

Steroid effects on human endometrial glycoprotein biosynthesis

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Summary. Human endometrium from the secretory phase of the menstrual cycle was incubated with ^3H - and ^{14}C -labelled glucosamine and [^3H]leucine. Incorporation into secreted extracellular glycoprotein and accumulation of the label into the microsomal fraction were measured. When oestradiol or progesterone were added to the medium, medroxyprogesterone acetate (MPA), ethynodiol diacetate and chlormadinone acetate reduced incorporation of glucosamine and MPA reduced incorporation of leucine into glycoprotein. MPA reduced the amount of glucosamine in the microsomal fraction and also had an effect on amino acid transport within the endometrial cells, as indicated by intracellular α -aminoisobutyric acid space measurements. These results and the ratios of ^3H and ^{14}C in the microsomal fraction and secreted protein suggest that MPA has a primary effect in decreasing amino sugar incorporation and a secondary effect in reducing amino acid incorporation into glycoprotein.

Introduction

The histology of human endometrial glandular cell secretion has been described by Noyes, Hertig & Rock (1950). During the secretory phase the Golgi complexes are greatly developed and the distended endoplasmic reticulum contains glycogen and glycoprotein (Wynn & Harris, 1967; Wynn & Woolley, 1967). Pincus, Rock & Garcia (1958) reported alterations in endometrial histology in response to synthetic progestagens. Flowers, Wilborn & Enger (1974) studied the ultrastructure of the endometrium after treatment with quingestanol acetate for 3 months and observed that many aspects of protein synthesis were affected and that, despite ovulation, subnuclear vacuolation did not occur. They inferred from histochemical studies that the progestagen did not alter transport mechanisms across the cell surfaces, but that there was a reduction in the glycoprotein in the cells and that intracellular transport systems were affected. The histochemical and histological effects of oestradiol and progesterone on human endometrium in culture have been studied by Csermely, Demers & Hughes (1969) and Kohorn & Tchao (1969). Shapiro & Hagerman (1974) examined precursor uptake into protein and RNA. This paper examines the effects of the progestagens, medroxyprogesterone acetate, ethynodiol diacetate, and chlormadinone acetate, on glycoprotein synthesis by human endometrium in culture to elucidate further the mechanism of action of these potent antifertility agents.

Materials and Methods

Chemicals

The radiochemical purity of [$1\text{-}^{14}\text{C}$]D-glucosamine hydrochloride (sp. act. 55.0 mCi/mmol), [$1\text{-}^3\text{H}$]D-glucosamine hydrochloride (sp. act. 2.6 Ci/mmol), [$4,5\text{-}^3\text{H}$]L-leucine (sp. act. 19 Ci/mmol), [$1\text{-}^{14}\text{C}$]2 α -aminoisobutyric acid (sp. act. 58 mCi/mmol) and [$1\text{-}^3\text{H}$]D-sorbitol (sp. act. 5.44 Ci/mmol) was more than 99% and the chemicals were used as purchased from the Radiochemical Centre, Amersham.

The steroids used were: oestradiol-17 β (1,3,5, (10)-estratriene-3,17 β -diol: Sigma Chemical Co., St Louis, Missouri); progesterone (4-pregnene-3,20-dione: Sigma); medroxyprogesterone acetate (17 α -acetoxy, 6 α -methyl-4-pregnene-3,20-dione (MPA): Upjohn Co., Kalamazoo, Michigan); ethynodiol diacetate (19-nor-17 α -ethynyl-4-androstene-3,17 β -diol diacetate (ED): G. D. Searle and Co., High Wycombe, Bucks): chlormadinone acetate (6 β -chloro-17 α -acetoxy-4,6-pregnadiene-3,20-dione (CA): Syntex Pharmaceuticals Ltd, Maidenhead, Berks) and norethisterone (17 α -ethynyl-17 β -hydroxy-19-nor-4-androsten-3-one: Syntex). Radioactive steroids were: [2,4,6,7- ^3H]oestradiol (sp. act. 100 Ci/mmol, Radiochemical Centre); [1,2,3- ^3H]progesterone (sp. act. 41 Ci/mmol; Radiochemical Centre); [6,7- ^3H]ethynodiol diacetate (sp. act. 81.2 mCi/mmol: Searle); [7 α - ^3H]medroxyprogesterone acetate (sp. act. 30 mCi/mmol: Upjohn) and [1- ^3H]chlormadinone acetate (sp. act. 10.3 Ci/mmol: Syntex).

