Changes in Binding of [^{3}H] Concanavalin A to Mouse Blastocysts at Implantation

by

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ABBREVIATIONS

a	long radius of an embryo
b	short radius of an embryo
BSA	bovine serum albumin
C	centigrade
Ci	curie (2.22×10^{12} DPM)
CPM	counts per minute
DPM	disintegrations per minute
h	hours
ICM	inner cell mass
I.U.	international units
i.v.	intravenous
M	molar
mM	millimolar
min	minute
mg	milligram
ml	milliliter
mm	millimeter
m.w.	molecular weight
ng	nanograms ($\times 10^{-9}$ grams)
P	probability
pg	picograms ($\times 10^{-12}$ grams)
pH	-log of H^+ concentration
POPOP	ρ-bis-{2-(5-phenyloxazolyl)}-benzene
PPO	2,5-diphenyloxazolyl
s.c.	subcutaneous

SEM	standard error of the mean
μm	micrometers (X 10^{-6} meters)

I. BACKGROUND

A. Preimplantation Mouse Embryo Development

The preimplantation period of mouse embryos begins with the release from the ovary of an egg surrounded by a glycoprotein coat, the zona pellucida, and a mass of granulosa cells (cumulus mass). Fertilization occurs while the egg is in the ampulla of the oviduct (Austin, '61) and cell division begins. The first cleavage takes about 24 h (Kiessling and Weitlauf, '79) and by early on the fourth day the embryo has undergone several divisions and enters the uterus with about 40 cells (Lewis and Wright, '35: Kiessling and Weitlauf, '79). The embryo at this stage is called a blastocyst and consists of an outer single layer of cells - the trophectoderm; an inner disc of cells at one end of the blastocyst - the inner cell mass (ICM) which will form the embryo proper; and a fluid filled cavity - the blastocoel. With no complications in pregnancy, the blastocyst prepares for implantation as the zona pellucida is dissolved (Dickson, '66; Orsini and McLaren, '67; McLaren, '69, '70; Mintz, '71, '72), its metabolic activity increases (Greenwald and Everett, '59; Brinster, '67; Mills and Brinster, '67; Weitlauf and Greenwald, '67; Ellem and Gwatkin, '68; Woodland and Graham, '69; Menke and McLaren, '70), it superficially adheres to the uterine wall (Potts, '66, '68; Finn and McLaren, '67; Smith and Wilson, '74), and cytoplasmic processes of trophectoderm invade between the cells of the uterine epithelium (Potts, '68, '69; Finn and Lawn, '68; Smith and Wilson, '74). These final steps in implantation are initiated on about day 4½ of pregnancy by activity of the hypothalamo-pituitary-ovarian endocrine axis (Bindon and Lamond, '69; Orsini and McLaren, '67), and by late day 5 the blastocyst is attached firmly enough to the uterine epithelium to resist displacement when the

uterine lumen is flushed with a stream of saline (Mintz, '71, '72).

If a mother experiences a post-partum estrus and a successful mating occurs, implantation of the newly conceived embryos is delayed for a variable period if a suckling stimulus is provided by the previous litter (Daniel, '10; Kirkham, '16, '18). This slowing of the developmental process due to concurrent lactation is called lactational delay of implantation and can be terminated by either removal of the suckling young or the injection of estradiol-17β (Whitten, '55, '58; McLaren, '68, '70; Mintz, '71, '72). A similar delay of implantation can be experimently induced by removing either the pituitary (Bindon and Lammond, '69; Weitlauf, '71) or the ovaries (Yoshinaga and Adams, '66) of pregnant animals prior to noon of day 4 of pregnancy and giving subsequent daily injections of progesterone. This experimental delay of implantation can be terminated by the injection of estradiol-17β in addition to progesterone (Yoshinaga and Adams, '66). Embryos in a delay of implantation are metabolically inactive (Menke and McLaren, '70; Weitlauf, '73, '74), shed their zona pellucida by a hatching process (Orsini and McLaren, '67; McLaren, '67, '68, '70), and remain unattached in the uterine lumen. When delay is terminated, embryos resume their metabolic activity (Weitlauf and Greenwald, '68; Menke and McLaren, '70; Torbit and Weitlauf, '74) and implant into the uterus.

B. Ultrastructure of Implantation

Implantation of the normal mouse blastocyst can best be described as a 3-step process which begins with the positioning of the embryos along the uterus at regular intervals in such a way that each has its abembryonic pole (i.e., the end of the blastocyst opposite the ICM) oriented anti-mesometrially in the uterine lumen and the inner cell mass mesometrially (Potts and Wilson,

'67). This apposition phase is followed by the superficial adhesion of the embryo to the uterine wall along its mural trophectoderm (Potts, '66, '69; Nilsson, '74) and finally by the invasion of cytoplasmic processes of trophectoderm between uterine epithelial cells (Potts, '66, '68; Finn and Lawn, '68; Smith and Wilson, '74). During the apposition phase the uterine luminal surface has a serpentine appearance characterized by many microvilli (Potts, '66, '69; Nilsson, '67). The plasma membranes of the embryonic cells are more uniform with only occasional microvilli (Potts, '66, '69) and are closely apposed to the uterus with a single trophectoderm cell usually making contact with 3 or more epithelial cells (Potts, '68). During attachment, the uterine luminal surface changes dramatically; the microvilli disappear and the epithelial surface becomes sinuous (Potts, '66, '68, '69; Nilsson, '67, '74). The surface of the trophectoderm does not undergo any marked changes but becomes arranged in a close parallel fashion with the uterine luminal surface (Potts, '66, '68; Nilsson, '74). The juxtaposition of the blastocyst and uterine membranes leaves only a 200-250 angstrom space separating their surfaces. This occurs on about the middle of day 5 of pregnancy, or about 100-110 h post coitum (Potts, '66, '68), and it is at about this time that embryos are firmly adhered to the uterine epithelium.

Ultrastructural studies during delayed implantation have demonstrated that the uterine surface has many microvilli and a number of apical protrusions (Bergstrom and Nilsson, '72; Nilsson, '74). The trophectoderm surface is largely devoid of microvilli but has numerous crater-like imprints which are presumably due to close contact with the uterine epithelial surface that results from progesterone-dependent uterine closure (Bergstrom and Nilsson,

'72, '73; Bergstrom, '72b; Nilsson, '74). It is remarkable that the blastocyst is so closely apposed to the uterine surface during this time and yet adhesion has not taken place (Sherman and Wudl, '76). Within 4 h after termination of delayed implantation by injection of estradiol-17β, the surface of the trophectoderm begins to bulge and microvilli appear (Bergstrom and Nilsson, '70, '71, '75; Bergstrom, '72a), and by 16 h the uterus has relaxed its clasp on the embryo as microvilli cover the entire blastocyst surface (Bergstrom and Nilsson, '70). By 24 h the trophectoderm membrane is once again rather smooth, with only occasional surface projections (Bergstrom, '72a; Bergstrom and Nilsson, '70, '71), and is closely apposed to the now smooth uterine surface (Potts, '69; Nilsson, '74). This close apposition of the blastocyst and uterine epithelium is thought to be due to embryo expansion and uterine closure (Enders, '76), and by 30-36 h after estradiol-17β blastocysts are firmly adhered to the uterine wall (Hoversland and Weitlauf, '80).

