78. SERUM GONADOTROPHIN

I. PREPARATION OF A STABLE CONCENTRATE FROM PREGNANT MARE'S SERUM

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SEVERAL different methods have been proposed for the preparation of gonadotrophic concentrates from pregnant mares' serum, but the majority of these fail to achieve adequate concentration of activity in relation to total weight, without serious loss of active material. Goss & Cole [1931] employed sodium sulphate fractionation and reported a ten-fold concentration of activity in the pseudoglobulin fraction. Evans *et al.* [1933] proposed using aluminium hydroxide as an adsorbing agent and this was applied to fresh plasma by Gustus *et al.* [1936]. Catchpole & Lyons [1934] used acetone for fractionation but achieved little separation from the serum proteins, while Meyer's [1935] method, using butanol and acetone, proved troublesome owing to the interference of inorganic salts.

The method which has so far proved most acceptable is that of Cartland & Nelson [1937] with the modifications introduced by Goss & Cole [1940]. It depends upon fractional precipitation of the serum with acetone at different pH levels; very large volumes of solvent are required. Cartland & Nelson state that a product substantially free from serum proteins may be obtained, representing 65% of the initial activity. Goss & Cole find that recovery after the first stage, even when improved according to their directions, does not average more than 60%, and the overall yields of their final preparations are not stated, although high potencies are claimed.

The use of salicylsulphonic acid at pH 1.9–2.2 to remove the inert proteins from active sera has been recommended by Rinderknecht *et al.* [1939]. One would expect such strongly acid solutions to cause not only a decrease in activity but also some irreversible splitting of the serum proteins, and in our hands both these expectations have been realized. Severinghaus *et al.* [1938] have obtained promising preliminary results with the ultracentrifuge applied to pregnant mare's serum.

A study of the foregoing publications shows that a method is still awaited which will achieve an initial concentration of the gonadotropic hormone, providing, without serious loss, a stable, solid substance of moderate activity but sufficiently refined to be easily soluble and suitable for clinical use. Such a concentrate can be obtained by the method described below in a yield representing on the average 73% of the initial activity of the serum. The method is economical and easily operated on a large scale. It is proposed to use the concentrate so obtained for studies in further purification, and considerable progress has already been made in this direction.

Methods

Assays. All activities have been determined, in comparison with the International Standard, by the method described by Day & Rowlands [1940], using ten rats for each assay, and the results are expressed in International Units (I.U.).

Protein determinations have been calculated from the total nitrogen (micro-Kjeldahl) or determined by the colorimetric method of Greenberg [1929] using Folin's phenol reagent, a tyrosine standard and an empirically determined factor of 22.8 [see Rimington & van den Ende, 1940].

Bound carbohydrate has been determined by the orcinol method as described by Rimington [1940].

pH values have been determined electrometrically using the quinhydrone electrode or, where insufficient material was available, by indicators, using buffer solutions for comparison.

EXPERIMENTAL

Eight pregnant mares were available for this work and from each, after preliminary tests to confirm pregnancy, about six bleedings were made (see Table 3), each of approximately 2 l. of blood, the intervals between successive bleedings being a week and being so arranged as to cover the period during which gonadotrophic activity of the serum rises to its maximum [Day & Rowlands, 1940]. Although mating was not observed, the mares and stallion being pastured together, the estimated dates, based upon Day's [1940] method of rectal palpation for the diagnosis of pregnancy, are accurate to ± 5 days. The blood was drawn into sterile receptacles containing 22 ml. of 10 % potassium oxalate per 2 l. of blood, the cellular elements were removed on the centrifuge and the resulting plasma clotted by addition, with vigorous agitation, of 3 ml. of 4 % calcium chloride to each 100 ml. of plasma. After straining, the serum was passed through a Seitz filter.

For kindly performing all these operations we are greatly indebted to Mr F. T. Day of the School of Agriculture, Cambridge.

After a small sample of each serum had been removed for biological assay, the remainder was stored at -10° until required for chemical work.

