STUDIES ON THE TERATOGENIC MECHANISM OF ACTION OF TRYPAN BLUE

by

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INTRODUCTION

"From the beginning, look,
what thou desirest to see,
it shall be shewed thee"
II Esdras 4:46

The inquiries into the teratogenic mechanism of action of Trypan blue stemmed from a 1948 report (1) of congenital anomalies in the offspring of rats injected with this dye during pregnancy. Most of the subsequent suggestions of the teratogenic site of action could be characterized as support for a locale within either the maternal animal or the boundary between mother and embryo. A third locale, the embryonic, has received very little support, for the characteristic blue color of this dye has been notably absent from the tissues of the affected offspring. At the initiation of my interest in the biological effects of Trypan blue, little work had been accomplished relating to dye effects on enzyme activity, even though a few phenomena suggested the possibility of such effects. Some initial studies alerted the author to the difficulty of evaluating the degree of relevance of such effects to the teratogenic mechanism. This consideration, therefore, made obligatory a more general approach, one which could direct future studies toward the most likely of the three locales of teratogenesis.

As a basis for understanding the formulation of this immediate research question, let us consider in general terms a few features of the early embryology of the rat. Following implantation, the developing organism is enveloped by layers of endoderm which lie external to those of ectoderm, a relationship the reverse of the usual. This "inversion

of the germ layers", or entypy, is brought about by the pushing into the endoderm of the "inner cell mass", the structure destined to give rise to the embryo proper. The endodermal layers comprise the bulk of the omphalopleure, an invaginated sac subdivided into an outer (parietal) and an inner (visceral) layer. The omphalopleure is the initial framework used in the formation of the future "inverted yolk-sac placenta", an organ of maternal-embryonic exchange which antedates the more elaborate chorio-allantoic placenta. In the early post-implantation period, the developing blastocyst is nourished for the most part by nutrients which reach it through the parietal and visceral yolk sac layers without the assistance of the vitelline blood circulation, a system slated for somewhat later development on a plane inside that of the visceral sac. Trypan blue injected into the maternal animal during the teratogenic period is found to be heavily concentrated within the cells of the visceral, or inner, yolk sac; the outer layer is virtually unstained. The narrow range of the teratogenic period is singularly early in ontogeny in comparison with most other teratogens, and this period precedes the establishment not only of the chorio-allantoic placenta, but also of the vitelline circulation of the visceral yolk sac. Since this period is roughly coincident with that stage in which nourishment must traverse the two layers of endoderm to reach the embryo, the question arises as to the degree of interference with nutrition resulting from the entrapment of dye by the inner layer, the so-called "last barrier" to dye infiltration of the developing embryo. A second reason for concentrating attention on the yolk sac involves the wide variety of effects seen among embryos of the same experimental litter; one embryo could be completely normal,

an adjacent one malformed, the next successive one resorbed, and so forth. Such unpredictability would seem to be less easily reconciled with a maternal locale for the teratogenic mechanism. The question posed, then, was whether or not any correlation existed between the concentration of dye in the visceral yolk sac and the degree of malformation of the embryo protected by that sac. If the yolk sac serves as an important locus of abnormal metabolism, the heaviest dye concentrations should exist within membranes surrounding the most malformed embryos. To be tested, this hypothesis required the use of embryos in stages of development sufficiently mature to be categorized as either normal or abnormal, whereas ideally, younger embryos should be examined, at a time not so far removed from that of maternal dye injection. But before a choice was made between these two extremes, the practical and the desirable, an entirely new procedure had to be devised to facilitate accurate and sensitive measurement of the dye in an individual visceral yolk sac membrane. The few methods known for estimating dye could not be applied to an individual membrane because of its meager size. An entirely suitable procedure was developed and applied to 13- and 12-day visceral yolk sacs. In the preparations for expansion of this series of tests, the author's attention was diverted by a series of observations which culminated in the startling conclusion that the visceral yolk sac was not a complete barrier to dye, for a part of the embryo was colored blue. The integration of information from these two areas, that of quantitative data on dye concentration in individual visceral yolk sacs and that of observations on dye localization within embryos, served as the basis for the conclusions in this dissertation.

In 1906 Nicolle (3) discovered Trypan blue; the word "Trypan", derived from the Greek word for "borer", reflects employment of the dye in the treatment of infections of domestic animals by parasitic members of the genus, Trypanosoma. Subsequent to the heyday of its utilization by the veterinarian (4), Goldmann (5, 6) discovered its ability to vitally stain animal tissues. It now enjoys no therapeutic application, although an isomer, Evans blue, is sometimes featured in plasma volume determinations. Gillman et al. (1) are credited with discovering its capability as a teratogen in mammals, although the literature completely overlooks Nicol's (7) earlier notation of abnormal embryos in treated guinea pigs.

The consummate reader of reports of Trypan blue experimentation soon becomes aware of a number of factors which interfere with his evaluation of an individual report. For example, injections vary in number or site; the information on injection time relative to gestation age is often vague or absent. Furthermore, the teratogenic effects are altered by a variety of environmental and genetic factors, as we shall see. But the most prevalent factor is the disparity in chemical composition among the dye samples employed; the resulting devaluation can be severe (8, 9). Both Wilson (10) and the Beck-Lloyd group (11, 12) utilized commercial products which were later shown not to consist principally of Trypan blue; the sole means of authentication did not appear until 1964 (13). Even authentic commercial disazo dyes contained gross impurities (14), consisting both of salts used in dye precipitation (12) and of colored fractions, red and purple. The red impurity was perhaps the most conspicuous contaminant (9, 15, 16, 17, 18, 19); it received intensive study from Okuneff (20), Hartwell and Fieser (21)

and Kelly (15). Lloyd (22) noted a red dye after reductive cleavage of one of the two azo bonds of Trypan blue; other formula possibilities for the red component were described by Kelly (15). A single purple fraction was described by Leeson and Reeve (23), but two such fractions were listed by Dijkstra and Gillman (24). Formula possibilities have also been proposed for this fraction (25).

A number of reports have at least implied that the discrepancies among results of teratological experiments may have been caused by dye contamination (24, 26, 27, 28, 29, 30), and Beck (11) provided evidence for this supposition. This problem gave birth to procedures for separating the commercial product into pure fractions. One effort (24) in this respect preceded Kelly's (15) success in obtaining at least a red-free product, but the best procedure was developed in 1963 by Beck and Lloyd (31), who used paper chromatography. A second attempt by Kelly's group (14) produced doubtful results. The latest procedure (32) utilized a series of steps: (a) authentication, (b) ascending paper chromatography (13) for the removal of the colored impurities, (c) dialysis, (d) filtration through sintered glass, (e) utilization of a Dowex 50W (H^+ form) ion exchange resin (31) to remove the metal cations and to convert the dye to the free acid form, and (f) estimation of the dye content. The latter step can be accomplished in several ways: by titration of the azo linkages by titanous sulphate (33), by polarography (34), or by spectrophotometry (18). The problem remained as to which of the fractions were teratogenic. Tuchmann-Duplessis et al. (27) had ruled out the red fraction by the time Beck and Lloyd (31) characterized the blue fraction as the only teratogenic one; Barber and Geer (35) substantiated this claim, using the mouse. Lloyd (22) also recently

indicated that this fraction produced reticulosis, whereas Dijkstra and Gillman (24) had assigned that capability to one of their purple fractions.

Consider now the disposition pattern of the dye after its injection into the maternal animal. Rawson (36) initially found the dye to be wholly bound to the albumin fraction of the plasma proteins, and Brenner (37) noted such an association in the rat, guinea pig and baboon, but Hansen and Nielsen (38) suggested the possibility of additional binding sites in other fractions. Lang and Lasser (18) ascribed the binding of this dye to three sites, arranged in two "sets", whereas Allen and Orahovats (39) had previously postulated two. Klotz (40) predicted that the binding of such a dye to a protein would yield a spectral shift in maximum absorption, a prediction later substantiated by Gregersen and Gibson (41) and Brenner (37). The differing strengths of binding (36, 42) served to explain why dyes related to Trypan blue displayed differing disappearance rates from the blood stream. For example, an isomer of Trypan blue, Evans blue, was retained longer supposedly because of an additional number of binding sites (39), and one study (43) did note more free dye with Trypan blue than with Evans blue. Unbound dye appears in the urine, a fact accounting for the virtual lack of Evans blue in this filtrate (44). Since only 6.85% of the radio-activity of an I^{131}-labelled Trypan blue was found in the urine of mice within a three-day period following injection (45), dye clearance from the blood seems to occur chiefly by means of tissue uptake. Kelly (14) noted no gross coloration in maternal tissues after injection of a red dye fraction, an observation in keeping with von Mollendorf's (46) detection of blue color in the renal tubule cells of dye-treated

animals and of red color in the urine.

Protein-bound dye passing into the tissues of experimental rodents is captured chiefly by components of the reticulo-endothelial system and by certain epithelial tissues, but never by the central nervous system. Its lack of detection in the latter location was noted by Wislocki (47) in experimentation with the guinea pig; even injection into the amniotic sac failed to permit passage across the so-called "blood-brain barrier" of the fetus. Gillman's (1) original report of dye-induced malformations also included the observation of dye exclusion from the central nervous system. Grazer and Clemente (48) recognized the existence of such a barrier in the rat as early as one-half day after the vascular invasion of the central nervous system. The barrier proved to be unidirectional, for dye injection into the ventricles stained the embryo in its entirety.

Although Nicol (7) could detect no uptake of dye by phagocytic elements within the circulatory system of the guinea pig, all of the several remaining reticulo-endothelial components have been shown to phagocytize the teratogen. Although this particular dye has long been considered a reticulo-endothelial system blocking agent (49, 50, 51), a number of reports (52, 53) question its efficacy. The splenomegaly (54, 53, 55) of the experimental maternal animal is, however, a reflection of confinement of formed blood elements rather than of dye capture (56). The dye is secured in the liver not only by the Kuppfer cells, as implied above, but also by its epithelial parenchyma, yet liver weight is not altered by the dye's administration (55). A second epithelium mentioned prominently in the literature is that of the kidney's proximal convoluted tubule (46, 47, 57, 58). According to Beck (59), adrenal cortical epithelia also show dye deposits.

Hetherington (60) discusses a different pattern of uptake by cells in culture. In each of the capture sites mentioned, the intra-cellular dye is segregated and concentrated, as noted by Evans and Schulemann (61). The diffusion of dye through cells usually signifies cell death (61, 62, 63, 64); indeed, for cells which normally do not take in the dye, blue coloration serves as the basis for viability tests (76, 77). The patterns of doses larger than those necessary to produce malformations are not considered above, nor are the effects of repetitive injections, such as are used in the production of carcinogenesis.