Although the stages of implantation have been well characterized morphologically, the mechanism of initial blastocyst adhesion to the uterus is not known. However, evidence in other cell systems suggests that intercellular recognition and adhesion is a direct result of specific interactions between molecules on the cell surfaces. These molecular interactions are thought to be noncovalent and involve a stereospecific complementary fit between various parts of the macromolecules (Denburg, '78). This has led to the suggestion that adhesion of blastocysts to the uterine epithelium is accompanied by a reduction in thickness of the cell coats or an rearrangement of their components (Enders, '76), and may result from an interaction of glycoproteins on their surfaces (Enders and Schlafke, '74).

C. Intercellular Recognition and Adhesion

Most evidence suggests that the complex carbohydrate components on cell surfaces play an important role in determining the specificity of many membrane-related phenomena (Hughes, '75). The simple sugars most commonly found in these animal cell membrane components are galactose, fucose, mannose, N-acetyl-glucosamine, N-acetyl-galactosamine, and various forms of sialic acid (Hughes, '75). These monosaccharides may be arranged in a number of different ways to form the carbohydrate moieties that are covalently attached to membrane lipids or proteins. The carbohydrate moieties may contain up to twenty sugar residues and the number of possible arrangements of these sugars in complex carbohydrates on the cell surface is extremely large (Hughes, '75). Thus not only the biochemical composition but also the structural specificity of these oligosaccharide moieties contribute to help the carbohydrate-containing components of cell membranes determine the specificity of many biological recognition events (Hughes, '75).

The carbohydrate-containing components of cell membranes (i.e., glycolipids and glycoproteins) are present in great numbers on the surfaces of cells and oriented in such a way that their carbohydrate residues are exposed at the outer face of the cell (Nicholson and Poste, '76). They are therefore accessible to exogenous molecules as well as to surfaces of other cells. Another important feature of the organization of these components in cell membrane structure is that they can diffuse laterally within the plane of the membrane (Nicholson and Poste, '76). This could allow redistribution of surface components in response to external stimuli, and assist in determining various contact and recognition events. Of the two carbohydrate-containing components of the cell surface, the glycoproteins appear

to be especially important in mediating a number of membrane-modulated phenomena such as responsiveness to hormones, recognition by antibodies, and cell recognition and adhesion (Cook and Stoddart, '73; Hughes, '76; Nicholson, '76).

The important role that surface components play in cell recognition and adhesion has been demonstrated in several plant and animal cell systems. For example, syngamy in the species Strongylocentrotus purpuratus is dependent on the interaction of a protein, bindin (Vacquier and Moy, '77), localized on the acrosome process membrane (Moy et al., '77), with a glycoprotein receptor on the sea urchin egg surface which has species-specific binding affinity for bindin (Glabe and Vacquier, '78). In yeast, a glycoprotein can be isolated from one mating type that agglutinates cells of the opposite mating type (Yen and Ballou, '74; Crandall and Brock, '68). Infection of the root hair of white clover by the nitrogen-fixing bacteria, Rhizobium trifolii, is dependent upon a protein in clover seed and seedling roots, trifoliin (Dazzo et al., '78), that cross links similar antigenic determinants on the root hair cell wall and acidic heteropolysaccharides of the bacteria cell envelope (Dazzo and Brill, '79).

Therefore, considering the evidence for similar mechanisms in other intercellular adhesion systems, the hypothesis that blastocyst adhesion to the uterus is mediated by interactions of the surface carbohydrates - especially of glycoproteins - is not unreasonable. However, the surface coats of the blastocyst and uterus on day 4½ of normal pregnancy and during the time of delayed implantation are apparently inadequate to allow recognition and functional adhesion between their surfaces since embryos can be easily flushed from the uterus during this time. It is only by late day 5

of normal pregnancy or after activation of delayed implanting blastocysts with estradiol-17β that the embryos adhere to the uterus with sufficient force that they resist displacement by flushing. This suggests that there are modifications in the expression or organization of the blastocyst and/or uterine surface components near the time of embryo adhesion and implantation.

D. Blastocyst Surface Charge, Antigen Expression, and Complex Polysaccharides at Implantation

Several studies have attempted to investigate the biochemical nature of the trophectoderm surface of mouse blastocysts and determine if there are changes in the surface components near the time of embryo adhesion. For most of these studies the model of delayed implantation has been used. The presence of acidic polysaccharides and acidic carbohydrates on the blastocyst surface has been demonstrated (Enders and Schlafke, '74) using the ruthenium red (Luft, '71) and colloidal thorium dioxide procedures (Rambourg and Leblond, '67; Stoward, '67), respectively. The alcian blue technique, thought to stain the mucopolysaccharide extracellular coat (Shea, '71), demonstrated only faint staining of blastocysts in delay of implantation, but staining after their activation to implant was intense, particularly along the abembryonic pole (Naeslund and Nilsson, '79). With this same implantation model it has been shown that there is a decrease in the amount of positively charged colloidal iron particles that will bind to the outer trophectoderm membrane of delayed implanting embryos following activation to implant, indicating a decrease in the net negative surface charge, possibly due to a decrease in sialic acid residues (Nilsson et al., '73, '75; Jenkinson and Searle, '77). Similarly, it was shown that the expression of cell surface antigens recognized by rabbit anti-mouse antiserum

(Hakansson, '73) and by alloantisera (Hakansson and Sundqvist, '75; Hakansson et al., '75; Searle et al., '76) decreased following activation of delayed implanting blastocysts. Although these studies demonstrate modifications in the surface components of mouse blastocysts that are activated to implant, they do not characterize directly the expression of specific carbohydrates of the trophectoderm surface.

E. Lectins: Concanavalin A

One tool that has been extensively used to help elucidate the specific carbohydrate expression of cell surfaces is the group of compounds known as lectins. Lectins make up a class of proteins and glycoproteins isolated from a large number of different plants and some invertebrates that specifically bind to simple sugar molecules. Their carbohydrate binding specificities have made them very useful for purposes varying from clinical blood typing, analysis of surface structure of normal and transformed cells, specific isolation of glycoproteins and other carbohydrate-containing molecules, and as specific molecular probes for studying membrane, cell, and tissue structure and organization (Nicholson, '74). Our knowledge concerning the nature of the various receptors that bind to lectins are based on results from inhibition studies using simple sugars as hapten antagonists of lectin binding. However, it is important to keep in mind that the best hapten inhibitor of binding is not necessarily identical to the sugar receptor recognized by the lectin (Sharon and Lis, '75), and that some lectins can interact either with terminal residues or with internal core saccharides (Goldstein et al., '73).

