References

Albert, A., Armarego, W. L. F., and Spinner, E. (1961), *J. Chem. Soc.*, 2689.

Bruice, T. C., and Benkovic, S. (1966), "Bioorganic Mechanisms," Vol. I, New York, N. Y., W. A. Benjamin.

Bruice, T. C., and Bradbury, W. C. (1963) *J. Org. Chem. 28*, 3403.

Bruice, T. C., Main, L., Smith, S., and Bruice, P. Y. (1971), *J. Amer. Chem. Soc. 93*, 7327.

Brüstlein, M. (1971), Ph.D. Thesis, University of Konstanz, Germany.

Gassman, P., and Campbell, G. A. (1972), *J. Amer. Chem. Soc. 94*, 3891.

Hamilton, G. (1971), *Progr. Bioorg. Chem. 1*, 83.

Hayatsu, H., Wataya, Y., and Kai, K. (1970), *J. Amer. Chem. Soc. 92*, 725.

Hemmerich, P. (1964), *Helv. Chim. Acta 47*, 473.

Hemmerich, P. (1970), *Vitam. Horm. (New York) 28*, 467.

Hemmerich, P., Fallab, S., and Erlenmeyer, H. (1956), *Helv. Chim. Acta 39*, 1242.

Hemmerich, P., Ghisla, S., Hartman, U., and Müller, F. (1971), *in* Flavins and Flavoproteins, Kamin, H., Ed., Baltimore, Md., University Park Press.

Hendriks, R., Cronin, J. R., Walker, W. H., and Singer, T. P. (1972), *Biochem. Biophys. Res. Commun. 46*, 1262.

Johnstone, R. A. W., Payling, D. W., and Thomas, C. (1969), *J. Chem. Soc. C*, 2223.

Kearney, E. B., Salach, J. I., Walker, W. H., Seng, R., and Singer, T. P. (1971), *Biochem. Biophys. Res. Commun. 42*, 490.

Kierkegaard, P., Norrestam, R., Werner, P.-E., Csöregh, I., von Glehn, M., Karlsson, R., Leijonmarck, M., Rönnquist, O., Stensland, B., Tillberg, O., and Torbjörnsson, L. (1971), *in* "Flavins and Favoproteins," Kamin, H., Ed., Baltimore, Md., University Park Press.

Knappe, W. R. (1971), Ph.D. Thesis, University of Konstanz, Germany.

Main, L., Kasperek, G. J., and Bruice, T. C. (1972), *Biochemistry 11*, 3991.

Massey, V., Müller, F., Feldberg, R., Schuman, M., Sullivan, P. A., Howell, L. G., Mayhew, S. G., Matthews, R. G., and Foust, G. P. (1969), *J. Biol. Chem. 244*, 3999.

Michaels, G. B., Davidson, J. T., and Peck, H. D., Jr. (1970), *Biochem. Biophys. Res. Commun. 39*, 321.

Michaels, G. B., Davidson, J. T, and Peck, H. D., Jr. (1971), *in* "Flavins and Flavoproteins," Kamin, H., Ed., Baltimore, Md., University Park Press.

Müller, F., and Massey, V. (1969), *J. Biol. Chem. 244*, 4007.

Pitman, I. H., Shefter, E., and Ziser, M. A. (1970), *J. Amer. Chem. Soc. 92*, 3413.

Pitman, I. H., and Ziser, M. A. (1970), *J. Pharm. Sci. 59*, 1295.

Salach, J., Walker, W. H., Singer, T. P., Ehrenberg, A., Hemmerich, P., Ghisla, S., and Hartmann, U. (1972), *Eur. J. Biochem. 26*, 267.

Schroeter, L. C. (1966), "Sulfur Dioxide," Oxford, Pergamon Press.

Shapiro, R., Servis, R. E., and Welcher, M. (1970), *J. Amer. Chem. Soc. 92*, 422.

Singer, T. P., Salach, J., Walker, W. H., Gutman, M., Hemmerich, P., and Ehrenberg, A. (1971), *in* Flavins and Flavoproteins, Kamin, H., Ed., Baltimore, Md., University Park Press.

Swoboda, B. E. P., and Massey, V. (1966), *J. Biol. Chem. 241*, 3409.

Walker, W. H., Hemmerich, P., and Massey, V. (1967), *Helv. Chim. Acta 50*, 2269.

Walker, W. H., Kearney, E. B., Seng, R., and Singer, T. P. (1971), *Biochem. Biophys. Res. Commun. 44*, 287.

Walker, W. H., Singer, T. P., Ghisla, S., and Hemmerich, P. (1972), *Eur. J. Biochem. 26*, 279.

Preparation and Characterization of Free Cell Suspensions from the Immature Rat Uterus†

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ABSTRACT: This report describes a procedure for the preparation of free cell suspensions from the immature rat uterus. Characterization of the cell suspensions indicates that 40–50% of the cells present in the intact uterus can be obtained as free cells in suspension. These cells exhibit viabilities in excess in 95% and carry out a number of metabolic processes at constant rates for up to 10 hr in suspension. The estradiol binding properties of the uterus are quantitatively recovered in the dispersed cells. The cell suspensions appear to provide a useful experimental system with which to study the interaction of estradiol with the uterine binding proteins in the intact cell at physiological temperatures.

Although some properties of the estrogen binding proteins of the rat uterus have been examined in crude and partially purified extracts (Ellis and Ringold, 1971; Giannopoulos and Gorski, 1971a,b; Puca *et al.*, 1971), we have very little information about the behavior of these proteins in the intact cell at physiological temperatures. Information about the equilibrium and kinetic binding behavior of these pro-

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teins within the intact cell is requisite to an understanding of the role these proteins play in the development of the uterine growth response. Recent evidence has demonstrated the complications of extracellular factors when examining the binding of estradiol by the whole tissue *in vitro* (Williams and Gorski, 1971). An experimental system of uterine cells in suspension would enable one to eliminate some of these problems and afford a much greater latitude in terms of experimental design. The experiments reported here concern the preparation and metabolic characterization of uterine cell suspensions. In addition, these suspensions have been employed to obtain information about the equilibrium dissociation constant and the forward and reverse rate constants for the estrogen binding process in the intact cell.

Materials and Solutions

Common reagent grade chemicals were obtained from commercial sources. The dyes and indicators used and their sources are as follows: Nigrosin and Eosin Y (Matheson Coleman & Bell), Trypan Blue (Grand Island Biological), and Phenol Red (Hartman-Leddon).

Enzymes and related biochemicals used and their sources are as follows: twice-crystallized, salt-free trypsin and type CLS collagenase (Worthington Biochemical); Grade B Pronase, calf thymus DNA, and Type 11-L lima bean trypsin inhibitor (Sigma); 15 cP of methyl cellulose (Fisher Scientific); filter-sterilized hypophysectomized lamb serum (Animal Genetics Laboratory, University of Illinois); penicillin G (Squibb); and tissue culture medium 199 and minimal essential medium (MEM)[1] (Grand Island Biological). Medium 199 and MEM were obtained with a bicarbonate buffering system and employed under an atmosphere of O_2–CO_2 (95:5) to yield a pH of 7.4 at 37°. In some cases MEM was obtained without bicarbonate and was buffered with 0.025 M Hepes to yield a pH of 7.3 at 37°.

Radioisotopes used and their sources are as follows: D-glucose-*U*-^{14}C (280 Ci/mol), L-leucine-*4,5-t* (2.0 Ci/mmol), and L-leucine-*U*-^{14}C (312 Ci/mol) (Schwarz BioResearch); and 17β-estradiol 6,7-*t* (40 Ci/mmol) (New England Nuclear).

Miscellaneous materials and their sources are as follows: NCS solubilizer (Amersham/Searle); hydroxide of Hyamine-10X, POP, and POPOP (Packard Instrument); Triton X-100 (Rohm and Haas); and Siliclad (Clay Adams).

