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ANALYSIS OF TRYPAN BLUE INDUCED MALFORMATIONS IN FROG AND MOUSE EMBRYOS

by

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American Zoologist, 7:745), and at the 1969 meeting of the American Association of Anatomists (abstract in the Anatomical Record, 163:191).

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I. Introduction

A. Teratogenesis as a tool in the study of mammalian embryology.

Teratology is the branch of biology devoted to the study of the development, anatomy and physiology of congenitally abnormal individuals. In experimental embryology, agents capable of producing malformations have been important tools in the analysis of tissue interactions in development and their control mechanisms. Before tissue culture techniques were perfected the causal analysis of mammalian embryogenesis relied almost exclusively on the analysis of experimentally produced teratogenesis and on the investigation of gene mutations affecting development.

The list of agents that have been employed to produce congenital malformations in mammals is continually growing. Below are recorded in tabular form many of the various agents that have been shown to be teratogenic (tables 1 to 5).

Unfortunately, the amount of information derived from these studies is less than one might expect considering the number of agents that have been tried and the variety of abnormalities that can be produced. The few generalizations one may extract from these studies can be summarized as follows:

1. Teratogenic agents are most effective during the early period of differentiation. Each organ and system appears to have a susceptible period early in the differentiation of its primordium (Wilson, 1959). This phase, commonly referred to as the "critical period", corresponds to the time just after "induction or chemical differentiation has occurred" (Wilson, 1965) when the organ is usually developing most rapidly (Dagg, 1966). Susceptibility to teratogenesis generally decreases as differentiation and organogenesis proceed and progressively larger doses of an agent are required to produce comparable malformations as organ formation advances (Wilson, 1959, 1965). Upon completion of organogenesis, the embryo enters the fetal period and becomes progressively more resistant to teratogenic agents administered to the mother. Thus in mammals, malformations do not occur when teratogenic agents such as irradiation (Russell, 1950; Wilson, 1954), vitamin deficiencies (Nelson, et al., 1955) or vitamin excess are applied during the cleavage and blastocyst stages of development. Treatment with X-rays, hormones or certain radiomimetic drugs during the fetal period may inhibit growth or cause pathologic degeneration but rarely causes malformations (Hicks, 1954).

2. Each teratogenic agent acts in a specific way and produces a characteristic but not necessarily unique

pattern of malformations (tables 1 to 5). If the pattern of malformations produced by two agents that are applied at identical developmental stages are entirely different, it is reasonable to assume that the agents act by interfering with entirely different processes of embryonic metabolism (Wilson, 1959).

3. The response to a teratogenic agent is dependent upon the intensity and duration of the stimulus. Typically, the range of doses can be divided into a subthreshold, teratogenic, and lethal range (Wilson, 1965; Dagg, 1966). Beck and Lloyd (1964) found that treatment of pregnant rats with 5 mg/kg body wt of trypan blue resulted in only 1% malformation. Increasing the dosage to 50 mg/kg body wt increases the incidence of malformation to over 20%, but at 100 mg/kg body wt almost 95% of the fetuses were resorbed and few if any of the surviving fetuses were malformed.

4. Both the severity and the pattern of an embryo's reaction to a teratogenic agent is influenced by the genetic background. One demonstration of the importance of the overall genetic background is the often reported observation that different strains and substrains of experimental animals react differently to the same teratogenic agent. For example, when response to maternal injection of trypan blue was studied, both the percentage of abnormal embryos and the types of anomalies

observed varied with the particular strain of rat or mouse chosen for investigation (Gunberg, 1958; for other examples see Fraser and Fainstat, 1951; Beck, 1964; Hamburgh and Callahan, 1967). Conversely, it has also been shown that the expressivity and penetrance of a given mutant can be influenced by agents that are effective in inducing malformations. Thus Beck (1963) was able to distinguish mouse embryos heterozygous for the recessive gene ey (eyeless) from homozygous normal embryos on the basis of their response to maternal injection of trypan blue. Heterozygotes for the gene ey developed normally when untreated but showed various degrees of microphthalmia and anophthalmia when subjected to trypan blue treatment in utero. Wild type homozygotes were unaffected by the same dose of dye. Landauer (1957) and Runner (1959) have reported similar results with respect to defects caused by nutritional deficiencies.