One of the most widely used and studied lectins is Concanavalin A (Con A), a protein extracted from the jack bean and shown to have binding specificity

for mannose-like sugars (Iyer and Goldstein, '73). Con A is a globular protein composed of identical 25,500 m.w. subunits that can be arranged in dimeric, tetrameric and higher order forms (Agrawal and Goldstein, '68; Kalb and Lustig, '68). The arrangement of the subunits into the polymeric Con A molecule is dependent upon a number of variables. The dimer is composed of two subunits and favored by pH between 2.0 and 5.5 (McKenzie et al., '72; Edelman et al., '72), low temperatures (0-4° C, McKenzie et al., '72; Huet et al., '74), and low ionic strength (I=0.3, McKenzie et al., '72). With conditions of pH 5.5 to 7.0 (McKenzie et al., '72; Edelman et al., '72), temperatures of 20-37° C (McKenzie et al., '72; Huet et al., '74) and when ionic strength is greater than 1.0 (McKenzie et al., '72), the tetramer of four subunits is favored. Each subunit has one saccharide binding site - thus the dimer has two, and the tetramer has four binding sites - and the carbohydrate binding activity is dependent on the presence of bound calcium and manganese ions (Kalb and Levitzki, '68). Con A binds to saccharides containing α-D-mannose or α-D-glucose residues, but the sugar of choice is methyl α-D-mannopyranoside (α-methyl mannoside, So and Goldstein, '67a, 67b). Other sugars that will inhibit Con A binding are N-acetyl-D-glucosamine and methyl α-D-glucopyranoside (Kaneks et al., '72). Any of these sugars will interact with Con A whether they occupy a terminal or internal position on a series of oligosaccharides provided that the protein has free access to the determinant sugar (Goldstein et al., '73).

The saccharide binding specificity of Con A has enabled this protein to be utilized in a variety of ways including the purification, characterization and sequencing of polysaccharides, glycopeptides, and glycoproteins from various sources, and as a useful tool in the study of the cell surface

and plasma membrane structure (Nicholson, '76). Con A-techniques that have been employed to study the carbohydrate components of the cell surface include the use of tetravalent Con A in cell agglutination studies, radio-labelled Con A to quantitate the binding capactiy of cells, and Con A-peroxidase method for ultrastructural localization of mannose-like sugar receptor sites. These and other techniques allow the use of Con A for following changes in the structure, organization, and dynamics of cell surface molecules during development, cell transformation, cell movement, and cell-cell interactions.

F. Concanavalin A Binding to Blastocysts at Implantation

Enders and Schlafke ('74) demonstrated the presence of mannose-like sugars in the surface coat of mouse blastocysts using the Con A-peroxidase method and electron microscopy to localize the electron-dense reaction product. With this technique they were unable to detect any changes in the coat thickness with embryo activation and implantation. However, Wu and Chang ('78) using scintillation counting techniques, observed that there is a greater than two-fold increase in the amount of [^{3}H] Con A that will bind to delayed implanting mouse blastocysts that are activated to implant, and they suggested that this change was due to an increase in the number of Con A binding sites on the embryo surface. In addition, Naeslund and Nilsson ('79) reported that there is less binding of Con A-coated latex spheres to the embryonic trophectoderm than to the abembryonic trophectoderm of either implanting or delayed implanting mouse blastocysts, and they indicated that there is a decrease in binding of spheres to the abembryonic trophectoderm surface as embryos prepare to implant (Nilsson, '79). Other studies demonstrated similar changes in the Con A binding parameters of normal day 4½ and day 5 blastocysts. These findings include an increase in [^{3}H] Con A

binding (Wu and Chang, '78; Wu, '80) and a decrease in the ability of Con A-coated erythrocytes to bind to the embryonic trophectoderm (Sobel and Nebel, '76) as mouse blastocysts prepare to implant.

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II. STATEMENT OF THE PROBLEM

It has been suggested that attachment of blastocysts to the uterus is due to interactions between glycoproteins on their surfaces (Enders and Schlafke, '74). However, because embryos only attach under specific conditions, it is inferred that there are modifications in the blastocyst and uterine surface components near the time of implantation.

The demonstration by Wu and Chang ('78) that binding of [^{3}H] Concanavalin A ([^{3}H] Con A) to mouse embryos increases at implantation, suggests that such modifications are reflected as an increase in the number of mannose-like sugars on the embryo surface. In those experiments the amount of [^{3}H] Con A binding was determined by scintillation counting techniques. Questions left unanswered by this study are:

1) whether all the [^{3}H] Con A is bound to the surface of the blastocyst, and

2) whether the increase in binding represents a change in the number of mannose sugars per unit of embryo surface area.

In addition, Nilssons' report ('79) of a decrease in binding of Con A-coated spheres to the abembryonic or mural trophectoderm of blastocysts at implantation provides evidence for regional modifications in the membrane. However, it has not been determined whether this regional difference is due to a decrease in the density of mannose-containing components in specific areas of the embryo surface, or to changes in the physical characteristics of the membrane (Nicholson, '74).

In an effort to answer these questions, experiments were undertaken to determine whether the changes in binding of molecular [^{3}H] Con A to blastocysts at implantation are due to changes in the density of mannose

sugars on the embryo surface. Initial experiments were designed to compare the temporal changes in binding with changes in the surface area of mouse blastocysts that were activated to implant. This experiment is presented in Appendix B of this thesis. Subsequent experiments examined the binding of [^{3}H] Con A to implanting and delayed implanting embryos using the technique of light microscopic autoradiography. This study is presented as a manuscript in the body of this thesis.

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III. MANUSCRIPT

Regional changes in binding of [^{3}H] Concanavalin A to mouse blastocysts at implantation: Autoradiographic studies.

ABSTRACT

Implanting and delayed implanting mouse blastocysts were incubated in vitro with [^{3}H] Con A and the distribution of binding on their surfaces was determined by light microscopic autoradiography. It was found that the density of binding was uniform on delayed implanting embryos but that there were regional differences in its distribution on the surface of implanting blastocysts. Although binding to the polar trophectoderm was similar for both types of embryos, it was significantly reduced on the proximal mural and distal mural trophectoderm of implanting blastocysts (by 36 and 60%, respectively), demonstrating that there is a regional reduction in the density of mannose-like sugars on the blastocyst surface at implantation. It is not known if such changes in the surface carbohydrates play a role in blastocyst attachment to the uterus.

INTRODUCTION

It has been proposed that attachment of embryos to the uterus results from the interaction of glycoproteins on the surfaces of the blastocyst and uterine epithelium (Enders and Schlafke, '74). Support for this possibility comes from the results of studies showing that there are modifications in the surface coat of delayed implanting blastocysts as they are activated with estradiol-17β and prepare to implant. For instance there is a loss of histocompatability antigens (Hakansson, '73; Hakansson and Sundqvist, '75; Hakkansson et al., '75; Searle et al., '76), a reduction in the net negative surface charge, possibly due to a decrease in sialic acid residues (Nilsson et al., '73, '75; Jenkinson and Searle, '77), and an increase in the binding of molecular [^{3}H] Con A (Wu and Chang, '78) possibly due to an increase in the density of mannose-like sugars. However, the observation that there was a decrease in binding of Con A-coated latex spheres to the abembryonic trophectoderm of delayed implanting blastocysts that were activated to implant (Nilsson, '79) suggests that not only are there modifications in the composition of the surface components at the time of attachment, but that there are regional alterations as well.