Exploratory experiments with ammonium sulphate

The possibility of fractionation by means of ammonium sulphate was investigated by separating from 100 ml. of serum, diluted with an equal volume of water, the globulin fraction at 50 % sat. (further differentiated by dialysis into eu- and pseudo-globulin), crystalbumin, albumin I (50–66 % sat.), albumin II (66–75 % sat.) and albumin III (75–100 % sat.). Each fraction was dialysed and the protein, bound carbohydrate and gonadotrophic activity determined with the results shown for a typical experiment in Table 1.

Table 1. *Fractionation of active serum by $(\text{NH}_4)_2\text{SO}_4$*

Fraction	Activity		Carbohydrate Total N
	% of total	I.U./mg. protein	
Whole serum	100	1.58	0.212
Euglobulin	7.5	2.56	0.029
Pseudoglobulin	45	1.14	0.030
Crystalbumin	18	1.80	0.100
Albumin I	33.5	6.08	0.329
" II	0	0	0.538
" III	0	0	1.370

There is no sharp separation of activity in any one fraction. Albumin I possesses the greatest potency, but this fraction represents, in all, only one-third of the total activity. Precipitation of the active material is complete at 66 % sat. with $(\text{NH}_4)_2\text{SO}_4$, a fact confirmed in experiments upon purer material, although the albumins II and III are relatively more rich in bound carbohydrate.

Acidic protein precipitants

A sharp separation of gonadotrophic activity from inert material follows the use of salicylsulphonic acid, trichloroacetic acid or metaphosphoric acid as a deproteinizing agent. Rinderknecht *et al.* [1939] recommended adding a 33.3 % solution of salicylsulphonic acid to the serum until the *pH* lay between 1.9 and 2.2, and claimed a recovery of nearly 100 % of the activity in the filtrate. This is surprising in view of the known instability of serum gonadotrophin in acid solution, and in our hands yields of less than 60 % were obtained. We found, moreover, that under such drastic conditions some disintegration of the serum protein occurred, and the precipitate was consequently difficult to centrifuge, while the supernatant liquids exhibited a milk-like opalescence.

Trichloroacetic acid produced fairly complete deproteinization, but a high concentration of the reagent was required, with a correspondingly high acidity and rapid destruction of activity. A large proportion of the activity was found, further, to be carried down with the precipitate.

An ideal protein precipitant for the present purpose should operate in low concentration and without producing a high resultant acidity; it should also, if possible, combine with the protein so that any free excess is reduced to the minimum. These conditions are fulfilled by metaphosphoric acid, which we consider to be a reagent worthy of much more attention for the purpose of deproteinizing tissue fluids or extracts, as an initial stage in the isolation of comparatively labile chemical substances.

The protein-metaphosphoric acid reaction has been studied recently by Perlmann & Herrmann [1938] and by Briggs [1940]. The former authors pointed out that a salt-like combination occurs between the reactants and considered that the PO_3 bound reached a maximum when the $\text{PO}_3:\text{NH}_2$ ratio was 1; the protein is not denatured by the reagent. Briggs confirms the statement that metaphosphoric acid reacts with the basic groups of proteins to form salt-like combinations with very low dissociation tendency, but he finds the relationship between the quantity added, as Na metaphosphate, to the system and that bound by the protein to follow a Freundlich adsorption isotherm, so that the ratio $\text{PO}_3:\text{NH}_2$ in the flocculate increases continuously with increase in metaphosphate. No similar reaction occurs between metaphosphate and amino-acids or other substances of low molecular weight, containing single basic groups.