The exact mechanisms by which environmental agents interfere in developmental physiology and cause malformations have so far eluded identification. It is obviously difficult to find a common denominator by which agents differing as widely as those listed in tables 1 to 5 cause similar or identical errors in development. With the conquest of infectious disease, the identification of the causes of congenital malformations has become one of the major problems of

medical biology. It is generally concluded that the hazards that are known to interfere with normal development such as X-rays, viral infections, or drug poisoning account for only a fraction of the vital statistics of birth defects and that by far the greatest percentage of congenital abnormalities must be a consequence of either genetic influence or of very subtle changes of the fetal environment that remain yet to be identified (Arey, 1965).

One of the major obstacles facing teratologists in their search for mechanisms is that so little is known about the site of action or the localization within the embryo of the numerous chemical substances that have been found to produce teratogenic effects. The discovery in 1948 by Gillman, Gilbert, Gillman, and Spence that the vital dye, trypan blue, is a potent teratogen in rats raised the hope that at last a teratogenic agent had been found whose site of action and intraembryonic localization could be followed with relative ease. It was therefore expected that research with this dye would eventually yield information about the physiological mechanisms that operate in teratogenesis. These hopes have not been fully realized.

B. Trypan blue as a teratogenic agent

Trypan blue is a member of a group of diazo dyes

first synthesized in the late nineteenth century for use as textile dyes (fig. 1). The ease with which this dye becomes bound to proteins led to its use as a chemotherapeutic agent, notably against trypanosome infections in laboratory animals (Evans and Schulemann, 1914); hence the name trypan blue. Goldmann (1909, 1912) first demonstrated that when injected into animals, acid diazo dyes are selectively taken up by the reticulo-endothelial system, a circumstance that made them useful in revealing the extent of this tissue throughout the body. Goldmann (1909, 1912) also noticed that vital staining of the rat placenta was not shared by the fetuses. This observation was confirmed for the gravid rabbit, guinea pig, and cat by Wislocki (1920, 1921) and in the rat by Everett (1935) who noted that trypan blue accumulated in the visceral yolk sac epithelium of 11 to 16 day embryos.

Largely as a result of the widespread use of trypan blue as a vital stain, this dye has been found to possess a variety of pharmacological and pathological properties. Among the latter are carcinogenesis (Gillman, Gillman, and Gilbert, 1949), depression of thyroid activity (Yamada, 1960), and teratogenesis (Gillman, et al., 1948). Soon after the first report of trypan blue induced teratogenesis in rats by Gillman, et al. (1948) the teratogenic action of this dye was confirmed in rats (Fox and Goss, 1955, 1956, 1957; Gunberg,

1956; Warkany, Wilson, and Geiger, 1958) and extended to the mouse (Hamburgh, 1952, 1954; Waddington and Carter, 1952, 1953), to the rabbit (Harm, 1954), to the guinea pig (Hoar and Salem, 1961), and to the hamster (Ferm, 1958). Exposure of chick, amphibian, or fish embryos to trypan blue was also found to result in teratogenesis (Ancel and Lallemand, 1941; Beaudoin and Wilson, 1958; Waddington and Perry, 1956; Greenhouse and Hamburgh, 1968; Battle and Laale, 1960).