In the present experiments, implanting and delayed implanting mouse blastocysts were incubated in vitro with molecular [^{3}H] Con A and the distribution of binding to their surfaces was determined by light microscopic autoradiography in an attempt to establish whether there are regional changes in the density of mannose-like sugars on the embryo surface at the time of attachment.

MATERIALS AND METHODS

Mice and Collection of Embryos

Sexually mature, virgin female white Swiss mice were selected at random stages of the estrous cycle and induced to ovulate with intraperitoneal injections of pregnant mares serum gonadotropin (5IU), followed 48 h later by human chorionic gonadotropin (HCG, 5IU) (Fowler and Edwards, '57); immediately after receiving HCG they were placed with fertile males. The presence of a vaginal plug the following morning confirmed mating and was designated as Day 1 of pregnancy.

The pregnant animals were bilaterally ovariectomized between 0800-1100 h on day 4, allowed to recover for 2 days, and randomly assigned to 2 treatment groups. Animals in group 1 received progesterone (2 mg per day) on days 7-10 (embryos from this treatment will be referred to as delayed implanting); animals in group 2 received progesterone on days 7 and 8, and progesterone in combination with estradiol-17β (25 ng per day) on days 9 and 10. Embryos in this treatment attach to the uterus by about 30-36 h after estradiol administration (Hoversland and Weitlauf, '80) and will be referred to as implanting blastocysts. All hormones were suspended in 0.1 ml sesame seed oil and injected s.c. at 0800 h. Animals were killed at 0900 h on day 10, their uteri were excised and trimmed free of mesenteries, and then embryos were collected by flushing the uterus with 0.5 ml ice-cold phosphate buffered saline (PBS - 154 mM NaCl, 0.9 mM $CaCl_2$, 0.5 mM $MgCl_2$, 0.15% bovine serum albumin (w/v), 10 mM sodium phosphate, pH 6.8) from a blunted 20 gauge hypodermic needle and tuberculin syringe. The embryos were pooled in silanized (1% Silane,

PCR Research Chemicals) depression slides and transferred with a glass micropipette through 3 washes with cold PBS.

Lectin Binding Assay

Binding assays were carried out in 6 x 25 mm silanized glass culture tubes that contained 20 µl of [^{3}H] Concananvalin A ([^{3}H] Con A, > 30 Ci/mmol, New England Nuclear Corp.) in 200 mM NaCl, 1 mM $MgCl_2$, and 10 mM sodium phosphate (pH 6.8). To determine the level of nonspecific binding, control incubations were done in the presence of 0.1 M α-methyl mannoside. Preliminary experiments were undertaken to determine the effects of both the length of incubation, and the concentration of [^{3}H] Con A, on total binding per embryo. In these studies 11-30 embryos were incubated on ice (0^o C) either in various concentrations of [^{3}H] Con A for 60 min or in 200 µg [^{3}H] Con A per ml for 5-240 min of incubation. At the end of the incubation period, the embryos were washed, placed on nitrocellulose filters (Millipore), and the amount of radioactivity bound was determined with a liquid scintillation counter (LS 230 Beckman). It was found that the amount of Con A bound increased rapidly in the first 20 min and increased more slowly with longer incubations (data not shown). Binding approached saturation with concentrations of [^{3}H] Con A of about 200 µg per ml (Fig. 1). Therefore, for the autoradiographic studies reported here, 11-23 blastocysts were transferred into assay tubes and incubated on ice (0^o C) in 180-200 µg [^{3}H] Con A per ml for either 15 or 60 min. Six independent assays were done for each treatment group (i.e., 3 with 15 min and 3 with 60 min of incubation).

Light Microscopic Autoradiography

Blastocysts were recovered from the assay mixture, washed 5 times with PBS (0° C) to remove unbound [^{3}H] Con A, and then fixed in 2.0% paraformaldehyde-2.5% glutaraldehyde in 0.1 M phosphate buffer (pH 7.2) at 0° C for 30 min. Embryos were then washed for at least 2 h in cold 0.1 M phosphate buffer (pH 7.3), postfixed in cold 1% osmium tetroxide in the same buffer for 30 min and washed for 10 min in cold buffer. They were then dehydrated in a graded series of cold alcohols, passed through propylene oxide, and embedded in araldite (Durcupan, Polysciences). Sections (0.5 μm in thickness) were cut on a Sorvall MT-2 ultramicrotome, placed on glass microscopic slides and prepared for autoradiography by coating with NTB-2 nuclear track emulsion (Eastman Kodak; diluted 5:3 with H_2O). Slides were exposed for 11 days at 4° C, processed in Kodak D-19 developer and Ektaflo fixer, dried, covered with glass coverslips and sections of embryos were photographed with a Zeiss Photomicroscope III.

Photomicrographs (final magnification 1120x) were randomized and analyzed to determine the number of reduced silver grains distributed along the outer surface of three areas of trophectoderm. The polar (embryonic), proximal mural, and distal mural (abembryonic) trophectoderm, as defined by Copp ('78), are regions which differ in their position relative to the inner cell mass. Five centimeter segments were measured along the outer membrane surface of each area of trophectoderm on the micrographs using a chart meter (Tacro Inc., Germany) and the grains within that segment were counted. Occasionally the distribution of grains was uneven for a region of

trophectoderm on a particular section, so usually the grains in two segments were counted for each region of trophectoderm and the values averaged. Counts were made on 2-8 sections per blastocyst and the values combined to determine the average number of grains per segment of membrane for each of the subdivisions of trophectoderm on a blastocyst. Two to six embryos from each of the 6 assays in a treatment group were analyzed in this manner.

In addition, the number of embryos with grains along the inner wall of the blastocoel was determined. When grains were present in the blastocoel of an embryo, sections of that embryo were subjectively graded to determine the relative number of grains on the interior versus the number on the outer surface.

Student's t test (Snedecor and Cochran, '67) was used to determine if there was a significant effect of the length of incubation on the binding of [^{3}H] Con A to the three regions of trophectoderm and to compare the effect of hormone treatment on binding to each region of trophectoderm. The data within treatment groups were evaluated by a one-way analysis of variance followed by Duncan's multiple range test (Steel and Torrie,'60) to compare differences. Chi square analysis (Snedecor and Cochran, '67) was used to determine the effect of treatment on the frequency of blastocysts with Con A inside the blastocoel.

RESULTS

The length of incubation (15 or 60 min) had no significant effect on the number of grains along any area of trophectoderm for either treatment and the data were combined (Table 1). Binding of

[^{3}H] Con A to delayed implanting embryos was uniform with an average of 20-22 grains per segment of either polar, proximal mural, or distal mural trophectoderm (Table 1, Fig. 2). The pattern of grains on the surface of implanting blastocysts was different. The number of grains per segment decreased from 19.2 on the polar, to 14.7 along the proximal mural and 8.8 along the distal mural trophectoderm (Table 1, Fig. 3). The amount of [^{3}H] Con A bound to the polar trophectoderm of implanting embryos was not significantly different from that on delayed implanting embryos ($P > 0.1$). However, the binding to proximal mural and distal mural trophectoderm were significantly less on implanting embryos ($P < 0.001$). Incubations with α-methyl mannoside resulted in a decrease (greater than 90%) in the density of grains along the surface of either implanting or delayed implanting blastocysts (Fig 4).

