. The supernatant, after dialysis through a cuprophane membrane, was found to have retained 50 % of the total serum antiprolan. The antiprolan yield obtained in this method of precipitation is therefore rather low.

(c) *Fractionation by isoelectric precipitation.* The precipitate formed by native rabbit or goat prolant antiserum at the isoelectric point contains practically no antiprolan. By working with solutions poor in electrolytes (solution of acetone-powder in distilled water) and at high dilutions (1 : 25 or 50) and by acidifying gradually with acetic acid, however, a considerable concentration of antigonadotropic factor was possible. Success in the formation of a maximal precipitate at the isoelectric point is chiefly a matter of practice. With experience it is possible to follow the general progress of the precipitation by rough nephelometric estimation. Addition of bromocresol green facilitates both the nephelometric comparison and the determination of the optimum pH (approx. 5.5) for precipitation.

Table I shows that the antiprolan content of the isoelectric precipitate depends on the degree of dilution of the solution of acetone-powder.

Table I. *Recovery of antiprolan by isoelectric precipitation of solutions of acetone-powder from rabbit antiserum solution containing 50 mg. powder = 100 P.A.U. per ml.*

Dilution	Yield of antiprolan in isoelectric precipitate %	Dilution	Yield of antiprolan in isoelectric precipitate %
1 : 1	0	1 : 20	83
1 : 2	33	1 : 25	90
1 : 4	50	1 : 30	90
1 : 8	66	1 : 40	90
1 : 16	75	1 : 50	90

It follows from the table that for a maximal yield of antiprolan in the isoelectric precipitation of rabbit antisera, preliminary dilution of the acetone-powder solution to at least 1 : 25 is desirable. Dilution beyond 1 : 50 is not to be recommended owing to the difficulty of determining the point of optimal precipitation. The isoelectric precipitates obtained are not readily soluble and it may be preferable to take them up in Locke solution. With goat antisera acetone-powder the isoelectric precipitation method has failed as yet to give as favourable results as are given by the rabbit antisera; even at dilutions of 1 : 50, only 50 % of the total antiprolan content came down in the isoelectric precipitate. Isoelectric precipitation is therefore an effective method of concentrating antigonadotropic factor, but is not applicable to all species of antiprolan sera. It is specially suited, however, for the purification of rabbit antisera, a concentration to 6 P.A.U. per mg. of dry substance being obtainable.

(d) *Fractional precipitation by treatment with CO₂.* On passing CO₂ through antiprolan solutions, precipitation of part of the serum globulins occurs. The centrifuged precipitate contains at best about 40 % of the total serum antiprolan. Concentration of antiprolan by this method is therefore impracticable.

(e) *Fractional precipitation by dialysis.* Native rabbit antiserum was dialysed in a cuprophane membrane against running tap water for 24 hr. The removal of electrolytes caused partial precipitation of the globulins and with them of 50% of the serum antiprolan. With goat antisera also rapid precipitation of serum globulins containing 50% of the total antiprolan was obtained. Fractional precipitation by dialysis is therefore impracticable as a method of concentrating the antigonadotropic factor.

(f) *Combined methods of fractional precipitation:* Two combined methods were tested: method A in which the isoelectric precipitation of rabbit antisera as described under (2 (c)) is followed by dialysis of the precipitate dissolved in Locke solution as described under (2 (e)) and method B in which the isoelectric precipitate (2 (c)) is extracted with 25% sat. $(\text{NH}_4)_2\text{SO}_4$ (2 (a)) instead of with Locke solution.

(i) *Combined method A.* 50 mg. of antiprolan acetone-powder, corresponding to 1 ml. native rabbit serum (= 50 P.A.U.), were dissolved in 25 ml. distilled water and brought to maximal turbidity by addition of 1% acetic acid drop by drop in the presence of bromocresol green. After 1 hr. the suspension was centrifuged and the precipitate was taken up in 3 ml. of Locke solution. This was then dialysed in a cuprophane membrane against running tap water for 24 hr. Under this treatment, a hydrophobic fraction of the isoelectric precipitate separated. The isoelectric precipitate without the hydrophobic fraction was found to contain 83% of the total serum antiprolan. 1 mg. of this final material contained 10 P.A.U., the concentration attained being a tenfold one. This purified material when dissolved in 0.005 *N* NaOH and diluted to a concentration of 25 P.A.U. per 4 ml. gave slight ninhydrin and biuret reactions and but a slight clouding with acetone and salicylsulphonic acid; no coagulation occurred on boiling.

(ii) *Combined method B.* 50 mg. antiprolan acetone-powder, corresponding to 1 ml. native rabbit antiserum (= 50 P.A.U.), were dissolved in 25 ml. distilled water and brought to maximal turbidity by addition of 1% acetic acid drop by drop in the presence of bromocresol green. After 1 hr. the precipitate was centrifuged, suspended in 3 ml. 25% sat. $(\text{NH}_4)_2\text{SO}_4$ and shaken for 1 hr. The extract was dialysed against running tap water. A slight inactive precipitate separated. The remaining supernatant liquid contained 75% of the total serum antiprolan at a concentration of 10 P.A.U. per mg. of dry substance, and behaved in protein tests as did the solution obtained in combined method A.

