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Localization of Glycogen in the Opacity Characterizing Decidualization in the Cleared Hamster Uterus.* (28336)

G. A. FOSTER, JR., MARGARET WARD ORSINI AND F. M. STRONG
(Introduced by H. W. Mossman)
Departments of Anatomy and Biochemistry, University of Wisconsin, Madison

Uteri of pregnant hamsters, and of pseudopregnant hamsters in which decidualization has been induced, reveal areas of opacity when viewed by oblique light after clearing by the benzyl-benzoate process. These opacities have been shown to be associated with decidua(1-3), and the presence of glycogen in them has been suggested(2).

During gestation, the first of these areas which appear opaque after clearing develop coincidentally with decidua and mark implantation or decidualization regions prior to visible external swelling. They increase in mass, distending the uterus as localized swellings, late on the sixth day of gestation. They reach a peak in terms of mass per conceptual swelling on the seventh day of development, and begin to regress from the antimesometrial area on the eighth day, *i.e.*, at 7 days and 12 hours.

This paper reports results of chemical analyses of these tissues in comparison to the remainder of the uterus.

Materials and methods. At the relative peak of development of the areas in question (sixth and seventh days), it is possible to make a transverse slit in the uterus at the swollen gestational sites and, with fine forceps, peal from the endometrium the denser mass of the embryo and its surrounding decidua. Examination of material separated by the above technique and then cleared, showed that the peeled out region comprised almost all the opaque material (Fig. 1 and 2). Such "peeled out regions" subsequently are referred to as "opacities," whether or not they have been subjected to the clearing process and thus actually appear opaque. Samples of the remaining portions of the uterus, from which all mesometrium was removed, were analyzed for comparison.

One series of determinations was carried out on material which had been cleared prior to the separation of the opaque regions. This cleared material was pooled from the tracts of 2 animals killed at 5 days and 23 hours and 2 animals killed at 6 days and 12 hours developmental age. Subsequent determinations were made on material from animals freshly killed by exsanguination. Opacities and uteri from these animals were frozen with dry ice immediately after dissection but the tissues were not cleared except for one swelling from each animal which was used as a control (*i.e.*, cleared but not analyzed). Two animals, both at 6 days and 12 hours, were used for study; one showed 12 and the other 13 conceptual swellings.

Since deciduomata also appear opaque, they were produced by air injection (0.05 ml) into the uteri of 2 hamsters made pseudopregnant by mating with vasectomized males. This air injection was done at 3 days 20-21 hours post ovulation and the animals were autopsied at 6 days 21 hours post ovulation. One deciduomata from each hamster was retained as a control (*i.e.*, cleared but

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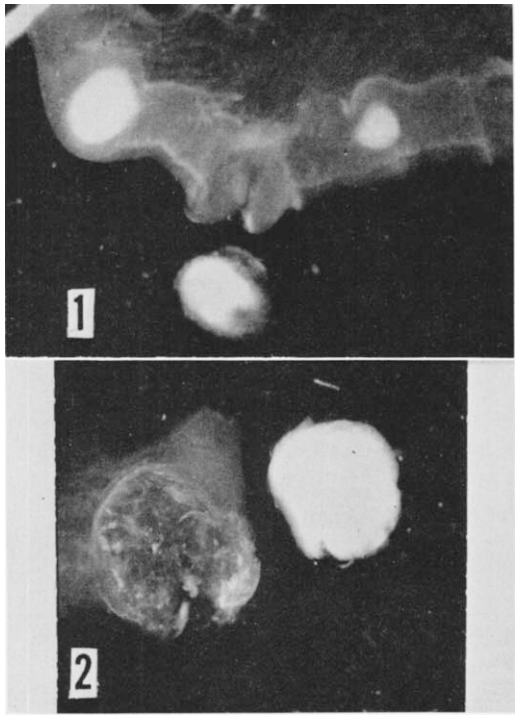

FIG. 1. Portion of cleared hamster uterus at 5 days and 16 hr developmental age. Three opacities are shown. The second was dissected out from the endometrium prior to clearing, and is shown beneath the cleared tract. × 7.