The purity of all steroids was checked by thin-layer chromatography in three systems.

Culture system

Human endometrium was obtained by curettage performed incidental to another procedure such as tubal ligation, cautery of the cervix or dilatation for dysmenorrhoea, when it was anticipated that the endometrium would be normal. Specimens obtained at hysterectomy were also utilized. The tissue was immediately placed in cold saline under sterile conditions and transported to the laboratory. A sample was taken for routine histology; abnormal tissues were discarded and only results from normal tissues are presented.

Medium T199 was used as purchased (Biocult Labs, Glasgow) but all other solutions were sterilized by passing through a Millipore filter (0.22 μm pore size). The tissue was set up immediately in organ culture (Trowell, 1959), each culture containing 8–10 2 mm³ explants. The explants were placed in the culture dishes (Falcon Plastics, Los Angeles) on lens paper supported on a grid over a central well containing 1 ml medium T199 with 10% calf serum (Biocult), 200 units penicillin (Glaxo Labs, London), 100 μg streptomycin (Glaxo) and 40 μg insulin (B.P., Weddel Pharmaceuticals, London). The cultures were placed in sealed boxes, gassed with 95% air, 5% CO₂ and incubated at 37°C. One culture dish was maintained for a histological control. The phenol red in the T199 was used to indicate the need for re-gassing during the course of the experiment.

Extraction of glycoprotein

Glycoprotein was extracted from 1 ml incubation medium aspirated from the culture dish, using ice-cold 10% trichloroacetic acid solution. The precipitate was washed three times with 1 ml 5% trichloroacetic acid, twice with 1 ml chloroform:methanol (2:1, v/v); twice with 2 ml acetone and twice with 1 ml ether (Molnar, Robinson & Winzler, 1965). The ether was evaporated and the residue solubilized for counting overnight with 0.3 ml hyamine hydroxide 10X (Packard Instrument Co., Illinois) at 55°C. Protein determinations were performed on 0.1 ml homogenate by the method of Lowry, Rosebrough, Farr & Randall (1951).

Preparation of the microsomal fraction

After the incubations were terminated and the media removed for glycoprotein precipitation, the explants were washed twice with 1 ml ice-cold 0.9% NaCl to remove any adhering medium. The excess fluid was removed by blotting and the explants homogenized in approximately 1 ml ice-cold 0.25 M-sucrose in a 180-mm A.G. tissue grinder. The homogenate was made up to 5 ml with 0.25 M-sucrose and then centrifuged at 800 g for 15 min. The supernatant was decanted and centrifuged at 105,000 g for 36 min. The supernatant was again decanted to obtain the microsomal pellet which was gently resuspended in 0.1 ml distilled water, transferred to a test-tube with a further 0.1 ml distilled water and was solubilized for counting by the addition of 0.8 ml hyamine hydroxide 10X and incubated overnight at 55°C.

Representative microsomal pellets were fixed in 3% glutaraldehyde, washed in 0.1 M-phosphate buffer, embedded in epoxy resin and sectioned at 60–90 nm. Examination with an AEI 6B electron

microscope at 60 kV at a final magnification of 30,000 showed a membrane preparation without nuclear or mitochondrial contamination.

Conditions of counting

The extract solubilized in hyamine hydroxide 10X was transferred to a counting vial; 0.2 ml hyamine hydroxide 10X washings were also transferred and 15 ml Bray's solution added (Bray, 1960). Counting was performed in either a Nuclear Chicago Mark I or Mark II Liquid Scintillation Counter. A quench correction was made by the addition of an internal standard or by channels ratio with an external standard. Counting times were such as to achieve less than 2% error.

Oxygen consumption measurements

Cultures were used after incubation for 0–4 days and 3 cultures were pooled to provide enough tissue for the electrode system. The tissue was blotted and placed in a reservoir containing fresh medium. This was gassed for 5 min, the gas shut off and the electrode placed in contact with the surface of the medium. With continuous gentle stirring to eliminate pockets of deoxygenated medium, the electrode was removed after a 15-min reading; the tissue was then dried to constant weight. In control runs medium only was placed in the reservoir. The measurements were carried out in a Y.S.I. Model 53 oxygen monitor (Yellow Springs Instrument Co. Ltd, Yellow Springs, Ohio).

