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Concentration of Azoproteins in Yolk Sac Placenta of the Rat.* (24660)

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The striking role of the rabbit yolk sac placenta in transmission of homologous gamma globulin from the maternal system into the embryo and the apparent non-permeability of this membrane to heterologous protein has been described by Brambell(1). Few attempts have been made to study the permeability of either the chorio-allantoic or the inverted yolk sac placentae of the rat to protein molecules although both homologous and heterologous protein compounds have been used extensively in various studies of maternal-fetal relationships in this animal. The present work is an attempt to study the fate of 2 different azoprotein molecules injected into the pregnant rat, specifically with respect to placental membranes at various stages of gestation.

Materials and methods. The azo dye, echt säure blau B, has been shown to be rapidly excreted in the bile and urine of animals injected with moderate amounts of this compound(2). It is not phagocytyzed by the reticulo-endothelial system and apparently does not combine with serum proteins *in vivo*. Echt säure blau B, an intensely blue azo dye, was diazotized and coupled to crystalline bovine serum albumin and rat serum albumin utilizing the technic described by Kruse and McMaster(2). Bovine serum albumin was obtained commercially and rat serum albumin was obtained by ammonium sulfate precipitation of pooled rat serum. After final dialysis and filtration, the bovine serum albumin-azo dye preparation (BSA-ESBB) contained 20 mg of protein and 1408 μg of dye/ml. The

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final rat serum albumin-azo dye preparation (RSA-ESBB) contained 30 mg of protein and 1270 μg of dye/ml. Four ml of the BSA-ESBB preparation and 5 ml of the RSA-ESBB preparation were injected intraperitoneally into young nulliparous Sherman rats weighing 250-280 g on days 7, 9, 11, 13, 15, 18, and 19 of pregnancy. Each animal received only one injection and was sacrificed 24 hours later at which time maternal cardiac blood was drawn. Specimens of maternal liver, spleen, mesenteric lymph node, and kidney were fixed in Carnoy's fluid, 10% formalin, and alcohol-formol-acetic acid. Placental membranes and fetuses were removed and fixed in an identical manner. Method of fixation was found not to affect the histological results, consequently tissues fixed in 10% formalin were sectioned at 10 μ, cleared in xylene, left unstained, and mounted with coverslips for histological localization of the colored azoprotein material. Some sections were stained with hemotoxylin and eosin for comparison. Control animals were divided into 2 groups. The first group received intraperitoneal injections containing 0.25% to 1.0% dye and 3.0% bovine serum albumin or rat serum albumin, mixed just prior to injection. For the second group of control animals. the azo dye was diazotized as in the first stage of the preparation of the azoprotein but tyrosine was substituted for the protein molecule. This solution was prepared to test the possibility of intermolecular coupling of dye molecules and to observe any effect that coupling might have on the histological results. Control animals received injections at corresponding stages of pregnancy, the maternal animals sacrificed at the same intervals, and the tissues treated in the same manner as the experimental animals. Maternal sera of the control and experimental groups were examined for gross blue coloration, precipitated with cold 10% trichloracetic acid (TCA) and centrifuged.

Results. In all control animals the maternal sera were free of any color at time of sacrifice. Some control animals, examined after only 5 hours exposure to the unbound dye-protein mixture, showed heavy dye concentration in the biliary tree of the liver, in the lumen of the small intestine and in the urinary bladder. No blue color could be seen in the maternal kidney, or within the fetuses or their placental membranes. In those control animals examined after 24 hours exposure to the control solutions, no evidence of any blue coloration could be found in maternal sera, maternal tissues or in embryonic tissues.

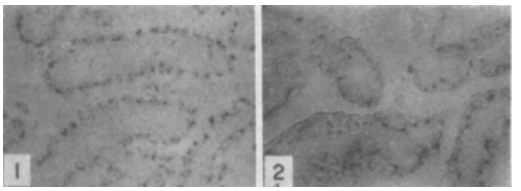

FIG. 1. Unstained cross-section of yolk sac placenta of a 19-day rat fetus, 24 hr after inj. of BSA-ESBB compound. Dark granules represent blue azoprotein material. 10 μ. 100×.

FIG. 2. Unstained cross-section of yolk sac placenta of a 19-day rat fetus, 24 hr after inj. of RSA-ESBB compound. Note the same general distribution of azoprotein material as in Fig. 1. 10 μ. 100×.

The maternal sera of the experimental animals receiving the BSA-ESBB preparation were quite blue at time of sacrifice and TCA precipitation yielded a definite blue precipitate with a clear supernatant. Histological examination of unstained sections revealed the blue azoprotein to be evenly distributed within the cells of the maternal renal tubules and in the mesenteric lymph nodes. Traces of the dye could also be found in maternal liver and spleen. Histological localization of the BSA-ESBB compound was apparent in all stages of the developing yolk sac placenta studied in this experimental group. As early as the eighth day of gestation the blue granules could be seen within the visceral layer of the yolk sac epithelium that lies next to the embryo proper. No azoprotein could be found in any other of the extra-embryonic tissues or in the embryo proper at this stage. At each succeeding stage studied, more obvious localization of the dye was apparent in the yolk sac endoderm and heavier concentrations appeared in the perinuclear cytoplasm of these cells (Fig. 1). At no stage of development was any of the BSA-ESBB compound found within the body of the embryo.