FIG. 2. Portion of cleared hamster uterus at 6 days and 12 hr developmental age; the opacity (at the right) was dissected out after clearing. × 7.

not analyzed); the rest were separated from the uteri, frozen with dry ice, and analyzed without having been cleared.

All tissues that were not cleared were lyophilized before analysis. Lyophilized tissues were analyzed directly but the cleared tissues were first washed with successive portions of benzene, absolute ethanol, 95% ethanol, and acetone and then dried in a vacuum desiccator. The opacities or uteri or fractions thereof were used as prepared above and no attempt at making a pooled homogeneous sample, as by grinding the tissue, was made.

Analytical methods. Glycogen was extracted from the tissue with hot potassium hydroxide and precipitated with ethanol according to the procedure of Seifter *et al.*(4). The precipitate was dissolved in sufficient water to give a concentration of about 0.5 mg/ml of solution, and glycogen determinations were made on 2 ml portions of this solution by the anthrone method according to Carroll *et al.*(5). Glucose was used as the standard.

Nitrogen determinations were made by a semi-micro Kjeldahl method following the procedure of the Association of Official Agricultural Chemists(6). Samples of approximately 3 mg from individual opacities or uteri were analyzed.

Phospholipid determinations were made on an ether extract of the tissues. Several opacities or a sample of uterine tissue were extracted overnight with ether in a Soxhlet apparatus. Total phosphorus content of the extract was determined(7) and converted to percent phosphatidyl choline (assumed mol. wt. 800) by multiplying by the factor 25.8.

Calcium determinations† were made on the ash of tissue samples weighing 50 to 100 mg (approximately 10 to 15 opacities or a large portion of a uterus). Ashing was carried out by charring the tissue on a hot plate and under heating lamps, then heating in a muffle furnace at 650°C for approximately 18 hours. The ash was dissolved in 6 N hydrochloric acid, the solution diluted 10-fold with distilled water and titrated with ethylenediamine-tetraacetic acid (EDTA) according to the method of Robinson and Rathbun as modified by Bunce(8).

Glycogen samples from the various tissues were tested for the presence of pentoses with Bial's reagent (1.5% orcinol in concentrated hydrochloric acid). Other samples were hydrolyzed by boiling in 3 N hydrochloric acid and the solutions were examined by paper chromatography for the possible presence of fructose or other sugars. The solvent system, butanol:ethanol:water (10:4:4 (v/v)) was used to develop the chromatograms and the sugar spots were localized by spraying with aniline phthalate.

Results and discussion. The glycogen concentrations found in the present study were surprisingly high. The absolute values (Table I) differ considerably for opacities prepared in different ways, and for different individual samples. However, the ratio of the

† Dr. Mark L. Morris, Jr. kindly performed the calcium determinations.

TABLE I. Results of Chemical Analysis of Hamster Tissue.

Tissue*	Developmental age of tissue	Glycogen (% dry wt)	Protein (% dry wt)	Phospholipid (% dry wt)	Calcium (mg/100 g tissue)
Cleared opacities dissected from pregnant animals	5d, 23 hr & 6d, 12 "	20.1, 22.6, 19.8 21.6, 19.9 Avg 20.8	66, 68, 68 66, 60, 60 Avg 65	†	185
Cleared uterine tissue from pregnant animals	5d, 23 " & 6d, 12 "	.95, .91, .87, .79 Avg .85	84, 83, 87 Avg 85	†	145
Lyophilized decidua (opacities) from pregnant animals	6d, 12 "	13.8, 14.0, 14.9 15.7, 16.7, 14.1 Avg 14.9	64, 64 Avg 64	1.55	250
Lyophilized uterine tissue from pregnant animals	6d, 12 "	.46, .45, .54, .45 Avg .48	79, 81, 83 82, 74, 79 Avg 80	1.44	256
Lyophilized deciduomata (opacities) from pseudopregnant animals	6d, 20 "	15.7, 15.0, 14.2 13.3 Avg 14.6	62, 63, 62 62, 67, 67 66, 56, 59 Avg 63	1.88	253
Lyophilized uterine tissue from pseudopregnant animals	6d, 20 "	.41, .61, .65, .27 Avg .49	66, 81, 73 77, 78 Avg 75	2.07	255

* See text for complete description and amounts used.