Of the epithelia which phagocytize the dye, the visceral yolk sac layer is probably of greatest interest to the teratologist. To better understand this interest, consider first some of the spatial relationships and characteristics of the layers surrounding the developing embryo. We have already visualized the yolk sac as consisting of two layers; (1) an inner, vascular visceral wall (visceral splanchnopleure, visceral yolk sac) composed of a simple columnar endodermal epithelium which rests on a mesenchymal base housing the vitelline vasculature, and (2) an outer, non-vascular parietal wall (bilaminar omphalopleure) formed of an incomplete layer of endodermal cells resting on the inner aspect of the basement membrane known as Reichert's. The latter appears early in ontogeny as a thick inner foundation for the trophoblastic giant cells, a reticulum through which flows the maternal blood. Although the rat trophoblastic giant cells actively engulf the dye (7, 59, 65), this meshwork of cells serves as no barrier to dye passage toward the rat embryo; several reports (66, 67) indicate maternal blood in direct contact with Reichert's membrane, and the latter report characterizes this blood as circulating. In fact, uninhibited passage

for the dye is assured as far as the yolk sac cavity (66, 67, 68), that space situated between the two yolk sac layers. Everett (67) noted a "bluish cast" in Reichert's membrane and in the parietal yolk sac, and we have already discussed the significance of such a lack of dye segregation within cells. Porter (69) supported this characterization of Reichert's membrane with observations on ectopic mouse embryos. The yolk sac is the only rodent extra-embryonic membrane which accumulates the dye (70, 71, 72); a tracer protein (horse-radish peroxidase) is also taken up by no other rodent extra-embryonic membrane (73). Waddington and Carter (74), using mouse embryos injected on the seventh day, identified the dye 36 hours later in the central region of the egg cylinder, which is the area of origin of the yolk sac. Other studies have also described the visceral yolk sac as phagocytic (67, 73, 75, 78, 79, 80, 81, 82); the phagocytized dye was detected in a matter of hours after injection into the mouse (70, 83), and Everett (67) viewed dye in the supra-nuclear region of the visceral yolk sac cells within 20 minutes of injection into maternal rats. This phagocytic capability was evidently fully retained both in cultures of rat yolk sac (71, 84) and in transplants of mouse yolk sac (69).

The literature contains a long list of negative reports of the search for evidence of dye in the embryo of the rodent (1, 47, 67, 68, 70, 82, 85, 86, 87, 88). Goldmann's (5, 6) introduction of Trypan blue as a vital stain specifically noted such lack of evidence, as did Nicol (7) in his superlative description of dye disposition around his "abnormal" guinea pig embryos.

The meager amount of evidence available suggests that the tissue dye deposits undergo little mobilization. Although the dye does not

have a leuco form (59), it can be rendered colorless by severance of both azo bonds (13). Stevens et al. (45) found no blue color in either the feces or bile of mice injected with a radioactive iodine-labeled dye, and since 54.4% of the label was recovered in the feces within a three-day period following injection, the intestine may well be a major site of dye breakdown. Roxon et al. (90) defined an azo reductase capability in the bacteria of the intestine, and Fouts et al. (91) characterized such an enzyme from rat liver. Lloyd et al. (22) applied Fout's enzyme to six disazo substrates, including Trypan blue, and noted disappearance of dye in each instance. Furthermore, in an additional experiment with three of the dyes, the disappearance of blue color correlated with appearance of red color. The latter was described as a mono-azo dye resulting from reductive cleavage of one of the two azo bonds. This mono-azo compound still qualified as substrate for the rat liver enzyme, yielding a disulphonic acid which they included in a list of compounds frequently found to be carcinogenic. Since we are dealing with a liver enzyme, it is pertinent to note here that both types of Trypan blue-induced cancerous growths occur only in the liver (26). Christie (92) found this disulphonic acid remnant of the Trypan blue molecule to be non-teratogenic, and the remaining Trypan blue fragment, o-tolidine, also was incapable of causing malformation. Although the works of both Ludford (58) and Smith(93) suggested the possibility of dye excretion via the bile, no great significance is attached to the presence of the liver azo reductase enzyme, for: (a) Stevens et al. (45) found that the radio-active label in mouse livers was as prevalent four to five days after injection as it was one day after, (b) the dye is very probably protectively sequestered within lysosomes, and (c) not

only was color not detected in bile during Stevens' (45) experiment, but also the literature records no instance of malformation resulting from dye passage through the gastro-intestinal tract. The very weak vestige of blue color in the urine of maternal animals at time intervals long after injection could conceivably be the result of degeneration of phagocytic elements.

Turning now to some of the parameters of teratogenesis resulting from dye usage, it is noted that work by Gillman and associates (1, 94) established that injection of Trypan blue on the eighth or ninth day of gestation, the primitive streak stage of development (59), produced the greatest number of malformations at term. Wilson et al. (95) confirmed this in the same species, rat, adding that teratogenic activity ceased on the tenth or eleventh day, at a time they considered coincident with the onset of function of the chorio-allantoic placenta. It has been postulated that this surprisingly early cessation, at approximately the 15 to 20-somite stage and corresponding to incompletion of organ formation (59), results from the protective influence of the visceral yolk sac rather than from any incapacity of the embryonic tissue proper to react to the dye directly (96). The time of cessation of the teratogenic effects of Trypan blue was confirmed by others (97, 98) in the rat, and by Nebel and Hamburgh (83) in the mouse. The latter experiment listed only embryonic resorptions or growth retardations subsequent to maternal injection on day 10.5-11.5. Although Smith (99) weakly documented a conclusion to the contrary, Beck and Lloyd (100) convincingly introduced a valuable concept in this respect in demonstrating that the vast majority of resorptions result from malformations which are incompatible with life. This implies that

resorptions resulting from dye injections outside of the critical time range represent normal wastage. The same authors (101) enlarged upon this concept with the introduction of valuable terminology relating to dosage-response graphs. Ferm's efforts with the hamster (81) demonstrated no early effects of the dye, for this teratogen failed to influence either the number of ova ovulated or fertilized, or the number of blastocysts implanting.

The teratogenic results of Trypan blue administration vary quite widely with any alteration of the maternal animal's genetic constitution, environment, or simultaneous treatment with other compounds, teratogenic or not. Several workers (27, 28, 102, 103) have documented the importance of consideration of the genetic factor. Gunberg (104) reported additional malformations, and variation in kinds of abnormalities, in embryos from rats maintained on less than optimal quantities of protein. Runner and Dagg (105) grouped the types of interaction between two teratogens under four descriptive titles: interference, non-additive, additive, and reinforcing. Wilson exemplified the worth of these groupings in the teratogenic interaction of the dye with vitamin A (106, 107) and 5-fluorouracil (106). The results of adding the teratogen thalidomide (108), thyroid-stimulating hormone (109), or vitamin A and cortisone (110) to the teratogenic insult induced by Trypan blue have also been reported. Kropp (57) used the non-teratogen penicillin G procaine to ameliorate the toxic effects of the dye in mice.

The literature is not unequivocal in its judgment of dye effect on the sex ratio of the offspring. Waddington and Carter (74) noted a greater frequency of death and resorption of the female mouse embryo, whereas Fox and Goss (111) concluded the opposite. Both Beck (32) and

Ferm (81) described a lack of sex-dependent difference in response in rats and hamsters.

The pattern of abnormalities seen in the rat, the animal most often used in studying the malforming effects of Trypan blue, is chiefly one of effects within three systems; central nervous, cardiovascular, and axial skeletal. Since Beck (59) has more than adequately chronicled such malformations in species other than the rat, using a variety of teratogenic disazo dyes related to Trypan blue, this dissertation will not specifically be concerned with these ancillary areas. The Gillmans' (1, 94) original descriptions of rat abnormalities were closely followed by other reports confirming a gamut of malformations (79, 112, 113). The original description (1) of abnormalities noted hydrocephalus as the defect most commonly seen, and this condition received intensive study from Wilson (79), Vickers (114) and Stempak (115). The latter pinpointed the aqueduct of Sylvius as the critical site of hydrocephalus, confirming the suspicions of the two previous workers. Incidentally, although a discussion of tooth malformation may seem out of order here, Knudsen (116) noted a correlation between the occurrence of brain malformation and the approximately 25% occurrence of various degrees of fusion of the upper incisors of the mouse; Kreschover et al. (117) had previously detected no rat tooth malformation. Of some association with central nervous system defects are the observations of Gilbert and Gillman (118) specifically on eye abnormalities. Beck and Lloyd (100) confirmed a conclusion from the Gilbert and Gillman report that no isolated eye defects existed at an early gestation stage (11½ days), yet Beaudoin (119) described his only surviving postpartum rats as being micro- or anophthalmic. Gunberg (120) studied and classified

types of spina bifida and axial skeletal defects in rats. Warkany, Wilson and Geiger (121) hypothesized an open neural tube as conditional for the development of myelomeningocoele. Hamburgh (87) also cited an open neural tube as one of the two major disturbances seen among experimental mice, the other being neural tissue hypertrophy, and Goda (122) reported that such failure of tube closure led to myeloschisis.

Prior to 1954, the only reports of induced cardiovascular abnormalities were those of Waddington and Carter (123) and Murakami (124). Then Wilson issued a series of four papers (10, 79, 125, 126) on this subject. His work was extended by four reports by Fox and her group (111, 127, 128, 129). Fox and associates considered the cardiac malformations observed to be caused by an abnormal looping of the cardiac tube; Christie (130) supported this theory, as did Mulherkar (131) in describing a delay in the formation of the cardiac loop in dye-treated chicks. Smith (132) associated malpositioning of rat atria either with such an abnormal looping or with a decrease in the amount of cardiac jelly. Finding a precocious occurrence of glycogen granules in these experimental hearts, Smith theorized that the dye acted directly on myoepicardial tissue, but Monie et al. (133) observed neither a decrease in the quantity of cardiac jelly nor a thinning of the myocardial layers in his experimental rats. Two additional studies on the cardiovascular effects of this teratogen were performed by Inoue (134) and Wegener (103). The latter described damage to the septal myocardium. Cardiovascular effects comprise an important segment of Trypan blue effects, for Richman et al. (135) in his catalogue of heart defects reported that 26 of 50 rats surviving for 22 to 33 days postpartum possessed major

cardiovascular anomalies. Finally, Myers (136) and Altmann (137) witnessed ear defects in experimental animals, and Goldstein (138) listed abnormalities of the urogenital system. The above list of abnormalities is not comprehensive, as it deals solely with malformations in common evidence.

MATERIALS AND METHODS

A. Procurement of embryos and visceral yolk sacs.

Rats of the Sprague-Dawley strain (Berkeley-Pacific Company; Berkeley, California) were supplied with pelleted rat food and water ad libitum. Nulliparous females exhibiting vaginal signs of the pro-estrous stage of the estrous cycle were mated with males of their own strain during a two-hour period. The age of an embryo, expressed in days, was computed by dividing by 24 the number of hours intervening between the midpoint of this two-hour mating period and the initiation of the maternal laparotomy. At 9 days the pregnant rats were injected intraperitoneally with a 2% aqueous solution of Trypan blue (Colour Index #23850, Direct Blue 14, 3,3'- [(3,3'-dimethyl-4,4'-biphenylene) bis(azo)] bis [5-amino-4-hydroxy-2,7-naphthalenedisulfonic acid]; Lot #2062P, National Aniline Division, Allied Chemical Corporation) at the dosage levels indicated below.