Anaerobic cultures

Cultures were placed in a Gaspack chamber (Becton–Dickinson U.K. Ltd, Wembley, Middlesex) which took 1.5 hr to become anaerobic.

Amino acid transport

The measured total aminoisobutyric acid space, the extracellular (sorbitol) space, and the derived intracellular aminoisobutyric acid space were calculated as described by Roskoski & Steiner (1967).

Results

Cultures without added steroids

After equilibration of cultures for 16 hr, the medium was replaced and to each culture dish was added 2.5 μCi [$1\text{-}^{14}\text{C}$]D-glucosamine. The cultures were terminated in pairs after 4, 8, 16, 24 and 48 hr, glycoprotein extracts were prepared from the media, microsomal preparations were made and the protein concentration of each dish was determined (Table 1). When the cultures of endometrium were exposed to an excess of glucosamine the uptake onto the microsomes was almost linear for 8 hr and then reached a plateau. Incorporation into the secreted glycoprotein reached a peak at 16 hr, was followed by a plateau, and then resumed activity, which in other experiments continued until 72 hr or more (Cooke, Hastings & Lambadarios, 1971). The results of adding 2 μCi [$4,5\text{-}^3\text{H}$]L-leucine and terminating the cultures in pairs after 1, 2, 3, 4, 8, 16 and 24 hr are shown in Table 1.

Tissue viability during culture

After equilibration at 1, 4, 8, 16 and 24 hr, paired cultures were given 2.5 μCi [$1\text{-}^{14}\text{C}$]D-glucosamine and harvested 2 hr later. Glycoprotein extracts and microsomal preparations were made and the protein concentration of the tissue determined. Peak glycoprotein production (680 d/min/mg protein)

Table 1. Incorporation of [¹⁴C]glucosamine and [³H]leucine (d/min/mg protein) onto microsomes and into secreted glycoprotein of human secretory endometrium in organ culture without added steroids

	¹⁴ C]glucosamine					³ H]leucine					
	4	Incubation time (hr)				1	Incubation time (hr)				
		8	16	32	48		2	4	8	16	24
Microsomes	2200	4900	6600	7210	7200	2600	2700	3400	6800	23,000	27,500
	3000	6100	7200	7296	8060	3000	4600	8000	8200	51,000	54,100
Glycoprotein	990	1500	8000	10,150	25,200	1200	1000	1800	4300	6200	6200
	1110	2000	9000	10,250	30,300	1700	2100	3400	6000	9500	11,000

occurred at 4 hr so it is unlikely that medium glycoprotein was derived from cell leakage, which would have increased with time.

As a further confirmation of viability in culture, oxygen consumption measurements were carried out on fresh tissue and tissue that had been in culture for 1–4 days in samples obtained at different times of the menstrual cycle. Basal consumption was 3.6–4.2 µl/hr/mg and the consumption on each day expressed as a mean (±S.E.M.) % of zero time was: Day 1, 118±38, (*n*=4); Day 2, 138±21 (*n*=4); Day 3, 194±92 (*n*=4); Day 4, 56±11 (*n*=4). Similar experiments showed that incorporation of 1.25 µCi [1-¹⁴C]2α-aminoisobutyric acid was <1000 d/min/mg protein throughout the 24 hr, excluding the possibility of contamination due to cell permeability and supporting the concept of active metabolism of the utilizable precursor by viable cells.

Anaerobic cultures

After equilibration for 16 hr the medium was changed and 0.25 µCi [1-¹⁴C]D-glucosamine and 2 µCi [4,5-³H]L-leucine were added. Half the cultures were incubated aerobically while the other half were incubated anaerobically. After 24 hr the cultures were harvested, glycoprotein and microsomal fractions prepared and protein concentrations measured for each dish. Under anaerobic conditions incorporation of both precursors was significantly reduced (*P*<0.005 for leucine incorporation into both fractions and *P*<0.0005 for glucosamine incorporation into both fractions), confirming the need for O₂ and the active nature of incorporation of amino sugar and amino acid.