† Removed by clearing process.

average glycogen concentration in the conceptual areas to that in the intergestational regions of the uterus is relatively constant, being 24.5 for the cleared and 31.0 for the lyophilized opacities from pregnant animals, and 29.8 for the lyophilized opacities from the pseudopregnant ones.

The possibility that some other carbohydrate might actually be present and be measured as glycogen in the analytical procedure used was investigated to some extent. Thus, Bial's test for pentoses was performed on a number of samples and was in all cases negative. An acid hydrolysate of a portion of the crude extracted glycogen was subjected to the resorcinol test for ketoses and was negative. Likewise, paper chromatography of the hydrolysate showed a very distinct spot in the expected position for glucose, but no other sugars could be detected on the papers. It consequently seems fairly certain that the polysaccharide measured was either glycogen or some other very closely related glucosan with similar solubility properties.

Preliminary work with the electron microscope(9) revealed in the opacity associated with deciduomata, and that of early pregnancy at stages comparable to those discussed here, extensive cytoplasmic aggregates of particulate "glycogen," so judged by the presence of 350 Å particles of irregular shape which showed a preferential affinity for lead stains(10).

No other striking differences were revealed by the other analyses carried out, namely for protein, phospholipid or calcium. The main variation encountered was a lowered protein content in the opacities, as would be expected from the fact that much of the dry weight in these samples consisted of carbohydrate material.

The similarity in composition between opacities due to normal pregnancy and those due to decidualization in pseudopregnancy indicates the close resemblance of these two tissues at this stage of development. It is doubtful, however, if this parallel would continue far beyond this stage (seventh day of pregnancy and seventh day of post ovulation life of the pseudopregnant ovum). On the eighth day, the decidual pattern of pregnancy changes.

The embryo at the seventh day of gestation is at the stage of the first appearance of mesoderm, and constitutes only a small portion of the total conceptual swelling. On

the seventh day post-ovulation the deciduomata may be smaller, equal to or larger than the normal conceptual swelling. They may increase in mass on the following day but necrosis is usually evident by the ninth day. Relatively little is known as yet of the life span of such deciduomata in the hamster.

The function of the glycogen may be to provide a rapidly available source of energy for the developing embryo and/or accessory structures, but no evidence on this point was obtained in the present study.

Whether the appearance of glycogen is due to a maternal or fetal stimulus is not certain. Since it appears in decidualization of pseudopregnancy when no fetal tissue is present a maternal origin seems most likely. Its accumulation may very well be triggered by the sequence of events associated with early gestation. These phenomena are common in pregnancy and pseudopregnancy, when the latter is accompanied by a stimulus sufficient to produce decidualization (*e.g.*, air injection), since simple pseudopregnancy without this additional stimulus results in no comparable opacity formation.

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EDTA, Antiserum, and Restim as Factors in Infection of Mice with *S. typhimurium.* (28337)

J. P. RANSOM
New England Institute for Medical Research, Ridgefield, Conn.

We presented evidence(1) that stimulation of the reticuloendothelial system (RES) by means of a yeast cell wall preparation (restim) did not result in enhanced resistance of mice to *Salmonella typhimurium* unless specific antibody were also present. The evidence suggested that some other activity of the RES, *e.g.*, intracellular digestion, must be involved in enhancement of resistance brought about by restim.

Elberg(2) reported that brucellae were made more susceptible to intracellular digestion by treatment with glycine. Repaske(3, 4) reported that Gram-negative bacilli which are normally insensitive to lysozyme were lysed at a rapid rate if they were pre-treated with versene (EDTA) in Tris buffer. In our hands, glycine has been ineffective in enhancing resistance of mice to *S. typhimurium* infection; however, EDTA has shown a significant effect when mice were pre-treated with restim. This report also shows that the blood of restim-treated mice has a higher content of lysozyme than that of normal mice.

Methods and materials. Salmonella typhimurium strain SR-11 was used as indicated in the previous report(1).

Mice of 20-25 g body weight were obtained from commercial sources and handled as previously reported.

Opsonization of bacterial cells. Rabbit anti-"O" serum against *Salmonella typhimurium,* having an "O" agglutination titer of 1:320, was heated at 56°C for 40 minutes to inactivate complement. To 9 ml of