Thirteen- and 12-day extra-embryonic membranes and embryos were obtained from rats anesthetized with sodium nembutal (60 mg/kg body weight). An anti-mesometrial incision of the uterus allowed an embryo to be gently separated from the contiguous endometrial tissue. While completely immersed in fresh saline, the embryo was relieved of its surrounding membranes. The decidua capsularis, with attached Reichert's membrane and parietal yolk sac cells, was swiftly stripped away with two pair of fine-pointed forceps, revealing the visceral yolk sac. With the aid of low-power magnification, this sac, with enclosed embryo, was carefully separated from the chorionic plate of the developing chorio-allantoic placenta. Then this visceral sac, freed of vitelline and allantoic vessels, amnion and embryo, was grasped with forceps and

removed to a thin plastic film supported by frozen carbon dioxide. Any remnants of other extra-embryonic membranes were promptly removed before transfer of the embryo to fresh saline for further examination under low-power magnification for external malformations. The embryos subsequently were stored in Bouin's fixative solution. The above procedure was repeated for each embryo. Saline-moistened gauze prevented any significant shrinkage of the unattended maternal uterus. After the maternal animal was sacrificed, the frozen yolk sac chips were stored at 0° C. in individual pre-frozen, glass shell vials. Six maternal animals were relieved of 20-day fetuses in similar fashion, except that the tissue envelopes external to the fetus were discarded.

B. <u>The individual visceral yolk sac: estimation of Trypan blue</u>.

A single, frozen yolk sac chip was placed in approximately 0.8 ml. of 33% aqueous n-butylamine contained within a 5-ml. Pyrex centrifuge tube. Complete dissolution of the tissue was accomplished by heating tube and contents for one hour in a 70° C. water bath. The tube mouth was closed by the tapered end of an identical centrifuge tube, an arrangement which served to condense the butylamine vapors and return them to solution. The contents of the tube usually sat overnight, after which an additional amount of the butylamine solution was added to bring the total volume to exactly one milliliter. This was achieved accurately and simply because each centrifuge tube had been previously calibrated to contain one milliliter by means of a volumetric tube apparatus sufficiently sensitive to detect evaporation from the contents of any tube left uncapped for as little as three minutes time.

This device, fashioned mainly of parts from a light microscope and a micrometer caliper, is illustrated in Figure 1. Exactly one

milliliter of water was placed in a clean 5-ml. centrifuge tube of selected diameter as two aliquots from a 500-ul. Levy delivery pipet (Microchemical Specialties Company; Berkeley, California). The tube (T) was raised or lowered within a closely-fitted plastic guide housing (H) by adjusting the micrometer screw (M), the end of which projected through the base of the guide housing. The micrometer screw was firmly affixed to the guide housing by means of Allen screws, and the guide housing was bolted to a section of 0.5-inch aluminum plate serving also as the base for the viewing lens system (L). The viewing lens system was constructed so that the observer's eye at (E) viewed a magnified, inverted image of the bottom of the liquid meniscus against an illuminated background of a cross-hair. The bottom of the meniscus was aligned with the horizontal member of the cross-hair by turning the micrometer screw, and the graduated head reading, in thousandths of an inch, was recorded for that particular tube. The tube was emptied, cleaned, and the calibration procedure repeated two additional times. These three readings for an individual tube usually ranged no greater than 1/2000th of an inch; an appropriate number was then etched on the tube. If the readings exceeded this range, the triplet of calibration procedures was repeated.

A short, solid glass rod sealed to the inside tapered bottom of one of the 5-ml. centrifuge tubes was aligned at its upper, pointed end with the horizontal member of the cross-hair. Previous to each utilization, the volumetric tube apparatus was checked for alignment with this standard tube. At no time did the instrument require adjustment. Enough of the butylamine solution was added to an experimental tube's contents by means of a 10-ul. eyedropper to bring the total

volume to exactly one milliliter. Tube and contents were spun for 15 minutes at 1610 x g in a clinical blood centrifuge. A slight quantity of insoluble lipid often floated on a portion of the surface of the clear, blue supernatant. This diminutive pellet, judged to be protein-negative by application of the Folin reagent, was left in the centrifuge tube by withdrawing only the supernatant with a previously unused glass Pasteur pipet. The supernatant was stored in a glass ampoule with heat-sealed top until the time for spectrophotometric analysis. A series of runs was accomplished using solubilized yolk sac solutions divided into two portions. One portion was immediately read in the spectrophotometer, the other read at several weekly intervals up to one month later. These runs showed (1) that the heat-sealing step did not affect the optical density reading and (2) that the optical density reading was stable over at least this period of time.

For the measurement of optical density, the solutions were transferred to 1-ml. silica absorption cells fitted with plastic closures. After the readings were performed in a Zeiss PMQII spectrophotometer at 6000 Angstroms, the individual solutions were returned to new glass heat-sealed ampoules by means of new Pasteur pipets. All of the solubilized yolk sacs from an individual potential litter were read at one sitting. The expected, slight daily fluctuations of the PMQII readings were compensated for by employment of a single sealed standard of aqueous Trypan blue.

C. Trypan blue in the embryo.

The following procedure enabled the dye to be seen (15 x magnification) in embryos stored in Bouin's solution for as long as one year. Embryos were put for 24 hours in 80% aqueous ethanol to which a few

drops of saturated lithium carbonate was added. After the picric salts were almost completely removed, the embryos were dehydrated by exposure to 95% and 100% aqueous ethanol and then cleared in cedarwood oil. All of the stored embryos from eight litters selected at random from investigations conducted during the past year were scrutinized upon completion of the above procedure. One of the eight maternal rats received 40 mg of dye/kg body weight and the other seven either 75 or 100 mg/kg. The photographs were made from Kodachrome II transparencies using a Zeiss automatic photomicroscope operated without filters.

RESULTS

A. 13-day embryos.

The following ten tables contain data obtained from rats bearing 13-day embryos. Both the weight and age data for the maternal animals refer to values at the time of injection of the teratogen. Each of the ten pregnant animals received 75 mg Trypan blue/kg body weight. The O.D. value in the first of three columns represents the corrected optical density at 6000 Angstroms of the 1-ml. butylamine solution containing all of the dye in the complete visceral yolk sac surrounding one living embryo. The second column lists values obtained by dividing each optical density by the average optical density for that particular incipient litter. The third column briefly notes the abnormalities revealed by the external examination of each embryo. The asterisks denote values referred to later in the discussion of these data.

1. Animal #14, 306 grams, 120 days old

Embryos: Living 11, resorbed 2, implants 13, abnormal 4, normal 7

O.D.	O.D./Av.O.D.	Description of abnormalities
0.16733	0.746	
.19696	.878	
.20960	.935	
.21132	.942	
.21700	.968	Slightest axial torsion
.22434	1.001	
.23132	1.031	Slightest axial torsion
.23281	1.038	
.24548	1.094	Slightest axial torsion; perhaps brain edema
.25029	1.116	Axial torsion
.27999	1.249	
.22422 = Av. O.D.		

2. Animal #4, 304 grams, 123 days old

Embryos: Living 15, resorbed 3, implants 18, abnormal 10, normal 5

O.D.	O.D./Av.O.D.	Description of abnormalities
0.23881	0.858**	Incipient bifida, tail V-shaped, lateral flexion behind anterior limb, abnormal heart shape, abnormal midgut closure
.24390	.876**	Left telencephalon absent, bifida, acute axial torsion posterior to anterior limb bud
.24451	.878	Moderate tail torsion
.25194	.905	Neural canal slightly irregular, plus expansion at level of hind limb bud
.25396	.913	
.26212	.942	
.26457	.950	Axial torsion, neural canal slightly irregular
.27620	.992	Neural canal moderately irregular, slightly in front of anterior limb bud
.27860	1.001	Acute angle in neuraxis, cardiac edema
.27964	1.004	Axial torsion, caudal
.29926	1.075	
.30679	1.102	Neural canal irregular
.30938	1.112	
.32740	1.176	
.33714	1.211	Acute offset in neuraxis posterior to anterior limb bud

.27830 = Av. O.D.

3. Animal #5, 272 grams, 116 days old

Embryos: Living 12, resorbed 1, implants 13, abnormal 5, normal 7

O.D.	O.D./Av.O.D.	Description of abnormalities
0.25135	0.881*	Neural canal torsion, cardiac enlargement, abnormal tail shape, white spots in yolk sac
.26244	.920	
.26986	.945*	U-shaped tail, small circular hematoma mid-axial opposite hind limb bud
.27381	.960	
.27941	.979	Neural canal torsion, cardiac edema
.28760	1.008	
.29667	1.040	
.29796	1.044	
.30828	1.081	
.32559	1.141	Neural canal torsion

.28530 = Av. O.D.

4. Animal #11, 312 grams, 117 days old

Embryos: Living 8, resorbed 5, implants 13, abnormal 4, normal 4

O.D.	O.D./Av.O.D.	Description of abnormalities
0.27004	0.720	Bloody yolk sac, pericardial edema, severe axial torsion, faint heart beat
.34132	.910	Very slightest neuraxial torsion
.34924	.931	Pericardial edema, slight torsion neural canal
.38498	1.027	
.38901	1.038	
.44458	1.186	
.44544	1.188	
.37490 = Av. O.D.		

5. Animal #15, 316 grams, 120 days old

Embryos: Living 10, resorbed 3, implants 13, abnormal 4, normal 6

O.D.	O.D./Av.O.D.	Description of abnormalities
0.32094	0.847	
.34063	.899*	Exencephaly
.36844	.973	
.37314	.985	
.37632	.993	Hollow spot in neural canal opposite hind limb bud
.37882	1.000	Entire embryo displays torsion
.38022	1.004	
.38285	1.010	Anterior neuropore open
.42738	1.128	
.43922	1.159	
.37880 = Av. O.D.		

6. Animal #9, 292 grams, 116 days old

Embryos: Living 9, resorbed 2, implants 11, abnormal 2, normal 7

O.D.	O.D./Av.O.D.	Description of abnormalities
0.28248	0.629	
.39881	.887	Twisted neural canal, hematoma ventral to caudal portion of neural canal
.40710	.906	
.44162	.983	
.46060	1.025	
.48240	1.074	
.49874	1.110*	V-shaped tail; vertebrae number possibly diminished
.51931	1.156	
.55240	1.230	
.44927 = Av. O.D.		

7. Animal #2, 306 grams, 116 days old

Embryos: Living 10, resorbed 2 plus 1 burst, implants 13, abnormal 0, normal 10

O.D.
0.45681
.45980
.46284
.52750
.53792
.54000
.54261
.54828
.57564
.57602
.52270 = Av. O.D.

8. Animal #4A, 274 grams, 118 days old

Embryos: Living 12, resorbed 4, implants 16, abnormal 0, normal 12

O.D.
0.36133
.37263
.38847
.39901
.40625
.40792
.40836
.41570
.41681
.41824
.42732
.40200 = Av. O.D.

9. Animal #7A, 300 grams, 122 days old

Embryos: Living 8, resorbed 4, implants 12, abnormal 2, normal 6

O.D.	O.D./Av.O.D.	Description of abnormalities
0.29625		
.30547	.875	Great brain schism
.31073		
.33789		
.35942		
.37485	1.074	Medullary schism
.37675		
.43082		
.34902 = Av. O.D.		

10. Animal #13A, 274 grams, 125 days old

Embryos: Living 9, resorbed 3, implants 12, abnormal 0, normal 9

O.D.
0.21309
.32692
.32850
.35655
.38454
.38822
.40386
.41295
.35183 = Av. O.D.

Averaging some of the data from these ten animals yields: living (L) 10.4, resorbed (R) 3.0, implants (I) 13.4, abnormal (A) 3.1, normal (N) 7.3; A/I = 0.23, R/I = 0.22, and A/L = 0.30.