Effect of steroid concentration

Replicate cultures were set up for 72 hr containing (i) 10⁻⁶ M-MPA and 10⁻⁶ M oestradiol+progesterone; (ii) 10⁻⁹ M-MPA and 10⁻⁹ M oestradiol+progesterone; (iii) 10⁻⁹ M-MPA and 10⁻⁶ M oestradiol+progesterone; (iv) 10⁻⁶ M-MPA, 10⁻⁹ M oestradiol+progesterone; (v) no steroids. A significant depression of glycoprotein production only occurred when 10⁻⁶ M-MPA was incubated in the presence of 10⁻⁹ M oestradiol+progesterone (9667±1000 d/min/mg versus 19,835±500 d/min/mg protein (control): *P*<0.005).

Addition of steroids

Samples of endometrium from 11 different patients, some in the proliferative and some in the secretory phase, were set up for 72 hr incubations. Samples of proliferative-phase tissue were set up as replicate dishes for the glucosamine control, the histology control, and added oestradiol, progesterone, oestradiol+progesterone, CA, MPA, ED, CA+oestradiol, MPA+oestradiol, or ED+oestradiol. Secretory-phase tissue was set up in replicate dishes as the glucosamine control, the histology control, and added oestradiol, progesterone, oestradiol+progesterone, CA+progesterone, MPA+progesterone, ED+progesterone, CA+oestradiol+progesterone, MPA+oestradiol+progesterone, or ED+oestradiol+progesterone. The oestradiol and progesterone was used at a final concentration of 10⁻⁹ M and CA, MPA and ED were used at a final concentration of 10⁻⁶ M. The results indicated that no proliferative-phase endometrium incorporated more than 2600 d/min/mg secreted protein,

Table 2. Incorporation of [¹⁴C]glucosamine and [³H]leucine (d/min/mg protein) onto microsomes and into extracellular glycoprotein of human secretory endometrium in organ culture, under the influence of added steroids (10⁻⁹ M oestradiol and progesterone, and 10⁻⁶ M-MPA)

	[¹⁴ C]glucosamine					[³ H]leucine						
	Incubation time (hr)					Incubation time (hr)						
	1	2	4	8		1	2	4	8			
Microsomes												
Oestradiol + progesterone	1900	2500	3500	7500		14,500	37,000	54,500	80,500			
Oestradiol + progesterone + MPA	1500	1700	2000	3000*		16,000	40,000	55,500	84,500			
Glycoprotein												
Oestradiol + progesterone	500	1100	1400	4500		11,000	35,000	56,300	75,500			
Oestradiol + progesterone + MPA	450	600	700	1500†		12,500	36,500	57,500	80,000			

Significantly different from control, *P<0.01; †P<0.05.

Table 3. Incorporation of [¹⁴C]glucosamine and [³H]leucine (d/min/mg protein), after 16 hr preincubation with [³H]leucine, onto microsomes and into secreted glycoprotein of human secretory endometrium in the presence of added steroids (10⁻⁹ M oestradiol and progesterone, and 10⁻⁶ M-MPA)

	[¹⁴ C]glucosamine					[³ H]leucine						
	Incubation time (hr)					Incubation time (hr)						
	16	17	18	20	24	16	17	18	20	24		
Microsomes												
Oestradiol + progesterone	0	12,000	10,000	16,000	14,000	34,200	5000	4600	8000	7900		
Oestradiol + progesterone + MPA	0	6000	18,000	16,000	14,000	24,000	2600	7600	7600	7600		
Glycoprotein												
Oestradiol + progesterone	0	11,400	7000	8000	8000	9000	40,000	24,400	26,000	26,000		
Oestradiol + progesterone + MPA	0	5000	9000	9000	7700	6000	18,600	30,000	30,000	24,000		

whereas secretory-phase endometrium incorporated up to 20,000 d/min/mg protein. Only in the cultures containing 10^{-9} M oestradiol+progesterone and 10^{-6} M progestagen with secretory-phase endometrium did significant depression of incorporation occur (CA, 776 versus 1900 d/min/mg protein: $P<0.005$; MPA, 7672 versus 10,866 d/min/mg protein: $P<0.005$; ED, 10,283 versus 14,690 d/min/mg protein: $P<0.05$).