In addition, it was found that although 43% of the delayed implanting embryos had [^{3}H] Con A in the blastocoel, up to 90% of the implanting blastocysts had grains along the inner wall. These ratios were statistically different ($P < 0.01$) indicating that after delayed implanting blastocysts are activated to implant, there is an increase in the number that will allow Con A into the blastocoel following their recovery from the uterus. When embryos with grains in the blastocoel were studied to determine the relative number of grains inside the blastocoel versus the number outside, it was found that 50-75% of the grains were along the inner, and only 25-50% was on the outer trophectoderm surface. This observation was true for both implanting and delayed implanting embryos.

DISCUSSION

The results of these experiments demonstrate that there is a change in the pattern of binding of [^{3}H] Con A to the surface of specific regions of trophectoderm as mouse embryos prepare to attach to the uterus. Thus while the surface of embryos recovered from delayed implanting animals has a uniform distribution of mannose-like sugars available for lectin binding, the density along the mural trophectoderm surface of implanting blastocysts is reduced.

Recently it was reported that the amount of [^{3}H] Con A that binds to blastocysts increases as the embryo prepares to implant, and it was suggested that this change was due to an increase in the number of Con A binding sites on the embryo surface (Wu and Chang, '78). In those experiments the amount of [^{3}H] Con A bound was determined by scintillation counting techniques. Using similar assay conditions, the data shown in Figure 1 were obtained. These results confirm that the amount of Con A that will bind to delayed implanting embryos increases nearly 3-fold after they have been activated to implant with estradiol-17β. However, when similar embryos were analyzed with autoradiography, it was found that some of the delayed implanting (43%) and most of the implanting (90%) embryos had more Con A (50-75%) bound along the inner blastocoel wall than was bound on the outer surface. This demonstrates that the capacity of the outer surface for binding [^{3}H] Con A cannot be accurately estimated by scintillation counting techniques because the lectin bound on the outer embryo surface cannot be distinguished from that which had access to the blastocoel and is bound to its inner wall. It appears

that the large increase in amount of [^{3}H] Con A that will bind to mouse blastocysts preparing to implant is not due to an increase in the number of binding sites on the embryo surface but is more likely related to an increase in the number of embryos that will allow Con A into the blastocoel.

The route of access of Con A into the blastocoel is not known. It is unlikely that the lectin is actively transported across trophoblast cells at the 0-4^o C temperatures maintained during the experiment because endocytosis is negligible at these temperatures (Noonan and Burger, '73). In addition, blastocysts recovered from the uteri of pregnant mice on day 4 or early day 5 of pregnancy have tight junctions of the zonula occludens type between trophoblast cells (Ducibella et al., '75; Magnuson et al., '77). These junctions effectively prevent the diffusion of lanthanum tracer (Ducibella et al., '75; Magnuson et al., '78) into the blastocoel, and the access of Con A-peroxidase (Konsinski et al., '77) and anti-mouse anti-serum to cells of the inner cell mass (McLaren and Smith, '77; Magnuson et al., '78). Thus it seems unlikely that molecular Con A could pass through the intercellular spaces of the trophectoderm and into the blastocoel of normal, intact blastocysts. However, it has been reported that the Con A-peroxidase staining procedure can result in a heavy staining of the basal lamina beneath the trophectoderm of some normal day 5 and delayed implanting embryos. This access of the stain complex into the blastocoel was associated with damaged trophectoderm cells (Enders and Schlafke, '74). It is possible that some delayed implanting and most of the implanting blastocysts in

the present experiments were damaged when flushed from the uterus, making them leaky, and allowing Con A to diffuse into the blastocoel. However, if this is the case, the damage to most implanting blastocysts appears to be reversible because if they are cultured in vitro for 2 h (37° C in 5% CO_2 in air) prior to incubation in [^{3}H] Con A there is a decrease in the number of embryos (from 90% to about 20%) which allow Con A into the blastocoel (data not shown).

The observation that there was a lower density of [^{3}H] Con A binding to the distal mural trophectoderm of implanting blastocysts than to the same region on delayed implanting blastocysts is in agreement with a reported decrease in binding of Con A-latex spheres to the abembryonic end of delayed implanting blastocysts that are activated to implant (Nilsson, '79). However, Naeslund and Nilsson ('79) also observed that the density of binding of Con A-spheres to the embryonic pole was lower than that at the abembryonic pole of both delayed implanting and implanting blastocysts. That observation is in contrast with the present finding that the density of binding of molecular [^{3}H] Con A on the polar trophectoderm is equivalent to, or greater than, that bound to the mural trophectoderm of delayed implanting and implanting blastocysts, respectively. A possible explanation for this discrepancy is that the binding of Con A-coated spheres to the polar trophectoderm may depend on factors other than the number of mannose-like sugars on the trophectoderm surface (i.e., the mobility of glycoproteins in the trophectoderm membrane, or membrane rigidity or deformability, Nicholson, '74).

The decrease in the availability of mannose-like sugars on the

embryo surface at the time of implantation could be due to a reduction in the actual number of sugars present per unit surface area; either as a result of an increase in embryo surface area (Carollo and Weitlauf, '79) while maintaining the same number of mannose-like sugars, the removal of surface glycoproteins by enzymatic factors in the uterine fluid (Pinsker et al., '74; Hoversland and Weitlauf, '78), or because carbohydrate-containing components of the blastocyst surface are left adhered to the uterine attachment site when the embryos are flushed from the uterine lumen. Alternatively, the decrease in availability of sugars could be due to masking of mannose-containing components on the trophectoderm surface either by uterine fluid factors or detached uterine surface components. The present experiments cannot distinguish between these possibilities.

The results presented here demonstrate that there is a modification in specific carbohydrates on the surface of delayed implanting blastocysts that are activated to implant. This change parallels those in the surface charge (Nilsson et al., '73, '75; Jenkinson and Searle, '77) and antigen expression (Hakansson, '73; Hakansson and Sunqvist, '75; Hakansson et al., '75; Searle et al., '76) of blastocysts at implantation. These findings are compatable with the possibility that modifications of the blastocyst surface coat are important for blastocyst-uterine recognition and adhesion. However, before this hypothesis can be accepted it will be necessary to demonstrate the appearance at implantation of specific components in the blastocyst membrane which, if modified, result in blockade of embryo attachment.

