The Teratogenicity of Congo Red in Rats.* (29528)

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Several of the disazo dyes have been tested for teratogenic activity in rats(1) and in the hen's egg(2). In each experiment it was concluded that Congo red had questionable teratogenic activity. A study of the relationship between chemical structure and teratogenicity of compounds related to trypan blue (3) led to the conclusion "that of all compounds tested only the intact trypan blue molecule is capable of such a marked effect upon embryonic development in the rat and that any structural modification is likely to affect the teratogenic properties of the resultant compound." No change in the trypan blue molecule was reported to enhance its teratogenicity.

Two of the disazo dyes, trypan blue and Evans blue, have been found to produce alterations in the proteins of maternal serum and 9-day embryo yolk sac fluid in rabbits(4,5). The primary alteration in both was a significant elevation in the beta globulin fraction. Trypan blue also has been found to cause changes in fetal rat serum proteins(6).

Congo red is an acid disazo dye with a molecular weight of 696. Trypan blue is also an acid disazo dye whose molecular weight is 960. The structural formulae of the 2 dyes are presented in Fig. 1. Congo red differs from trypan blue in the absence of 2 CH_3 groups, 2 OH groups and 2 $NaSO_3$ groups and differs also in the position of the 2 remaining $NaSO_3$ groups and the 2 NH_2 groups on the naphthalene rings.

Procedure. Virgin females of Wistar Albino rats (Albino Farms, Red Bank, N. J.) were used in this study. The animals were maintained on a Rockland Complete Rat Diet *ad libitum*. Day 0 of pregnancy was considered to begin on the morning sperm was found in the vaginal smear. Pregnant rats were given a single intraperitoneal injection

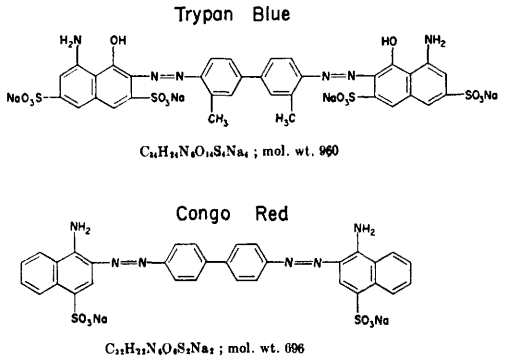

FIG. 1

of a 2% aqueous solution of Congo red† during the 8th day of gestation. The dosages employed were either 14 mg/100 g, 20 mg/100 g or 40 mg of dye/100 g maternal body weight. Forty-eight hours after injection blood was withdrawn via cardiac puncture and serum obtained. Blood was again drawn on the 20th day just prior to autopsy. The serum was analyzed by the paper electrophoretic method in a Spinco cell using Spinco B-2 buffer (Veronal at pH 8.6; 0.075 ionic strength). The paper strips were run for 17 hours at 3.5 ma. The strips were subsequently stained with bromphenol blue and their density was measured in a Spinco Analytrol (recording densitometer) to determine the relative concentration of each component. Total protein content of the serum was measured with a Bausch and Lomb Serum Protein Meter.

On the 11th, 15, and 20th days of gestation samples of maternal spleen, liver, abdominal lymph node and kidney, as well as representative placentae, were fixed in Bouin's fluid or cold acetone for histological examination. These tissues were examined as unstained sections for the presence of the injected Congo red. Fetuses recovered from pregnancies terminated on the 20th day were examined for malformations. Random fetuses

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† Congo red obtained from Matheson, Coleman & Bell Co., Norwood, Ohio.

TABLE I. Effect of Intraperitoneal Injection of a 2% Aqueous Solution of Congo Red into Female Rats on 8th Day of Gestation.

Dosage (mg/100 g)	No. of mothers	No. of implantation sites	No. and (%) resorbed	No. and (% survivors) malformed	% Implantation sites affected by treatment
14	9	103	10 (9.7)	3 (3.2)	12.6
20	24*	267	20 (7.5)	38 (15.4)	21.7
40	6†	—	—	—	—
Control	5	67	1 (1.5)	0	1.5

* Of which 2 died before autopsy.
† Five rats receiving this dose died between 11th and 13th day of gestation. The sixth rat survived to autopsy, but was not pregnant.

from each litter were prepared for histological examination of the soft tissues or for alizarin red staining of bone. Control animals were run concurrently with the experimentals.