Solutions used and their compositions are as follows: Tris (T) buffer (0.05 M Tris-HCl, pH 7.4, 25°); Tris-KCl (TK) buffer (T buffer containing 0.10 M KCl, pH 7.4, 25°); Tris-EDTA (TE) buffer (T buffer diluted 1:5 and containing 0.0015 M EDTA, pH 7.4, 25°); modified Krebs–Ringer (KRB) buffer (in grams per liter, NaCl (6.923), KCl (0.353), KH_2PO_4 (0.162), $NaHCO_3$ (2.1), glucose (1.0), methylcellulose (1.0), and Phenol Red (0.005), pH 7.4, 37°, under an atmosphere of 5% CO_2); KRB lacking $NaHCO_3$ but buffered with 0.528 g/l. of $NaH_2PO_4 \cdot H_2O$ and 4.352 g/l. of $Na_2HPO_4 \cdot 7H_2O$, pH 7.3, 0°; scintillation fluid A (5.0 g/l. of POP and 0.3 g/l. of POPOP in toluene); and scintillation fluid B (27.2% Triton X-100, 9.1% absolute ethanol, and 63.7% scintillation fluid A). Except where noted all samples were counted in scintillation fluid A. Methylcellulose (1.0 g/l.) was added to the KRB and the tissue culture media. The methylcellulose was dissolved by stirring for 8–10 hr at 5°. The media were then vacuum filtered through membrane filters (0.45-μ diameter pore size) to remove undissolved methylcellulose. This compound was included in the media because of its property of protecting mammalian cells in suspension culture from hydrodynamic fluid forces and centrifugal forces (Bryant, 1966).

Glassware used with the cell suspensions was cleaned with alcoholic NaOH, rinsed in tap water, soaked in dilute HCl, and rinsed in deionized water. The glassware was then treated with 1% Siliclad to provide a nonstick surface, thoroughly rinsed in distilled water, and oven dried. For experiments requiring incubations longer than a few hours, the glassware was autoclaved and all solutions were filtered sterilized.

Experimental Procedures

Uteri were excised from 20- to 24-day-old Holtzman rats, finely minced with razor blades, and incubated for 30 min at 37° in KRB containing 2% trypsin and 0.0115 M $CaCl_2$. Incubations were carried out in 50-ml tubes with silicone rubber stoppers and a tissue to volume ratio of 2 uteri/ml. Tubes were held horizontally and shaken at 60–80 cycles/min while submerged in a water bath. The tissue pieces were then centrifuged and washed twice with KRB at room temperature. All centrifugations in the cell preparations and in the experiments to be described were for 5 min at 300*g*. With this force there is no loss of cell viability. Centrifugation at greater forces resulted in a marked decrease in viability as judged by exclusion dye staining. The washed tissue pieces were resuspended in KRB containing either 0.75% collagenase plus 0.20% Pronase or 1.5% collagenase plus 0.35% trypsin. After incubation at 37° for 30 min, the tissue pieces were drawn into and slowly expelled from a series of disposable pipets with tip openings of decreasing size. This procedure was found to be effective in breaking up the tissue pieces (Liu and Gorski, 1971). Repetition of this process several times in a 20- to 30-min period resulted in the nearly complete dissociation of the tissue pieces into individual cells. The resulting suspension was centrifuged and washed six times in KRB. The last four washes contained trypsin inhibitor at 200, 20, 10, and 10 μg per ml, respectively. All media or solutions to which the cells were subsequently exposed contained 10 μg/ml of trypsin inhibitor. Undissociated tissue pieces or cell clumps were removed by aspiration.

Determination of Cell Viability. Cell viability was determined by the method of exclusion dye staining with 0.05% Nigrosin (Kaltenbach *et al.*, 1958). Similar results (±5%) were obtained with Eosin Y (Schrek, 1936; Hanks and Wallace, 1958) and Trypan Blue (Merchant *et al.*, 1960). Cell viability is expressed as the per cent of cells excluding the dye with total cell number determined on at least two dilutions of the suspension. For any determination, 500–1000 cells were counted.

Measurement of Glucose Metabolism. Samples of the cell suspensions or excised uteri were incubated with glucose-^{14}C in the designated medium and the conversion of glucose-^{14}C to $^{14}CO_2$ was determined by the method of Cuppy and Crevasse (1963). In one experiment the acid treated cell pellet remaining after the CO_2 collection was extracted for neutral lipids by the method of Dole (1956). The lipid extract was washed four times to remove residual glucose (Folch *et al.*, 1957) and counted with 75% efficiency. In one experiment the incorporation of ^{14}C from glucose-^{14}C into acid-insoluble material was determined. After the CO_2 collection, the uteri or cells were homogenized in 5% trichloracetic acid. Aliquots of the homogenate were washed onto glass filters, washed five times

[1] Abbreviations used are: MEM, minimal essential medium; POP, 2,5-diphenyloxazole; POPOP, 1,4-bis[2-(5-phenyloxazolyl)benzene; Hepes, *N*-2-hydroxyethylpiperazine-*N'*-2-ethanesulfonic acid.

with 5% Cl_3CCOOH, dried, digested with 2.0 ml of NCS overnight, and counted with 65% efficiency.

Measurement of Leucine Uptake and Incorporation into Cl_3CCOOH-Soluble and- Insoluble Fractions. Samples of free cells or uteri were incubated at 37° in the designated medium containing leucine-*t*. At the end of the incubation period, samples were diluted with five volumes of KRB at −5°, washed twice with 5 ml of KRB at 0°, suspended in 1.0 ml of 7% Cl_3CCOOH, and homogenized. The homogenate was diluted to 3 ml with 7% Cl_3CCOOH containing 50 μg/ml of leucine, stored overnight on ice, and centrifuged to yield acid-insoluble and -soluble fractions.

Acid-Insoluble Fraction. The Cl_3CCOOH-insoluble pellets were washed two times with 7% Cl_3CCOOH, solubilized with 2 ml of NCS, and counted with 25% efficiency. In one experiment the incorporation of leucine-*t* into acid-insoluble material by the cell suspension was terminated by the addition of five volumes of 5% perchloric acid followed by washing the pellet with 5% perchloric acid, ethanol, and diethyl ether.

Acid-Soluble Fraction. Aliquots (0.50 ml) of the acid-soluble supernatants were added to scintillation fluid B (11 ml) and counted with 14% efficiency to give an estimate of the total acid-soluble radioactivity. The remainder of the supernatant was extracted with diethyl ether to remove the Cl_3CCOOH. The aqueous phase was then concentrated and subsequently chromatographed on 1 × 35 cm strips of Whatman No. 1 filter paper in a descending system of 1-butanol saturated with 2.0 N NH_4OH. Leucine-^{14}C was added as an internal marker.

Determination of Estradiol Binding Capacity. CELL-FREE EXTRACTS. For the determination of estradiol binding sites in cell-free extracts, all operations were carried out at 0–4°. Samples of free cells were suspended in TK or T buffer and broken by passage through a French pressure cell at approximately 8000 psi. The low-speed supernatant (LSS) was prepared by centrifugation of the burst suspension for 20 min at 800*g*. The high-speed supernatant (HSS) or cytosol fraction was prepared by centrifugation of the LSS or the burst suspension for 45 min at 226,000*g*. Excised uteri were homogenized in T or TK buffer as described (Giannopoulos and Gorski, 1971a). LSS and HSS were prepared from the homogenate as described above. On several occasions the uterine homogenate was passed through the French pressure cell before centrifugation. This procedure had no effect on the quantity of estradiol binding sites recovered from the tissue.