When 0.25 μ Ci [14 C]D-glucosamine and 2 μ Ci [4,5- 3 H]L-leucine were added to secretory-phase endometrium in the presence of 10^{-6} M MPA and 10^{-9} M oestradiol+progesterone (Table 2), MPA depressed the incorporation of [14 C]glucosamine into the microsomal fraction ($P<0.01$) and secreted glycoprotein ($P<0.05$). Incorporation of [3 H]leucine into the secreted glycoprotein was depressed after 4 hr ($P<0.01$) but there was no effect on the incorporation into the microsomal fraction.

Preincubation with [3 H]leucine

After equilibration for 16 hr, the medium bathing secretory-phase endometrium was changed and 2 μ Ci [3 H]leucine were added. The tissue was equilibrated for a further 16 hr and the medium replaced by fresh medium containing 2 μ Ci [3 H]leucine and 0.25 μ Ci [14 C]glucosamine and steroids. Half the cultures were exposed to 10^{-9} M oestradiol+progesterone and half to 10^{-9} M oestradiol+progesterone and 10^{-6} M-MPA. Cultures were harvested at 1, 2, 4 and 8 hr. Glycoprotein was extracted, microsomal fractions prepared and protein content determined for each dish (Table 3). Since leucine is a component of basic cell structures and is incorporated more rapidly this was added first. On later addition of [14 C]glucosamine the incorporation of [3 H]leucine into the microsomes fell sharply during the first hour and then remained at this low level. Incorporation of [3 H]leucine into secreted glycoprotein rose dramatically 1 hr after exposure to [14 C]glucosamine. When MPA was present a similar picture was observed for the microsomes but the rise in incorporated [3 H]leucine in glycoprotein was slower in the first hour. The incorporation of [14 C]glucosamine into the microsomes rose sharply over the first hour and then reached a plateau. Incorporation into secreted glycoprotein reached a peak at 1 hr, then fell to a steady level. No changes were seen when MPA was present.

Pulse-chase experiments

Although [14 C]glucosamine incorporation into secreted glycoprotein was depressed by 72 hr after exposure of oestradiol, progesterone and progestagens, the timing of the onset of the depressive effect was not known. Preliminary experiments showed that depression was maximal within the first 24 hr and later experiments that the incorporation was greatest within the first 8 hr.

Tissue was exposed to 1.25 μ Ci [3 H]glucosamine for 2 hr, the medium was removed, the tissue washed and fresh medium containing an equimolar amount of [14 C]glucosamine added. Two hours later the medium was removed, the tissue washed and 1 ml medium without labelled glucosamine added. Oestradiol+progesterone (10 μ l at 10^{-9} M) and 10 μ l oestradiol+progesterone (10^{-9} M) and MPA (10^{-6} M) were added at zero time, and at 2 and 4 hr the medium was replaced. Cultures were terminated in replicate after 1, 2, 3, 4, 5, 6 and 8 hr of incubation.

Microsomal preparations, glycoprotein extractions and protein determinations were carried out (Table 4). There was an early and well-maintained reduction in the uptake of [3 H]glucosamine onto the microsomes in the presence of 10^{-6} M-MPA ($P<0.01$). The chase of [14 C]glucosamine showed a depression ($P<0.01$) after 4 hr. The uptake of precursor into glycoprotein was also considerably depressed by MPA as shown by the 3 H-labelled ($P<0.01$) and the 14 C-labelled pulses of glucosamine ($P<0.001$) (Table 4). More [14 C]glucosamine than [3 H]glucosamine was taken up in the first hour of exposure.

Ratio of [14 C]glucosamine and [3 H]leucine incorporation

The presence of MPA lowered the ratio of glucosamine to leucine on the microsomes significantly ($P<0.0005$) at 2–4 hr (Table 5), indicating that less glucosamine than leucine was incorporated and this difference was maintained. The ratio of glucosamine to leucine in secreted glycoprotein was not

Table 4. Incorporation (pmol precursor/mg protein) of a 2-hr pulse of [³H]glucosamine followed by a 2-hr chase of [¹⁴C]glucosamine onto the microsomes or into secreted glycoprotein of secretory endometrium, under the influence of added steroids (10⁻⁹ M oestradiol and progesterone, and 10⁻⁶ M-MPA)

