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Isolation of a Gonadotropin (PMEG) From Pregnant Mare Endometrial Cups: Comparison with PMSG (40207)

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The isolation of pregnant mare serum gonadotropin (PMSG) has been previously described as well as some of its properties (1–3). It is a glycoprotein hormone possessing about 45% carbohydrate and exhibits activity both *in vivo* and *in vitro* as a follicle stimulating hormone (FSH) and a luteinizing hormone (LH) (4–6). Like the pituitary gonadotropins and human chorionic gonadotropin (hCG), PMSG has been shown to consist of two nonidentical subunits (4) and one of the subunits (PMSG-α) is able to combine with the beta subunit of other pituitary gonadotropins or hCG with the resultant generation of the activity expected of the beta subunit employed. PMSG is a circulating form of gonadotropin whereas the pituitary gonadotropins are probably storage forms and hCG is a urinary excretion product of placental origin. Allen and Moor (7) have shown that the uterine endometrial cups from which PMSG originates during the pregnancy of a mare are of fetal trophoblast derivation. Isolation of the hormone from the endometrial cups (designated PMEG) would allow comparison with PMSG. The studies reported here represent an approach to this problem.

Materials and methods. Highly purified PMSG (15,000 IU/mg) was prepared as previously described (2) and used for the comparative studies described herein. A series of endometrial cup preparations were obtained from Shetland ponies whose date of breeding was known. The animals were anesthetized with sodium pentobarbital and then sacrificed at 70 to 90 days of pregnancy. The desanguinated cups and adhering secretion were immediately removed from the ponies and placed in frozen storage and kept there until fractionation. Up to 15 cups were obtained from each animal and the combined weight of the cups and secretion ranged from 1 to 12.5 gm per animal. Table 1 lists data on each of the preparations fractionated (days of pregnancy, wts., serum levels of PMSG, etc.). Final preparations were evaluated by both *in vivo* and *in vitro* assays. The hCG augmentation test (8) was employed for in vivo FSH activity. *In vivo* LH assays were by the ovarian ascorbic acid depletion (OADD) method (9), and *in vitro* by stimulation of testosterone production in isolated rat Leydig cells (10, 11). Other methods employed will be referred to in the results section.

The procedures previously described for the purification of PMSG (1, 2) were employed with modification for the preparation of PMEG from the individual batches of endometrial cups listed in Table I. In brief, the cups were homogenized in a Waring blender for 1–2 min with 50 ml of 0.9% saline per g (wet wt) tissue. The homogenate was stirred at 4° for 4 hr after which the insoluble residue was removed by centrifugation. Inert material was precipitated from the supernatant fluid by adjusting to pH 3.0 with 0.2 *M* HPO_3 and centrifuged off. The hormonal activity was precipitated from this latter supernatant by the addition of ethanol to 50% (v/v) at pH 4.5, removal of the precipitate (inert) and increasing the alcohol content to 75% (v/v). The formed precipitate was dissolved in water, dialyzed, lyophilized, and subjected to chromatography on columns of sulfoethyl Sephadex C-50, and finally gel filtration on Sephadex G-100 in 0.05 *M* NH_4HCO_3. The Ve/Vo values of the various preparations were close to 1.32 in most cases, a value near that obtained with PMSG under the same conditions (2, 5). Final yields of purified PMEG are listed in Table I and ranged from 0.05 to 1.9 mg/g of starting tissue.

Results. Biochemical characterization. The various PMEG preparations were analyzed by disc electrophoresis in columns of 7½% polyacrylamide at pH 8.3 (12) and compared with PMSG. PMSG is characterized by a

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diffuse band (Rf. 0.27), suggestive of microheterogeneity. The PMEG preparations were similar in character (Rf. 0.29) but showed increased evidence of heterogeneity.