Attempts to elucidate the mechanism of action of this teratogen have been complicated by the repeated observation that the dye either does not penetrate the embryonic tissue proper, or if it does, accumulates only in minute amounts (Goldmann, 1909, 1912; Everett, 1935; Gillman, et al., 1948; Hamburgh, 1954; Wilson, Shepard, and Gennaro, 1963; Waddington and Perry, 1956; Greenhouse and Hamburgh, 1968). Although Ferm (1956) was able to demonstrate the presence of trypan blue in the fluid of blastocysts obtained from rabbits injected with the dye, he did not find any evidence of dye within embryonic tissues. Davis and Gunberg (1968) have reported traces of trypan blue in gut cells of rat embryos whose mothers had been injected with dye on the 12th to 14th day of gestation. Barber and Geer (1964) reported that 7 to 9 day old mouse egg cylinders exhibited a faint and diffuse blue coloration of the embryonic mass.

However, in the most sensitive study so far undertaken, Wilson, et al. (1963) failed to demonstrate radioactivity in rat embryos treated with ^{14}C-labeled trypan blue and concluded that it is highly unlikely that even minute quantities of the dye can penetrate past the placenta into the mammalian embryo.

In recent years, interest has centered on the hypothesis that interference with a nutritive function of the yolk sac placenta is the primary event in trypan blue induced teratogenesis. Several factors have combined to focus attention on this organ: (1) There is considerable evidence implicating the rodent yolk sac placenta in the etiology of other types of experimentally induced congenital malformations. Johnson and Spinuzzi (1966, 1968) found changes in the pattern of appearance of certain isozymes within cells of the yolk sac in response to teratogenic doses of 9-methyl pteroylglutamic acid, a folic acid antagonist. Slotnick and Brent (1966) reported the accumulation of maternally injected teratogenic anti-rat-kidney antiserum within this organ but not within the other embryonic tissues. (2) It has been repeatedly observed that the yolk sac epithelium is the only embryonic tissue that accumulates trypan blue (Everett, 1935; Wislocki, 1920, 1921; Hamburgh, 1954). (3) The teratogenic effect of the diazo dye is virtually confined to the period

when this organ is the sole source of embryonic nourishment (Beck, Lloyd, and Griffiths, 1967a).

The most cogent evidence in support of the hypothesis implicating the yolk sac placenta in teratogenesis has come from observations of F. Beck and coworkers. Prompted by Trump's (1961) demonstration of intracellular segregation of trypan blue in lysophagosomes of kidney cells, Beck (1965) and Lloyd, Beck, Griffiths, and Parry (1968) have shown that in chick and rat embryos trypan blue in the yolk sac epithelium is segregated into lysophagosomes. They have also demonstrated (Beck, et al., 1967a) that in vitro, trypan blue is a potent inhibitor of several lysosomal enzymes such as acid phosphatase, RNAase, DNAase, and β-glucuronidase.

On the basis of these observations, Beck, et al. (1967a) advanced the hypothesis that trypan blue might exert its teratogenic effect by inhibiting the lysosomal enzymes of the visceral yolk sac at a time when this organ functions in the digestion of macromolecules essential for embryonic nutrition. The conceptus would consequently be deprived of materials essential for normal development. However, Beck's theory of trypan blue activity is based solely on observations obtained from mammalian and chick embryos and does not explain reports suggesting that the dye also induces malformations in amphibian and fish embryos, organisms not dependent on a yolk sac placenta.

Furthermore, it is obvious that the *in vitro* studies demonstrating enzyme inhibition by trypan blue must be supplemented with studies *in vivo*, in which nonlethal but teratogenic doses of this dye are utilized.

C. Specific aims of the present study

The present study was therefore undertaken to reinvestigate the problem of the localization and site of action of the trypan blue in both amphibian and mammalian embryos. Specifically, the answers to the following questions were sought: (1) Do the types of malformations observed in amphibian embryos following exposure to trypan blue resemble the spectrum of abnormalities reported in mammalian embryos? (2) In amphibians, does the dye actually penetrate into the embryonic tissue? (3) Do the jelly coats and vitelline envelope of the amphibian egg operate in any way so as to influence the action of the dye on amphibian morphogenesis? (4) Does the stage of development at which the amphibian egg is sensitive to the teratogenic action of the dye correspond to the critical period of susceptibility in mammals? (5) Is the teratogenic effect of the exposure to the dye reversible or irreversible? (6) In mammals, does the injection of nonlethal but teratogenic doses of trypan blue into pregnant females alter the activities of hydrolytic

and oxidative enzymes in cells of the yolk sac placenta *in vivo*? (7) Is there any correlation between the effect of the dye on the yolk sac and the incidence of malformations in mouse embryos?