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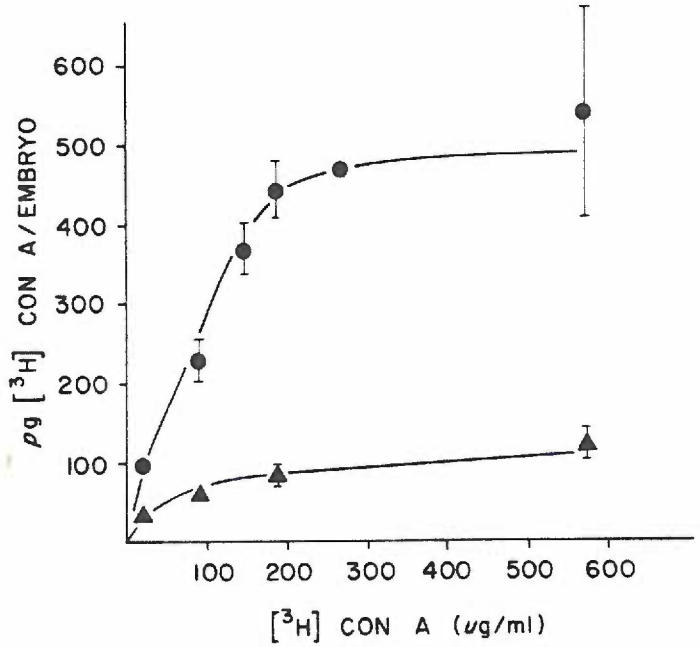
Fig. 1. Specific binding of [^{3}H] Con A to delayed implanting (▲) and implanting (●) mouse blastocysts as a function of Con A concentration. Embryos were incubated in the [^{3}H] Con A for 60 min, either with or without 0.1 M α-methyl mannoside. Specifically bound Con A is the difference in amount bound in the absence or presence of α-methyl mannoside. Values are mean ± SEM (2-8 assays/mean).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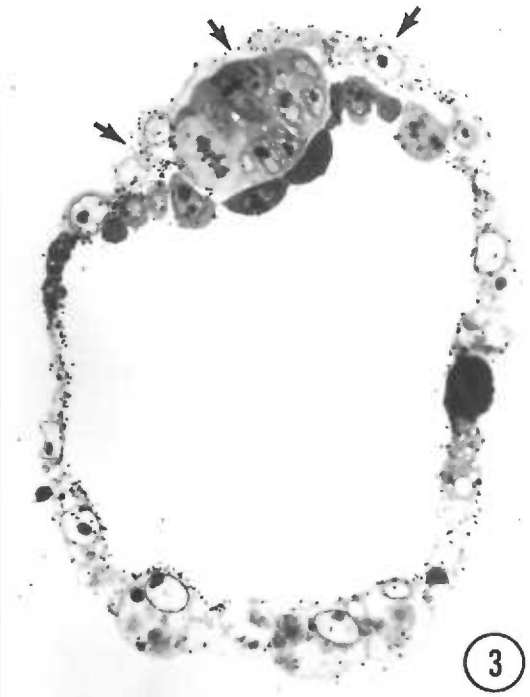

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TABLE 1. Distribution of autoradiographic grains on the surface of delayed implanting and implanting blastocysts after incubation in [^{3}H] Con A.

Treatment group*	Number of grains per segment of trophectoderm		
	Polar	Proximal mural	Distal mural
Delayed implanting	20.6 ± 1.6 (21)^{ab}**	22.9 ± 1.4 (23)^{a}	22.1 ± 1.3 (21)^{a}
Implanting	19.2 ± 1.2 (17)^{b}	14.7 ± 1.2 (18)^{c}	8.8 ± 0.7 (17)^{d}

*See text for details.

**Mean ± SEM (number of blastocysts) Superscripts with a letter in common designate means that are not significantly different ($P>0.05$); conversely the lack of a common letter in the superscript indicates means that are statistically different ($P<0.01$).

IV. APPENDIX A

[^{3}H] Concanavalin A

Each batch of [^{3}H] Concanavalin A (Con A, >30 Ci/ mmol, New England Nuclear Corp.) was analyzed to verify its purity, and determine the concentration of protein and amount of radioactivity. It was first subjected to electrophoresis on 15% polyacrylamide vertical slab gels using the buffer system described by McKenzie et al. ('72). Electrophoresis was terminated when the methyl green marker had reached the bottom of the gel. The gel was then immersed in 12.5% trichloroacetic acid for 60 min, stained with 0.1% coomassie blue (50% methanol, 10% glacial acetic acid) and destained with a solution of 10% ethanol, 7% acetic acid and water. Portions of the gel were prepared for scintillation autography (Bonner and Laskey, '74) and some were cut into 2 mm sections, solubilized in Protosol (New England Nuclear) and radioactivity estimated using a Beckman LS 230 scintillation counter.

The [^{3}H] Con A was found to co-migrate with native Con A (Sigma) showing two bands of protein; a heavily stained band running between chymotrypsinogen (m.w. 25,000) and bovine serum albumin (BSA, m.w. about 65,000), and a lightly stained band running behind BSA. It was assumed that these bands represented dimer and tetramer Con A of about 53,000 and 106,000 m.w. respectively (McKenzie et al., '72). Scintillation autography (fluorography) and counting of radioactivity of gel strips demonstrated that all [^{3}H]-label was associated with the two observed bands of protein and was taken as evidence that the [^{3}H] Con A was pure.

The protein concentration was determined by absorbance spectrophotometry at 280 nm with a Beckman model 35 spectrophotometer using the

molar extinction coefficient for Con A (13.7, Yarif et al., 1968). Samples of each lot were placed on nitrocellulose filters (Millipore) and the radioactivity determined by liquid scintillation counting. Specific activity was expressed as CPM per μg [^{3}H] Con A; it varied between 7.94 - 14.90 X 10^4 CPM/μg protein depending on the lot used, and these values were used in all the appropriate calculations in this thesis.

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V. APPENDIX B

INTRODUCTION

It has been reported that there is an increase (i.e., 2X) in the amount of [^{3}H] Con A that will bind to delayed implanting mouse blastocysts that have been activated to implant. Further, it was suggested that this change represented an increase in the number of Con A binding sites on the embryo surface (Wu and Chang, '78). If this is true, then such an increase could be due to either 1) an increase in the density of mannose-like sugars on the embryo surface (i.e., number of sugars per unit surface area), or 2) a change in the total membrane area with an accompanying increase in the number of mannose-containing components on the embryo surface.

The present experiments were initiated to compare the binding of [^{3}H] Con A with changes in embryo surface area as delayed implanting mouse blastocysts were activated to implant.

MATERIALS AND METHODS

Mice and Collection of Embryos

Sexually mature, virgin female white Swiss mice were induced to ovulate and mate as described in the accompanying manuscript.

Animals in group 1 received progesterone (2 mg per day) on days 7- 10; group 2 received progesterone on days 7 and 8, and progesterone in combination with estradiol-17β (25 ng per day) on days 9 and 10; group 3 received sesame seed oil (i.e., vehicle only) on days 7-10; and animals in group 4 received sesame seed oil on days 7 and 8, and estradiol-17β (25 ng per day) on days 9 and 10. All hormones were suspended in 0.1 ml sesame seed oil and injected s.c. at 0900 h.

Embryos were collected as described in the accompanying manuscript.

Lectin Binding Assay

Embryos were assayed in [^{3}H] Con A as described in the accompaning manuscript.