	Microsomes								Glycoprotein							
	1	2	3	4	5	6	8	1	2	3	4	5	6	8		
[³H]glucosamine pulse																
Oestradiol + progesterone	0.10	0.60	1.40	1.41	1.94	1.21	0.81	0.7	1.0	0.4	0.6	0.7	1.0	0.6		
Oestradiol + progesterone + MPA	0.05	0.15	0.40	0.80	0.40	0.25	0.50	0.3	0.3	0.2	0.2	0.1	0.2	0.2		
[¹⁴C]glucosamine chase																
Oestradiol + progesterone	0	0	6.0	10.1	10.0	9.0	7.0	0	0	1.8	3.7	11.3	17.9	8.0		
Oestradiol + progesterone + MPA	0	0	1.8	9.3	4.5	3.1	5.5	0	0	0.7	0.9	1.0	0.4	0.9		

significantly altered up to 4 hr by the presence of MPA, but from 4–8 hr there was a significant increase ($P < 0.025$).

Table 5. Ratio of incorporated [^{14}C]glucosamine to [^3H]leucine onto microsomes and into secreted glycoprotein of human secretory endometrium, under the influence of steroids (10^{-9} M oestradiol and progesterone, and 10^{-6} M-MPA)

	Microsomes				Glycoprotein			
	Incubation time (hr)				Incubation time (hr)			
	1	2	4	8	1	2	4	8
Oestradiol + progesterone	1.36	1.30	1.44	0.7	0.10	0.36	0.76	0.22
	1.44	1.46	1.66	0.8	0.21	0.50	0.86	0.31
Oestradiol + progesterone + MPA	1.22	0.42	0.56	0.38	0.20	0.10	0.37	1.30
	1.32	0.52*	0.70*	0.50	0.32	0.20	0.44	1.60†

Significantly different from control, * $P < 0.0005$; † $P < 0.025$.

Thin-layer chromatography of added steroids

Endometrium from the secretory phase was equilibrated in culture and labelled oestradiol, progesterone, MPA, CA and ED were added to each dish. Cultures were terminated 1, 4, 8, 16, 24 and 48 hr later. The media and the 105,000 g supernatant were collected and TLC performed. No metabolism of any steroid except oestradiol could be detected. Increase in the oestradiol metabolite increased with time: at 1 hr, 0%; 24 hr, 33%; and at 48 hr, 50% in the medium; and at 1 hr, 42%; 24 hr, 50%; and 48 hr, 67% in the cytosol. The metabolite appeared in the cytosol after 1 hr but not in the medium until 4 hr. This suggests that the metabolite is being produced within the tissue and not formed by enzyme leakage into the medium. Chromatographic mobility in three systems (A, C and K of Lisboa & Diczfalusy, 1965) suggested that the metabolite was oestrone, but no further identification was attempted. Cytosol levels of oestradiol, progesterone and the progestagens varied from 2.0–7.5% of the administered concentration. This was reached by 1 hr and remained relatively constant for the remainder of the 48 hr incubation. The uptake did not appear to vary with the day of the cycle.

Amino acid transport

After equilibration for 16 hr the culture medium was changed and 0.5 μCi [^{14}C]aminoisobutyric acid (AIBA) and 0.5 μCi [^3H]D-sorbitol with carrier D-sorbitol to make a final concentration of 1.0 mM were added. Cultures were harvested at 1, 4, 8, 16 and 24 hr. Some cultures received no steroids; others received 10^{-9} M oestradiol+progesterone and either 10^{-6} M norethisterone or 10^{-6} M-MPA. After harvesting, a 10 μl sample medium and a 100 μl sample homogenate (of total volume 5 ml) were taken for counting. Both samples were digested overnight at 55°C with 0.3 ml hyamine hydroxide 10X.

The measured total AIBA space, the extracellular (sorbitol) space and the derived intracellular AIBA space were calculated as described by Roskoski & Steiner (1967):

$$\text{Total AIBA space } (\mu\text{l/mg protein}) = \frac{^{14}\text{C d/min/mg protein in homogenate}}{^{14}\text{C d/min}/\mu\text{l medium}}$$

$$\text{Sorbitol space } (\mu\text{l/mg protein}) = \frac{^3\text{H d/min/mg protein in homogenate}}{^3\text{H d/min}/\mu\text{l medium}}$$

Since the extracellular space = the sorbitol space, intracellular AIBA space = total AIBA space – extracellular space.