Figure 2 depicts a summation of some of the above data from these ten 13-day animals. The abscissa represents for each potential litter the quotient of the number of abnormal embryos divided by the number of implantation sites. The ordinate represents the average optical density for each potential litter. In addition, the latter contains three additional points representing the arithmetic means of individual optical densities of those potential litters possessing identical (or, in one case, very similar) abscissal values. These three additional points, appearing graphically as △, were calculated as follows:

Rat #	A/I	Average O.D.	Mean, individual optical densities
2	0/13 = 0	0.52270	.42978
4	0/16 = 0	.40200	
13	0/12 = 0	.35183	
7	2/12 = 0.167	.34902	.40210
9	2/11 = 0.182	.44927	

Rat #	A/I	Average O.D.	Mean, individual optical densities
11	4/13 = 0.308	0.37490	
14	4/13 = 0.308	.22422	.31711
15	4/13 = 0.308	.37880	

Figure 2 displays a dashed line connecting five points, three being the arithmetic means of individual optical densities and two being average optical densities. It is readily seen that the higher the average optical density of a potential litter, the lower is the proportion of abnormal embryos.

B. 20-day fetuses.

The following six tables contain data obtained from rats bearing 20-day fetuses. These maternal rats were members of the same Sprague-Dawley population sample to which the first seven of the above ten 13-day maternal rats belonged. Both the weight and age data for the maternal animals refer to values at the time of injection of the teratogen. Each of the six pregnant animals received 75 mg Trypan blue/kg body weight.

1. Animal #10, 292 grams, 110 days old

Embryos: Living 11, resorbed 6, implants 17, abnormal 6, normal 5

Abnormal embryo #	Description of abnormalities
1	Hind legs crossed, slightest bifida
2	Micro-ophthalmia, left
3	Torsion of fetus
4	Bifida
5	Slightest meningocoele
6	Bifida, micro-ophthalmia, right

2. Animal #6, 272 grams, 114 days old

Embryos: Living 7, resorbed 6, implants 13, abnormal 4, normal 3

Abnormal embryo #	Description of abnormalities
1	Shortened tail
2	Shortened tail, circular flexion; probable short trunk
3	Tailless; large meningocoele
4	Exencephaly; tongue extrusion

3. Animal #7, 306 grams, 115 days old

Embryos: Living 6, resorbed 8, implants 14, abnormal 2, normal 4

Abnormal embryo #	Description of abnormalities
1	Abnormal right eye
2	Left eye missing

4. Animal #12, 302 grams, 123 days old

Embryos: Living 8, resorbed 4, implants 12, abnormal 1, normal 7

Abnormal embryo #	Description of abnormalities
1	Agenesis of trunk; exencephaly; heartbeat vigorous

5. Animal #13, 310 grams, 124 days old

Embryos: Living 2, resorbed 9, implants 11, abnormal 0, normal 2

6. Animal #8, 318 grams, 124 days old

Embryos: Living 11, resorbed 2, implants 13, abnormal 1, normal 10

Abnormal embryo #	Description of abnormalities
1	Left eye missing

Averaging some of the data from these six animals yields: living (L) 7.5, resorbed (R) 5.8, implants (I) 13.3, abnormal (A) 2.3, normal (N) 5.2; $A/I = 0.175$, $R/I = 0.44$, and $A/L = 0.31$.

C. 12-day embryos.

The following four tables contain data obtained from rats bearing 12-day embryos. Both the weight and age data for the maternal animals refer to values at the time of injection of the teratogen. Each of

the four pregnant animals received 75 mg Trypan blue/kg body weight. The explanations for the three columns are identical to those for the three columns of 13-day embryo data of section A.

1. Animal #1, 292 grams, 121 days old

Embryos: Living 11, resorbed 3, implants 14, abnormal 2 plus 2 probable, normal 7

O.D.	O.D./Av.O.D.	Description of abnormalities
0.09085	0.645	Mid-gut open, smaller embryo, abnormal curling
.12436		
.12494	.888	Caudal body torsion, large caudal hematoma, mid-gut open, probable diminution of blood
.14939		
.15021		
.15334		
.15724		
.15740		
.15840		
.14068 = Av. O.D.		

2. Animal #6, 308 grams, 121 days old

Embryos: Living 13, resorbed 1, implants 14, abnormal 2, normal 9 plus 2 probable

O.D.	O.D./Av.O.D.	Description of abnormalities
0.16467		
.18333		
.19179		
.20395		
.20824	0.966	Slightest irregularity in neuraxis
.21097		
.21610		
.24010		
.24325	1.128	Slightest irregularity in neuraxis, probable pericardial edema
.25119		
.25697		
.21551 = Av. O.D.		

3. Animal #8, 290 grams, 122 days old

Embryos: Living 12, resorbed 0, implants 12, abnormal 1, normal 11

O.D.
0.16150
.17006
.17631
.18002
.18535
.18890
.19067
.19179
.20551
.18335 = Av. O.D.

4. Animal #12, 302 grams, 129 days old

Embryos: Living 12, resorbed 1, implants 13, abnormal 2, normal 10

O.D.	O.D./Av.O.D.	Description of abnormalities
0.12031		
.12263		
.12990		
.13035		
.13083		
.13131		
.14200		
.14331	1.075	Irregularity in neural canal
.14919		
.13331 = Av. O.D.		

Figure 3 depicts a summation of some of the above data from these four 12-day animals. The abscissa represents for each potential litter the quotient of the number of abnormal embryos divided by the number of implantation sites. The ordinate represents the average O.D. for each potential litter. No particular association exists between average optical density of a potential litter and the proportion of abnormal embryos. It is to be noted, however, that the next to lowest average optical density is displayed by the group with the highest proportion of abnormal living embryos.

D. Observations on additional 12-day embryos.

A review of the experimental results on 12-day embryos suggested that the dosage level employed was insufficient for the production of an acceptable proportion of abnormalities per maternal rat. Therefore, the previous group contained only four animals. So an additional group of 12-day rats, injected with the increased dosage of 100 mg Trypan blue/kg body weight, was to be analyzed for dye content in the visceral yolk sacs of their developing offspring. Table 1 A summarizes much of the data taken from these eleven animals. The lower portion of this table compares this information with that procured from the previous group. As expected, the elevated dosage level increased the prevalence of abnormalities and resorptions.

Table 1 A

Maternal rat #	Embryos (L) Living	(R) Resorbed	(I) Implants	(A) Abnormal	(N) Normal
2	13	2	15	5	8
10	12	4	16	2	10
20	4	2	6	1	3
27	9	5	14	2	7
23	13	3	16	5	8
19	13	1	14	3	10
13	13	4	17	4	9
17	12	3	15	5	7
6	11	4	15	4	7
8	10	2	12	3	7
3	9	4	13	7	2
Average	10.8	3.1	13.9	3.7	7.1

	A/I	R/I	A/L
Av. values, eleven 12-day, 100 mg/kg	0.27	0.22	0.35
Av. values, four 12-day, 75 mg/kg	0.17	0.095	0.19

The dye content analyses were never performed, however, for the gross examination of the first embryos of this series strongly suggested the totally unexpected presence of dye in their caudal portions. Beginning with the fourth maternal rat, #27, the developing offspring of the eight remaining animals were carefully scrutinized for blue coloration as well as for malformations. Table 1 B lists for these eight incipient litters the incidence of data notations for each of twelve general classes of abnormality. The incidence is entered in either of two columns, depending on whether or not the abnormal embryo contained blue coloration.

Table 1 B

	Number of data notations	
Abnormalities, 12 day	In the 26 embryos lacking color	In the 7 embryos possessing color
Neural canal	13	5
Caudal hematoma	11	
Extra-caudal blister	4	
Trunk torsion or flexion	3	1
Neuropore, posterior	3	
Bifida	2	1
Brain	2	1
Tail	2	1
Size, diminished	2	1
Heart	2	1
Mid-gut	2	3
Somite	1	

Blue coloration appeared in 18 of the normal embryos and in seven of the abnormal embryos. This represents coloration in 32% (18 of 57) of the normal embryos, 21% (7 of 33) of the abnormal embryos. Each of the color-containing normal and abnormal embryos displayed this color within the tail region. Examination of Table 1 B reveals 15 notations of hematomas or blisters in the 26 abnormal embryos lacking blue coloration. This incidence of 58% is in marked contrast with the

total lack of either clear or blood-filled blisters among the seven abnormal embryos possessing blue coloration.

E. Observations on a final sample of 12-day embryos: improved lighting conditions.

Twelve-day embryos of a different strain of rats were examined after improving viewing conditions by the utilization of a white translucent plastic wafer beneath the glass vessel containing the saline-immersed embryo. Three Sprague-Dawley rats were compared with four Long-Evans rats. These Long-Evans animals, obtained from Dr. F. Robert Brush of this institution's Department of Medical Psychology, were considerably more physically active and purportedly (2) more resistant to the teratogenic effects of the dye than those of the Sprague-Dawley strain. Table 2 A illustrates that the Long-Evans animals were indeed more resistant.

Table 2 A

Averages:	Sprague-Dawley rats	Long-Evans rats
Living (L)	10.3	10.25
Resorbed (R)	4.0	0.75
Implants (I)	14.3	11.0
Abnormal (A)	5.7	1.5
Normal (N)	4.7	8.75
A/I	39.5%	13.6%
R/I	27.9%	6.8%
A/L	54.8%	14.6%

Table 2 B summarizes more of the data obtained from these seven animals, which were injected with the quantity of dye (100 mg/kg body weight) shown in the previous 12-day series to yield an acceptable proportion of abnormal embryos.

Table 2 B

Rat #; LRIAN data	Normals: Comments on color	Abnormals: Comments on color	Abnormals: Sites of abnormalities
Sprague #2; 10,2,12,5*,5	3 of 5 have color, in tail	1. No color	Neural canal; bifida
		2. Color, in tail	Neural canal
		3. Color, in tail	Exencephaly; mid-gut; tail; neural canal
		4. Color, in tail	Neural canal
Sprague #9; 13,4,17,4**,9*	8 of 8 have color, in tail	1. Color, in tail	Tail; neural canal
		2. Color, in tail	Tail; neural canal; somites
Sprague #13; 8,6,14,8**,0		1. No color	Caudal blister; neural canal; mid-gut
		2. Perhaps, in branchial pouches	Caudal blister perhaps over bifida; neural canal has sharp irregularities
		3. Some, in tail	Neural canal has appreciable bend
		4. Perhaps, in branchial pouches. Very strong in tail	Neural canal has many sharp irregularities
		5. Very strong in caudal portion of tail	Mid-gut; right-angled trunk flexion
		6. Extends cephalad into embryo	Mid-gut; right-angled trunk flexion
Long-Evans #3; 11,0,11,1,10	8 of 10 have color, in tail	1. Very large, dark streak in tail, below the blister	Caudal blister
Long-Evans #11; 9,0,9,2,7	6 of 7 have color, in tail. One of six has color in branchial pouches	1. No color	Mid-gut; neural canal
		2. Color, in tail	Exencephaly

Rat #; LRIAN data	Normals: Comments on color	Abnormals: Comments on color	Abnormals: Sites of abnormalities
Long-Evans #30; 11,1,12,2,9**	7 of 7 have color, in tail	1. Slight color, in tail	Neural canal, slightest irregularity
		2. Color, in tail	Neural canal, several small irregularities
Long-Evans #21; 10,2,12,1,9**	7 of 7 have color, in tail. Color of one is very strong	1. Color, very strong, in tail	Neural canal, slight irregularity

* = One embryo was damaged and discarded prior to examination
** = Two embryos were damaged and discarded prior to examination

Color was detected in 87% (54 of 62) of the embryos examined; 28% (25 of 90) of the embryos in the previous group of eight 12-day rats displayed such color. The dramatic improvement in detection was obviously due to the alteration in the viewing apparatus. The color was distributed equally between normal and abnormal embryos, for 89% (39 of 44) of the normals and 83% (15 of 18) of the abnormals contained the evidence of dye deposition.