Optimal conditions for the HPO_3 precipitation

The relations between the quantity of metaphosphoric acid added, the proportion of protein precipitated, the final *pH* of the solution, and the recovery of activity in precipitate and supernatant respectively, were explored as follows. To 10 ml. lots of an active serum diluted 1:3 with distilled water were added differing quantities of a freshly prepared 0.3*N* HPO_3 solution. We have used throughout this work 'metaphosphoric acid, glacial in sticks' of British Drug Houses Ltd, and have found it convenient to work with the concentrations indicated. The addition was made dropwise to the well-stirred, ice-cold liquid; the precipitates were collected on the centrifuge, redissolved in water with the help of dilute alkali and made up to 10 ml. The *pH* of the supernatant fluids was determined on portions electrometrically, and on the remainders of these, and the redissolved precipitates, biological assays and determinations of protein (by phenol reagent) were performed. The results are shown in Fig. 1. At *pH* 3.5 the proportion of the total protein which is precipitated is 84 % and at *pH* 3.2 it has risen to 93.6 % with a corresponding improvement in the potency (activity per

mg.) of the unprecipitated active fraction; on the other hand, at reactions more acid than pH 3.5 loss of hormone from the filtrate becomes appreciable. While, therefore, separation of inert protein is not completed at pH 3.5, this end-point has been chosen as that giving the best all-round results for the reaction. The quantity of HPO_3 required to reach this end-point varies slightly, in accordance, no doubt, with the total protein content of the sera, but averages 475 ml. of the 0.3N solution per l. of serum. When working up large batches of material, the

Fig. 1. Relations in the system: Pregnant mare's serum—metaphosphoric acid.

precipitated protein metaphosphate has been removed by passing the mixture through a Sharples super-centrifuge and the cake has been washed by resuspending it in 0.01N HPO_3 , using about 350–400 ml./l. of original serum, and again separating in the super-centrifuge. The combined filtrate and washings are neutralized by adding 20% NaOH drop by drop with vigorous mechanical stirring.

The average recovery at this stage has been 95% of the original activity.

Adsorption of the active material upon benzoic acid

Katzman & Doisy [1934] were able to adsorb gonadotrophic hormone from pregnancy urine by adding to it a saturated solution of benzoic acid in acetone, whereupon benzoic acid was precipitated and could be filtered off, carrying a considerable proportion of the activity with it.

We endeavoured to achieve a similar adsorption from our metaphosphoric acid filtrates, but the yields were disappointing; not only was adsorption incomplete and variable, but there was also appreciable inactivation of gonadotrophic hormone. Benzoic acid suspensions have a pH of about 3, and we suspect that destruction is a result of this comparatively high acidity. Much better results were obtained by liberating benzoic acid in the solutions by adding a mineral acid to sodium benzoate. The system benzoic acid:sodium benzoate possesses buffering power, and a closer study showed that we could precipitate a sufficient amount of the free acid in a finely divided form in our solutions, both

effectively and economically, without allowing the pH to fall below 4.5, a point at which there is little fear of inactivation of the hormone. Trials were made by using differing quantities of adsorbent at the same final pH , and also by varying the pH when the amount of benzoic acid in suspension was kept unchanged, from which it was found that adsorption takes place efficiently, from filtrates of widely varying activities, when the concentration of benzoic acid is about 5% and the final pH is 4.5–5.0 but not greater than 5.5. These conditions are reached in practice by adding, to the filtrates, 60 g. sodium benzoate per l. (equivalent to 5% benzoic acid) and then running, drop by drop, into the ice-cold, mechanically stirred solution, rather more than three-fourths of the equivalent quantity of 3*N* HCl. The final pH may be checked electrometrically or colorimetrically.

When working with large batches, the resulting precipitate has been collected by passing the mixture through a Sharples super-centrifuge, and unprecipitated benzoic acid has been recovered from the supernatant fluid, for purification and re-use, by addition of an excess of mineral acid.

We have omitted any aqueous washing of the benzoic acid with its adsorbed active material. The precipitate was scraped out and stirred with a quantity of acetone approximately equal to the volume of serum originally employed. This dissolves the benzoic acid and leaves the adsorbate as a finely flocculent material, which can be collected on the centrifuge, washed free from benzoic acid and moisture with three successive quantities of anhydrous acetone and one of dry ether, and then dried *in vacuo*.