II. Trypan blue induced teratogenesis in Rana pipiens embryos

A. Materials and Methods

Female Rana pipiens were induced to ovulate by Rugh's method (1962). The eggs were stripped into dry finger bowls, fertilized by pipetting sperm suspension over them, flooded with spring water, and incubated at 18°C±2°C. Only clutches of eggs which proved to be 95% fertile or better were used for experiments. Embryos were raised in finger bowls and staged according to Shumway (1940). Unless otherwise indicated, Great Bear spring water was used in maintaining the embryos. After fertilization, the jelly coats of the eggs were manually removed with watchmaker's forceps before placing them into trypan blue solution. In some cases, the vitelline envelope was removed at the two cell stage. Eggs were exposed to concentrations of trypan blue ranging from 0.1% to 0.5%. The actual concentration used in any series of experiments was adjusted to compensate for the variability in response among different batches of eggs. In order to determine the proper concentration of dye to be used samples of eggs from each female were placed into solutions of 0.5%, 0.25%, or 0.1% trypan blue and allowed to develop to neurula stage at room temperature. Nonlethal concentrations causing high incidence of abnormality were chosen for subsequent

experiments. During these preliminary determinations the remainder of the eggs in each batch were allowed to develop at reduced rates on a cold water trough. In designing experiments, each experimental group was paired with control eggs obtained from the same female. In amphibians, occasional batches of eggs are encountered which exhibit a high frequency of spontaneous malformations. If, during the course of an experiment it became apparent that the incidence of spontaneous malformations exceeded ± 5% the experiment was discarded.

A series of experiments were carried out to test the effect of varying the duration of exposure to the dye and the stage at which treatment with the dye was initiated. In one series (1) of experiments, eggs were placed into trypan blue solution at the two cell stage (Shumway stage 3) and treatment was continued until control embryos reached Shumway stage 21 (mouth open, cornea transparent). Experimental embryos suffered developmental arrest at this stage. In another series of experiments (2), eggs were placed into trypan blue at Shumway stage 3 (2 cells), and small groups were removed at successive intervals and allowed to continue development in fresh spring water. Embryos were transferred to fresh spring water at the following stages: mid-cleavage (Shumway stage 8), dorsal lip (Shumway stage 10), mid-gastrula

Shumway stage 11), neural plate (Shumway stage 13), neural folds (Shumway stage 14), rotation (Shumway stage 15), and stage of muscular response (Shumway stage 18). In a third series of experiments (3), normal embryos were initially reared in spring water and trypan blue treatment was initiated at successively later stages of development starting with stage 8 (mid-cleavage) and continuing through stages 9 (late cleavage), 10 (dorsal lip), 11 (yolk plug), 13 (neural plate), 14 (neurol folds), and 21 (open mouth, cornea transparent).

Commercial dye lots of trypan blue usually contain high quantities of NaCl (Lloyd and Beck, 1963). In order to determine whether the teratogenicity in our sample resided in this contaminent, both dialyzed and undialyzed samples of trypan blue were tested. A 0.5% solution of dye in distilled water was dialyzed against distilled water for 5 days. Additional tests included the raising of frog eggs in distilled water and in 0.25% NaCl solution.

