XLVIII. PRELIMINARY EXTRACTION OF GONADOTROPHIC PRINCIPLE FROM PREGNANT MARE SERUM

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THE serum of the mare contains a relatively high concentration of gonadotrophic material during the second and third months of pregnancy [Cole & Hart, 1930]. This substance resembles anterior pituitary extracts in causing development of the follicles, luteinization and interstitial cell proliferation in the ovary of normal and hypophysectomized rats [Evans *et al.* 1933, 1, 2; Rowlands, 1938] while in the hypophysectomized male spermatogenesis is maintained or restored and the interstitial cells are stimulated [Evans *et al.* 1933, 3; Liu & Noble, 1939]. As a result of gonad stimulation secondary effects are produced in both sexes.

In the present paper a simple method is described for separating the active principle from the bulk of inert material in the serum.

Methods

Serum was obtained from the freshly clotted blood of mares at a suitable stage of pregnancy. It was stored at 0° and maintained at as low a temperature as practicable during all procedures.

The gonadotrophic activity was assayed by the increase in weight produced in the ovary of immature (40–50 g.) female rats. Since the supply of serum was limited groups of only 3 rats could be used for each test. Daily injections were made for 5 days and the animals killed on the sixth. The organs were dissected, fixed in Bouin's fluid and weighed from 70% alcohol.

Results

Precipitation with salicylsulphonic acid. An attempt was made to discover a method of removing the inactive material in the serum from the active substance. According to a British patent (specification No. 440,530 (19. ii. 1935)) protein precipitants such as picric acid, salicylsulphonic acid and trichloroacetic acid have been used for this purpose. Preliminary experiments in which picric acid or salicylsulphonic acid was added to the serum to complete precipitation gave inactive filtrates, but by suitably adjusting the concentration, and consequently the pH of the salicylsulphonic acid, it was found possible to obtain the required separation. The following standard method was employed for each precipitation, the concentration of acid being altered systematically. To 20 ml. of serum at 0° varying amounts between 1 and 2 ml. of 33.3% salicylsulphonic acid were added with constant stirring. The mixture was kept in the refrigerator for about 10 min., and was then centrifuged in cooled buckets. The clear supernatant fluid was decanted; its pH was determined by glass electrode and it was neutralized immediately with 2N NaOH. The precipitate was washed with 5 ml. of acid solution. After centrifuging the supernatants were pooled and adjusted to pH 7.5 using bromothymol blue as indicator.

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The results are summarized in Fig. 1, the weights of the ovaries being plotted against the pH of the different precipitations. Each test was performed on the equivalent of 2 ml. of the original serum which gave ovaries of 316 mg. when tested. From an examination of the curve it can be seen that the yield of activity

Fig. 1. Relationship between yield of gonadotrophic substance in salicylsulphonic acid supernatant and pH .

at pH 1.9–2.2 is almost 100%. At $pH > 2.2$ the decrease in potency is very rapid. Below pH 2.2 it is more gradual and probably due to destruction by the acid rather than to partial precipitation of the active material. It is important to use fresh yellow serum for this method since haemolysed serum requires much more salicylsulphonic acid to obtain the required pH and even then there is a considerable loss of potency in the extraction. A similar series of precipitations was carried out with normal horse serum to obtain the quantitative relationship between the pH of the precipitation and the weight of total solids in the supernatant. The supernatant solutions after dialysis to remove salicylsulphonic acid and salts were therefore evaporated to dryness and the solid content determined.

Fig. 2. Relationship between yield of dry substance in salicylsulphonic acid supernatant and pH .

From Fig. 2 it may be seen that a minimum yield of solid material is obtained from the supernatant at that pH where there is a maximum yield of gonadotrophic hormone, when pregnant mare serum is used.

Further concentration of the gonadotrophic principle was obtained following dialysis of the supernatant in a collodion thimble for 15–20 hr. at 4°. No loss of activity followed this treatment, and the solution after dialysis was evaporated to dryness *in vacuo* at low temperature, or precipitated with 80 % acetone. The yield of dry substance after such procedure was 100–150 mg. per 100 ml. of serum. It was found that if the solution after dialysis was concentrated to a small volume a precipitate was formed which could be removed by centrifuging. As this precipitate contained no activity the weight of dry active material could be reduced to 30–50 mg. per 100 c.c. of serum.