The sorbitol or extracellular space showed no consistent alteration by MPA or norethisterone. The intracellular AIBA space was relatively constant in control cultures after the first hour. The progestagens however caused major fluctuations (Table 6), but the nature of the significant reductions and significant increases seen within a 24 hr period is obscure.

Table 6. Mean (\pm S.E.M.) intracellular and extracellular endometrial spaces (μ l/mg protein) and steroid influence on human endometrium in culture (6 observations/group)

	Time (hr)				
	1	4	8	16	24
Extracellular (sorbitol) space					
Control	14.3 \pm 8.7	13.8 \pm 4.1	18.0 \pm 12.7	26.6 \pm 11.1	30.5 \pm 11.1
Oestradiol + progesterone + MPA	10.4 \pm 1.1	24.9 \pm 7.8†	9.8 \pm 0.8	28.2 \pm 6.8	26.9 \pm 0.8
Oestradiol + progesterone + norethisterone	10.4 \pm 4.3	14.3 \pm 6.5	20.8 \pm 2.3	26.8 \pm 11.2	27.9 \pm 1.9
Intracellular space					
Control	6.4 \pm 6.6	23.3 \pm 12.9	20.1 \pm 13.1	27.4 \pm 17.2	22.8 \pm 17.1
Oestradiol + progesterone + MPA	24.5 \pm 1.3†	23.3 \pm 16.8	39.2 \pm 4.0§	10.0 \pm 10.0	4.1 \pm 4.1§
Oestradiol + progesterone + norethisterone	1.2 \pm 0.1*	11.3 \pm 0.4	21.3 \pm 6.2	11.6 \pm 8.0*	54.7 \pm 11.5†

Significantly different from controls, * $P < 0.05$; † $P < 0.025$; ‡ $P < 0.01$; § $P < 0.005$.

The ratio of intracellular AIBA space to extracellular AIBA space in control cultures exceeded unity from 4–16 hr. Since AIBA is not metabolized this may indicate that it is concentrated in the cell, entry occurring by means of an active process even though the changes were not striking. There were no consistent effects of MPA or norethisterone.

Discussion

The culture periods in these experiments varied from 16–96 hr but 16 hr was usual. Histological examination of tissue cultured for longer times sometimes showed necrotic changes, but this was less likely if the explants were kept small. The longer total incubation times also resulted in some tissue distortion due to PAS-positive material distending the interstices of the stroma. The explants became more friable and tended to grow into the matrix of the lens paper after long periods of culture; they were therefore difficult to remove and less tissue was recovered at these times. The tissue was, however, viable after long culture as partly friable explants still exhibited measurable oxygen consumption after 4 days of culture. The results obtained with anaerobic cultures also confirmed the validity of the culture system.

The concentrations of oestradiol and progesterone used in the experiments approach normal plasma levels and are similar to those used by Csermely *et al.* (1969) and Kohorn & Tchao (1969). Pietras & Szego (1975), using isolated rat endometrial cells, found that the same oestradiol concentration elicited a primary target cell action of sodium and water increments within 2 hr. The concentrations of the progestagens in the culture medium were probably higher than in plasma. Nash, Bopp, Shreve & Schols (1971) reported that chlormadinone acetate reached a concentration of 10^{-8} M (2 ng/ml) in plasma after oral administration of contraceptive dosages, although Guerrero, Landgren, Montiel, Cekan & Diczfalusy (1975) emphasized the discrepancy between plasma and endometrial levels of endogenous steroids which were up to 20-fold greater in the tissue and mostly higher in the proliferative phase.

Sweat & Bryson (1970) have demonstrated metabolism of progesterone in human endometrium, but no major metabolic product was identified in the present experiments, presumably because of the very small amounts of tissue incubated. Metabolic products of the synthetic progestagens have not been identified. Sweat, Bryson & Young (1967) and Gurpide & Welch (1969) have shown that oestradiol is extensively converted to oestrone in human endometrium.

