CCLIII. FRACTIONATION OF ANTITHYROTROPIC AND ANTIGONADOTROPIC SERA

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THE rapidly growing literature on "antihormones" contains little reference to the fractionation of antithyrotropic or antigonadotropic sera. Thompson [1937] states that the whole activity of antigonadotropic serum is contained in the pseudoglobulin fraction. He gives few details however and the question of the precise distribution of antihormonal activity in such sera remains undecided.

The object of the present work was to make a general survey of serum fractions in relation to their antihormonal activity and to devise a method for the preparation of a stable concentrate of the active substance.

I. Antithyrotropic sera

Production and assay of sera. The antisera were obtained by the daily subcutaneous injection into adult rabbits of 25 mg. of an extract of ox anterior pituitary tissue (acetone-desiccated) prepared by extraction with 50% aqueous pyridine followed by precipitation of the solution with alcohol and ether; 4 mg. of this preparation given once daily to immature guinea-pigs of 200 g. body weight caused an average increase of about 35 mg. in the weight of the thyroid [cf. Rowlands & Parkes, 1934].

After the injections had been continued for ten weeks 2 ml. of the rabbit serum entirely inhibited the thyrotropic effect of the standard 4 mg. dose of anterior pituitary extract when simultaneously administered; after 16 weeks' injections 1 ml. of serum was adequate. The various serum fractions were assayed by observation of the degree to which the response to the standard dose of thyrotropic hormone was inhibited by their simultaneous administration. Groups of five guinea-pigs were employed for each assay.

Fractionations. Exp. 1. In the absence of all definite knowledge of the nature of the antihormone an orientating experiment was first carried out to determine its distribution between alcohol-soluble and alcohol-insoluble components of the serum. 80 ml. of serum were stirred into 400 ml. 95% alcohol and the mixture kept for 24 hr. at room temperature; the precipitate was collected, re-extracted with 80% alcohol (200 ml.), separated, dried and suspended in 1% NaCl (80 ml.) (1 A).

The combined extracts were freed from alcohol by distillation *in vacuo* (bath not over 50°) and the residue extracted repeatedly with benzene; the aqueous layer was freed from benzene by brief vacuum distillation and brought to 30 ml. (1 B) while the benzene extracts were evaporated and the residue taken up in 20 ml. olive oil (1 C).

Table I shows that this experiment entailed considerable loss of total activity, such of the latter as was left being mainly associated with the denatured protein precipitate (1 A).

Exp. 2. Aliquot portions of a second batch of antithyrotropic serum diluted with an equal vol. of distilled water were precipitated with $(\text{NH}_4)_2\text{SO}_4$ at 35 and

Table I. *Effects of antithyrotropic serum and fractions thereof on response of guinea-pigs to 5 daily doses of 4 mg. standard thyrotropic extract*

Exp. no.	Fraction	Equivalent of original serum given ml.	Increase in weight of thyroids mg.
1	—	0	37
	Whole serum	1	1
	1 A; alcohol-insol.	2	9
	1 B; alcohol-sol.—benzene-insol.	2	23
	1 C; alcohol- and benzene-sol.	2	40
2	—	0	37
	Whole serum	2	7
	2 A; 35 % sat. $(\text{NH}_4)_2\text{SO}_4$ ppt.	2	1
	2 B; 50 % sat. $(\text{NH}_4)_2\text{SO}_4$ ppt.	2	8
	2 C; albumin	2	22
	2 D; dilution ppt.	2	34
	2 E; not pptd. by $(\text{NH}_4)_2\text{SO}_4$	2	33
	2 F; whole serum dialysed	2	2
3	Whole serum	2	0
	Whole serum	1	8
	3 A; water-insol. globulin	2	36
	3 B; CO_2 -pptd. globulin	2	14
	3 C; pseudoglobulin	2	4
	3 B and 3 C	1	8
4	Dried globulin (3 B and 3 C)	0.5	12
5	Whole serum	1	1
	5 A; total globulin	1	1
	5 B; heat ppt. (aqueous)	1	28
	5 C; mother liquor of 5 B	1	31
	5 D; pepsin-digested	1	30
	5 E; acid-treated (control of 5 D)	1	26
	5 F; heat ppt. (alcoholic)	1	33
	5 G; mother liquor of 5 F	1	32
	5 L; alcohol-ether mother liquor	1	25
	5 M; alcohol-ether ppt.	1	5

50 % saturation; each precipitate was collected, redissolved in water and dialysed until free from $(\text{NH}_4)_2\text{SO}_4$, the volume of the final solution being made equal to that of the original serum and NaCl being added to 0.9 % (2 A and 2 B). The mother liquor from the 50 % $(\text{NH}_4)_2\text{SO}_4$ precipitation was saturated with solid $(\text{NH}_4)_2\text{SO}_4$ and the precipitate similarly treated (2 C).