In the preparation of amphibian material for microscopic analysis special precautions were taken to avoid leaching out any trypan blue which might have entered embryonic tissues. Embryos were fixed in a solution containing 2 parts Bouin's fluid to 1 part dioxane. Tissues were dehydrated and cleared in dioxane, embedded in paraffin, and serially sectioned at 8-10μ. Substitution of dioxane for alcohol offered

two advantages. First, dioxane facilitates the sectioning of yolk laden tissues (Puckett, 1937) and second, whereas trypan blue is soluble in ethanol, the dye is insoluble in dioxane. The choice of Bouin's as a fixative was dictated by previous observations (Thilander, 1964) that use of this fixative causes neither decolorization of trypan blue nor dissolution of the dye in vitally stained tissues. Sections were stained with either carmalum, hematoxylin and eosin, or Patay's trichrome method for connective tissue.

B. Results

1. Comparison of undialyzed and dialyzed samples of trypan blue.

Both the undialyzed and dialyzed trypan blue solutions were found to be teratogenic to Rana pipiens embryos (tables 6 and 7). However, dialysis reduced considerably the teratogenic activity of the dye from an incidence of 100% to approximately 35%. This loss in potency of the dialyzed trypan blue sample could not be prevented by addition of NaCl or spring water to the dye. The subsequent finding (on the basis of O. D. measurements) that dialysis reduced the dye concentration by about 20% may explain the loss in teratogenicity.

2. Stage dependency and reversibility of the teratogenic action of trypan blue (undialyzed sample).

Eggs placed into a solution of trypan blue shortly after fertilization but removed to pure spring water prior to stage 9 (late blastula) developed normally. On the other

hand, the full syndrome of malformations was observed to occur in eggs that were placed into trypan blue solution as late as Shumway stages 10, 11, 12, or 13 (figs. 2, 4, 5, 7, and 8). Embryos placed into trypan blue at stage 14 (neural folds) developed relatively normally although tail outgrowth was sometimes retarded (fig. 7). Exposure to trypan blue at still later stages had no observable effect on subsequent development of embryos.

Abnormalities already in the process of formation as a consequence of exposure to trypan blue could not be reversed when such embryos were subsequently removed from dye solution to fresh spring water. Embryos which were placed into trypan blue solution at Shumway stage 10 and removed to fresh spring water at Shumway stage 11 exhibited the same abnormalities at Shumway stage 14 as did embryos which had been developing in trypan blue throughout the critical period (fig. 8, table 7).

3. The syndrome of malformations caused by treatment with trypan blue (undialyzed sample).

a. Macroscopic Analysis (fig. 2). At the concentrations of trypan blue used throughout the course of these experiments, there was no apparent increase in mortality prior to gastrulation. Rana pipiens eggs exposed to 0.5% trypan blue solution at the two cell stage could not be distinguished

from untreated embryos until late stage 13 or early stage 14, at which time the neural folds normally appeared, and the first elongation of the antero-posterior axis became observable. In the experimental animals, the elongation of the main body axis seemed to be inhibited and neural folds in the presumptive brain region were clearly abnormal in appearance. The latter, instead of flaring outward to encompass the entire anterior dorsal region, remained close together in experimental embryos. The furrow separating the two folds did not extend into the brain region, which appeared as a rather narrow, raised, solid block of cells. Subsequent development of these embryos resulted in larvae with foreshortened trunks and microcephaly. The extent of microcephaly varied in degree from minor decreases in head size to complete absence of the head (fig. 2).

The outgrowth of the posterior axis was also affected in experimental embryos. Elongation of the tail was often completely inhibited or, when the tail did emerge, the resulting structure was often kinked and aberrant in shape and proportion (figs. 2, 7, and 8). In some cases, the posterior neural folds never completely fused and a remnant of the blastopore persisted. Many dye treated embryos became edematous after reaching stage 15 or 16. Trypan blue treated embryos never exceeded 5 mm in length, nor did they

ever survive beyond Shumway stage 21. All control embryos exhibited spontaneous muscular contractions by 96 hours post-fertilization. By contrast, experimental embryos never exhibited muscular contraction, even when vigorously pricked with a dissecting needle.

b. Microscopic analysis. Examination of 75 serially sectioned embryos revealed that both the chordamesoderm and the central nervous system were severely damaged in embryos that were cultured in trypan blue.