The specific estradiol binding sites present in the LSS were measured by the glass pellet assay (Clark and Gorski, 1969). The specific binding sites in the HSS were measured by gel filtration on 7 × 0.6 cm Sephadex G-25 columns. For both methods the respective supernatants were incubated at 0° for 90 min with 2 × 10^{-8} M estradiol-*t* prior to assay. For the gel filtration assay, bound hormone in the Blue Dextran marked void volume was collected in four-drop fractions directly into scintillation vials containing scintillation fluid A (10 ml) and 100% ethanol (4 ml). The bound estradiol emerging with the void volume is completely separated from the free hormone which is included in, and retarded by, the gel. The Blue Dextran does not bind the hormone or alter the counting efficiency of the samples. Radioactivity was assayed with 20–25% efficiency.

In contrast to the glass pellet assay (Clark and Gorski, 1969), some nonspecific binding is seen with the gel filtration assay. Nonspecific binding represents hormone which is associated with components other than the specific estradiol binding proteins. With the conditions and protein concentrations employed here, nonspecific binding is usually negligible up to estradiol concentrations of 10^{-9} M. With hormone concentrations required to saturate the specific binding proteins during the brief incubations used for the assays, however, the nonspecific or nonsaturable component may represent 10–15% of the total bound hormone. One can distinguish hormone bound to the nonspecific sites as opposed to the specific estradiol binding proteins by taking advantage of the fact that the nonspecific sites are a low-affinity binding system present in very large numbers while the estradiol binding proteins are a high-affinity binding system present in very limited numbers (Ellis and Ringold, 1971; Toft *et al.*, 1967). Consequently, the nonspecific component can be determined at any estradiol concentration by incubating a parallel sample with estradiol-*t* and a 100-fold excess of unlabeled estradiol. The sample containing labeled and unlabeled estradiol estimates the amount of nonspecifically bound estradiol in the sample containing the labeled hormone alone. By difference, therefore, one obtains the quantity of hormone bound to the specific estradiol binding proteins. For this procedure to be valid, the nonspecific component must behave as a linear function of the hormone concentration within the employed concentration range. With the method of assay employed in these studies, the nonspecific component behaves in a linear fashion up to estradiol concentrations of at least 2 × 10^{-6} M. This has been found to be true when working with uterine cytosol, intact uteri, or free cell suspensions (Williams, 1972). Once the linear behavior of the nonspecific component has been ascertained, one can employ a fixed level of unlabeled estradiol as competitor.

INTACT CELL CAPACITIES. For the determinations of estradiol binding capacities of the intact samples, free cells or uteri were incubated for 60 min at 37° with 2 × 10^{-8} M estradiol-*t*. Cell samples were then diluted with three volumes of iced, phosphate-buffered KRB, centrifuged at 0°, and washed three times with 5 ml of iced, phosphate-buffered KRB. The washed pellet was extracted with 100% ethanol and the radioactivity in the extract was assayed with 20–25% efficiency. Uteri were homogenized in TE buffer, and the washed crude nuclear fraction was prepared (Williams and Gorski, 1971). This fraction was extracted with ethanol and assayed for radioactivity as above. Nonspecific binding was determined as described above. These values have been subtracted from total bound hormone to give the reported values. For the determinations of estradiol binding as a function of time, at 37° the binding process was terminated by rapidly lowering the temperature to 0–4°. This was accomplished by adding six volumes of phosphate-buffered KRB at −5° to one volume of the cell suspension. The samples were then washed as described above. Experiments reported here and previously (Williams, 1972; Williams and Gorski, 1971) show the binding process to occur at a negligible rate at 0° relative to 37°.

MISCELLANEOUS MEASUREMENTS. All radioactivity was measured by liquid scintillation spectrometry. Counting efficiencies for each type of sample were determined by the method of internal standardization.

DNA was measured on appropriate aliquots with *p*-nitrophenylhydrazine by the method of Webb and Levy (1955), employing hydrolyzed calf thymus DNA as the standard. The media and solutions in which the cell or tissue samples were incubated were checked for interference with this assay and found to be negative.

Results

When designing the procedure for the preparation of dispersed uterine cells and evaluating the success of the proce-

TABLE I: Analysis of Uterine Cell Suspensions.

Tissue Mince → (30 min, 37°; 2% trypsin) → Wash → (45–60 min, 37°; + 1. 0.75% collagenase–0.20% pronase or 2. 1.5% collagenase–0.35% trypsin) → Wash → Free Cell Suspension

Parameter	Value ± SEM	N^a
DNA/uterus (g)	$279.0 \pm 8.0 \times 10^{-6}$	17
DNA/cell in suspension (g)	$6.5 \pm 0.4 \times 10^{-12}$	8
Cells/uterus	$4.4 \pm 0.3 \times 10^{7}$	8
Per cent recovery of cells in preparation	43.5 ± 2.8	28
Per cent viable cells	97.3 ± 0.4	11

[a] N = Number of experimental means used in the calculations.

TABLE II: Metabolic Stability of Cell Suspensions.

Incubation (hr)[a]	Viability (% ± SEM)[b]	$^{14}CO_2$[c,d]	Lipid-^{14}C[c,d]	Leucine-t[c,e]
0–1	97.3 ± 0.4	5540 ± 78	760 ± 17	
2–2.5				5487 ± 216
3–4	95.5 ± 0.1			
4–5		5120 ± 155	890 ± 13	
5–5.5				5813 ± 162
6–7	94.2 ± 0.8			
9–10	91.7 ± 0.8	5092 ± 61	895 ± 18	

[a] Incubations were carried out with 0.6–2.0 × 10^7 cells/flask in MEM or Medium 199, containing 100 U/ml of penicillin. Hypophysectomized lamb's serum (10%) was included in all media except for the leucine determinations. [b] Viabilities are means of several determinations during the designated interval. SEM = standard error of the mean. [c] Cpm/flask ± SEM. [d] At the designated times, an equal volume of medium containing glucose-^{14}C (10 μCi) was added to each flask, and the incubations were terminated 1 hr later. Experimental values refer to radioactivity subsequently recovered in the CO_2 and lipid fractions. Background controls for the glucose metabolism experiments were provided by samples incubated with the isotope but without tissue. After the CO_2 collection, acid-treated cell pellets or uteri (Table III) were added to the medium and carried through the procedures for lipid extraction or the determination of Cl_3CCOOH-insoluble radioactivity (Table III). Background values for the three time intervals were respectively 660, 770, and 860 cpm per flask (for $^{14}CO_2$) and 25, 31, and 26 cpm per flask (for lipid-^{14}C). These background values have been subtracted from the gross values to give those shown above. [e] At the designated times, an equal volume of medium containing leucine-t (10 μCi) was added to each flask, and the incubations were terminated 30 min later by the addition of five volumes of 5% perchloric acid. Background values for samples incubated without cells were respectively 75 and 90 cpm.

dure, a number of criteria were considered essential. (1) The procedure should provide reasonable yields of free cells with a minimum of experimental manipulations. (2) The dispersed cells should exhibit a high degree of viability and metabolic integrity, and should survive in suspension for at least several hours. (3) The specific estradiol binding sites of the uterus must be quantitatively recovered in the cell suspensions. (4) It must be possible to use the system to study the interaction of estradiol with these binding sites.

The data in Table I summarize the results from a number of cell preparations. The procedure outlined in the flow diagram is relatively simple to execute and can be completed in about 2.5 hr. The use of either enzyme combination provides free cells in approximately the same yield and exhibiting the same degree of viability. Suspensions prepared with pronase are somewhat more free of debris than those prepared with trypsin. The use of trypsin in the second stage of dissociation has the advantage, however, that specific inhibitors are available for this enzyme. Consequently, when cell free extracts were prepared from the suspensions, the trypsin–collagenase combination was the method of choice.

With either enzyme combination the cell suspension appeared to be a uniform population of cells exhibiting less than 5% contamination with cell debris. Greater than 90% of the dissociated cells exist as individual cells with very large prominent nuclei. The remainder of the cells exist in clumps of 2–10 cells. The ratio of nuclear volume to cell volume was estimated to be 0.6–0.8. This ratio is characteristic of that seen in photomicrographs or electron micrographs of the immature or ovariectomized rat uterus (Bo *et al.*, 1968; Stumpf, 1968; V. Bell, W. H. Beers, J. Gorski, and A. B. Taylor, unpublished observations). No attempt was made to identify the individual cell types present in the cell suspensions.