Results. Table I presents the results of treating rats on the 8th day of pregnancy with a 2% aqueous solution of Congo red. At the 2 lower dosages used there appears to be no correlation between dye concentration and number of implantation sites resorbed. However, there is an apparent direct correlation between amount of dye injected and number of survivors malformed. The highest concentration of Congo red used in this study (40 mg/100 g) proved lethal to all pregnant rats 3-5 days after receiving the injection. The only rat to survive treatment with this high dose was not pregnant.

A pink coloration was observed in cells of the convoluted tubules in kidneys fixed in cold acetone on the 11th day of pregnancy, but not in those fixed on the 15th or 20th day of pregnancy. It is assumed that this color was due to the presence of Congo red. The dye was not visible, at any time, in the other maternal tissue examined nor in epithelial cells of the yolk sac placenta.

An analysis of the incidence of malformations found in fetuses whose mothers had received a 20 mg/100 g dose of Congo red revealed the following: 13 cases of hydronephrosis, 12 of hydrocephalus, 7 of microphthalmia, 6 of apparent anophthalmia and 1 short tail. Only 4 fetuses showed multiple defects; the remaining 34 had only a single malformation. No heart or skeletal defects were seen.

The effect of Congo red on maternal serum protein components is shown in Table II. Following injection each rat exhibited a lowering of total protein concentration. At the time of autopsy total protein concentration had returned to pre-injection levels. Unlike trypan blue, a teratogenic dose of Congo red did not cause marked changes in material serum proteins. The only statistically significant alteration in maternal serum protein following a dose of 20 mg/100 g was an elevation in alpha-1 globulins on the 20th day ($P=0.05$). The beta globulins were not elevated, as is the case following trypan blue treatment. Forty-eight hours after injection of a lethal dose (40 mg/100 g) animals exhibited significant changes in concentration of gamma ($P=0.05$), alpha-2 ($P=0.01$), and alpha-1 ($P=0.01$) globulins; and in albumin ($P=0.01$).

Discussion. Attempts to determine the reactive site(s) on teratogenic disazo dye molecules have been unsuccessful(3). The chemical formula for Congo red (Fig. 1) indicates that it it less like trypan blue than any other of the related teratogenic disazo dyes. Thus, Congo red offers no new information relating chemical structure to disazo dye teratogenicity. The appearance of trypan blue (and related blue dyes) in maternal macrophages and in epithelial cells of the yolk sac placenta is a common finding. It is not known whether it is significant that Congo red could not be located in any of these sites. Since trypan blue is not teratogenic in rats after the 10th day of gestation, Wilson *et al*(7) have suggested the possibility that the accumulation of dye by the yolk sac epithelium could serve to protect the embryo. Thus,

TABLE II. Total Protein and Protein Fraction Concentration in Control Rats and Rats Treated with Congo Red.

Treatment	No. of mothers	Total protein*	Globulins*					Albumin*
			Gamma	Beta	Alpha-3	Alpha-2	Alpha-1	
Serum from 10th day								
Congo red, 20 mg/100 g†	22	5.90 ±.47	.78 ±.26	1.00 ±.24	.31 ±.06	.38 ±.05	.84 ±.28	2.54 ±.46
Control	13	6.25 ±.54	.87 ±.26	1.08 ±.12	.32 ±.04	.30 ±.06	.76 ±.14	2.86 ±.36
Congo red, 40 mg/100 g‡	5	5.80 ±.62	.54 ±.10	1.10 ±.09	.41 ±.09	.56 ±.20	1.05 ±.08	2.28 ±.40
Serum from 20th day								
Congo red, 20 mg/100 g†	22	6.40 ±.42	.55 ±.22	.91 ±.10	.30 ±.04	.38 ±.07	1.76 ±.10	2.52 ±.30
Control	13	6.40 ±.52	.49 ±.14	.93 ±.13	.36 ±.06	.40 ±.10	1.54 ±.22	2.65 ±.30

* Concentration expressed as g per 100 ml with standard deviation.
† All P values >.5 except alpha-1 on day 20 (P = .05).
‡ Significant P values were obtained for gamma (.05), alpha-2 (<.01), alpha-1 (<.01) and albumin (.01).

the dye would only have access to the embryo prior to the completion of the yolk sac.

The defects observed following treatment with Congo red are similar to the major defects seen following trypan blue administration, except that the incidence of hydronephrosis is higher in this study than in any previous report for trypan blue. Excluding the one case of short tail, only 3 types of malformation were observed; hydrocephalus, hydronephrosis and ocular defects. The distribution of these defects in fetuses was interesting in that 34 of the 38 malformed fetuses had only one abnormality. The 4 remaining had multiple defects. The teratogenicity of Congo red was not evident in the study by Wilson(1) because of the dosage employed. Wilson used 30 mg/animal (200-250 g) which corresponds closely to the 14 mg/100 g dose used in this investigation. The present study shows that it is not until the dose reaches 50-60 mg per animal that the teratogenic activity of Congo red is expressed. Doses of Congo red greater than 100 mg were lethal to pregnant Wistar rats.