Colorimetric procedures which were scaled down to analyze small samples (13) were employed for the carbohydrate determination. The results are summarized in Table II. None of the four preparations analyzed possess as much total carbohydrate as PMSG and range from about 13–30% compared with 45% for PMSG. The lower total content of carbohydrates is not due to lesser amounts of any single sugar constituent, but decreased quantities of every type of sugar, although in each case there appears to be somewhat greater percent losses of the hexosamine and sialic acid than of the hexose when compared to PMSG.

Amino acid analyses of the PMEG preparations were performed by the method of Spackman *et al.* (14) in a Beckman Model 120 B amino acid analyzer. The results are tabulated in Table III. All of the four PMEG preparations are similar to one another. Compared to PMSG, values for histidine and arginine are slightly lower, and the aspartic acid and valine values somewhat higher. The lower half-cystine and methionine content of the PMEG's are probably due to the fact that the preparations were not oxidized with performic acid prior to hydrolysis.

Amino terminal residues of the PMEG preparations were determined by the dansyl procedure (15, 16). Analysis of PMSG (4) reveals phenylalanine (PMSG-α) as a major terminal amino acid and smaller quantities of serine (PMSG-β) and glycine, the latter arising presumably from partially degraded peptide chains. In the case of the PMEG preparations a greater degree of heterogeneity was observed than in PMSG. Phenylalanine was detected as a major terminal residue in all of the preparations examined. One sample, EC15B, also possessed major quantities of valine and tyrosine. Except for preparation EC14B, where only phenylalanine and leucine were detected, the other preparations each had several amino acids present in minor quantities, mainly leucine, valine, isoleucine, serine and alanine.

Biological characterization. The PMEG preparations were assayed for LH and FSH activity by the standard *in vivo* assays (OAAD and hCG augmentation test, respectively) and the results are summarized in Table II.

TABLE I. DATA ON ENDOMETRIAL CUPS FRACTIONATED AND YIELDS OF PMEG OBTAINED.

Preparation	Days pregnant	Serum PMSG (IU/ml)	g tissue	PMEG yield mg	PMEG yield, mg/g tissue
EC10B	?	200	17	27.3	1.6
EC11B	93	180	7	13.5	1.9
EC12B	95	200	10.5	0.7	0.07
EC13B	88	40	8	0.4	0.05
EC14B	82	170	3	5.0	1.7
EC15B[a]	—	—	10	8.0	0.8

[a] EC15B was a pool of small amounts of endometrial cups from several mares in which pregnancy and serum PMSG data was lacking.

TABLE II. CARBOHYDRATE CONTENT[a] AND BIOLOGICAL ACTIVITY OF PREGNANT MARE ENDOMETRIAL CUP GONADOTROPN PREPARATIONS COMPARED TO PMSG.

Preparation	Hexose	Hexosamine	Sialic acid	Total	Activity	
					LH[b]	FSH[c]
PMSG	14.3	20.6	10.2	45.1	100	100
EC10B	9.9	8.9	5.8	24.6	54 (37–97; 0.12)	41 (20–97; 0.21)
EC11B	4.6	6.0	2.1	12.7	23 (17–35; 0.09)	11 (5–28; 0.20)
EC14B	11.9	10.4	7.7	30.0	54 (37–95; 0.12)	25 (16–64; 0.13)
EC15B	8.8	7.4	4.1	20.3	24 (15–37; 0.13)	11 (0.2–21; 0.16)

[a] g/100 g glycoprotein.
[b] Assayed by OAAD relative to PMSG taken as 100%; in this assay PMSG is 1.5–2.5 × NIH-LH-Sl.
[c] Assayed by hCG augmentation test; PMSG taken as 100%; in this assay PMSG is 75–135 × NIH-FSH-Sl. 95% confidence limits and index of precision in parentheses.

TABLE III. AMINO ACID COMPOSITION[a] OF PMSG COMPARED WITH ENDOMETRIAL CUP PREPARATIONS.