The recoveries of cells in the preparations averaged about 43% (Table I). This value is based on the DNA content of the immature rat uterus and the DNA recovered in the suspensions. The magnitude of recovery is sufficient to make the routine use of the procedure feasible. Measurements of DNA content per cell are based on direct cell counts and DNA determinations of the cells in suspension. The value of 6.5×10^{-12} g/cell is in agreement with diploid DNA values for other rat tissues (Sober, 1968). This observation provides assurance that the measured recoveries of DNA are true reflections of cell recovery.

Cell Viability. The data in Table I show the free cells to exhibit viabilities in excess of 95%. This criterion of viability is based on the ability of the cells to exclude particular dyes (Schrek, 1936; Hanks and Wallace, 1958; Kaltenback *et al.*, 1958; Merchant *et al.*, 1960). As indicated in Table II, the initial viability decreases only slightly during 10-hr incubation.

The data in Table II show the free cells in suspension to retain their ability to convert glucose-^{14}C to CO_2 and a neutral lipid fraction at essentially constant rates for at least 10 hr. Similarly, the incorporation of leucine-t into acid-insoluble material occurs at the same rate after 2- or 5-hr incubation. Other experiments have shown the incorporation of leucine-t into acid-insoluble material to proceed at a constant rate for the first 2.5 hr after the cells have been isolated from the tissue (Williams, 1972).

A number of experiments have been carried out to examine the uptake of leucine-t into an acid-soluble fraction of the dispersed cells (Williams, 1972). These studies show a rapid uptake of this amino acid which reaches an equilibrium plateau

TABLE III: Metabolic Comparison of Free Cells and Whole Uteri.[a]

Parameter	Sample	Dpm/μg of DNA ± SEM	Cells/ Uterus
Glucose-$^{14}C \rightarrow {}^{14}CO_2$ 60-min incubation[a]	Free cells	116.9 ± 4.9	
	uteri	183.3 ± 19.8	0.64
Glucose-$^{14}C \rightarrow Cl_3{}^{14}CCOOH$ insoluble 60-min incubation[a]	Free cells	93.2 ± 1.3	
	uteri	99.0 ± 10.0	0.94
L-Leucine-*t* incorporation[b] 30-min incubation			
Cl_3CCOOH soluble	Free cells	233.7 ± 4.6	
Cl_3CCOOH insoluble	Free cells	131.1 ± 5.0	
Cl_3CCOOH insoluble/ Cl_3CCOOH soluble	Free cells	0.561	
Cl_3CCOOH soluble	Uteri	168.8 ± 4.0	
Cl_3CCOOH insoluble	Uteri	98.6 ± 5.2	
Cl_3CCOOH insoluble/ Cl_3CCOOH soluble	Uteri	0.584	0.96

[a] For the glucose-^{14}C determinations, 4×10^7 cells or two whole uteri were incubated in 2 ml of Medium 199 containing 10 μCi of glucose-^{14}C. [b] For the leucine-*t* determinations, 1.1×10^7 cells or two whole uteri were incubated in 1 ml of Medium 199 containing 2 μCi of leucine-*t*. The Cl_3CCOOH-soluble radioactivities are the means of values obtained after 10-, 20-, and 30-min incubation. The Cl_3CCOOH-insoluble values represent the accumulation of radioactivity in this fraction between 10- and 30-min incubation. Background controls were provided by adding cells or uteri to iced medium containing the isotope and immediately processing as described in Experimental Procedures.

TABLE IV: Free Cells *vs.* Uterus: Comparison of Estrogen Binding Protein Content and Specific Uptake of 17β-Estradiol-*t*.

	17β-Estradiol-*t* Dpm/280 μg of DNA ± SEM		Cells/ Uterus
	Cells	Uterus	
A. Binding protein[a]			
I. Glass pellet assay			
Experiment 1	78,540 ± 1860	80,130 ± 896	0.98
Experiment 2	78,193 ± 5910	85,090 ± 6350	0.92
II. Sephadex G-25 assay	98,093 ± 5024	91,980 ± 4410	1.07
B. Specific uptake[b]	83,448 ± 6320	85,787 ± 5960	0.97

[a] Binding protein content refers to the quantity of specific binding sites assayable in cell free extracts at 0°. [b] Specific uptake refers to the quantity of a specifically bound estradiol present in the cell suspensions or the washed nuclear fraction of the uterus after the suspensions or intact uteri were incubated with 2×10^{-8} M estradiol-*t* for 60 min at 37°.

in 10–12 min at 37°. This uptake is inhibited by reduced temperatures and essentially eliminated by exposing the dispersed cells to 1 mM dinitrophenol and 2.5 mM iodoacetate. These inhibitors also eliminate the incorporation of leucine-*t* into acid-insoluble material.

Metabolic Comparison with the Uterus. Several additional experiments have been performed to compare the metabolic activity of the isolated cells with the intact uterus *in vitro*. In the first experiment the conversion of glucose-^{14}C to CO_2 and Cl_3CCOOH-insoluble material was measured in a 1-hr incubation. As indicated in Table III, the free cells are somewhat less active than the whole tissue with respect to CO_2 production, but are essentially as active in the conversion of glucose to acid-insoluble material.

The second experiment concerned the uptake and incorporation of leucine-*t* into acid-soluble and -insoluble fractions for 10-, 20-, and 30-min incubation. As indicated previously, the Cl_3CCOOH-soluble radioactivity was essentially constant between 10- and 30-min incubation for the free cells and uteri. The incorporation into Cl_3CCOOH-insoluble material was linear over this time period for both samples. The data from this experiment are shown in the bottom portion of Table III. The Cl_3CCOOH-soluble radioactivity is expressed as the mean of the determinations at the three time points. The Cl_3CCOOH-insoluble radioactivity represents the incorporation between 10 and 30 min; that is, these values represent the insoluble accumulation during the time when the acid-soluble radioactivity was at a constant level. The data show the dissociated cells to have been somewhat more active than the isolated tissue with respect to both acid-soluble and -insoluble parameters. If the incorporation of leucine-*t* into the insoluble fraction is expressed as a function of the radioactivity present in the acid-soluble fraction, however, the free cells and the isolated uteri appear to have been equally active.

In all the experiments concerned with the uptake and incorporation of leucine-*t*, the Cl_3CCOOH-soluble fractions were examined chromatographically to ensure that extensive metabolism of the label had not occurred. In the chromatographic system employed, 93–96% of the acid-soluble radioactivity cochromatographed with authentic marker leucine and leucine-^{14}C was added as an internal standard.

Estradiol Binding Properties. The data in Table IV show a comparison of estradiol binding capacity between the dissociated uterine cells and uteri. As judged by the glass pellet assay or gel filtration chromatography, extracts from the free cells contain essentially the same number of estrogen binding sites per cell as extracts from the uterus (Table IV, portion A). Measurements of specific binding to the intact cells at 37° show the same quantity of binding sites as are present in extracts from the cells (Table IV, compare A and B values). This is essentially the same number of binding sites that can be found in the nuclear fraction of the intact uterus after incubation for 2 hr at 37° with 2×10^{-8} M estradiol-*t* (Table IV, portion B). These conditions have been shown to result in the nuclear localization of approximately 85–90% of the specific binding sites in the intact uterus (Giannopoulos and Gorski, 1971a). The measurement of total binding sites as shown here corresponds to 12,000–15,000 estradiol binding sites/cell. This value is in agreement with values obtained in numerous laboratories by a great variety of assay techniques

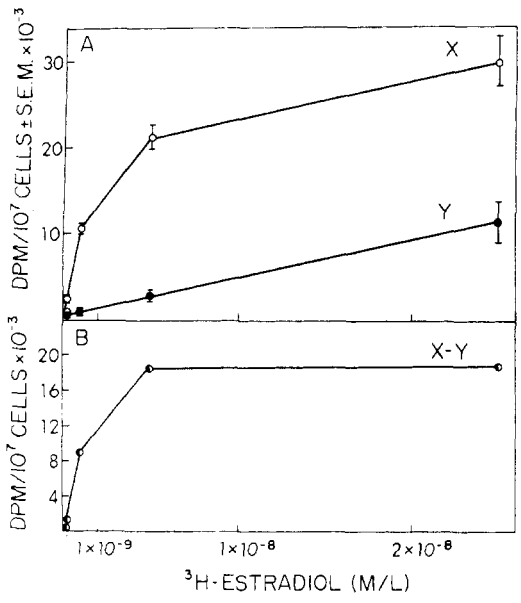

FIGURE 1: Estradiol binding by free cell suspensions. (A) Cells were incubated in KRB at 2×10^7 cells/ml for 60 min at 37° with the estradiol-*t* concentrations given on the abscissa. Incubations were carried out with estradiol-*t* (○) or estradiol-*t* plus a 10-fold excess of unlabeled estradiol (●). (B) This curve shows the difference between the two curves in part A.