A further portion of serum was diluted with distilled water (7.5 vol.) and the solution saturated with CO_2 ; after keeping overnight at 0° the precipitate was collected, washed with CO_2 -saturated water and dissolved in 0.9 % NaCl (2 D). The mother liquor from 2 C was freed from $(\text{NH}_4)_2\text{SO}_4$ by dialysis and concentrated *in vacuo* (2 E).

Finally 65 ml. of original serum were dialysed against running tap water for 24 hr., treated with NaCl to 0.9 % and adjusted to 75 ml. (2 F).

It appears from this experiment (see Table I) that antithyrotropic activity is associated with pseudoglobulin (activity of 2 A and 2 B), but scarcely at all with albumin (inactivity of 2 C) or euglobulin (2 D); the active substance is completely precipitated by $(\text{NH}_4)_2\text{SO}_4$ (2 E) and is non-dialysable (2 F).

Exp. 3. Total globulin was prepared as in Exp. 2; the small precipitate which separated on dialysis against distilled water at 0° was collected and dissolved in 0.9 % NaCl (3 A); a further (gummy) precipitate obtained by saturation of the mother liquor from 3 A was similarly collected (3 B); pseudoglobulin was reprecipitated from the mother liquor of 3 B by half-saturation with $(\text{NH}_4)_2\text{SO}_4$ (3 C).

As may be seen from Table I the antithyrotropic activity is again associated with the globulin and particularly with the pseudoglobulin (3C).

Exp. 4. The residues remaining from 3B and 3C after physiological test were combined and evaporated to dryness in a vacuum desiccator, first over H_2SO_4 and then over P_2O_5 . The organic matter of the residue represented about 2% of the original serum, and the dry preparation retained its full activity; moreover, it has remained fully active for a year since its preparation.

Exp. 5. Total globulin was prepared as before from a batch of 880 ml. of antithyrotropic serum. An aliquot of the solution having been reserved (5A) further portions were treated as follows: 5B, 60 ml. adjusted to pH 5.7, heated at 80–85° for 30 min., precipitate collected, suspended in 0.35% NaCl, warmed to 45° and treated with NaOH to pH 8.5; 5C, mother liquor from 5B; 5D, 120 ml. adjusted to pH 1.8 and divided into equal parts, one being incubated with pepsin (0.14 g. of Parke Davis 1:10,000) for 2 hr. at 37° (5D) and the other (5E) similarly treated with water instead of pepsin solution (both these fractions were neutralized to pH 6.8 at the end of the experiment); 5F, 60 ml. adjusted to pH 5.7, treated with alcohol (250 ml.) and brought to boiling; precipitate treated as in 5B; 5G, mother liquor from 5F after freeing from alcohol; 5M, 70 ml. chilled to 0° and poured slowly with stirring into 500 ml. absolute alcohol at –8°, precipitate collected and washed repeatedly with dry ether at 0°, finally being extracted with dry ether in a Soxhlet apparatus for 5 hr.; 5L, mother liquors from 5M freed from alcohol and ether.

Here (see Table I) activity is confined to 5A and 5M; i.e. it has been destroyed by heating at the approximate isoelectric point of serum globulin both in presence and absence of alcohol and by exposure to dilute acid both with and without addition of pepsin. On the other hand dehydration with cold alcohol and ether (5M) is no more destructive of activity than is simple desiccation.

Exp. 6. The last conclusion was confirmed by cold alcohol-ether dehydration of the total globulin from a further batch of antithyrotropic serum. This preparation (representing 2.5% of the original serum) has remained fully active in the dry state for 9 months; it is almost completely soluble in 0.9% NaCl (6).

II. Antigonadotropic sera

(a) *Pituitary gonadotropic substance.* The thyrotropic pyridine extract of ox pituitary described on p. 2049 was also gonadotropic as tested on oestrous rabbits, and the antiserum prepared against the extract had antigonadotropic activity as shown by its power to inhibit the ovulation which normally occurs 10–11 hr. after mating of the oestrous rabbit [Parkes & Rowlands, 1936]. Whereas 5 ml. of the original serum reduced the percentage ovulation in a group of 10 mated rabbits to 30, an amount of fraction 6 equivalent to 5 ml. original serum inhibited ovulation in 3 out of 6 rabbits. The dry globulin powder therefore retains much, if not all, of the antigonadotropic activity.

(b) *Urine of pregnancy gonadotropic substance.* A human urine of pregnancy extract ("Pregnyl"—UP12) was obtained. Assayed by the ovarian response of the immature rat, 0.25 mg. given once daily over 5 days produced ovaries weighing 35 mg. which represents nearly the maximum growth attainable in the time by this type of preparation.