Properties of 'initial powder'

The material obtained at this stage is a snow-white, light powder which dissolves slowly in water or buffer solution at pH 7.0–7.5, forming opalescent solutions which give positive biuret and Molisch tests, but contain no heat-coagulable protein. We have not been able to detect any change in the activity of samples kept during 8 months in stoppered bottles at room temperature. The yield of this material is about 6 g./l. of serum, and the activity ranges from 10 to 50 I.U./mg. It will be seen that only two simple chemical operations are necessary to obtain this comparatively stable product from active mares' serum which normally deteriorates unless stored under special conditions.

Factors affecting yields

From thirty-nine different sera, the activities of which varied over a wide range, we have been able to recover on the average 79% of the original activity in the form of the stable initial powder. Details of the volumes, activities, yields etc. at each stage are presented in Table 2, and the mean data for each individual mare are summarized in Table 3. Our conclusions may be summarized as follows:

(1) *Volume of serum and weight of initial powder.* There is a fairly constant relationship between these quantities, irrespective of the activity of the serum employed, the mean yield being approximately 6 g./l. As will be seen later, we believe that this powder is made up, for the greater part, of some constituent, probably glycoprotein in nature (see p. 746), which carries the gonadotrophic hormone with it.

(2) *Activity of serum and activity of initial powder.* If the entrainment of gonadotrophically active substance by the inert material accompanying it in the initial powder were complete, or always in the same proportion, a straight-line relationship would be expected between the potencies of powder and serum.

Table 2. *Data on gonadotrophic activity and recovery up to stage of 'initial powder' from thirty-nine sera*

Mare and no. of sample	Stage of pregnancy days	Volume of serum ml.	Activity of serum I.U./ml.	HPO ₃ filtrate Recovery of activity* %	Initial powder		
					Weight g.	Activity I.U./mg.	Recovery of activity* %
J 6	44	665	270	119	4.464	42	106
7	51	1375	390	86	†4.207	40	65
8	58	1150	510	75	†4.647	38	
9	65	1135	450	105	†3.677	56	72
10	72	1080	300	102	†3.936	54	
L 3	45	975	100	91	8.838	40	91
4	53	1190	90	113	7.072	42	92
5	58	1980	210	77	6.302	20	110
6	67	850	180	93	7.084	14	93
7	74	1590	180	75	12.113	24	70
8	81	1355	120	89	5.846	30	116
M 3	49	940	170	102	10.738	20	87
4	56	1065	280	91	7.817	20	98
5	63	1320	360	96	5.084	26	84
6	70	1080	360	80	5.442	28	51
7	77	960	290	114	12.179	20	54
8	84	1090	230	78	6.235	30	48
N 3	53	1055	150	100	6.251	32	74
4	60	1042	330	105	8.211	26	88
5	67	1430	390	84	5.152	30	98
6	74	1250	300	116	6.267	36	66
7	81	900	210	106	†3.877	57	
8	88	1275	72	90	†4.227	52	79
P 3	45	1290	140	78	†3.941	38	81
4	53	1070	220	100	†4.246	36	
5	58	2340	420	116	5.069	30	81
6	67	1240	450	111	8.788	9	87
7	74	1130	240	113	6.745	15	64
8	81	1215	255	85	6.758	28	82
Q 5	80	1040	100	74	13.285	28	41
5A	80	620	64	—	9.236	24	40
R 2	59	1940	145	117	7.117	26	69
3	65	2170	155	106	6.876	29	65
S 4	47	817	145	101	6.632	7	44
5	54	1165	180	92	3.230	11	90
6	61	1390	240	94	8.803	30	95
7	68	1435	190	101	12.348	24	88
8	75	1220	145	92	3.484	34	102
9	82	1250	65	83	4.093	40	79
					7.683	34	78
					7.539	38	105
					6.324	18	65
					6.347	8	62

* Allowance has been made for samples withdrawn from the diluted serum for assay.

† 5% benzoic acid suspension.

‡ 10% benzoic acid suspension.