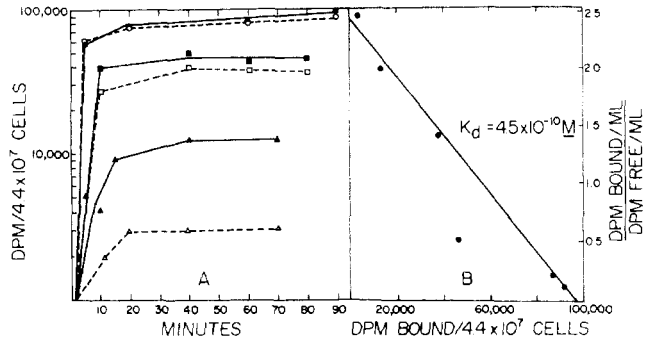

FIGURE 2: Time course of binding at 37° with various estradiol concentrations. (A) Cells were incubated in Hepes-buffered MEM at $0.8–1.0 \times 10^7$ cells/ml. The quantity of bound hormone not subject to competition was determined in parallel incubations and subtracted from the gross values to give the data plotted here. Each data point is the mean of duplicate determinations. Initial estradiol concentrations were 1.9×10^{-11} (Δ), 9.3×10^{-11} (▲), 3.2×10^{-10} (□), 9.9×10^{-10} (■), 4.3×10^{-9} (○), and 9.0×10^{-9} (●). (B) Scatchard plot of the mean plateau values in part A. K_d refers to the dissociation constant for the binding process determined from the slope of the line.

(Toft *et al.*, 1967; Clark and Gorski, 1969; Feherty *et al.*, 1970; Notides, 1970; Giannopoulos and Gorski, 1971a).

An important demonstration in Table IV is the equivalence of binding sites measured in extracts from the cells with the number of sites measured by specific binding of estradiol to the intact cells. This suggests that one can monitor the binding of estradiol to these sites on an equilibrium or rate basis by measuring the specific binding to the cells in suspension. The limit of sensitivity for this approach is given by the nonspecific binding of estradiol to sites other than those on the specific estradiol binding proteins. Figure 1 shows the results from an equilibrium binding experiment with the free cells. In this experiment, cell samples were incubated for 60 min at 37° with increasing estradiol-*t* concentrations. Nonspecifically bound hormone was determined from a parallel set of samples containing the estradiol-*t* and an excess of unlabeled estradiol as discussed previously. As shown in the upper panel of Figure 1, the samples which contained the estradiol-*t* alone exhibit a biphasic binding curve with increasing hormone concentrations (curve *X*). There is a large increase in the quantity of bound hormone at the lower concentrations, and a more gradual increase toward the upper end of the concentration range employed here. The samples containing the estradiol-*t* and the excess unlabeled hormone (curve *Y*) show only the component which exhibits the gradual increase. This nonspecific component appears to increase as a linear function of the hormone concentration. The difference between the two curves is plotted in the lower panel of Figure 1. It is evident that saturation binding curves can be generated with the dissociated uterine cells in this fashion.

The data in Figure 2A show the time courses of estradiol binding at different initial estradiol concentrations. For all the estradiol concentrations, equilibrium binding plateaus are attained after 40- to 60-min incubation at 37°. This behavior is similar to that seen with the intact uterus *in vitro* at 37° (Giannopoulos and Gorski, 1971a). Figure 2B shows a Scatchard plot of the mean plateau values from Figure 2A, and indicates that an incubation period of 60 min at 37° is sufficient to attain binding equilibria in the range of approximately 5–90% saturation of the specific estradiol binding sites. The slope of the line yields an apparent dissociation constant, $K_d = 4.5 \times 10^{-10}$ M.

The data in Figure 3A provide a more detailed examination of the binding kinetics of the free cells at 37°. For this experiment the system was set up to produce approximately 60% saturation of the specific binding proteins at equilibrium. This extent of binding site saturation has been shown to occur *in vivo* during the estrous cycle (Lee and Jacobson, 1971; Clark *et al.*, 1972) and also after physiological hormone doses have been adminstered to immature animals (Jensen *et al.*, 1969; Sarff and Gorski, 1971). As seen in Figure 3A, the formation of bound hormone proceeds without any apparent lag period. The binding of estradiol to the cytosol binding protein has been shown to follow second-order kinetics when measured in cell-free extracts at 0, 10, and 18° (Erdos *et al.*, 1971). It was of interest to determine whether the binding process also followed a simple scheme when examined in the intact cell at 37°. Figure 3B shows a second-order plot of the binding data in Figure 3A. For this analysis the binding process was treated as a second-order reaction opposed by the first-order dissociation of the estradiol binding site complex. The data points appear to fall on a straight line, suggesting that the binding process can be described by a simple kinetic scheme in the intact cell. The slope of the line yields an apparent second-order rate constant, $k_1 = 1.52 \times 10^6$ M^{-1} sec^{-1}. Figure 4 shows the result of adding excess unlabeled estradiol to a cell suspension which has been prelabeled with estradiol-*t*. Displacement of previously bound estradiol-*t* occurred with a half-life of 71 min. If exchange accurately reflects the dissociation of estradiol, these data yield a dissociation rate constant, $k_{-1} = 1.62 \times 10^{-4}$ sec^{-1}.

Effect of Temperature on the Binding Process. A number of previous studies have suggested that estradiol rapidly binds to the cytosol binding protein when the immature rat uterus is incubated with hormone at 0° (Jensen *et al.*, 1968; Shyamala and Gorski, 1969; Giannopoulos and Gorski, 1971a). Other experiments (Williams and Gorski, 1971) have indi-

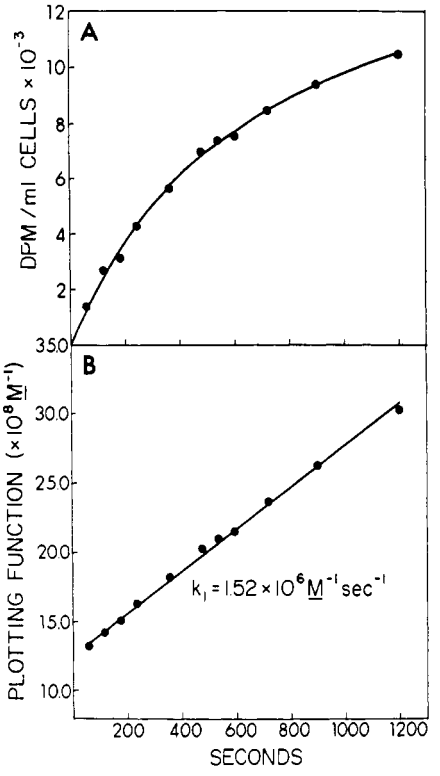

FIGURE 3: Kinetics of estradiol binding to dissociated cells at 37°. (A) Initial estradiol concentration was 5.61×10^{-10} M. Each data point represents the mean of duplicate determinations. (B) The binding process in part A was treated as a second-order reaction opposed by the first-order dissociation of the complex.