Acetone precipitation. A small amount of the active material was obtained by the above process and treated by fractional precipitation with acetone. The active substance was quite soluble in 50 % acetone or alcohol, but was precipitated quantitatively in concentrations above 80 % acetone. In one experiment a solution containing 6.5 mg. of the dry powder per ml. was adjusted to pH 7.5. Following the addition of an equal volume of acetone the precipitate was allowed to flocculate in the cold and was then centrifuged. The precipitate (PMS 8083) on assay was found to contain from 20 to 30 % of the total activity. The acetone concentration of the supernatant fluid was raised to 80 % and the further precipitate washed with acetone and ether and dried *in vacuo*. This powder (PMS 8082) contained 70 to 80 % of the total gonadotrophic activity. This experiment was repeated using a more dilute solution (PMS 83—3 mg. per ml.) since the activity in the 50 % acetone precipitate might be due to adsorption. An equal volume of acetone added to this solution only produced a faint opalescence and no definite precipitate was obtained until the pH was brought to 5.0. There was no activity in this precipitate but that obtained with 80 % acetone contained 100 % (PMS 831).

The dry weight of the final products represented 0.13 mg. and 0.09 mg. per ml. of the original serum respectively. Table I gives the activities of these various

Table I

No.	Preparation	Dose per rat			
		Equiv. to ml. of serum ml.	Wt. mg.	Ovaries mg.	Uterus mg.
PMS 801	Salicylsulphonic acid supernatant	1	(1)	64	154
PMS 8081	Insoluble part of supernatant	5	—	15	30
PMS 8083	50 % acetone precipitate	1	—	26	147
PMS 8082	50 % acetone supernatant	1	0.13	44	107
PMS 83	Salicylsulphonic acid supernatant	1	(0.25)	66	267
PMS 831	50 % acetone supernatant	1	0.09	67	141

samples, the comparatively low ovary weights being due to the use of haemolysed serum and consequent loss of activity during the salicylsulphonic acid precipitation. The final product is a stable powder readily soluble in water. In concentrations of 3 mg. per ml. it gives neither Millon nor biuret tests. Since these tests are faintly positive when applied to serum proteins in the same concentration, this indicates that the final material is partly non-protein.

Lack of material prevented further research into this acetone fractionation but it appears that the optimal conditions for precipitation are dependent on the pH and on the concentration of material.

Under the conditions described it is possible, therefore, to accomplish an approximately 1000-fold concentration (on the basis of total solids) when compared with the crude serum, by a relatively simple and rapid chemical process.

In spite of the marked purification which can be produced, the greater part of the product is inactive material since the above process yields approximately the same weight of dry substance when applied to non-pregnant horse serum.

DISCUSSION

A number of chemical procedures have been described for the purification of the gonadotrophic factor of pregnant mare serum. Goss & Cole [1931] fractionated pregnant mare serum by precipitation with sodium sulphate but only obtained a 10-fold purification. Evans *et al.* [1933, 4] used acetone-ammonia extraction of a solution of the acetone precipitate from the serum. This was applied by Catchpole & Lyons [1934] to fresh serum without any appreciable separation from the serum proteins. Later Evans *et al.* [1933, 5] obtained a purer product by adsorption of the same material on aluminium hydroxide; this procedure was modified and adapted to fresh serum by Gustus *et al.* [1936]. Finally, Evans and his co-workers [1936] claimed to effect a separation of the follicle-stimulating and luteinizing fractions by fractional precipitation with ammonium sulphate. A laborious method of fractional precipitation with acetone or alcohol designed by Cartland & Nelson [1937] yielded a 130- to 1800-fold purification of the active principle. Severinghaus *et al.* [1938] obtained promising results using the ultracentrifuge. The results here described indicate that salicylsulphonic acid precipitation of the inert material is a simple and rapid means of concentration. The further purification following dialysis, and the fractional precipitation with acetone yields the active substance in a form in which it would appear to be well suited for further chemical study.

SUMMARY

A method is described by which a purified fraction of gonadotrophic hormone may be obtained from pregnant mare serum, the essential steps being the precipitation of the inert serum proteins by a suitable concentration of salicylsulphonic acid, and subsequent purification by dialysis and fractional precipitation with acetone. The material obtained was a white powder readily soluble in water, and represented approximately a 1000-fold purification of the hormone in terms of solid content of original serum.

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