All assays was done on ice (0° C), and transfer of embryos to and from the tubes were accomplished in less than one min. The volume needed to transfer the embryos was less than 0.5 µl and thus resulted in a dilution of the Con A concentration of less than 2.5%. After recovery from the assay mixture the blastocysts were transferred through a series of 5 washes of PBS (0° C) to remove unbound [^{3}H] Con A.

Embryo Surface Area

While embryos were in wash number 5, the long and short diameters of each blastocyst were measured with the aid of a compound microscope and an ocular micrometer. The long (a) and short (b) radii of each individual embryo were used to calculate the surface area using the formula:

$$\text{Surface Area of a Spherical Ellipsoid} = 2\pi b\left(b + \frac{a^2}{(a^2 - b^2)^{\frac{1}{2}}} \arcsin\left(\frac{a^2 - b^2}{a^2}\right)^{\frac{1}{2}}\right).$$

The reliability of these estimates is dependent upon the embryo having a circular cross section and a smooth surface architecture. Therefore, all embryos that were obviously flattened or broken were discarded from analysis. Blastocysts collected 30 h after the administration of progesterone plus estradiol-17β (i.e., group 2) were frequently flattened in appearance. Only 10% or less of the embryos collected at other times and from other treatments appeared to be flattened. Bergstrom ('71; Bergstrom and Nilsson, '73) observed that the architecture of the trophectoderm surface during delayed implantation is relatively smooth, with sparse microvilli and crater-

like imprints. However, 16 h after activation with estradiol-17β the blastocyst surface has numerous microvilli, but by 24 h the surface is again rather smooth with few microvilli (Bergstrom and Nilsson, '70). These observations suggest that the estimates of surface area are reasonably accurate for delayed implanting blastocysts and blastocysts collected 25 h after activation. The estimates for embryos collected after 15, 20 or 30 h of activation are probably conservative.

Scintillation Counting

Embryos were placed on a 3 x 15 mm piece of nitrocellulose filter (Millipore). The filters were dried for 12 h at 60° C, placed in glass scintillation vials with 5 ml of scintillation cocktail (4% PPO, 0.04% POPOP, in toluene), and counted to determine the amount of radioactivity bound. A 2 µl aliquot of the 5th wash was placed on a similar filter and handled as above to determine the amount of unbound radioactivity that was transferred to the filter with the embryos from the last wash. This "background" activity (less than twice machine background) was subtracted from the experimental values and the total amount of isotope bound to blastocysts on each filter was converted to µg of [^{3}H] Con A bound (i.e.,

$$\frac{\text{CPM } [^3\text{H}] \text{ Con A bound}}{\text{specific activity of } [^3\text{H}] \text{ Con A}}).$$

Statistics

The data were evaluated by a two-way analysis of variance (Snedecor and Cochran, '67). Duncan's multiple range test (Steel and Torrie, '60) was used to compare differences between treatments at each time point.

RESULTS

Binding of [^{3}H] Con A to Implanting and Delayed Implanting Embryos

Length of incubation - Figure 1 shows the amount of [^{3}H] Con A that was bound to delayed implanting and implanting blastocysts as a function of length of incubation at 0° C. With delayed implanting embryos (Fig. 1a) there was a rapid binding in the first 20 min, with no further increase at up to 4 h ($P > 0.05$). Although the amount of [^{3}H] Con A that was bound to implanting blastocysts (Fig. 1b) also increased rapidly in the first 15 min, it continued to increase slowly with longer incubations; the level of binding was not significantly increased by 60 min ($P > 0.05$) but did reach statistical significance by 120 min ($P < 0.01$). To obtain the binding data in this study, 60 min was selected as the length of incubation because there was little change in binding with changes in incubation time.

Binding specificity - When binding assays were done in the presence of 0.1 M α-methyl mannoside, 85-90% of the total binding to either delayed implanting or implanting embryos was inhibited (see Figs. 1a and 1b). Also, addition of 0.1 M α-methyl mannoside to embryos previously exposed to [^{3}H] Con A caused the release of the bound radioactivity (68% in 120 min). In contrast, there was no inhibition of binding when embryos were placed in vitro with [^{3}H] Con A that had been preincubated with 0.1 M D-galactose (i.e., a non-specific hapten sugar of Con A binding). These observations were taken as evidence that the Con A was specifically bound to mannose-like sugars on the blastocyst surface. Control incubations were done throughout these studies and all binding data are reported as specific binding (i.e., the difference between the amount of [^{3}H] Con A bound in the absence and presence of 0.1 M α-methyl mannoside).

Effect of concentration of [^{3}H] Con A - The amount of [^{3}H] Con A that bound to delayed implanting and implanting blastocysts as a function of the concentration of Con A is shown in fig. 2. With either delayed implanting or implanting blastocysts, the amount of lectin bound increased with concentrations up to about 200 μg of [^{3}H] Con A per ml, with no significant increase in binding with higher concentrations tested. Therefore, concentrations of 180-200 μg [^{3}H] Con A per ml were used to obtain the binding data in the following treatment groups.

Effect of Hormone Treatment on Binding to Delayed implanting Embryos

The effect of estradiol-17β and progesterone on binding of [^{3}H] Con A per embryo is summarized in Table 1. With progesterone alone (i.e., group 1), the amount of Con A bound specifically to the blastocyst was low and remained low for up to 78 h of treatment (i.e., day 7 at 0900 h to day 10 at 1500 h). In contrast, with blastocysts from animals receiving progesterone in combination with estradiol-17β (i.e., a treatment that leads to implantation, group 2), the amount bound per embryo increased with time after treatment. The change was small after the first 15 h of estradiol (i.e., day 9 at 0900 h to day 9 at 2400 h), but binding increased more than 3-fold during the second 15 h, with 5 times the amount of Con A bound to embryos recovered at 30 h (i.e., day 10 at 1500 h) than at 0 h of estradiol (i.e., day 9 at 0900 h).

With blastocysts from animals given oil alone (i.e., group 3) or estradiol-17β alone (i.e., group 4), the amount of Con A bound per embryo was low and did not change significantly with time.

Effect of Hormone Treatment on Surface Area of Delayed Implanting Embryos

Changes in the estimated surface area of embryos from the various treatment groups are summarized in Table 1. There was little or no change with time in the total surface area of blastocysts from animals treated with either progesterone alone, oil, or estradiol-17β alone. However, following administration of progesterone in combination with estradiol-17β (i.e., group 2), the area increased with a temporal pattern similar to the changes in [^{3}H] Con A binding. Thus, embryos recovered 15 h after estradiol had 40% more (i.e., day 9 at 0900 h) and at 25 h 100% more surface area (i.e., day 10 at 1000 h) than did delayed implanting embryos (i.e., group 1, day 10 at 1500 h).