$$E + B \underset{k_2}{\overset{k_1}{\rightleftharpoons}} EB$$

where E and B refer to the concentrations of unbound estradiol and binding sites, respectively. The rate equation is then $d(EB)/dt = K_1(E)(B) - k_{-1}(EB)$. At any time, $(E) = (E_t) - (EB)$, $(B) = (B_t) - (EB)$, and $k_{-1} = k_1K_d$, where (E_t) = total estradiol concentration, (B_t) = total binding site concentration, and K_d = equilibrium dissociation constant for the binding process. After inserting these parameters into the rate equation and expanding, the following substitutions can be made: $X = (E_t)(B_t)$, $Y = -((E_t) + (B_t) + (K_d))$, and $Z = 1$. Defining $q = 4XZ - Y^2$, integration yields

$$k_1t = -(q)^{-1/2} \ln \left[\frac{2Z(EB) + Y - (-q)^{1/2}}{2Z(EB) + Y + (-q)^{1/2}}\right] - (-q)^{-1/2} \ln \left[\frac{Y - (-q)^{1/2}}{Y + (-q)^{1/2}}\right]$$

where the last term on the right side of the expression is the integration constant evaluated at $t = 0$ with $(EB) = 0$. For a second-order–first-order reversible reaction, a plot of t against the expression on the right side of the equation should yield a straight line having a slope equal to k_1. In this figure, $k_1 = 1.52 \times 10^6$ M^{-1} sec^{-1}. The appropriate values for the experiment are: $(E_t) = 5.61 \times 10^{-10}$ M, $(B_t) = 2.52 \times 10^{-10}$ M, $K_d = 3.1 \times 10^{-10}$ M. The K_d used here is a mean value taken from a series of binding experiments (Williams, 1972).

cated, however, that the initial binding process in the intact tissue *in vitro* is very temperature dependent. The temperature dependence of the initial binding process was apparently not observed in previous studies because of the accumulation of extracellular hormone at reduced temperatures. This temperature dependence is readily seen with the dissociated uterine cells since the complications of extracellular factors can be experimentally eliminated. The data in Figure 5A show the

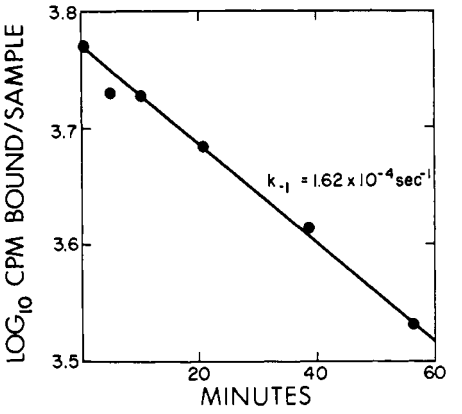

FIGURE 4: Displacement of bound estradiol from free cells. Incubation was carried out with 1.5×10^7 cells/ml in Hepes-buffered MEM for 40 min at 37° with 5×10^{-10} M estradiol-*t*. After samples were removed to determine the quantity of bound estradiol-*t*, unlabeled estradiol was added to give a concentration of 5×10^{-8} M. Samples were then removed at 5, 10, 21, 39, and 55 min. The quantity of bound hormone not subject to competition was determined in parallel incubations and subtracted from the gross values to give the data plotted here. Each data point represents the mean of duplicate determinations. The slope of the line yields an apparent first-order dissociation constant, $k_{-1} = 1.62 \times 10^{-4}$ sec^{-1}.

binding process to occur much more slowly at 0° than at 37°. For this experiment the cells were incubated in Hepes-buffered MEM which has a pH of 7.30–7.35 at 37° and a pH of 7.60–7.65 at 0°. The reduced binding rate at 0° is also seen if the cells are incubated in bicarbonate-buffered MEM, KRB, bicarbonate-buffered Eagle's HeLa medium, or phosphate-buffered KRB. These media have pH's in the range of 7.25–7.35 at 0°.

The data in Figure 5A show the shift from 0 to 37° to yield a large increase in the rate of hormone binding resulting in the rapid attainment of a binding equilibrium. Incubations at 0° have not been carried out for sufficient periods of time to determine whether the extent of binding and the equilibrium dissociation constant are the same at 0° as at 37°. Experiments have been carried out to determine whether exposure of the cells to 0 or 37° in the absence of hormone has any effect on the number of binding sites assayable in a subsequent 60-min incubation at 37°. Preincubations at either temperature for periods of at least 3 hr do not influence the number of assayable binding sites.

An interesting aspect of the binding of estradiol by the uterine cells is revealed by the data in Figure 5B. These data concern the binding of estradiol to the nonspecific or nonsaturable binding component present in the dissociated cells. When the cell suspension was shifted to 37°, there was only a small increase in the quantity of hormone bound to these sites as opposed to the specific sites. To examine this difference an experiment was carried out to determine the effect of temperature on the initial rate of binding to both sets of binding sites in the 0–37° temperature range. Figure 6 shows the initial rates for the specific and nonspecific binding processes in the form of an Arrhenius plot. The data for the specific binding sites yield an apparent activation energy of 20.7 kcal/mol while those for the nonspecific or nonsaturable component give a value of 2.5 kcal/mol.

Discussion

Cell Preparation and Viability. The procedure described here for the preparation of uterine cell suspensions appears to

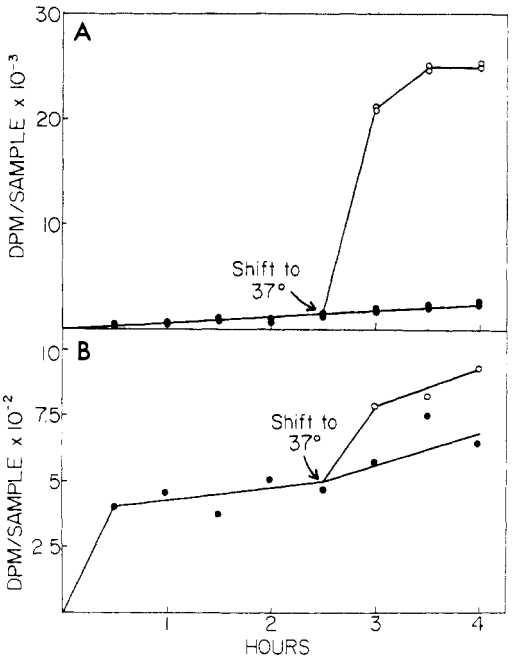

FIGURE 5: Estradiol binding at 0 and 37°. (A) Cells were incubated at 0° in Hepes-buffered MEM with 5×10^{-10} M estradiol-*t*. Data represent specifically bound estradiol after subtracting the quantities of bound hormone not subject to competition (determined in parallel incubations containing 5×10^{-10} M estradiol-*t* and 5×10^{-8} M unlabeled estradiol) from the gross values. The arrow indicates the time at which half the suspension was shifted to 37°. (B) These data represent the quantities of bound hormone not subject to competition in the experiment shown in part A. Details are given above.