A closer examination of Table 2 B reveals two interesting relationships. (1) Consider the first Sprague-Dawley animal, #2. One of the four abnormal embryos lacked blue coloration; this embryo displayed a bifida. Furthermore, one of the six abnormal embryos of Sprague-Dawley #13 also lacked blue coloration; this embryo possessed a caudal blister. The second abnormal embryo of the latter rat was perhaps colored in the area of the branchial pouches, not in the usual caudal region, and it possessed a caudal blister. As noted, this blister may have obscured a bifida. Some additional observations on embryos of similar characteristics have suggested some causal relationship between

a blister and the subsequent appearance in that site of a spina bifida. Therefore, here are three instances of a correlation between the absence of caudal coloration and the presence of a caudally situated blister or bifida. This correlation coincides with the relationship previously noted in Table 1 B. Even though the capacity to detect dye was reduced in that set of observations, it was noted that 58% of the embryos lacking the caudal color had caudal or extra-caudal blisters, whereas blisters were absent whenever blue color was seen. Two examples from a more resistant strain seem to contradict this relationship. Among the few abnormal embryos of the Long-Evans rats, the only one with a caudal blister also possessed a very noticeable blue streak ventral to the abnormality. The only abnormal Long-Evans embryo lacking caudal color displayed an abnormal midgut and a neural canal irregularity. (2) The maternal animal (Sprague-Dawley #13) with the highest proportion of abnormal embryos displayed the highest proportion of embryos containing dye in loci cephalic to the tail region. Of six abnormal embryos, two perhaps contained dye in the branchial pouches, and one definitely contained dye forward of its usual site of deposition. It is to be noted that one of the apparently <u>normal</u> Long-Evans embryos contained dye in the branchial pouch area, while another <u>normal</u> contained a high concentration of caudally situated dye.

Observations on the embryos of three additional rats should be incidentally recorded here. The embryos of two experimental (100 mg/kg) 11-day Sprague-Dawley animals presented very faint, but unmistakable, evidences of tail color. A third experimental (100 mg/kg) Sprague-Dawley, several months older than those previously studied, exhibited

12-day embryos; the majority of these contained caudal coloration.

F. Localization of dye within the embryo (Bouin's-cedarwood procedure).

The paucity of the dye and the opacity of the tissues had thus far interfered with accurate localization of the intra-embryonic color, and additional failures were recorded in this attempt. Photographic definition was little improved by micro-resection of interfering portions of fresh or fixed embryos. Butylamine solutions of fresh embryo tails from dye-injected maternal animals displayed no optical density differences (6000 Angstroms) when compared with similar control preparations; the visual sensation of color was produced by dye concentrations which were obviously very low. So a technique was needed for increasing the accuracy of demarcation of the sites of blue color, a technique which could be applied to embryos already stored in Bouin's fixative solution. The clearing procedure outlined in this dissertation's section dealing with methodology satisfied these two criteria. The procedure was applied to Bouin's-fixed embryos judged heretofore as either containing or lacking dye, as well as to Bouin's-fixed embryos fixed prior to the initial observation of embryonic blue coloration. The embryos varied as to age (11-day to 13-day) and strain (Sprague-Dawley or Long-Evans); the maternal animals varied as to dosage level (40 to 100 mg/kg). All of the fixed embryos from eight potential litters were scrutinized upon completion of the clearing procedure. A summary of these observations, grouped according to embryonic age, is presented below along with some observations on color-containing embryos of the mouse and the hamster. Controls consisted of embryos from uninjected maternal animals; in no instance was blue coloration detected in any embryonic or extra-embryonic region.

Gross observations: rat

Eleven day (averaging 18 somites): The ten examined embryos of this age group were obtained from a rat that had received 100 mg/kg Trypan blue. Only one of them displayed malformations (bilateral head blisters and widely-patent anterior neuropore), but its pattern of dye deposition was not atypical. Dye, seen in all ten, extended in an uninterrupted line from an area near the tip of the tail to a point slightly cephalic to the line of attachment of the gut to the yolk sac. Slight alterations in this pattern appeared to be the result of imperfect clearing. Dye concentration seemed strongest in the tail-gut, somewhat reduced in the anterior portion of the line of distribution, and weakest in the intervening area, that of the mid-gut.

Twelve-day: The 34 embryos of this age group came from four females, two of which received 75 mg/kg dye and two 100 mg/kg. All displayed unmistakable evidence of dye in the gut. The pattern of dye deposition was remarkably constant; Figure 4 illustrates a typical embryo of this age group. The heaviest dye concentrations were localized in a portion of the tail-gut opposite the allantoic diverticulum; the dye extended ventrad for a short distance into the allantoic stalk. From this area of heavy deposition a blue line continued caudad ending near the tip of the tail. Again from the allantoic "patch", a line of dye of lesser concentration extended cephalad within the gut lumen to a point just caudal to the liver. This blue color faded markedly in the region of juncture with the yolk stalk.

One embryo (Fig. 5) displayed a markedly different dye pattern. This embryo, from a Long-Evans female injected with 100 mg/kg, exhibited an extensive blue coloration in the fore-gut. The dye was easily seen

in the branchial pouches of this living embryo during examination in saline. The fixation and clearing procedure, which permitted the display of the pattern invariably seen in the caudal segment of the fixed gut, did not alter the distribution of the dye seen in this embryo prior to death. It must be emphasized that this instance was the only radical departure from the pattern of dye distribution described above.

As noted in the description of 11-day embryos, no significant differences in dye distribution could be detected between the apparently normal and the abnormal embryos. However, comparisons of embryos of two rats, both injected with 100 mg/kg, showed that dye was more apparent in the embryos from the rat with the higher percentage of abnormal offspring.

Thirteen-day: The 31 embryos of this age group came from three females; two, of different strains, received 75 mg/kg dye, and one 40 mg/kg. All displayed unmistakable evidence of dye in the gut. The dye was visible in two areas in nearly every embryo. The allantoic "patch" persisted, as did its caudal prolongation; variable amounts of dye extended for a short distance cephalad from the allantoic "patch". In addition, dye was repeatedly seen in the proximal segment of the caudal limb of the mid-gut loop. Scattered deposits of dye were often detected in both the cephalic and the caudal limbs of the mid-gut loop. The embryos from the maternal animal injected with 40 mg/kg displayed identical patterns of dye distribution but the coloration was very faint. The abnormal and the normal embryos had similar patterns of distribution.

Gross observations: mouse and hamster

The bulk of observations on members of these two species will

constitute a line of inquiry apart from the goals of this dissertation, but a portion of these observations are of direct relevance here. Briefly, embryos of these species also displayed evidence of intra-embryonic dye, disposed in locales similar to those seen in the rat. Of specific interest is a typical example of the observations recorded on the mouse embryos. Adult Swiss-Webster mice, injected at approximately seven and three-tenths days with 0.25 cc. of the teratogenic solution (= 5 mg. of dye), were sacrificed at approximately 10.5 days. The mouse visceral yolk sacs contained the dye as larger, less uniformly distributed flakes than those of the rat yolk sacs. Of 12 implantation sites, two were mis-handled during laparotomy and two were resorbed, leaving eight living concepti. Each of the eight embryos presented several malformations and blisters. The clearing procedure revealed dye in each of the eight as large, non-confluent globules. In three instances, the caudally located color extended approximately to the trunk mid-point, in two instances to the caudal border of the heart, and in three instances to points cephalic to the heart. In comparing mouse with rat, this elongation of the antero-posterior extent of color is of particular interest.

DISCUSSION

The data on dye concentration in individual 13-day visceral yolk sacs does not lend support to the proposed likelihood that the site of action of the dye is within the visceral yolk sac, for the higher the litter average concentration of color, the less the percentage of abnormality within that particular litter. At least three possible explanations could be thought of for such a relationship. Firstly, a sufficient amount of the harmful compound may be phagocytized by the visceral sac cells so that an insufficient concentration of dye is present to act directly on the embryonic tissue. Stated conversely, any diminution in phagocytic efficiency results in an increase in the amount of dye in the fluids bathing the embryonic cells; such an increase in excess of the concentration threshold for direct harm would yield a pattern of malformation. Secondly, if one could have examined at an earlier stage the yolk sac surrounding a 13-day malformed embryo, an analysis might have shown a dye concentration higher than, or as high as, that of a sac surrounding an adjacent, normal conceptus, as originally proposed. This hypothesis is based on the supposition that by the 13th day, certain of the cells of the sac of the abnormal embryo may have either desquamated intact, as suggested by the observations of the 12-day embryos reported by Davis and Gunberg (139), or partially or wholly disintegrated. The literature notes several instances of the disintegration of cells which have been exposed to Trypan blue, but for the sake of continuity, let us briefly postpone our consideration of these instances. Some estimate of the value of this second explanation would be provided by the assay of sacs of 12-day, or earlier, embryos. Since the Bouin's-cedarwood oil procedure showed dye in each and every

embryo, even of the youngest stages examined (11-day), regardless of the presence or absence of malformed tissues, no judgment could be made as to the probable relevance to the teratogenic mechanism of desquamated cells, cell fragments, or proteins as carriers of dye. A third explanation for the inverse relationship noted above would direct attention toward the maternal animal as the site of teratogenic action. Since the litter average concentration of color is a direct reflection of serum dye concentration, a comparatively large uptake of dye by components of the maternal reticulo-endothelial system would deprive the visceral yolk sac cells of dye, and the malfunction of these maternal components could have a teratogenic impact on the developing offspring.

Let us now briefly examine our postponed consideration of reported instances of the degeneration of cells which phagocytize Trypan blue. Disintegration was described in the uterine decidua of the guinea pig (7) and in members of the genus, Paramoecia (1). Cell degeneration was evident in the epithelium of the proximal convoluted tubule of the rat six hours after dye injection, and frank cellular necrosis was described two days after treatment (54). It has also been reported (53) that Kupffer cells of rat liver degenerate after holding the dye. Other colloids may also injure phagocytic cells, as noted (65) in the uterine cavity of the rat injected with lithium carmine.

Several assumptions have received no previous expression. In computing the ratio of abnormality to implantation, the presence of one slight departure from normal marks that embryo as abnormal, whereas an adjacent embryo, scoring as no less normal, might display a multitude of apparently severe external malformations. The only justification for such a scoring system is that it has no substitute. Secondly, the pos-

sibility exists that the color uptake in the envelope surrounding a malformed embryo is affected by the abnormal metabolism of the latter. Mitosis or phagocytic activity could be examples of the yolk sac parameters affected. Again, analyses of yolk sacs of embryos younger than 13 days should provide pertinent information. Finally, tighter controls should be applied in future efforts. For example, the utilization of an "average optical density" value fails to fully compensate for size differences among yolk sac membranes both within and between litters; simultaneous protein determinations, for example, would provide a desirable standard for comparisons.