The CA, MPA and ED were only added to proliferative-phase tissue with oestradiol since this would have been the steroid to which the tissue had been exposed *in vivo*. Similarly, secretory-phase tissue had been exposed to oestradiol and progesterone *in vivo*. The fact that the 72 hr culture of secretory tissue showed a depression of incorporation into secreted glycoprotein when CA, MA and ED were added with oestradiol and progesterone, but not when oestradiol or progesterone or the synthetic progestagens alone were used, suggests a competitive mode of action by the progestagens.

MPA had an effect on the amino acid and amino sugar components of glycoprotein. The effect was first seen at 2 hr as impaired uptake of glucosamine onto microsomes, but was no longer present at 8 hr. Leucine uptake onto microsomes was not influenced, but leucine uptake into glycoprotein was reduced at 8 hr. The absence of depression of microsomal leucine uptake suggests that leucine also acts as a precursor for many other structural proteins that are not influenced by this progestagen. Glucosamine incorporation into secreted glycoprotein was more obviously influenced by the progestagens; reduction in glucosamine incorporation occurred from 2 hr but did not have a critical effect on leucine incorporation until 8 hr (Tables 2 and 5). Sugar residues in glycoprotein are probably added after the polypeptide has been synthesized and released from the ribosomes, although a small amount of bound glucosamine probably occurs in nascent polypeptides (Ginsberg & Neufeld, 1969). However, available glucosamine must be critical for continued completion of the polypeptide chain, as shown by the results of leucine preincubation in the absence of glucosamine (Table 3) and in the reduction of leucine incorporation seen at 8 hr (Table 2). The effect on leucine incorporation was striking, as shown by the increased ratio of incorporated glucosamine to leucine. If the only

effect were a reduction in glucosamine, this ratio should not have risen. Since it rose significantly, the amino acid reduction must have been greater than the molar glucosamine reduction and there must have been an additional depressive effect on amino acid incorporation. This was not shown by a change in microsomal ratio, probably because it was masked by leucine taken up for synthesis of structural proteins. The primary effect therefore of MPA and perhaps the other progestagens is a reduction of the microsomal amino sugar uptake, and hence of passage through into secreted glycoprotein, and a secondary reduction in amino acid incorporation into glycoprotein because this is critically dependent on an adequate supply of amino sugar. Long-term administration of steroids would probably result in a progressive reduction in glycoprotein synthesis.

The depressive effects of MPA first noted on the endoplasmic reticulum may influence the quantity and, by changes in precursor ratio, the quality of secretion and it could result in the histological abnormalities described by Flowers *et al.* (1974). Thus specific endometrial glycoproteins, such as blastokinin (Krishnan & Daniel, 1967) which may have an important role in implantation (Arthur & Daniel, 1972), may be altered or critically reduced, resulting in potent antifertility effects of these progestagens by a local action quite apart from any suppression of ovulation.

The pulse-chase experiments showed that the progestagen effect occurred rapidly and was first seen at the microsomal level. The apparently anomalous point at 4 hr (Table 4) was frequently observed and this and the surprisingly rapid uptake of glucosamine after leucine preincubation may suggest that acute changes in medium concentration of precursor influenced the subsequent rate of uptake. Other experiments showing continuous exposure to labelled glucosamine did not show a sudden increase in uptake between 3 and 4 hr. The stimulatory effect of oestradiol and progesterone has already been demonstrated (Cooke *et al.*, 1971) and although the steroid cytosol content remains relatively constant over a 48 hr period the addition of fresh oestradiol and progesterone with the chase label could exert a further stimulatory effect.

Sunshine, Williams & Rabin (1971) and Blyth, Freedman & Rabin (1971) suggested that oestradiol and testosterone may be bound on intracellular membranes and may activate them, but the binding affinity of synthetic progestagens for these membranes and hence their potential blocking action is unknown. MPA and norethisterone influenced the transport of amino acid within the cell during a 24 hr period, but elucidation of the major fluctuations in space volumes requires further study. Oestradiol and progesterone have different effects on the components of endometrium, the glandular and luminal epithelial cells and the stromal cells, as shown by Tachi, Tachi & Lindner (1972) in the rat, and Marcus (1974) in the guinea-pig. Human secretory-phase endometrium contains a large proportion of glandular cells; since secreted glycoprotein was assessed in the present study it is likely that the results refer principally to the action of progestagens on the glandular cells.

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