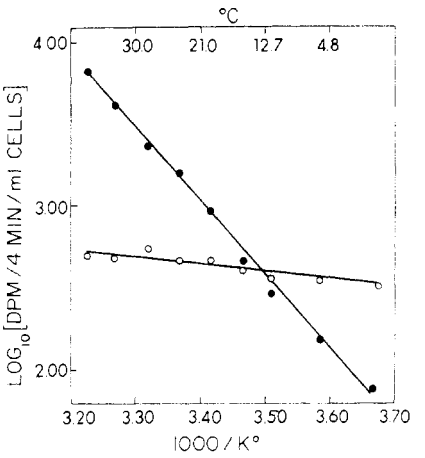

FIGURE 6: Temperature dependence of estradiol binding. Samples were incubated with 6×10^{-10} M estradiol-*t* or 6×10^{-10} M estradiol-*t* plus 6×10^{-8} M unlabeled estradiol for 4 min at the designated temperatures. Specifically bound hormone (○) represents the difference between the total bound hormone and that not subject to competition with excess unlabeled hormone (●). The initial binding to the specific binding sites yields an apparent activation energy of 20.7 kcal/mol while the binding which is not subject to competition yields an apparent activation energy of 2.5 kcal/mol. Each data point is the mean of triplicate determinations.

satisfy the basic criteria required to justify the routine use of this method. Free cells in reasonable yields and virtually free of debris can be obtained in several hours with a minimum of manipulation. After the isolation procedure, the free cells exhibit a high degree of viability as judged by exclusion dye staining. This observation and the maintenance of high viabilities for up to 10-hr incubation suggest that the isolation procedure has not seriously altered the ability of these cells to selectively exclude particular solutes.

The ability of the cell suspensions to convert glucose-^{14}C to a CO_2 and a lipid fraction at essentially constant rates for at least 10-hr incubation supports the conclusion that the isolated cells are quite viable. The maintenance of their ability to incorporate leucine-*t* into acid-insoluble material at a constant rate for at least 5 hr after their isolation from the tissue provides another indication of their viability. Similarly, the inhibition of leucine-*t* uptake into the acid-soluble fraction by inhibitors of oxidative phosphorylation and glycolysis suggests the maintenance of functional integrity in the transport of this amino acid into the isolated cells.

The free cells carried out the measured conversions of glucose-^{14}C and leucine-*t* at rates essentially the same or very comparable to those exhibited by intact tissue incubated under the same conditions. As a criterion of viability, these comparative data make unlikely the possibility of the observed activity being due to a small per cent of the isolated cells.

Estradiol Binding Properties. The data in Table IV show essentially the same number of specific estradiol binding sites per cell in the cell suspensions as can be found in the intact tissue. This similarity in the number of binding sites per cell whether assayed in cell-free extracts (Table IVA) or in the intact cells (Table IVB) indicates a quantitative recovery of these binding sites in the isolated uterine cells. The extreme susceptibility of these binding sites to a variety of proteolytic enzymes in cell-free extracts (Noteboom and Gorski, 1965; Toft and Gorski, 1966) appears to eliminate the possibility that the uterine estrogen binding proteins are of extracellular origin. Similarly, if associated with the cell membrane, these binding sites are at least not accessible to the proteases used for the isolation.

Saturation binding curves can be generated using the cell suspensions and competitive methods to correct for the amount of hormone bound to the nonsaturable binding components. The nonspecific binding is low enough that it does not impose serious constraints on the sensitivity with which the specific binding sites can be examined. The sensitivity of the measurements and the ease of manipulating the cell suspensions make it possible to examine the binding process with as much precision as is possible in cell free extracts.

The data in Figure 2B yield an apparent dissociation constant for the binding process, $K_d = 4.5 \times 10^{-10}$ M. To consider this value as the true K_d, one must assume the unbound hormone concentration within the cell at equilibrium to be the same as that present in the incubation medium. The similarity between this K_d value and that obtained from the ratio of the on and off rate constants, $k_{-1}/k_1 = 1.1 \times 10^{-10}$ M, suggests that this assumption may be true. On the other hand, in the absence of definite information about the hormone concentration within the cell, it seems best to consider the apparent K_d value as simply the concentration of unbound hormone present in the incubation medium at equilibrium when 50% of the specific binding sites contain hormone. The apparent K_d values obtained here are very close to the values obtained in an elegant series of perfusion experiments with the mature and immature rat (DeHertogh *et al.*, 1971). In these studies estradiol was infused into the circulation of the animal to produce different levels of circulating hormone. After measuring the quantity of hormone in the uterus and correcting for the metabolic clearance of estradiol from the circulation, half-

maximal saturation of uterine binding sites was observed at circulating estradiol concentrations of 1–3 $\times$ 10^{-10} M. Further studies with the cell suspensions should allow an examination of the binding process in the presence of various humoral agents under conditions which approximate the physiological situation. Of particular interest are the dynamics of hormone transfer from binding sites on serum proteins to the specific binding sites within the uterine cells.

The observation that the data of Figure 3A fit a simple second-order–first-order scheme suggests that the binding process is not significantly altered by the behavior of the binding sites subsequent to the initial formation of the hormone–protein complex. When the intact uterus is incubated at 37° in the absence of estradiol, all the specific binding sites are found in the cytosol fraction of the tissue (Giannopoulos and Gorski, 1971a). When estradiol is added to the uterus at 37°, the majority of the filled binding sites are transferred to the nuclear fraction. After the first few minutes of incubation. nuclear-bound hormone constitutes 85–90% of the total bound hormone in the tissue (Williams and Gorski, 1971). The bound hormone measured with the free cells represents the sum of cytosol and nuclear bound hormone. If the filled cytosol sites are precursors to the nuclear sites, as proposed (Jensen *et al.*, 1968; Shyamala and Gorski, 1967, 1969), the observation that the binding process appears to follow a simple second-order–first-order scheme suggests that the redistribution of filled binding sites within the uterine cell does not alter the binding of estradiol to these sites or the dissociation from the sites.

Effect of Temperature on the Binding Process. The temperature sensitivity of the binding process is readily apparent from the data in Figures 5 and 6. The presence of the reduced binding rate at low temperatures when the cells are incubated in a variety of media suggests that this behavior is a characteristic of the uterine cells themselves. Similarly, the presence of the reduced binding rate at pH 7.25–7.35 as well as at 7.60–7.65 indicates that the slight pH increase of Hepes-buffered MEM at 0° is not responsible for the slower binding rate. It is readily apparent that one can use a rapid temperature drop to effectively terminate the binding process at 37°. Application of this procedure should permit a detailed kinetic examintaion of the interaction of estradiol with the uterine binding proteins in the environment of the intact cell at physiological temperatures. Such studies have not been possible in cell-free extracts. Similar temperature sensitivity has been demonstrated with the intact tissue *in vitro* (Williams and Gorski, 1971). The significance of this temperature sensitivity in relation to the interpretation of previous experimental data concerning the cytosol to nuclear translocation of filled binding sites has been previously discussed (Williams and Gorski, 1972).

A point of interest here is the basis of the temperature sensitivity in the binding process. The data in Figures 5 and 6 suggest a fundamental difference in the binding of estradiol to the specific binding sites as opposed to the nonsaturable component present in the uterine cells. An examination of the initial rate of binding to the nonsaturable component as a function of temperature yields an apparent activation energy of 2.5 kcal/mol. This value is in a range which might be explained on the basis of diffusion alone. The value of 20.7 kcal/mol for the specific binding process, on the other hand, is somewhat more difficult to interpret. The magnitude of this value suggests the presence of significant conformational contributions to the binding process. The question of interest is whether such contributions may reflect the behavior of the binding sites within the cell or a temperature dependent penetration of estradiol into the cell. It is evident that both possibilities are consistent with the available data. If the temperature dependence is not simply a reflection of hormone penetration into the cell, further studies may provide some useful information about the behavior of the binding sites in the intact cell.

References

Bo, W. J., Odor, D. L., and Rothrock, M. (1968), *Amer. J. Anat. 123*, 369.

Bryant, J. C. (1966), *Anal. N. Y. Acad. Sci. 139*, 143.

Clark, J. H., Anderson, J., and Peck, E. J. (1972), *Science 176*, 528.

Clark, J. H., and Gorski, J. (1969), *Biochim. Biophys. Acta 192*, 508.

Cuppy, D., and Crevasse, L. (1963), *Anal. Biochem. 5*, 462.

DeHertogh, R., Ekka, E., Vanderheyden, I., and Hoet, J. J. (1971), *Endocrinology 88*, 165.