As indicated previously, the first seven of the ten 13-day maternal rats belong to the population sample encompassing the rats bearing 20-day fetuses. The amount of resorption of the six 20-day fetuses is 43.7% (R/I = 35/80 = 0.437). If this percentage is applied against the first seven maternal members of the 13-day group, it is seen that 41 of the total number of implants (94) should be resorbed by the 20th day. Since only 19 resorptions were actually seen in the 13-day group, 22 more resorptions would probably have occurred had this group been allowed to pursue gestation until the 20th day. It seems probable that these 22 additional resorptions would be derived entirely from the 27 fetuses of the 13-day group which bear some abnormality. This probability is based on two facts: (1) the vast majority of the 13-day abnormalities were not described in the 20-day fetuses, and (2) the work of Beck and Lloyd (100) strongly supports the concept that the majority of resorptions are preceded by malformation. Each of the 27 abnormal embryos in the 13-day group received a decimal value in the data column entitled "O.D./Av. O.D.". This value reflects the color

concentration of the yolk sac in relation to the other sacs of that one potential litter. A value of 1.000, for example, represents an exactly average optical density value. The average of all 27 of these values is 0.97748 (= 26.392/27), which hints that 13-day yolk sac color content values do not serve as indices of whether or not their contiguous embryos will be labelled abnormal, for this average of the 27 values is near unity. So we now see another relationship that detracts from the choice of the yolk sac as the site of teratogenic metabolism. This equal distribution of malformed embryo optical density values on either side of the average of optical density values of normal and malformed embryos also suggests that embryos slated for resorption during an additional seven-day period would not be indicated by their yolk sac assay values. This suggestion of course necessitates our previously expressed assumption that the future resorptions would be those displaying some 13-day abnormality. And furthermore, the value of 0.97748 poses the question as to why, on the average, the average of the optical density values of the normals of a predominantly abnormal potential litter is less than the average of the optical density values of the normals of an all-normal potential litter. This is another way of stating that sacs of 13-day normals from an all-normal potential litter usually contain more color than the sacs of 13-day normals from a predominantly abnormal potential litter.

Table 3

Type of abnormality	Incidence 20-day*	Incidence 13-day	13-day O.D./Av. O.D.+
Spina bifida	5	2	0.858, 0.876
Tail	3	4	0.858, 0.881, 0.945, 1.110
Micro-ophthalmia	3	-	
Anophthalmia	2	-	
Exencephaly	2	2	0.876, 0.899

*Incidences of one were arbitrarily deleted from consideration.
+The origin of these values is indicated by an asterisk in the data section. A double asterisk refers to the utilization of that value in each of two categories.

A final point of minor persuasiveness can be made utilizing the 13-day data. Table 3 summarizes the incidences of each kind of malformation noted in the six 20-day fetuses. It also summarizes the incidences of those 13-day abnormalities which might have developed into the aforementioned malformations had the embryos been allowed an additional week of growth. Each of the 13-day malformations displays its "O.D./Av. O.D." value. The average of these eight values is 0.912, whereas the average of the remaining values is 0.974. This difference suggests that the 13-day malformed embryos of Table 3 might have survived until the 20th day by reason of lesser concentrations of dye in their yolk sacs. To phrase this relationship differently, seven of these eight "position values" (O.D./Av. O.D.) are found at or near the low end of ranked optical density concentrations in their respective litter rankings. To be sure, many of these "position values" are calculated for embryos which display a multitude of malformations; because of this, and of the low number of malformations seen at 13 days which are common at 20 days, the argument might be a specious one. The proposal is, though, that within any one 13-day maternal animal the yolk sacs more heavily dye-laden envelope embryos destined to resorb, whereas the less

heavily dye-laden surround embryos which will survive for an additional week or longer as malformed feti. And importantly, this idea of elevated sac concentration being linked with future resorption (or normality) does not necessarily conflict with our former correlation between high litter average yolk sac concentration and high normality if we continue with the heretofore suggested premise of a direct mode of action--in the all-normal 13-day potential litter, the heavily dye-laden sacs have provided almost a perfect shield for the embryo, whereas in the 13-day potential litter displaying many abnormalities, the yolk sac barrier has earlier been comparatively less perfect, so additional capture of dye from the serum by any one yolk sac dooms its embryo to future death. In other words, the effect of color concentration ranking within the litter must be considered as an entity separate from that of the litter average ranking between litters. The degree of validity of these several arguments should be clarified by analyses of 12-day sacs. Incidentally, note that Table 3 illustrates that eye abnormalities, which were not detectable at 13 days, share the lead in prevalence at 20 days with spina bifida. In this dissertation's introductory section, it was noted that Beck and Lloyd (100) confirmed a conclusion from the Gilbert and Gillman report (118) that no isolated eye defects existed at an early gestation stage (11½ days), yet Beaudoin (119) described his only surviving postpartum rats as being micro- or anophthalmic. Table 3 mirrors these observations reported in the literature, thus reinforcing the worth of the tabular data.

The four 12-day analyses suffer from the small size of the sampling, but as noted, the results do not necessarily conflict with relationships discerned in embryos 24 hours older. The data is included to illustrate

the magnitude and variance of the optical density values, as well as to illustrate in such rapidly growing and highly variable embryos the need for a standard of comparison for yolk sac assays, such as protein concentration. The 12-day results demonstrate the paucity of detectable external malformation resulting from usage of the same dye dosage which produced adequate numbers of malformations in 13-day embryos. In this respect, if a future concept of teratogenesis should require, for the initiation of the disturbance in metabolism, an ontogenetic stage which is more advanced than heretofore supposed, it is important to recall the presence of dye in the caudal portions of the embryo long after maternal serum dye levels have fallen to near-zero. Finally, yolk sacs as young as 11-days have been assayed, but the 12-day data discloses a very real problem in their utilization, which is that a maternal dye dose sufficient to produce moderate abnormality at early stages is quite likely to produce total resorption at later stages. Such early aberrant structures may rather be earmarks of an incipient death which was not preceded by the usual dye-induced malformation. The embryo in such cases was, instead, susceptible to the direct toxic action of the large dye quantity administered to the maternal rat, a possibility admitted by Beck and Lloyd (59).

Attention should now be given to the second of the two areas of results, that of observations of dye within the embryo. A summarative view of the data presented in sections D, E and F reveals an increasing capability in the visualization of intra-embryonic dye deposits. The "Bouin's-cedarwood oil" procedure of section F unmasked the blue color in <u>each</u> of the embryos of eight experimental maternal animals, irrespective of previous categorization of the embryo as normal or abnormal,

"colored" or "not colored". This simple treatment, which permitted dye detection in embryos stored either briefly (24 hours) or for as long as one year in Bouin's fixative, obviously enhanced the resolution of color seen during examination of the 12-day living embryos of sections E and D, in which dye was observed in 87% (54 of 62) and 28% (25 of 90), respectively, of those embryos examined. Beck and Lloyd (59) have stated that "any theory of direct action would strive to establish the presence of dye in the tissues of the embryo". Since this phenomenon has now been adequately established by the previous observations, let us re-examine these observational notations from that point of view. Wislocki's (47, 140) experiments establishing the presence of some intra-embryonic color in species other than rats were conducted on maternal animals which had received excessively large amounts of dye. The color seen in the present work is probably not a reflection of the abnormal breakdown of the barriers to embryonic infiltration by the teratogen, for the Bouin's-cedarwood oil procedure revealed dye in embryos from rats which had received as little as 40 mg/kg body weight. In order to harmonize with a theory of direct action, however, it would be desirable to establish a difference in either depth of color or distribution of color (or both) between normal and abnormal embryos. Yet much of the data is equivocal or in contradistinction to this desirable feature. The evidence in section D is equivocal: color appeared in 21% (7 of 33) of the abnormal, 32% (18 of 57) of the normal. The evidence in section E is even less encouraging in this respect: 85% (11 of 13) of the Sprague embryos, and 90% (28 of 31) of the Long-Evans were normal, 83% (10 of 12) of the Sprague and 83% (5 of 6) of the Long-Evans were abnormal. Even the Bouin's-cedar-

wood oil data of section F revealed no substantial differences.

The question is posed, then, as to the accuracy of the observations of such a lack of color difference. If a true color difference between normal and abnormal embryos does not exist at the time of teratogenic activity, and if the teratogenic mechanism is effective before the time of examination of the above embryos, either the presence or the absence of such a difference at examination time would suggest a mechanism other than a direct one. As another possibility, postulate that a true difference does not exist at the time of examination but did exist at an earlier time, a time of teratogenic activity; this relationship would be compatible with a mechanism of direct dye action. As a third possibility, one again in support of direct action, our difference between normal and abnormal color is postulated to exist during both the teratogenic period and the examination period, but the difference could not be detected by the subjective observations made. If one postulates that at least some of the embryos were examined during part of the period of teratogenic capability, two final choices remain, a fourth and a fifth. If the color difference did not exist, the theory of direct action would suffer; if it did exist, obviously the difference was not detected. Now, in surveying these five possibilities, two inferences emerge. Firstly, the need for a sensitive and accurate method for estimation of dye in the embryo is readily apparent. This need has not been satisfied by radio-active tagging (142). Optimally, in order to determine the degree of correlation between tissue malformation and tissue dye content, such a technique should allow quantitation of dye in discrete portions of an individual embryo. Secondly, if a true difference between normal and abnormal color content does exist, we have to postulate that this

necessarily small difference, this unseen dye, can directly cause damage to embryonic cells. The inability of these embryonic cells to segregate the dye into sharply demarcated sites is a phenomenon in agreement with observations on the chick (141). Based both on these present results and on those of Wilson, Shepard and Gennaro (142), it is obvious that the concentration of Trypan blue in the rat embryo is not high. Also, recall from section F that butylamine solutions of fresh embryo tails from injected animals displayed no optical density differences when compared with similar control preparations. However, since reports of teratogenic effects of Trypan blue on chick (143, 144), amphibian embryos (145) and cultured rat embryos (96) indicate that a low concentration of this dye in direct contact with embryonic tissues is capable of eliciting malformations, a direct mode of action is not excluded by the difficulty of seeing the dye, this "necessarily small difference". In fact, two of the above papers (144, 145) reported lack of color both in embryos and in malformed young.

The previous paragraph lists a number of possible explanations for the apparent lack of difference in blue color intensity between normal and abnormal embryos. Admittedly, the teratogenic relevance of this blue color is unknown, as is its carrier, but a hypothesis of direct action via dye absorption within the embryonic gut can be supported by several observations now to be more carefully considered. (1) The maternal animal of section E (Sprague #13, Table 2A) which displayed by far the highest percentage of abnormal embryos also scored highest in embryos featuring blue coloration in areas cephalic to the usual caudal sites of deposition. (2) The data exemplified the correlation between caudal blisters, clear or blood-filled, and a lack of color in

the tail region; yet in each of these examples, the supposed lack of color was shown by the Bouin's-cedarwood oil procedure to be equated rather with the diminution of color. This lowering of dye concentration resulted in lack of color detection by the less desirable methods used. It was as if the cells of these embryos had accepted some of the dye from these caudal areas usually capable of disallowing dispersion of the visible deposits; this acceptance led to necrosis and blistering. In Table 1 B of section D blisters were listed in 58% of the "no blue" group, 0% of the "blue" group. In Table 2 A of section E, the only embryo of Sprague #2 which "lacked" color displayed a bifida. Of Sprague #13 of that section, the only embryo which "lacked" color displayed a caudal blister; another embryo which perhaps had branchial pouch color but no tail color possessed a caudal blister partly obliterating a developing spina bifida. Of a Long-Evans rat of that section, the only abnormal embryo "lacking" color presented severe mid-gut and neural canal malformations. The markedly different patterns of dye distribution among the three age groups of section F bear further witness to the possibility of dye absorption via the embryonic gut. The above statements demonstrate the need for an examination of the ultrastructure of the embryonic gut at these early stages.