Fig. 2 demonstrates that this expectation is not fully realized. With sera containing up to 250 I.U./ml., the activity of the powder is very approximately a linear function of the activity of the serum, but with more highly active sera there is a progressive deviation from this ideal relationship. The experimental data can be more readily interpreted if the process of concentration of the activity be regarded as one of adsorption or, more strictly, of co-adsorption; gonadotrophin being first adsorbed by the inert serum constituent and this latter, with its attached active material, becoming secondarily adsorbed upon the benzoic acid precipitate liberated in the system. The latter adsorbent must be

Table 3. *Summary of data for individual mares*

No. of mare	No. of bleedings	Volume of serum extracted l.	Mean activity of serum i.u./ml.	Recovery in HPO_3 filtrate %	Initial powder		
					Total weight g.	Mean activity i.u./mg.	Recovery %
J	5	5.405	385	97	36.841	45	85
L	6	7.940	147	90	49.900	21	96
M	6	6.455	282	94	43.400	27	67
N	6	6.952	242	100	41.585	36	82
P	6	8.285	287	101	50.020	25	60
Q	2	1.660	82	74	3.394	9	67
R	2	4.110	150	112	21.151	27	91
S	6	7.277	161	94	35.469	29	82
39		48.084		95	281.760	27	79

present, however, in such quantity that adsorption upon it is virtually complete. The factor limiting the final yield of gonadotrophin would then be the relative quantity of the inert constituent supplied by the serum; increasing the amount of benzoic acid used should have little, if any, effect. This is borne out by the

Fig. 2. Relation between activity of serum and potency of initial powder.

results of experiments in which the HPO_3 filtrates from highly active sera were divided, and one-half worked up as usual with 5% of benzoic acid, the other half with twice this amount—see Table 4.

Table 4. *Effect of concentration of benzoic acid on recovery of gonadotrophin from highly active sera*

Serum	Activity i.u./ml.	Conc. of benzoic acid %	Wt. of powder g.	Activity i.u./mg.	Recovery of activity %
J 7	390	5	4.207	40	63
		10	4.647	38	66
J 8	510	5	3.677	56	70
		10	3.936	54	72
N 5	390	5	3.877	57	79
		10	4.227	52	79
N 6	300	5	3.941	38	80
		10	4.264	36	82

Fig. 3. Relation between activity of serum and percentage recovery in initial powder from individual mares. Abscissa = I.U./ml. of serum. Ordinate = percentage recovery.

Fig. 4. Relation between activity of serum ●-●-●, percentage recovery in initial powder ○-○-○ and stage of pregnancy for individual mares. Abscissa = stage of pregnancy in days. Ordinates = left: percentage recovery; right: I.U./ml. of serum.

(3) *Activity of serum and percentage recovery in initial powder.* Fig. 3 shows for each mare separately (omitting Q and R from which two bleedings only were obtained) the relationship between the original activity of the serum samples and the percentage of this activity finally recovered in the form of the 'initial powder'. In four out of six mares there is a regular fall in percentage recovery as the activity of the serum increases; in the remaining two cases, mares S and L, the peak activity usually encountered about the 65th day of pregnancy was much less marked, and the bleedings, therefore, provide insufficient evidence for such a comparison. These relationships are also brought out in Fig. 4, which relates stage of pregnancy to activity per ml. of serum and percentage recovery of activity.

The choice of a highly active serum as starting material for the preparation of gonadotrophic concentrate is not without its drawbacks. By pooling sera of higher and lesser activities, the optimal conditions for obtaining maximum recovery are likely to be more nearly approached.

A second feature discoverable from Fig. 3 is the difference between individual mares, some yielding sera from which a consistently better percentage yield of activity was obtained than others, e.g. the mean yield from the five bleedings of mare J was 85% and that from the six bleedings of mare P only 60% of the original activity of the sera. Such individual differences may, possibly, be due to differences in the proportion of 'inert protein adsorbent' in the sera.