A 45 kg. goat was injected daily for 6 weeks, subcutaneously, with 10 mg. of this preparation; afterwards the daily dose was raised to 25 mg. for another 13 weeks. Samples of serum were obtained weekly after 11 weeks of injections.

The antiserum was assayed against a standard amount of another urine of pregnancy extract (UP10) of which 1 mg. over 5 days caused an increase in the

ovary of the immature rat from the normal weight of 10 to 35 mg. Groups of 10 rats weighing 40–50 g. were used. They were injected once daily for 5 days, the gonadotropic extract and the antiserum being injected on opposite sides of the animal. These were killed 24 hr. after the last injection; the ovaries were fixed in Bouin's fluid and subsequently weighed on a torsion balance after immersion in 70 % alcohol for a few hours.

The fractionation and assay work were performed on serum taken after 4 months or more of injections, when 0.0125 ml. of the antiserum inhibited 1 mg. of the gonadotropic extract of urine of pregnancy (UP 10) [Rowlands and Parkes, 1937].

170 ml. of the serum were treated as usual and the solution of the $(\text{NH}_4)_2\text{SO}_4$ precipitate dialysed against running tap water for 24 hr. and then for 48 hr. against changes of distilled water at 0°. The dialysed solution was adjusted to pH 5.5 with dilute acetic acid and the precipitate of euglobulin separated at the centrifuge and dried in a vacuum desiccator over H_2SO_4 . 0.935 g. was obtained, of which 5.5 mg. were equivalent to 1 ml. of serum. *Fraction AGG 1A*. Pseudo-globulin was precipitated from the mother liquor with cold alcohol and ether as usual. 4.045 g. powder were obtained, of which 24 mg. were equivalent to 1 ml. serum. *Fraction AGG 1B*.

These fractions were dissolved in saline and injected together with a total of 1 mg. urine of pregnancy extract, UP 10. The results of the assays of the whole serum and of the fractions are given in Table II. When a total of 0.0125 ml. of

Table II. *Effects of antigonadotropic serum and fractions thereof on response of immature rats to 1 mg. of urine of pregnancy extract (UP 10)*

Fraction	Amount mg.	Equivalent of original serum ml.	Weight of ovaries mg.	Macroscopic effect on reproductive organs
AGG 1A	2.50	0.46	12	Uterus not stimulated, vagina closed
	1.25	0.23	11	Uterus not stimulated, vagina closed
	0.62	0.115	15	Vagina open in all. No ovulation
	0.31	0.056	27	Vagina open in all. Corpora lutea in all ovaries
AGG 1B	12.5	0.52	13	Vagina closed
	6.25	0.26	10	Vagina closed
	3.12	0.13	10	Vagina closed
	1.56	0.065	12	Vagina of 2 rats open and corpora lutea in ovaries
	0.78	0.032	14	Vagina open in 2 rats, only one had ovulated
	0.39	0.016	20	Vagina open in all, 8 had ovulated
Whole serum	—	0.1	8	Uterus not stimulated, vagina closed
	—	0.05	10.5	Vagina closed
	—	0.025	11	Vagina closed
	—	0.0125	14.5	Vagina open in 9 rats, 3 had ovulated
	—	0.00625	17.5	Vagina of all open, 9 ovulated

the whole serum was injected with 1 mg. of the urine of pregnancy extract the reproductive organs (particularly the uterus) of the injected rats showed some signs of stimulation, although the effect was scarcely noticeable in the weight of the ovary. The administration of half this amount of serum (0.00625 ml.) over 5 days together with the same amount of gonadotropic extract, allowed of a significant increase in the weight of the ovaries.

The vagina was open in all the rats receiving 0.00625 ml. of the serum, whilst nine of the ten rats had corpora lutea in the ovaries.

It will be seen that, compared in equivalents of original serum, the pseudo-globulin fraction is 4-5 times as active as the euglobulin fraction. Since the former constitutes about four-fifths of the total globulin content of the serum, the two fractions are about equally active per mg.

The two fractions were also tested for their power to inhibit ovulation in the oestrous rabbit. In an equivalent of 0.35 ml. or less of original serum, both fractions inhibit the activity of an amount of urine of pregnancy extract (UP 10) which, given alone, would cause ovulation in 100 % of a group of oestrous rabbits. The result shows that separation of the euglobulin and pseudoglobulin fractions offers no advantage.

After 5 months of injections, the goat was bled to death under anaesthesia. About 1200 ml. of serum were collected, mixed with another 500 ml. obtained from the weekly bleedings and Seitz-filtered. The total globulin fraction was then prepared by Dr Hartley.