The small amount of data from strains and species other than the Sprague-Dawley rat can also be interpreted as supporting the same mechanism of direct dye action via gut absorption. One Long-Evans embryo of section E revealed color in the branchial pouches, another revealed an unusually high concentration of caudally located dye; each of these embryos was apparently normal. A third embryo showed a new combination, color in the tail region ventral to a caudal blister. In

view of the fact that Long-Evans embryos were much more resistant to effects of the dye (see Table 2 A), the above information suggests the capability of these gut cells to resist the potentially harmful effects of the dye. Again, an ultrastructural examination of gut from a similar embryo of this species would be highly desirable. We find additional support in the data from the mouse, in which the gut deposits were quite different from those of the rat, as is the response of the mouse to Trypan blue. Each of eight embryos of the potential mouse litter discussed in detail in section F displayed blistering and multiple malformations, and in all eight the antero-posterior range of color exceeded that normally seen in the rat embryo of comparable age.

A number of reports substantiate one of the implications presented above, that dye-induced blisters can produce malformation. Hamburgh (87) could detect no mouse embryo somite abnormalities other than those caused by blisters. Goda (122) blamed the failure of neural tube closure in rodent embryos on blisters or hematomas in the adjacent mesenchyme. Beck and Lloyd (100), in observing 11.5-day rat embryos, noted that six of eight instances of spina bifida were associated with the displacement of the caudal end of the neural tube by hematomata or fluid-filled vesicles. Two reports of work on the chick provide additional support for our implication. Kaplan and Grabowski (147) painstakingly documented the train of events leading from caudal hematoma to rumplessness. Grabowski (148), after stressing his chick tissue with hypoxia rather than with dye, concluded that brain, eye and limb malformations resulted from the brief presence of a hematoma.

The most readily apparent accumulation of intra-embryonic dye, as noted in the data of the present work, existed within the tail region.

One might wish this accumulation, as well as the often invisible deposits further cephalad in the embryo's gastro-intestinal tract,' to be considered in light of any literature notations of tail malformation. Surely blistering can disturb tissues (vide supra), but blisters are not always apparent in association with malformation. In this respect, however, Beck and Lloyd (100) stated that dye-induced tail defects in rodent embryos do not occur together with abnormalities of the anterior part of the neural tube, indicating the possibility of at least two kinds of response to the dye. Several papers (143, 149) have discussed the peculiar dye sensitivity of such tissues as the tail, and statements in the preceding paragraph support this suggestion. Furthermore, Lyngdoh (113) listed the highest percentage of dye-induced rat defects as that of the tail group. Hamburgh noted (150) tail abnormalities in newborn mice, and observed in embryos that the occurrence of hematomata was most frequent in the tail region (87). Waddington and Carter (74) viewed hematomata particularly on the tails and heads of 12.5-day mouse embryos.

As implied heretofore, a brief examination of the discussion centering on color analyses of 13-day yolk sacs engenders support for a direct mechanism of action. The first two of the three previously listed explanations for the major correlation seen in these analyses provide this support; the third possibility fits the maternal teratogenic locale, a locale choice made doubtful by the arguments of Beaudoin et al. (146). The argument utilizing ranked values of intra-litter yolk sac color concentration additionally prevails against the yolk sac as the principal locale for teratogenesis.

A number of investigators have postulated a direct mechanism of

action for Trypan blue, perhaps in recognition of the fact that the production of malformation by direct contact of embryonic tissue by dye (vide supra) could either obviate the need for the other two mechanisms of action or relegate these two mechanisms to roles as modifying influences. Wilson et al. (95) postulated that Trypan blue affects development by its direct application to embryonic tissues during a period of time prior to completion of yolk sac envelopment of the embryo. Turbow's data (96) satisfies that postulate, but aside from a criticism on the basis of technique, his results could also be construed as favorable to a mechanism centered on the yolk sac. Three considerations are basic to a postulate of direct action. Firstly, it has now been established (31) that the dye and not an impurity is the causative agent. Secondly, any breakdown of dye is probably unrelated to the teratogenic mechanism, and since Trypan blue is not part of a re-dox system, no colorless form exists (59). Thirdly, investigation must disclose it within the embryo; this report satisfies that necessity.

Although investigators have suggested the possibility of a direct teratogenic action of Trypan blue on the mammalian embryo, the inability to demonstrate dye within the rodent embryo or fetus discouraged acceptance of this hypothesis (see 59). Of particular interest is the report by Wilson et al. (142) of an experiment utilizing Trypan blue labelled with radio-active carbon. These investigators were unable to demonstrate significant amounts of labelled dye in 9-, 10-, 11-, 12-, or 20-day rat embryos of females treated with dosages of the tagged dye that were higher than the maximum concentration utilized in the present work. The two opposing conclusions are difficult to reconcile in view of the ease with which dye was seen in the embryos of the pre-

sent work. Fortunately, a number of considerations could account for the failure of light microscope investigations, other than that (139) based on the present work, to note the presence of intra-embryonic dye. Thilander (19) tested the dissolving and decolorizing power of a number of reagents against Trypan blue. Carnoy's solution produced a most pronounced bleaching of the color, and 10% neutral formalin, a fixative commonly used in Trypan blue studies, yielded a dissolution sufficiently pronounced to provoke a suggestion against its utilization. Tincture of iodine and sodium thiosulphate, compounds commonly employed in the removal of crystals precipitated by the mercuric chloride component of some fixatives, also bleached this dye. Thilander (19) agreed with an observation made during the present work that mixtures of formalin and alcohol decolorize the dye. Pertusa (151) also noted that ethyl alcohol destroyed or dissolved vital dyes. The present work fortuitously chose Bouin's fixative, a formulation to which Thilander (19) awarded the label of "best fixing agent".

As Beck and Lloyd (59) pointed out, a number of investigators have presented evidence suggesting the presence of Trypan blue in mammalian embryos. Ferm (80) supported the theory of direct action on mammalian tissue with his demonstration of dye in the blastocyst fluid of Trypan blue-injected rabbits. Barber and Geer (35) reported that mouse embryos sectioned on the seventh, eighth, or ninth day of gestation displayed a faint blue coloration in a portion of the embryonic mass. Kelly et al. (14) suggested a mechanism of direct toxicity based on a red fluorescence noted in the tissues of embryos of treated rats. His dye fractions were of doubtful purity, however, and it is to be remembered that abnormal development is induced only with usage of the

blue fraction. Of particular note, however, is the previous lack of any report of the observation of distinct dye deposits in embryonic cells. The successful employment of the Bouin's-cedarwood oil procedure now constitutes such a report, along with that (139) of light microscope examinations of tissue sections obtained from the embryos described in this dissertation.

In the final analysis, the two types of inquiry reported herein have provided solid support for a direct mode of action of Trypan blue, a support which heretofore lacked a keystone. The color assays of 13-day visceral yolk sacs solubilized in butylamine shifted attention from the yolk sac to the embryo; the data from future assays of 12-day and younger sacs promises additional clarification of the assay value pattern exhibited by yolk sacs from 13-day embryos. The Bouin's-cedarwood oil procedure allows the visualization by other investigators of intra-embryonic dye in fresh or stored tissues of the rat, mouse and hamster. The future use of Lloyd and Beck's (13) identification procedure on blue color solubilized by butylamine from embryonic tissues should provide the ultimate proof, if the penultimate is insufficient, that such color demarcates Trypan blue. The need to establish the importance of the visible and invisible embryonic color deposits to teratogenesis requires a search for the correlation between tissue malformation and tissue color, assuming that the majority of the dye is not metabolized. Since low-concentration dye identification is very difficult with the light microscope, the cell components of the embryonic gut and of contiguous malformed tissue should be the recipient of future ultrastructural studies in light of the probable absorption of dye by this splanchnopleure. Finally, once the teratogenic site has been

delimited by approaches perhaps similar to the above, the disrupting influence of Trypan blue on metabolic systems for growth within the cell can be scrutinized with a greater assurance that the phenomena uncovered do indeed relate to teratogenesis.

The pursuit of a mechanism of action for Trypan blue is not solely an academic one. Hamburgh (150) concluded from his study of embryonic mice aberrations that the effects of Trypan blue resembled some natural mutations. Monie et al. (133) remarked after an exhaustive study in the rat of Trypan blue-induced heart malformations that all of the noted cardiovascular abnormalities had their counterpart in man. Knudsen's (116) upper incisor aberrancies in the mouse were considered by the author as not morphologically distinguishable from those effects produced by maternal injection of vitamin A. Geber (152), by application of audio-visual stress to pregnant rodents, produced some malformations which were similar to those resulting from maternal rodent injection of Trypan blue. The point of the above is not only that Trypan blue effects are similar to those produced either by nature or by other teratogens, but also that all these disruptive influences may at least partially share components of mechanisms of teratogenesis. What that common denominator could be is not the question posed in this dissertation, but it is the essence of the quest being formulated on its foundation.

SUMMARY

The teratogenic site of action of the disazo dye Trypan blue is unknown. It has been suggested that it may be located within the maternal animal, the yolk sac placenta linking maternal and embryonic tissues, or the embryo proper. Two techniques were employed in the attempt to decide among these possibilities. First, a technique was developed for assaying the dye phagocytized by the yolk sac envelope of the 13-day embryo, to ascertain if direct correlation existed between dye and embryonic abnormality. This technique featured (a) the direct solubilization of individual visceral yolk sacs in n-butylamine solutions, and (b) the utilization of a volumetric tube apparatus for the accurate procurement of a 1-ml. portion of the dye solution extracted from an individual sac. Second, a simple schedule for clearing tissues was utilized in search of intra-embryonic dye.

The yolk sac assays unexpectedly supported an inverse correlation between dye concentration and embryonic abnormality. The direct observation of embryos revealed unequivocal evidence of dye within the developing gastro-intestinal tract.

The results of the two approaches provided significant support to the hypothesis of a direct mechanism of action of Trypan blue in the production of malformed rodent embryos.

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Figure 1

Volumetric tube apparatus (3/4 actual size)

E = position of eye
H = plastic guide housing
L = lens system
M = micrometer screw
T = centrifuge tube, 5 ml.