Further concentration by fractional alcohol precipitation

The crude initial powder resulting from the benzoic acid adsorption can be further purified by alcohol precipitation at suitable pH levels; in particular, the sparingly soluble impurities can be eliminated and a material obtained assaying up to 625 I.U./mg. At this stage of the process we have been guided by the experience of Gurin *et al.* [1939] with urinary gonadotrophin, but we have modified their technique by adding alcohol to the neutral solution and then titrating with alcoholic acetic acid to pH 4.8, instead of shaking the powder directly with buffered alcohol. By this simplification, one complete set of manipulations, the extraction with 50% alcohol at pH 6.0, is eliminated; moreover, by titrating instead of washing repeatedly with solvent a great saving of time is effected and, in our experience, higher yields are obtained. The method may be described as follows.

The initial powder is brought into solution by grinding it with forty times its weight of water containing 4.4 ml. 0.1N NaOH per g. of powder, which is sufficient to bring the pH to about 7.4. It is advisable to stir the resulting mixture mechanically for some hours and to leave it standing in the ice chest overnight. An equal volume of alcohol ('Methcol') is then added and, while stirring mechanically, a 5% solution of acetic acid in 50% alcohol is added drop by drop until pH 4.8 is reached;¹ this requires approximately 1.1 ml./g. of initial powder.

The heavy precipitate which is formed is collected on the centrifuge after chilling the mixture for 1-2 hr., and washed three times by stirring with 50% alcohol buffered at pH 4.8 (60 ml. $N \text{CH}_3\text{COONa}$ + 40 ml. N acetic acid + 100 ml. water + 200 ml. alcohol) and recentrifuging. To the combined supernatant fluids an equal volume of alcohol is added (to 75%), whereupon a fine flocculent precipitate is formed. The mixture is left overnight at 0° and the precipitate then

¹ A small drop of the solution is mixed with a drop of water on a glazed plate; bromocresol green is added and the colour compared with that given by a similarly diluted drop of 50% alcohol containing acetate buffer at pH 4.8. Values so obtained have been checked by the quinhydrone electrode.

collected on the centrifuge, washed once with each of the following: 80 % alcohol, absolute alcohol, acetone and dry ether; it is finally dried *in vacuo*.

We have found the yields by our method to be very good, approximately 92 % by weight of the initial powder being removed, leaving, on the average, 92 % of the activity in the concentrate (see Table 5).

Table 5. *Purification of initial powder by 50 % alcohol at pH 4.8*

No.	Initial powder		Concentrate		Recovery of activity %
	Wt. g.	Activity I.U./mg.	Wt. g.	Activity I.U./mg.	
L 5 (8)	0.189	36.4	0.0446	148	95.9
N 8 (1)	7.18	10	0.362	190	94.4
N 7 (1)	5.06	30	0.414	320	87.3
PMS 33 (1)	19.36	50	1.639	625	105.1
PMS 30	7.83	26	0.619	311	94.5
PMS 32	14.35	33.5	1.272	287	76.1
					Mean 92.2

During large-scale operations, a further economy might possibly be introduced by reworking the alcohol-insoluble residues for the modicum of activity they retain. As an example, the fraction of PMS 33 (1) insoluble in 50 % alcohol (see Table 5) and containing 5.3 I.U./mg. was redissolved in dilute alkali and the process of alcohol fractionation repeated. This material, which we designated PMS 33 (2), thus yielded a fraction soluble in 50 % alcohol, representing 81.3 % of the total activity and having a potency of 227 I.U./mg., while the residue assayed only 1.4 I.U./mg.

Properties of the product obtained after alcohol precipitation

The material obtained from the fraction soluble in 50 % alcohol at pH 4.8, but insoluble at 75 % concentration, is a white powder, very readily and completely soluble in water; solutions give positive biuret and glyoxylic tests. The gonadotrophic activity of our preparations has ranged from 150 to 625 I.U./mg., and there appears to be a correlation between the activities of the concentrates and those of the initial powders from which they were derived (see Table 5). The histological response elicited in the ovaries of hypophysectomized rats by the injection of the purified product is similar to that given by untreated serum.