Amino acid	PMSG[b]	EC10B	EC11B	EC14B	EC15B
Lysine	5.4	6.8	5.9	6.0	6.0
Histidine	2.5	2.4	1.7	1.9	1.8
Arginine	6.1	4.8	3.7	5.7	3.8
Aspartic	5.5	7.2	7.5	7.2	7.6
Threonine	8.6	8.0	8.7	8.3	6.8
Serine	8.0	9.1	11.5	8.9	7.4
Glutamic	8.4	8.7	8.8	8.8	9.2
Proline	12.5	8.8	6.8	10.3	7.1
Glycine	5.1	6.6	7.0	5.6	5.4
Alanine	7.5	6.9	7.1	7.1	7.2
Half-cystine	7.5	4.5	4.3	5.6	4.5
Valine	4.6	6.9	8.2	5.9	6.5
Methionine	1.7	1.5	1.0	1.6	1.0
Isoleucine	4.8	4.2	4.0	4.8	3.6
Leucine	6.3	7.4	7.8	5.5	8.2
Tyrosine	2.4	3.0	3.4	2.2	2.9
Phenylalanine	4.0	3.3	3.0	3.7	3.4

[a] Calculated as residues/100 residues analyzed.
[b] Taken from Schams and Papkoff (2).

In no case was the activity of any of the preparations equal to PMSG. Each preparation, however, possessed both LH and FSH activity. LH activities ranged from 23% (EC11B and EC15B) to 54% (EC10B and EC14B) that of PMSG. The FSH activities were 11–41% that of PMSG, and paralleled the LH activities. PMSG has been shown (5, 6, 11, 17) to be very active in stimulating the production of testosterone *in vitro* in preparations of isolated rat Leydig cells. Figure 1 shows the results obtained in this system with the PMEG preparations compared to PMSG. The results are similar to those obtained in the OAAD in that none of the four PMEG preparations are as active as PMSG. Individual preparations appeared to be from 14% (EC11, 15B) to 80% (EC10B) as active as PMSG. No determinations for other hormonal activities were made on these preparations.

Immunological. We previously prepared in rabbits an antiserum against purified PMSG (2). The antiserum was recently employed to show that the beta subunit of PMSG but not the alpha subunit cross reacted with PMSG in agar double diffusion tests (5). Figure 2 shows a double diffusion test in which the various PMEG preparations form lines of precipitation with the antiserum which are identical to that formed with PMSG. The formation of 'lines of identity' between PMSG and PMEG suggests a high degree of

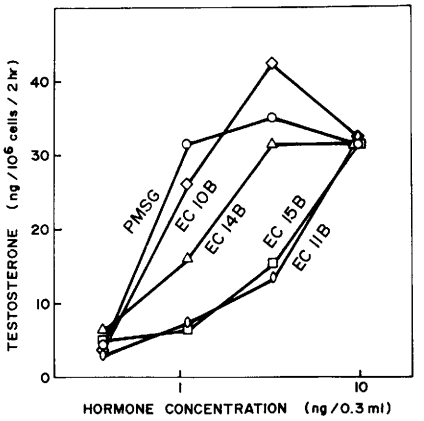

FIG. 1. *In vitro* stimulation of testosterone production in dispersed rat testis Leydig cells by PMSG and several PMEG preparations.

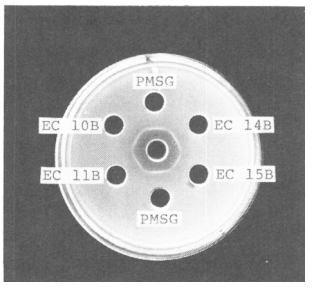

FIG. 2. Immunodiffusion plate showing precipitin reactions of PMSG and PMEG preparations with antiserum against PMSG (central well); 20 μg samples in peripheral wells.

immunological relatedness between the preparations.