DISCUSSION

The results of these experiments demonstrate that the amount of [^{3}H] Con A that binds to delayed implanting embryos is increased by 300% following treatment with progesterone in combination with estradiol-17β. In contrast, the blastocyst surface area increases by only 100%. Apparently these changes occur only when the hormonal status of the embryo donor is sufficient to support blastocyst adhesion and implantation as they did not occur when animals were treated with either progesterone alone, oil, or estradiol-17β alone. The initial interpretation of this data was that the increase in binding of [^{3}H] Con A at implantation represents an increase both in the total membrane area and in the number of mannose-like sugars per unit of embryo surface area. However, subsequent experiments initiated to determine the distribution of [^{3}H] Con A bound to delayed implanting and implanting blastocysts (see manuscript) demonstrated:

1) a decrease in the density of mannose-like sugars along the mural trophectoderm surface of delayed implanting blastocysts that are activated to implant; and

2) that more Con A was bound on the juxtacoelic surface of the trophectoderm than on the outer surface of 43% of the delayed implanting and up to 90% of implanting blastocysts.

In view of these findings, it appears that the temporal change in binding of [^{3}H] Con A to blastocysts at implantation is not due to an increase in the density of mannose sugars on the embryo surface. Rather, this change represents an increase in the number of embryos with Con A in the blastocoel.

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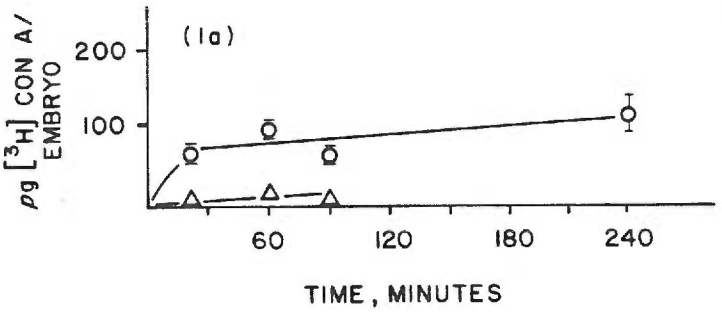
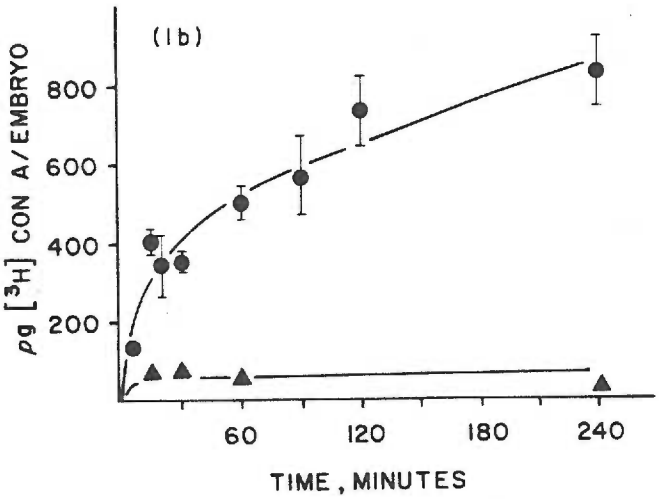

(1a)
200
100
ρg [3H] CON A/ EMBRYO
60
120
180
240
TIME, MINUTES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800
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ρg [3H] CON A/EMBRYO
60
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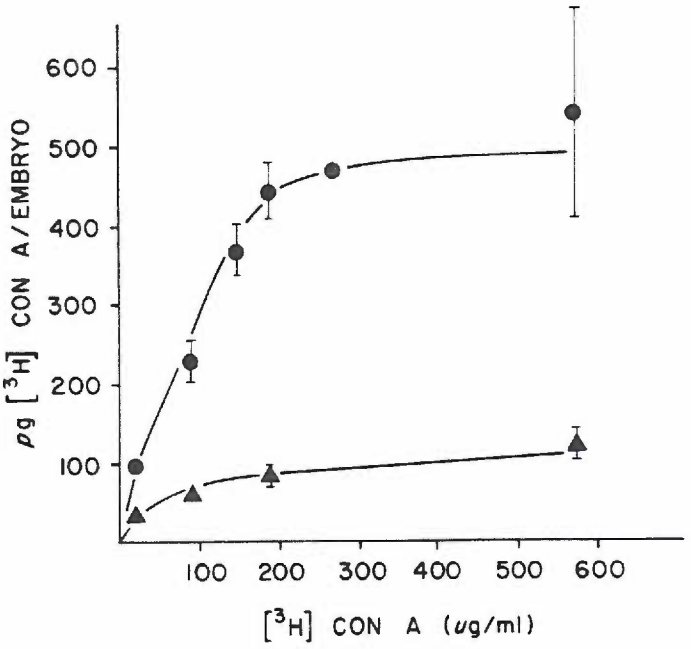
240
TIME, MINUTES

1


600
500
400
300
200
100
pg [3H] CON A/EMBRYO
100
200
300
400
500
600
[3H] CON A (ug/ml)

(2)

TABLE 1. Effect of Estradiol-17β and Progesterone on the binding of [^{3}H] Con A and surface area of Mouse blastocysts.

Hormone Treatment group*	Progesterone 2.0 mg/day	Estradiol-17β 25.0 ng/day	Embryos Collected Day	Embryos Collected hour	[^{3}H] Con A bound pg/embryo	Surface Area $\mu m^2 \times 10^4$
Group 1	Day 7	--	7	0900	36.9 ± 4.1^{a}** (5)	3.28 ± 0.16^{e}*** (106)
	Days 7-10	--	10	1500	64.8 ± 7.5^{a} (6)	3.67 ± 0.15^{e} (92)
Group 2	Days 7-9	Day 9	9	0900	66.6 ± 9.7^{a} (5)	3.66 ± 0.11^{e} (109)
	" "	"	9	2400	114.1 ± 15.0^{b} (5)	4.92 ± 0.22^{f} (65)
	" "	"	10	0500	184.5 ± 15.4^{c} (5)	5.14 ± 0.19^{f} (70)
	Days 7-10	Days 9-10	10	1000	371.7 ± 27.9^{d} (5)	7.45 ± 0.18^{g} (51)
	" "	" "	10	1500	394.2 ± 20.8^{d} (5)	6.83 ± 0.22^{g} (75)
Group 3	Day 7 (oil only)	--	7	0900	37.9 ± 7.4^{a} (5)	3.95 ± 0.31^{e} (93)
	Days 7-10 (oil only)	--	10	1500	32.7 ± 4.2^{a} (5)	3.45 ± 0.10^{e} (105)
Group 4	Days 7-8 (oil only)	Day 9	9	0900	48.1 ± 9.1^{a} (5)	3.96 ± 0.16^{e} (89)
	" "	Days 9-10	10	1500	60.5 ± 14.5^{a} (6)	4.13 ± 0.26^{e} (78)

*Hormones injected s.c. in 0.1 ml sesame seed oil at 0900 hr.

**Mean ± SEM (number of assays).

***Mean ± SEM (number of blastocysts).

Duncan's multiple range test was used to detect significant differences between treatments at each collection time: superscripts with a letter in common designate means within a column that are not statistically different ($P>0.05$); conversely, the lack of a common letter in the superscript indicates means in a column that are statistically different ($P<0.05$).

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