(i) Notochordal abnormalities (figs. 3 and 4). Of 35 experimental animals examined at various ages including neural fold, tail bud and free swimming stages, only two had normal notochords. In 20 animals, three different staining procedures employing either carmalum, hematoxylin, or Patay's trichrome method failed to reveal the presence of any notochord in either the anterior or posterior regions of the body axis. In 13 embryos notochordal structures developed in the anterior half of the body only, but these were sometimes off center and/or barely distinguishable from surrounding mesoderm (figs. 3 and 4).

(ii) Mesodermal abnormalities (figs. 3 and 4). The state of differentiation of the somitic mesoderm usually reflected the extent of notochordal damage. In regions where the notochord was present in its normal position,

the mesoderm was separated into two lateral sheets with metameric segmentation. In those areas where there was no notochord, the lateral mesoderm on each flank formed an unsegmented mass with fusion of the left and right sides beneath the neural tube. In cases where an anterior notochord was present, the anterior mesoderm was segmented and approximated that of untreated embryos in appearance, whereas in the posterior regions lacking a notochord, the mesoderm formed an unstructured mass of tissue whose left and right sides fused beneath the neural tube (figs. 3 and 4).

(iii) Abnormalities of the neural tube (figs. 3, 4, 5, and 6). Examination of serial sections of 50 experimental embryos showed abnormalities of the neural tissue in all cases (figs. 3 and 4). In the forebrain the abnormalities included absence of the diencephalon, the optic cups, and cranial ganglia (fig. 5). Mid- and hind-brain structures were usually present but frequently in a disorganized state. The extent of differentiation of the neural tube posterior to the hind-brain varied with the state of differentiation of the adjacent notochord. A normal notochord was almost always accompanied by a fairly well-developed neural tube, whereas in areas in which the notochord was absent or only poorly developed, the neural tube usually resembled a narrow solid cylinder or was absent altogether (fig. 6).

4. Penetration of dye into the embryo.

a. The embryo proper. Special precautions were taken in the fixation and dehydration of the tissue to avoid leaching-out of trypan blue that might have accumulated in the embryo proper (see materials and methods). Careful study of 50 serially sectioned embryos failed to reveal any trace of the dye within embryonic cells.

b. The jelly coats and vitelline envelope. A comparison of eggs in which the jelly coats were left intact with a selected batch of eggs in which a special effort was made to remove the vitelline envelope after fertilization and before trypan blue exposure revealed that the presence of either one of these envelopes did not substantially affect the development of amphibian eggs. Furthermore, eggs denuded of their vitelline envelope and/or jelly coat and subsequently reared in trypan blue solution developed the same syndrome of malformations as did embryos whose outer membranes were left intact.

III. Analysis of trypan blue induced malformations in mouse embryos (Mus musculus)

A. Materials and Methods

1. Animal maintenance and general protocol.

Swiss albino mice of the CD1 strain from the Charles River Breeding Laboratories were used throughout the course of this study. This strain has a low incidence of spontaneously occurring abnormalities (table 8). Females were placed in mating cages, 4 females to 1 male, each evening and checked for vaginal plugs on the following morning. The date on which a vaginal plug was observed was regarded as the first day of pregnancy. Pregnant females were given one 0.25 ml intraperitoneal injection of 1% trypan blue (National Aniline) in 0.9% NaCl on the morning of either the 7th, 8th, 9th, 10th, 11th, 12th, 13th, or 14th day of gestation. This dosage was chosen because preliminary experiments had indicated that when injected on either the 7th, 8th, or 9th days of pregnancy it yielded a maximum number of malformations and a minimum of resorptions. Control animals were injected with 0.25 ml of 0.9% NaCl solution. The critical period of trypan blue induced abnormalities in the mouse has been well documented by several investigators (see Hamburgh 1952, 1954; Waddington and Carter 1952, 1953; Hamburgh and Callahan 1967).