Carbohydrate content of fractions prepared by 50 % alcohol

Determinations of carbohydrate content have been made at each of the principal stages in the concentration of serum gonadotrophin, but until the stage of alcohol fractionation was reached no reliable information could be gained concerning the nature of the hormone, owing to the large proportion of impurity still present. It was noted, however, that the initial powders afforded the relatively high figure of 7–8 % of bound hexose, resembling, in this respect, seromucoid and other typical glycoproteins.

The carbohydrate contents have now been determined of the 50 % alcohol concentrates in five out of the six samples listed in Table 5, and also in their corresponding fractions insoluble in 50 % alcohol at pH 4.8. The results show a striking difference between the two materials, the relatively inert fractions containing about 8 % of bound hexose and the active fractions from 2.5 to 3.5 % (see Table 6).

Table 6. *Carbohydrate content of fractions separated by 50% alcohol at pH 4.8*

No.	Insoluble fraction		Soluble fraction	
	Activity I.U./mg.	Hexose %	Activity I.U./mg.	Hexose %
N 8 (1)	0.7	8.32	190	2.94
N 7 (1)	2.7	8.42	320	2.54
PMS 33 (1)	5.3	7.40	625	3.44
PMS 33 (2)	1.4	7.80	227	2.41
PMS 30	1.9	8.38	311	2.84
PMS 32	1.9	7.78	287	3.87

This result is somewhat surprising, in view of the suggestion of Li *et al.* [1940] that their highly purified gonadotrophic extract from pregnant mare's serum is a glycoprotein, and the figure of 14.1% hexose quoted in respect of it by them. Their statement that 'the carbohydrate determination may be taken as a measure of PMS potency in the purification of the hormone' is clearly invalid. Our preparations of about 600 I.U./mg. undoubtedly still contain much impurity, but carbohydrate content and gonadotrophic activity are, at this stage, running in opposite directions. An interesting illustration is afforded by sample PMS 33 (2). This was the alcohol-insoluble fraction of the large batch PMS 33 (1), and was refractionated by the same process (see above). The active fraction obtained (2% of the weight) contained 81.3% of the activity and 2.4% hexose, while the insoluble fraction, containing 23.1% activity, had a hexose content of 7.8%, slightly higher than the initial figure of 7.4% before fractionation.

DISCUSSION

The method most usually adopted for the preparation of active concentrates from pregnant mare's serum is that of Cartland & Nelson as modified by Goss & Cole [1940]. The latter authors state, however, that the yield from the initial acetone precipitation is less than 60% of the original activity of the serum, and for their final products no yields are quoted. The method employs large volumes of acetone which would be difficult to recover, since they contain the water, salts etc. of the serum, and large vessels are required to handle the big bulk of fluids.

The method described in the present paper is simple and comparatively economical; the acetone and benzoic acid used can be recovered with ease, thus lessening working costs. To work up 100 l. of serum we calculate the requirements of the two methods as follows:

Cartland and Nelson's method

500 l. acetone (not easily recoverable)
Smaller quantities of alcohol, acids, etc.

Method here described

1.4 kg. metaphosphoric acid
20 kg. benzoic acid } Recoverable
Approx. 100 l. acetone } for re-use
Smaller quantities of alcohol, acids, etc.

By our method, a material is obtained representing 73% of the original activity of the serum and with an activity of 150-625 I.U./mg. For therapeutic use it may be mixed with a suitable proportion of an inert diluent.

Investigation is proceeding upon the further purification of the hormone with a view to its ultimate isolation and chemical characterization.

Protocol

As an example of the working of the method, a single preparation will be described in detail.

Serum N 7 from mare N when 81 days in foal; volume 900 ml.; activity assayed at time of preparation 210 I.U./ml.