The 1700 ml. of serum were diluted with an equal volume of 0.9 % NaCl and 3400 ml. of saturated $(\text{NH}_4)_2\text{SO}_4$ added. After 2 hr. the precipitate of total globulin was filtered off, redissolved in 2000 ml. water and again precipitated by the addition of 2000 ml. of saturated $(\text{NH}_4)_2\text{SO}_4$. The globulin precipitate was collected on a filter paper, pressed into a soft mass, transferred to a collodion bag and dialysed, under slight pressure, against distilled water until free from $(\text{NH}_4)_2\text{SO}_4$. Solid NaCl was added to the dialysed globulin so that the final volume of the solution was 500 ml. and the NaCl concentration was 0.85 %. The globulin solution was sterilized by filtration through a Seitz filter and the filter washed with 0.85 % NaCl, the final volume of the solution of globulin being 500 ml. Exactly 5 ml. quantities of this solution were delivered by means of a precision distribution apparatus into a series of previously sterilized ampoules and the contents of the ampoules reduced to the dry condition by the method described by Hartley [1937]. The ampoules were filled with pure dry nitrogen and sealed. When required for use, solution of the dry globulin is effected by the addition of 5 ml. of sterile 0.9 % NaCl to each ampoule.

For assay, an ampoule of the dried globulin was reconstructed in sterile saline by heating to 37° for 2 hr. After solution was complete, the globulin was diluted with sterile saline and various doses were injected into groups of 10 rats for 5 days together with 1 mg. of the gonadotropic extract of urine of pregnancy (UP 10). Complete inhibition was afforded by the equivalent of 0.0125 ml. of original serum. A quantitative concentration of the antigonadotropic activity in the dry globulin fraction had therefore been achieved.

(c) *Gonadotropic substance of pregnant mare serum.* Rabbits chronically injected with 0.5 mg. rising to 3.0 mg. daily of an extract of mare pregnancy serum (Antex PMS2) yielded a serum inhibitory to a different preparation of pregnant mare serum (Antex PMS3) when tested on immature rats (Table III). Tests were carried out on groups of 5 immature rats.

This anti-“Antex” serum was fractionated by Dr Hartley as follows:

Table III. *Effects of antigonadotropic serum and globulin fraction thereof on response of immature rats to 2.5 mg. of extract of pregnant mare serum (Antex-PMS3)*

Nature of preparation of antiserum	Amount	Weight of ovaries mg.
—	—	58
Whole antiserum	0.5 ml.	41
Whole antiserum	1.25 ml.	20
Total globulin	15 mg.	48
Total globulin	50 mg.	20

300 ml. of 0.9 % NaCl were used to dilute 100 ml. of this serum and 400 ml. of saturated $(\text{NH}_4)_2\text{SO}_4$ were then added and the precipitated globulin separated by filtration. The amount of precipitate obtained was small and there was a considerable unavoidable loss in transferring the globulin precipitate to the collodion dialysing bag. The globulin was dialysed for 2 days against distilled water and the dialysed globulin brought finally to a volume of 15 ml. in 0.85 % NaCl. The solution was centrifuged to remove shreds of filter paper and the clear solution (15 ml.) was added drop by drop to a well cooled mixture of alcohol (100 ml.) and ether (50 ml.). The precipitated globulin was collected, dried and weighed (1.265 g.).

Table III shows that the activity of the antiserum can be recovered in the globulin fraction, but as 12.5 mg. of the globulin fraction corresponded to 1.0 ml. of the original serum, there has obviously been some loss.

SUMMARY

Antithyrotropic serum was obtained in rabbits by the prolonged daily injection of ox anterior pituitary extract. An antigonadotropic serum to human urine of pregnancy extract was prepared in a goat and an antiserum to a gonadotropic extract of pregnant mare serum was prepared in the rabbit by similar means.

The results show that the antithyrotropic and antigonadotropic activities of sera are associated with the serum proteins.

The antithyrotropic substance is non-dialysable, is almost completely precipitated by one-third to one-half saturation with ammonium sulphate but not by dilution and adjustment to pH 5.5. The activity therefore appears to be associated with the pseudoglobulin fraction. The pseudoglobulin fraction has been dried and the powder has retained its full activity for over a year.

Experiments on the stability of the antithyrotropic substance show that heating at 80–85° for 30 min., or boiling in aqueous-alcoholic solution completely destroys the activity.

The antigonadotropic activity of the antiserum to the gonadotropic extract of human urine of pregnancy, prepared in a goat, can be quantitatively recovered from the globulin fraction. There is an equal distribution between the pseudo- and euglobulin fractions.

Our best thanks are due to Dr P. Hartley who carried out the fractionation of the goat serum, and the anti-“Antex” serum.

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