2. Collection and preparation of egg cylinders and yolk sacs for histological analysis.

Females were sacrificed by cervical dislocation on the 8th, 9th, 10th, 11th, 12th, or 13th day of pregnancy. Uteri with embryos implanted were removed and fixed in Bouin's solution. Randomly selected implants were dehydrated, cleared, embedded in paraffin, serially sectioned at 7-10μ, and lightly stained with Romeis' carmalum. In some cases dioxane was substituted for alcohol in order to prevent any possibility of leaching trypan blue from the tissues during fixation and dehydration. Sections were examined microscopically in order to determine whether trypan blue had accumulated within the embryonic cells or fetal membranes. Special emphasis was given to an examination of the yolk sac epithelium to ascertain whether the distribution of trypan blue within cells of this organ was uniform, or whether there were regional variations with respect to its deposition.

In addition, the histological appearance of randomly selected embryos on the 8th and 9th days of gestation 24 and 48 hours after their mothers had been injected with trypan blue was compared with that of control embryos which had not been exposed to the teratogen.

3. Collection and preparation of yolk sacs for histochemistry.

At intervals between the 9th and 16th day of gestation experimental and control females were sacrificed by cervical dislocation. Pregnant uteri were immediately dissected out and either frozen on dry ice or fixed in buffered neutral formalin at 4°C, depending on the requirements of the histochemical procedure to be used. Routinely the first three implants of either uterine horn were chosen for histochemistry. All frozen implants were sectioned at 14μ in a cryostat at -14°C, mounted on warm coverslips and kept in the cryostat until used. Formalin fixed yolk sacs were stored in 30% sucrose solution at 4°C until used.

4. Histochemical procedures.

Four histochemically demonstrable enzymes were compared in trypan blue loaded and untreated yolk sacs at different stages of gestation. Acid phosphatase, a lysosomal constituent, thiamine pyrophosphatase, an enzyme associated with the Golgi apparatus, succinic dehydrogenase, a mitochondrial enzyme, and glucose-6-phosphate dehydrogenase, a cytoplasmic enzyme, were chosen for study. For each run, control and experimental animals were sacrificed together. All sections to be compared were incubated simultaneously "back to back" in the same dish. Substrate free controls were run for all

histochemical procedures.

(a) Acid phosphatase. Three different methods were employed for the demonstration of acid phosphatase activity. In addition to the lead sulfide method developed by Gomori (1950), the simultaneous coupling azo dye methods of Burstone (1958) and of Barka (1960) were employed.

Tissues under investigation by the Gomori (1950) technique were sectioned in the cryostat, mounted on warm coverslips, and kept at -14°C until used. Immediately prior to incubation the sections were removed from the cryostat and fixed in cold (4°C) buffered neutral formalin for 1 hour. They were then rinsed in cold distilled water and incubated at 37°C for 2 hours in a medium containing excess of the substrate Na-β-glycerophosphate (0.01M), $MnCl_2$ as enzyme activator (Barka and Anderson, 1963), acetate buffer at pH 5.3, and $PbNO_3$. The product of the reaction is $PbPO_4$, an insoluble colorless precipitate. After incubation, sections were rinsed in distilled water and immersed in 2% NH_4S solution for 1 minute to convert the $PbPO_4$ to the easily visualized brown PbS. Sections were washed again, and mounted on slides with glycerine jelly. In controls consisting of sections boiled for 5 minutes no enzyme activity was apparent.