To 900 ml. of serum were added 1800 ml. of water, and 20 ml. were removed for assay. The remainder was surrounded by ice and mechanically stirred while 430 ml. of 0.3 *N* HPO₃ were added drop by drop at the rate of about 1 drop/sec. The pH, determined electrometrically, was then 3.3. The mixture was passed through a Sharples super-centrifuge and the cake of protein metaphosphate resuspended by grinding in 1 l. of 0.01 *N* HPO₃. This mixture was passed through the machine and the clear fluid added to the main filtrate, which was neutralized to pH 7.0 by addition, with mechanical stirring, of sufficient 20% NaOH. The volume of filtrate was 4140 ml. 20 ml. were removed for assay. A solution of sodium benzoate was prepared by boiling 207 g. benzoic acid in 200 ml. water and 136 ml. of 50% NaOH, and cooling the mixture to room temperature.

The HPO₃ filtrate was surrounded by ice and mechanically stirred; the sodium benzoate solution was added and then 3 *N* HCl was allowed to drop into the briskly stirred mixture at a rate not greater than 1 drop per sec. 450 ml. of acid were required to bring the pH to 4.55. The mixture was put through the Sharples super-centrifuge and the cake of benzoic acid scraped out and stirred with about 1 l. of acetone.

When all the lumps had disintegrated, the fine precipitate was allowed to settle and the bulk of the supernatant acetone removed. The precipitate from the remainder was then collected on the centrifuge (using 200 ml. capacity glass bottles), washed three times on the centrifuge with about 400 ml. in all of anhydrous acetone and finally once with about 150 ml. of anhydrous ether. The last traces of ether were removed from the 'initial powder' by exposing it over sulphuric acid in a vacuum desiccator overnight.

Weight of powder recovered 5.069 g.

Activity 30 I.U./mg.

Recovery of original activity of serum 81%.

5.06 g. of initial powder were ground up with 200 ml. of water containing 2.2 ml. of *N* NaOH and the opalescent solution which had pH 7.4, was stirred mechanically for 2 hr. and then left in the ice chest overnight. 200 ml. of alcohol were added and then dropwise, while stirring, 7 ml. of 5% acetic acid in 50% alcohol, which brought the pH to 4.7. After chilling for 1.5 hr. the mixture was centrifuged and the precipitate washed on the centrifuge three times with 50% alcohol at pH 4.8 (see p. 744). To the combined supernatant fluids an equal volume of alcohol was added and the mixture left at 0° overnight. The small precipitate was collected on the centrifuge, washed once with 80% alcohol, absolute alcohol, acetone and dry ether, then freed from solvent *in vacuo* over sulphuric acid.

Weight of powder recovered 0.4141 g.

Activity 320 I.U./mg.

Recovery of activity 87.3%.

Overall recovery from serum 71%.

The material insoluble in 50% alcohol at pH 4.8 was dehydrated with acetone and ether. It weighed 4.475 g. and contained 2.7 I.U./mg., accounting for 8% of the total activity.

SUMMARY

A method is described for the preparation from the serum of pregnant mares of a stable, dry concentrate. The method is economical and is suitable for large-scale operation and depends firstly upon precipitation of inert serum proteins by metaphosphoric acid. Very little loss in gonadotrophic activity occurs at this stage. The active principle is then adsorbed upon benzoic acid and by solution of the latter in acetone a stable initial powder is obtained averaging 27 I.U./mg.

and accounting for 79% of the activity (data compiled from thirty-nine individual preparations totalling 48 l. of serum).

The initial powder is further fractionated by means of titration in 50% alcohol to pH 4.8; an insoluble portion representing 92% by weight and practically devoid of activity is rejected, while from the solution the active fraction is recovered by further addition of alcohol. This material contains 150–625 i.u./mg. and is readily soluble in water. The average yield at this stage is 92%, making an overall yield of 73% of activity from the original serum.

The chemical properties of the active material have been investigated: it contains 2.5–3.5% of hexose (orcinol method), while the inert fraction insoluble in 50% alcohol at pH 4.8 contains 7.5–8.5% of hexose.

Investigation is proceeding upon the further purification of the hormone.

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