Dole, V. P. (1956), *J. Clin. Invest. 35*, 150.

Ellis, D. J., and Ringold, H. J. (1971), *in* The Sex Steroids, McKerns, K., Ed., New York, N. Y., Appleton-Century-Crofts, p 73.

Erdos, T., Bessada, R., Best-Belpomme, M., Fries, J., Gospadarowicz, D., Menahem, M., Reti, E., and Veron, A. (1971), *Advan. Biosci. 7*, 119.

Feherty, P., Robertson, D. M., Waynforth, H. B., and Kellie, A. E. (1970), *Biochem. J. 120*, 837.

Folch, J., Lees, M., and Sloane-Stanley, G. H. (1957), *J. Biol. Chem. 226*, 497.

Giannopoulos, G., and Gorski, J. (1971a), *J. Biol. Chem. 246*, 2524.

Giannopoulos, G., and Gorski, J. (1971b), *J. Biol. Chem. 246*, 2530.

Hanks, J. H., and Wallace, J. H. (1958), *Proc. Soc. Exp. Biol. Med. 98*, 188.

Jensen, E. V., Suzuki, T., Kawashima, T., Stumpf, W. E., Jungblut, P. W., and DeSombre, E. R. (1968), *Proc. Nat. Acad. Sci. U. S. 59*, 632.

Jensen, E. V., Suzuki, T., Numata, M., Smith, S., and DeSombre, E. R. (1969), *Steroids 13*, 417.

Kaltenback, J. P., Kaltenback, M. H., and Lyons, W. B. (1958), *Exp. Cell Res. 15*, 112.

Lee, C., and Jacobson, H. I. (1971), *Endocrinology 88*, 596.

Liu, T. C., and Gorski, J. (1971), *Endocrinology 88*, 419.

Merchant, D. J., Kahn, R. H., and Murphy, W. H. (1970), Handbook of Cell and Organ Culture, Minneapolis, Minn., Burgess, 188pp.

Noteboom, W. D., and Gorski, J. (1965), *Arch. Biochem. Biophys. 111*, 559.

Notides, A. C. (1970), *Endocrinology 87*, 987.

Puca, G. A., Nola, E., Sica, V., and Bresciani, F. (1971), *Advan. Biosci. 7*, 97.

Sarff, M., and Gorski, J. (1971), *Biochemistry 10*, 2557.

Schrek, R. (1936), *Amer. J. Cancer 28*, 389.

Shyamala, G., and Gorski, J. (1967), *J. Cell Biol. 35*, 125A.

Shyamala, G., and Gorski, J. (1969), *J. Biol. Chem. 244*, 1097.

Sober, H. A., Ed. (1968), Handbook of Biochemistry, Cleveland, Ohio, Chemical Rubber Co., Section H.

Stumpf, W. E. (1968), *Endocrinology 83*, 777.

Toft, D., and Gorski, J. (1966), *Proc. Nat. Acad. Sci. U. S. 55*, 1574.

Toft, D., Shyamala, G., and Gorski, J. (1967), *Proc. Nat. Acad. Sci. U. S. 57*, 1740.

Webb, J. M., and Levy, H. B. (1955), *J. Biol. Chem. 213*, 107.
Williams, D. (1972), Ph.D. Thesis, University of Ill., Urbana, Ill.
Williams, D., and Gorski, J. (1971), *Biochem. Biophys. Res. Commun. 45*, 258.
Williams, D., and Gorski, J. (1972), *in* Biology of Reproduction: Basic and Clinical Studies, Velardo, J. T., and Kasprow, B. A., Ed., New York, N. Y., Academic (in press).

Isolation and Characterization of 3-*O*-α-D-Xylopyranosyl-D-glucose and 2-*O*-α-L-Fucopyranosyl-D-glucose from Normal Human Urine†

Arne Lundblad* and Sigfrid Svensson

ABSTRACT: Two disaccharides, 3-*O*-α-D-xylopyranosyl-D-glucose and 2-*O*-α-L-fucopyranosyl-D-glucose, have been isolated from normal human urine. Their structures have been established by sugar analysis and methylation analysis. Gas–liquid chromatography and mass spectrometry of the permethylated disaccharide alditols confirmed their homogeneity and structures.

Normal human urine contains a considerable number of low molecular weight carbohydrate components (Boas, 1956; Lundblad, 1966, 1970; Lundblad and Berggård, 1962; Miettinen, 1962, 1963; Bourillon, 1970; Bourillon *et al.*, 1962; Hakomori *et al.*, 1962; Huttunen, 1966). Some of the excreted oligosaccharides are related to the ABO blood group and the secretor status of the individual (Lundblad, 1966, 1970; Björndal and Lundblad, 1970; Lundblad and Kabat, 1971). Secretors, in contrast to nonsecretors, have fucose-containing components in their urine, in addition to ABH-specific oligosaccharides. These fucose-containing components are eluted as disaccharides in gel chromatography. Previously, L-fucopyranosyl-*myo*-inositol, a new disaccharide, was isolated from human urine (Lundblad, 1970). The present study reports the isolation and characterization of two new disaccharides, 3-*O*-α-D-xylopyranosyl-D-glucose and 2-*O*-α-L-fucopyranosyl-D-glucose, from the urine of normal human ABH secretors.

Materials and Methods

Urine was collected from 14 healthy male, secretor individuals belonging to different ABO blood groups and from five healthy nonsecretors of blood group O. The urines were pooled in the following way: (I) ten O (H) secretors starved for at least 16 hr. This pool was produced during 98 hr of starvation; (II) three nonstarved secretors of blood group A, volume 8 l.; (III) one nonstarved blood group B secretor, volume 1.5 l.; and (IV) five nonsecretors of blood group O, starved for at least 16 hr. Urine was collected during 20 hr of starvation.

Preservation. Bacterial growth was prevented by the addition of phenylmercuric nitrate (30 ml of saturated solution/l. of urine).

Analytical Methods. Colorimetric methods for determination of 6-deoxyhexose and hexose and the enzymatic assay for D-glucose have been described earlier (Lundblad, 1966, 1967).

Gel chromatography, preparative zone electrophoresis, and preparative paper chromatography of oligosaccharides were performed as previously described (Lundblad, 1966, 1967) using the following buffers, solutions, and solvent mixtures: 2 M acetic acid (pH 1.9) (a), pyridine–acetic acid–water (100:6:894, v/v, pH 6.5) (b), 1-butanol–pyridine–water (3:2:1.5, v/v) (c), ethyl acetate–acetic acid–water (3:1:1, v/v) (d), 1-butanol–acetic acid–water (4:1:5, v/v) (e), and 1-butanol–formic acid–water (8:2:1, v/v) (f).

Sugar analysis was performed by gas–liquid chromatography (glc) (Sawardeker *et al.*, 1965) and mass spectrometry (Golovkina *et al.*, 1966). The absolute configuration of the sugars was determined by optical rotation.

Methylation analysis was performed as previously described (Björndal *et al.*, 1970).

Analysis of the disaccharides as permethylated alditols by glc was done using the column 5% XE-60 on Chromosorb W 80–100 mesh) at 200°. For mass spectrometry, a Perkin-Elmer 270 GLC-MS instrument fitted with the above column was used. Mass spectra were recorded at an ionization potential of 70 eV, an ionization current of 80 μA and an ion source temperature of 80°.

Results

Isolation of the Disaccharides. The four pools of urine were filtered and ultrafiltered at 4° using Visking $^{23}/_{32}$-in. dialysis tubing (Union Carbide Corp., Chicago, Ill.) and a negative pressure of 660 mm of Hg. (The tubing retains protein molecules larger than 10^4; Berggård, 1962.) The ultrafiltrates were concentrated ten times and applied to a Sephadex G-25 (fine) column (10 × 109 cm). The eluates were analyzed for 6-deoxyhexose. The elution patterns for pool I and IV are shown in Figure 1.

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