In those experimental animals injected with the RSA-ESBB preparation there was wide-

spread distribution of the blue color at time of sacrifice. This color was evenly distributed throughout the body, coloring especially the subcutaneous tissues and fat deposits. The maternal liver, kidney and lymph nodes were grossly quite blue. There was detectable color in the maternal serum at time of sacrifice, but the coloration was not as intense as with the BSA-ESBB series. There was much more marked deposition of the blue azoprotein within the sinusoids of the maternal liver and within the lymph nodes of this experimental series than in the BSA-ESBB series. The renal tubules also showed concentration of blue pigment but in this series there was a tendency for it to be concentrated within the proximal part of the tubules. A strikingly similar concentration and distribution of the RSA-ESBB and the BSA-ESBB compounds was found within the visceral layer of the yolk sac placenta at all stages of gestation (Fig. 2). No histological evidence of the labeled rat serum albumin could be found within the embryo proper at any stage of gestation studied.

Discussion. The findings presented here offer direct visual evidence that the yolk sac placenta of the rat concentrates and apparently tends to block the transfer of both the BSA-ESBB and the RSA-ESBB preparations into the embryonic system at all stages of gestation studied. We cannot be certain that in labeling the rat serum albumin it has retained its species identity. It appears to behave differently in the maternal system than the BSA-ESBB compound, in that it is diffusely spread throughout the system, coloring subcutaneous tissues, mesenteries, and fat deposits as well as appearing in the renal tubules and yolk sac endoderm.

The problem of permeability of the placenta of rodents and closely related forms to protein molecules has been examined by various technics. Brambell(1) has shown that the permeability of the rabbit blastocyst to maternal serum albumin apparently ceases after the ninth day of gestation. Winkler(3), using radio-iodinated homologous serum albumin in the near-term rabbit, found some evidence of a slow transfer of the homologous albumin to the fetus after 24 hours of maternal exposure to this compound. Dancis(4) has reported that the guinea pig fetus is capable of synthesizing all of its own serum proteins except gamma globulin. He also presents data suggesting that there is some placental transfer of maternal serum proteins to the guinea pig fetus as measured by *in vivo* and *in vitro* radio-labeling of maternal serum. However, the main difficulty in interpreting data using radio-labeled proteins in studies of placental permeability is that there may be a breakdown and resynthesis of the protein-label in one or more of the placental tissues.

The limiting factor in this technic of tracing the fate of colored azoproteins is one of color dilution, and the technic does not permit final conclusions as to completeness of the placental block since it may be that very small amounts of protein get across the placental membrane into the embryo. The strength of the bond between the azo dye and the protein molecule, and the rapid elimination of any unlinked dye makes this technic a reliable one within the limitations mentioned.

In view of the above data, it would seem advisable to review the experimental data in which placental transfer of heterologous or homologous protein molecules has appeared to be an important factor in making definite conclusions. Such situations might exist in the experimental data accumulated in use of protein hormones in the study of the fetal endocrine system and, also, in the recently described phenomenon of actively acquired tolerance of the fetal system to antigenic material.

Summary. Bovine serum albumin and rat serum albumin were coupled to an azo dye, echt säure blau B to form colored azoproteins. Female rats at various stages of gestation were exposed 24 hours to these compounds and to control solutions. Both azoprotein molecules could be found within the kidney tubules, lymph nodes, maternal serum, and in all stages of the developing yolk sac placenta. No evidence of the dye in either case could be found in the embryonic tissues proper or within any of the other extra-embryonic membranes at any stage of gesta-

tion studied. The significance of this apparent impermeability of the rat placenta to these azoproteins is discussed.

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Proposed Method for Determining Biological Value of Protein in Ruminants. (24661)

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Little attention has been directed to the biological value of protein for ruminant animals because of the ability of the rumen microflora to synthesize amino acids. With the advent of nutritional interest in non-protein nitrogen, and more recently the interest in chemical stimulation of growth in ruminants, the need for an adequate simplified measure of biological value of protein has become important. Protein digestion and nitrogen retention have been used as methods for determining protein utilization by ruminants because no other satisfactory methods have been available. The Thomas-Mitchell method(1) for biological value determination, which has been generally used as the criterion in monogastric animals, has not proven satisfactory for ruminants. Because of the unique physiological functions of the ruminant digestive tract, accurate measurements of metabolic and endogenous nitrogen, required by the Thomas-Mitchell method, are not obtainable. However, estimates of endogenous and metabolic nitrogen calculated by Harris and Mitchell(2) from body weight and feed consumption, respectively, have been used in biological value determinations in ruminants.

In this investigation, alteration of blood and urinary nitrogen constituents from lambs was studied to develop a simplified method of: (a) predicting the biological value of protein, and (b) determining magnitude of stimulation from anabolic drugs.

TABLE I. Constituents of Experimental Rations, %.

Constituents	CSM	CSM-urea	Urea
Molasses beat pulp	36.5	36.5	36.5
Cotton-gin waste	35	35	35
Barley		12.05	24.1
Cottonseed meal	27.5	13.75	
Urea		1.7	3.4
Salt	1	1	1

Method. Twenty-six lambs were fed 3 rations (Table I) which varied only in source of nitrogen: (a) cottonseed meal, (b) 50% cottonseed meal : 50% urea, and (c) urea.

One-half of the lambs fed each ration was subcutaneously implanted at the base of the ear with 12 mg diethylstilbestrol. Rations were fed *ad libitum* for 10 days, followed by a 10-day period in which rations (17% crude protein) were fed in accord to 2.3% body weight. Blood samples, plus 24-hour urine and fecal samples were obtained from all lambs for analyses of nitrogen constituents. Apparent nitrogen (N) digestion was determined by means of the chromic oxide ratio technic. Thereafter, lambs were fed their respective rations *ad libitum,* and 45-day weight gains were calculated.

Observations. Illinois workers(3) reported that rations containing 11% protein equivalent satisfied the N requirements for maintenance and growth of lambs. Diets that contained 12% protein equivalent derived from urea N resulted in similar biological values

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