By combined fractional precipitation methods therefore, i.e. (a) isoelectric precipitation followed by dialysis, or (b) isoelectric precipitation followed by fractionation with sat. $(\text{NH}_4)_2\text{SO}_4$, a tenfold concentration of the antiprolan content of rabbit antiserum is possible.

(3) Adsorption and elution

(a) *Adsorption with kaolin and Lloyd's adsorbent.* The adsorbent was added to prolan antiserum at various pH values (4.5; 7.0; 9.5) until the reaction of the supernatant fluid to salicylsulphonic acid became negative. The suspensions were centrifuged and filtered and elution by means of glutamic acid, glycine and phosphate buffers at different pH values (9.5; 7.0; 4.9) was carried out. Elution of antiprolan from kaolin proved feasible only at alkaline or neutral reaction. Protein and antiprolan in the eluates gave parallel values. Elution from Lloyd's adsorbent failed consistently. These adsorption methods are therefore impracticable as methods of preparation of concentrated antigonadotropic factor.

(b) *Adsorption with Willstätter $Al(OH)_3$ A and B.* Antiprolan was adsorbed by alumina in alkaline, neutral and acid solution. Elution with alkaline, acid and neutral buffer solutions was tested. The acid eluents practically dissolved the aluminium compound giving toxic eluates which were useless for the purpose in hand; neutral buffers failed to elute antiprolan whilst alkaline buffers eluted varying amounts but at best not more than 30 % of the serum antiprolan. The methods described in this section are therefore of little practical value.

(c) *Adsorption with franconite and "Fasertonerde".* Adsorption by these two adsorbents occurred at every pH tested. Elution was possible only with alkaline buffers. The protein and antiprolan contents of the eluates gave parallel values, however, and it must be concluded that the method described is impracticable for the preparation of antiprolan in concentrated form.

(d) *Adsorption with benzoic acid.* Antiprolan solution was shaken with benzoic acid for 3 hr. The precipitate obtained was centrifuged, freed from benzoic acid by washing three times with acetone and tested for antiprolan. It was found to be inactive.

(4) *Preparation by coagulation and elution*

(a) *Coagulation with acetone and elution with dilute acetone.* As Zondek & Sulman have shown [1937], 80 % acetone precipitates the antigonadotropic factor. If water is then added to the suspension so that an acetone concentration between 40 and 45 % is obtained the antiprolan is eluted in quantities ranging between 33 and 50 % of the total available antiprolan. Simultaneously at least 33–50 % protein is redissolved. This method is therefore of no value for the preparation of antiprolan concentrates.

(b) *Coagulation with alcohol and elution with dilute alcohol.* Use of alcohol as described under 4 (a) for acetone gave similar results.

(c) *Coagulation by heat and elution with water.* The method here utilized has been used and is recommended by Besredka [1929] for the preparation of antisera concentrates. The antiprolan acetone-powder from rabbit serum was taken up in water in a concentration of 5 : 1. The mash obtained was shaken well and placed in the oven at 65° for 8 hr. to effect coagulation. The coagulate was extracted with distilled water. Since considerable quantities of protein accompanied the eluted antiprolan, such heat-coagulation followed by elution with water is not feasible as a method of separation of the antigonadotropic factor from serum protein.

DISCUSSION

The results presented in this paper show that the antigonadotropic factor cannot be obtained free from globulins by the common methods of adsorption and elution. This property distinguishes the antigonadotropic factor from numerous hormones and in particular from the gonadotropic factor itself. Evans *et al.* [1933], it will be recalled, adsorbed the gonadotropic factor on Willstätter alumina A and B and obtained highly purified hormone preparations on subsequent elution.

The antigonadotropic factor is always contained in the serum globulin, a property which places this factor in the same class as the antibodies. These experiments constitute further support for the view advanced that antiprolan is similar to an antibody and is not a hormone.

SUMMARY

Different methods of concentrating antiprolan are described.

I. Methods which appear to be of little practical value are:

(1) Direct precipitation with alcohol, benzoic acid, flavianic acid or tannic acid.

(2) Fractional precipitation utilizing potassium iodide plus sodium chloride, carbon dioxide or dialysis.

(3) Adsorption and elution, utilizing kaolin, Lloyd's adsorbent, Willstätter alumina A and B, franconite, "Fasertonerde" or benzoic acid.

(4) Coagulation and elution utilizing acetone, alcohol or heat.

II. The methods which were found to give results of significant practical value are:

(5) Precipitation and preservation by treatment with acetone and ether.

(6) Fractional precipitation with saturated ammonium sulphate.

(7) Isoelectric precipitation from dilute solutions poor in electrolytes.

The purest preparations were obtained by combined methods of preparation, viz.:

(8) Combined method A. Isoelectric precipitation of diluted antiserum, followed by solution of the precipitate in Locke solution and dialysis.

Combined method B. Isoelectric precipitation of diluted antiserum, followed by partial solution of the precipitate in 25 % sat. $(\text{NH}_4)_2\text{SO}_4$ and dialysis of the filtrate.

The concentrated antiprolan solutions obtained by the combined methods of preparation gave only a faintly positive reaction in the ninhydrin and biuret tests, developed only slight turbidities with acetone and salicylsulphonic acid and did not coagulate on boiling; 1 mg. purified antiprolan dry substance contained 10 antiprolan units.

The combined methods of preparation were not of equal efficacy with the two species tested, the results obtained with rabbit antiserum by the combined methods being far more promising than those obtained with goat antiserum.

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