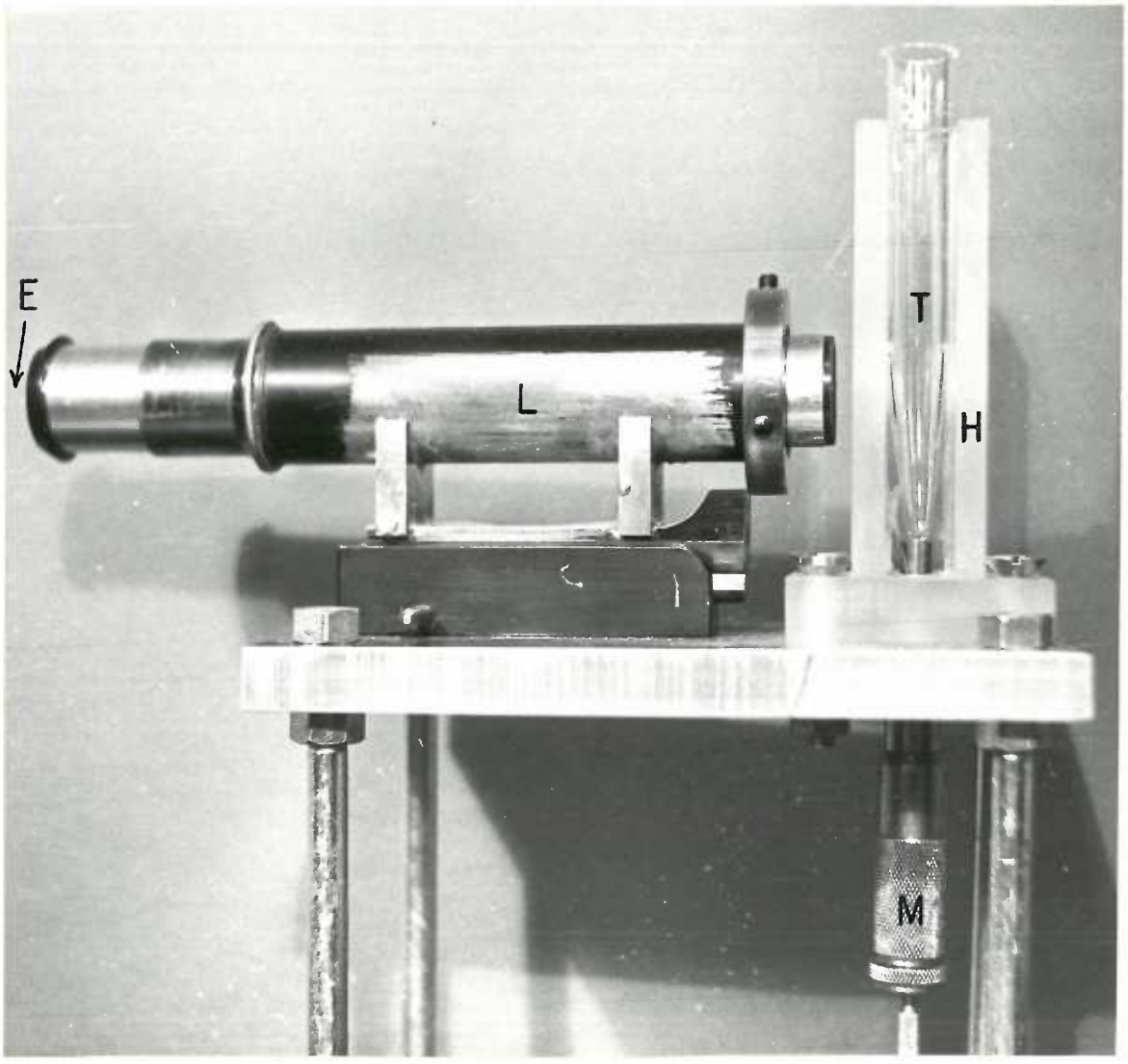
E
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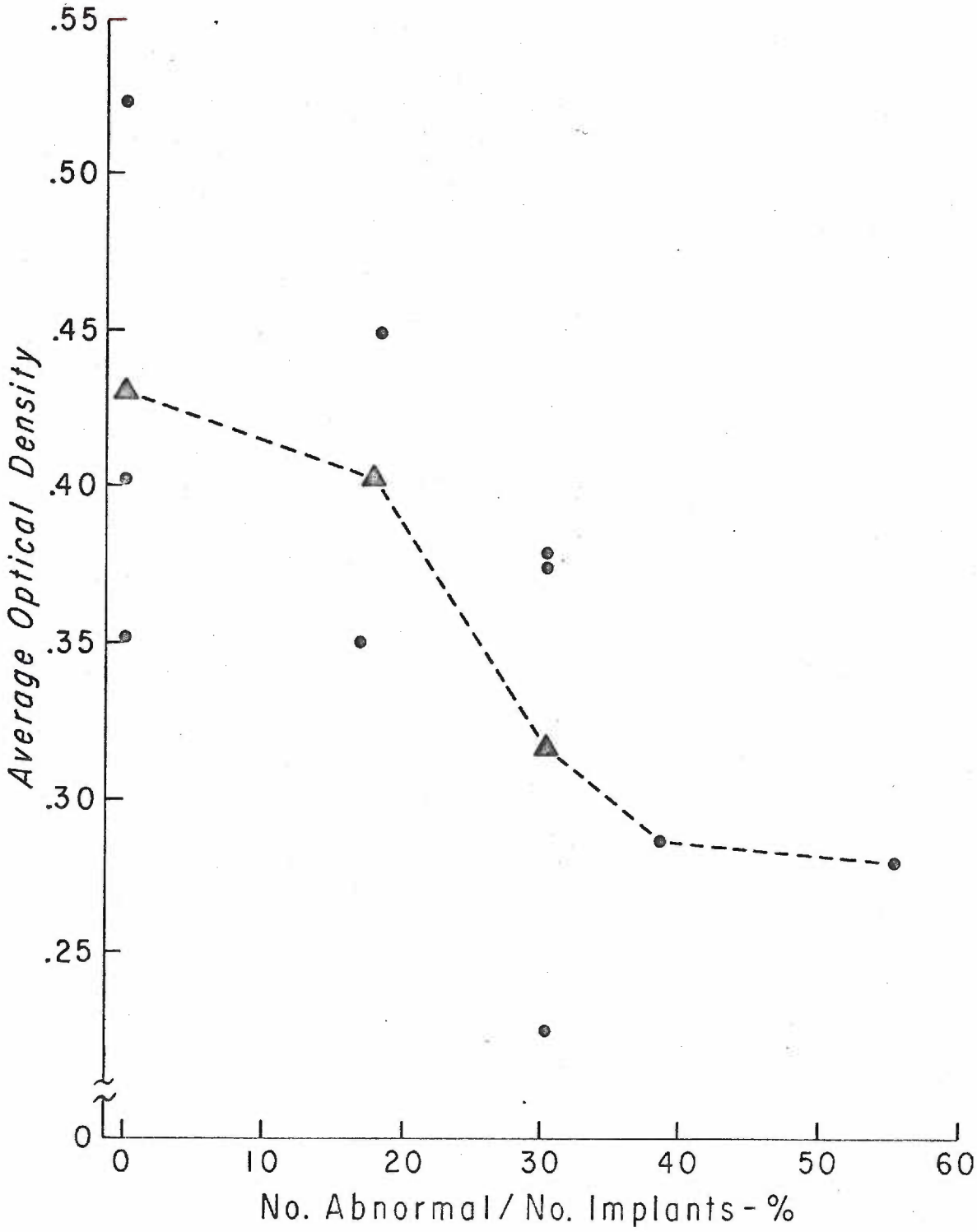
Figure 2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Average Optical Density
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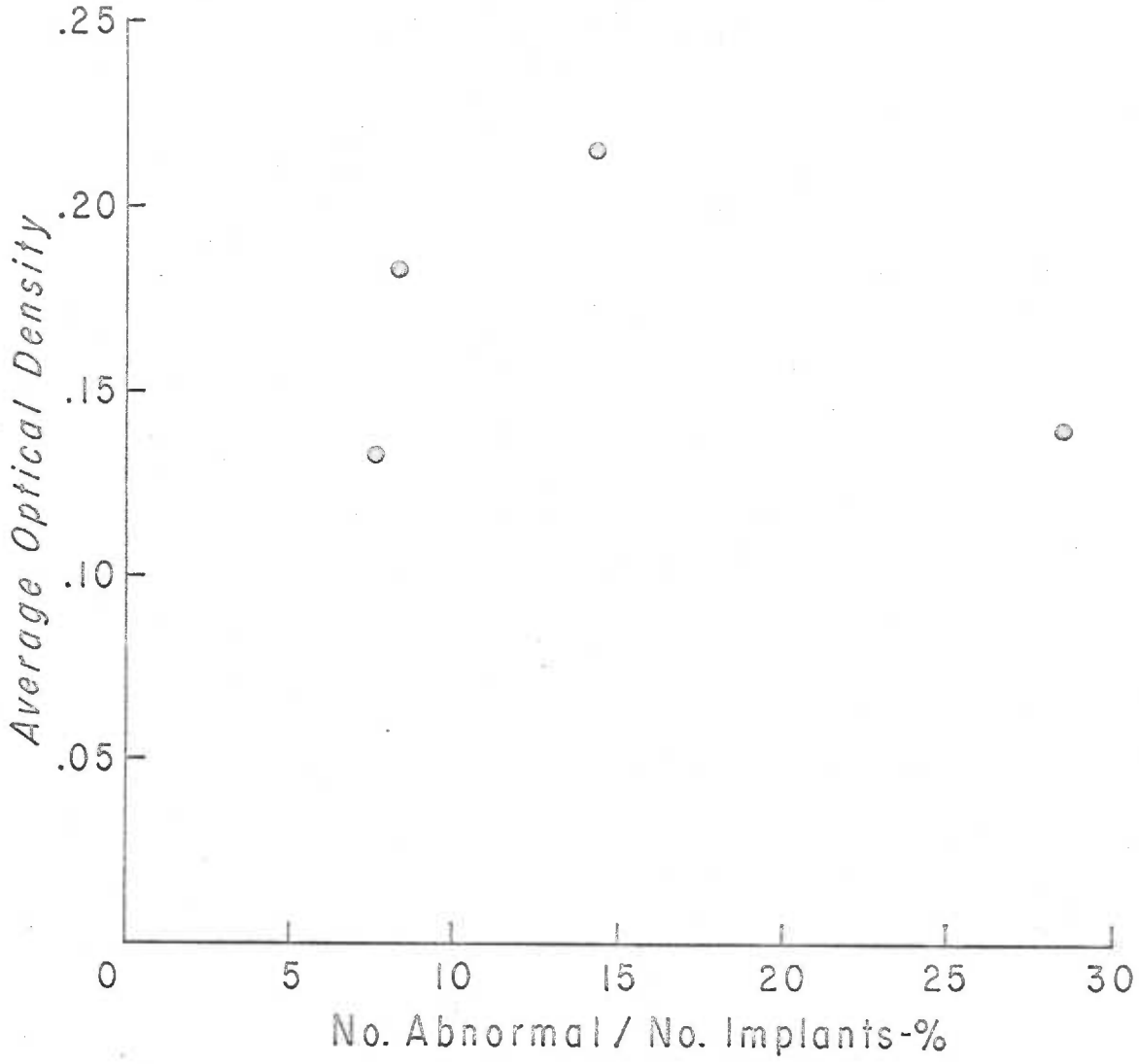
No. Abnormal / No. Implants - %

Figure 3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No. Abnormal / No. Implants-%

*Figure 4**

*Twelve-day rat embryo, 100 mg/kg group. The embryo was fixed in Bouin's, embedded in celloidin, and cleared in cedarwood oil. This apparently normal embryo displays the typical pattern of distribution of Trypan blue in the gut. A portion of the dorsal surface of the tail was removed to assist in seeing the dye. 23 X. *(139)*

*Figure 5**

*Twelve-day rat embryo, 100 mg/kg group. The embryo was fixed in Bouin's, embedded in celloidin, and cleared in cedarwood oil. This abnormal embryo displays the typical pattern of distribution of Trypan blue in the hind-gut. In addition, the dye has atypically invaded the fore-gut and is especially well seen through the pharyngeal clefts. 23 X. *(139)*