Discussion. There has been increasing interest in recent years in comparing the properties of the circulating form of protein and polypeptide hormones with those of the hormone isolated from its tissue of origin. The very low concentrations of pituitary hormones in the blood has precluded thus far their isolation in quantity sufficient for biochemical analysis. PMSG, however, because of its high circulating levels during early pregnancy, is readily isolated in a high state of purity (1–3). The studies reported here demonstrate that the endometrial cup tissue of the pregnant mare can be fractionated and the hormone (PMEG) isolated by essentially the same procedure as employed for the preparation of PMSG. This would suggest that PMEG is similar in its properties to PMSG.

The PMEG preparations examined all appeared to be somewhat more heterogeneous than PMSG as evidenced by both disc electrophoresis and examination of NH_2-terminal amino acids. There was considerable variation observed from preparation to preparation in the NH_2-terminal residues present, although one (EC14B) had only two residues as in the case of PMSG. It is now generally accepted that the pituitary glycoprotein hormones all exhibit a similar heterogeneity which appears to be related to variations in carbohydrate content and to a limited proteolytic attack primarily at the termini of the polypeptide chains (18–21) which in part arises during purification. This may be the case as well with the PMEG preparations examined in this study.

The PMEG preparations showed variation in biological activity and carbohydrate as well (Table II). This is in contrast to our experience with PMSG in which the products obtained from several pools of sera or crude commercial preparations were very similar to one another with respect to carbohydrate content and biological activity. It is possible that the PMEG preparations isolated represent populations of molecules in various states of biosynthesis. If this is the case, it would appear from the analyses performed that this possibility is reflected largely in terms of the amount of carbohydrate present (Table II) as the amino acid compositions are fairly uniform (Table III). If only completely biosynthesized molecules are secreted into the circulation, this would account for the greater homogeneity and higher sugar content seen in PMSG. On the other hand, if a spectrum of molecules, as represented by the PMEG preparations, are secreted into the circulation, it is reasonable to assume that different molecules would have varied clearance rates, and with time a steady state would be achieved with a more homogeneous population of molecules. In particular, sialic acid content would appear to be of importance, as studies on other glycoprotein hormones show that loss of sialic acid results in a much more rapid clearance of the hormone from the circulation (23, 24). All of the PMEG preparations possess less sialic acid than is found in PMSG (Table II).

The amino acid composition of the PMEG preparations are very similar one to another. Also, the agar diffusion experiment shows that PMEG preparations are indistinguishable from PMSG. It seems clear, however, that a major difference between PMEG and PMSG is in the lower, and varied carbohydrate content exhibited by the former.

In all cases, PMEG preparations appear to be lower in activity than PMSG (11–54% as active) both with respect to *in vivo* and *in vitro* assays and in terms of both LH and FSH activity. The *in vivo* assays (Table II) show that when there is a decrease in LH activity, there is a similar decrease in FSH activity. Thus, the ratio of LH to FSH activities in the various preparations are about equal. Recent results by Stewart *et al.* (25), using radioreceptor assays and crude PMSG and PMEG extracts, were similar in showing a constant ratio of LH to FSH activity, although they had data suggesting that the ratio was somewhat lower in endometrial cup extracts than in PMSG. The *in vitro* experiment reported here also shows that PMEG preparations are lower in activity than PMSG. Thus, the lower potency is not just a function of *in vivo* vs *in vitro* assays, but is a property of PMEG preparations and possibly relates to the smaller quantity of carbohydrate present compared to PMSG.

Summary. The gonadotropin (PMEG) in pregnant mare endometrial cups was purified and compared to pregnant mare serum go-

nadotropin (PMSG). Purification methodology applicable to PMSG was employed. *In vivo* and *in vitro* assays for FSH and LH were used to evaluate PMEG preparations. In all cases, lower activities (11–54%) were observed with PMEG compared to PMSG. Antiserum raised in rabbits against PMSG cross-reacts fully with PMEG in agar double diffusion tests. The amino acid composition of PMEG is similar to PMSG, but amino terminal group analyses show PMEG preparations to be more heterogeneous than PMSG. The carbohydrate composition of all PMEG preparations examined was considerably less (13–30%) than found in PMSG (45%).

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