The fact that maternal serum protein changes following treatment with Congo red are not the same as those following treatment with trypan blue raises a question as to the relationship between serum changes and disazo dye teratogenicity. Trypan blue has been reported to cause an elevation in alpha and beta globulins and albumin in the pregnant rabbit(4,5,8). These serum protein changes are one of the few physiologic alterations reported in trypan blue treated pregnancies, and the two events have been linked causally, at least by implication. Evidence supporting this view has been reported by Langman *et al*(9), who found abnormal electrophoretic patterns in serum proteins from 19 selected human pregnancies, the outcome of which was abnormal in 15 of the 19 cases. The abnormality found in the serum proteins was a change in the alpha and/or beta globulin pattern.

It is possible that Congo red may act in a different manner than the other teratogenic disazo dyes. Two observations could be interpreted so support this: (1) Congo red was not found in the yolk sac placenta, and (2) the infrequent appearance of multiple malformations. This question cannot be answered until other disazo dyes are examined for their effects on the serum proteins.

Summary. The teratogenicity of Congo red was investigated in the Wistar Albino rat. Female rats received a single intraperitoneal injection of a 2% aqueous solution of Congo red on the 8th day of pregnancy. Dosages used were either 14 mg/100 g, 30 mg/100 g or 40 mg/100 g maternal body weight. A dose of 20 mg/100 g was found to be teratogenic, 15.4% of the survivors being malformed. A dose of 40 mg/100 g was lethal, all pregnant rats dying within 5 days after injection. The malformations produced were hydrocephalus, hydronephrosis

and ocular defects. Multiple malformations were rare. The teratogenic dose of Congo red did not produce any alteration in maternal serum protein components during the expected period of teratogenic activity. This is contrasted to the situation found following treatment with trypan blue.

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Role of Calcium in Complement Dependent Hemolysis.* (29529)

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Mayer and his co-workers(1,2) have defined the role played by divalent cations in complement (C′) dependent immune red cell (EA) lysis. Their investigations clearly show that the need for Ca^{++} occurs earlier in immune hemolysis than the need for Mg^{++}. This observation enabled the same investigators to establish techniques for preparation and study of sensitized red cell complement component intermediates (EAC′....)(3,4). As a result it is now known that Ca^{++} participates in the attachment of the first component of C′ (C′1) to EA, while Mg^{++} is required for the attachment of C′2. C′4 and C′3 can function in the absence of both Mg^{++} and Ca^{++}. The various salts of EDTA have been of great practical importance in studies on EAC′.... lysis. Addition of Na_3HEDTA to a source of C′ binds both Mg^{++} and Ca^{++}; such a reagent, containing fully active C'_3, will support the lysis of $EAC'_{1,4,2}$. Addition of Na_2Mg EDTA to a C′ source binds Ca^{++}, but the Mg^{++} present preserves the ability of C′4, C′2, and C′3 to function; such a reagent will support the hemolysis of EAC'_1, $EAC'_{1,4}$, and $EAC'_{1,4,2}$, but will not support lysis of EA or EAC'_4 when C′ is employed in the usual concentrations(5,6).

The *in vitro* hemolysis of red cells (E) from patients afflicted with paroxysmal nocturnal hemoglobinuria (PNH) occurs best in undiluted human serum which has been slightly acidified (pH 6.5), but requires the presence of all 4 components of C′(7). In addition it can be shown that following exposure to acid serum, those cells which have resisted hemolysis bear on their surface components of C′(8). Nevertheless, PNH-E do not require Ca^{++} for lysis in serum depleted of Ca^{++} and Mg^{++} by IRC-50 resin treatment, since the readdition of Mg^{++} only will reconstitute the PNH hemolytic system(7). The appropriate use of EDTA salts establishes the same principle (Table I) since serum chelated with Na_2Mg EDTA is fully capable of supporting PNH-E lysis, while Na_3HEDTA is almost totally inhibitory.

If PNH-E lysis is truly C′ dependent, these results might suggest that PNH-E are in the functional state $PNH\text{-}EC'_1$, or $PNH\text{-}EC'_{1,4}$. If this were so, pre-exposure of PNH-E to

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