For the Barka (1960) procedure, sections prepared

as above were fixed for 1 hour in cold ($4^{\circ}C$) filtered formol calcium, rinsed in distilled water, and incubated for 1-5 hours at $37^{\circ}C$ in a medium containing as substrate naphthol AS-TR phosphate (Sigma) dissolved in N,N dimethylformamide, diazotized pararosanilin, and veronal acetate buffer, pH 5. After incubation, sections were rinsed, dehydrated by passing through a graded series of alcohols and xylene, and mounted on slides with Permount. In some cases, formalin fixed whole yolk sacs were incubated in the above medium and then sectioned in paraffin at 7μ. Sodium flouride (10^{-2}M) added to the incubation medium inhibited this reaction. The mechanism of this reaction involves the formation of an insoluble and highly substantive (Barka and Anderson, 1962) azo dye by the coupling of a diazonium salt to an aromatic compound. The diazonium salt is freshly prepared by the standard procedure of dissolving a primary aromatic amine, in this case pararosanilin, in dilute aqueous mineral acid and adding aqueous sodium nitrite. When added to the incubation medium, the diazotized pararosanilin spontaneously couples to the substituted α-naphthol (aromatic compound) which is liberated by the acid hydrolysis of the naphthol AS-TR phosphate.

The third method used to demonstrate acid phosphatase activity was that of Burstone (1958). Cryostat sections prepared as previously described were fixed for 1 hour

in cold (4°C) charcoal filtered acetone to remove free lipid. Rinsing in distilled water was followed by a 3 hour incubation at 37°C in a medium containing naphthol AS-BI phosphate (Sigma) as substrate, fast red violet salt LB (diazonium salt), $MnCl_2$ as activator, and acetate buffer, pH 5.4. The mechanism of this reaction is essentially the same as for the Barka procedure. An insoluble azo dye is formed by coupling of the diazonium salt, fast red violet LB, with the aromatic compound liberated by the acid hydrolysis of the naphthol AS-BI phosphate.

(b) Thiamine pyrophosphatase. The method of Novikoff and Goldfischer (1961) was employed for the study of this enzyme. Cryostat sections prepared for the study of thiamine pyrophosphatase activity were mounted on warm coverslips and kept in the cryostat at -14°C. Immediately prior to incubation the sections were fixed for 1 hour in cold (4°C) filtered formol calcium. After rinsing in cold distilled water, mounted sections were incubated for 1-2 hours at 37°C in a medium containing the substrate cocarboxylase (thiamine pyrophosphate [Sigma]), $MnCl_2$ as activator, lead nitrate, and tris maleate buffer, pH 7.2. The product of this reaction is lead phosphate, an insoluble colorless precipitate. After incubation the sections were rinsed, immersed for 1 minute in 2% NH_4S, rinsed again, and

mounted on slides with glycerine jelly.

(c) Succinic dehydrogenase. Freshly cut cryostat sections were mounted on warm coverslips and incubated for 30 minutes at 37°C in a medium containing the substrate sodium succinate, Nitro BT (Sigma), a tetrazolium salt which acts as a hydrogen acceptor, Ringer's solution, and phosphate buffer, pH 7.4 (Barka and Anderson, 1963, modification of Nachlas et al., 1957). After incubation sections were briefly rinsed in cold distilled water and postfixed for 1 hour in cold 10% formalin. The sections were rinsed again in distilled water and mounted on slides with glycerine jelly. In controls consisting of sections boiled for 5 minutes, no enzyme activity was discernible. The mechanism of this reaction depends on the reduction of the soluble Nitro BT to an insoluble blue formazan salt.

(d) Glucose-6-phosphate dehydrogenase. A modification of the technique suggested by Barka and Anderson (1963) was used for the study of this enzyme. Freshly cut cryostat sections were incubated at 37°C in a medium containing the substrate glucose-6-phosphate (Sigma), Nitro BT (Sigma), TPN (Sigma), $MgCl_2$ as enzyme activator, and phosphate buffer, pH 7.4. After incubation, the sections were rinsed and postfixed in formol alcohol for 1 hour, rinsed again, and mounted on slides with glycerine jelly. The

mechanism of the reaction is essentially the same as that for succinic dehydrogenase.

In order to determine whether there existed any correlation between trypan blue induced malformed embryos and histochemically demonstrable enzyme changes within the yolk sac placenta, embryos from 7 randomly selected trypan blue injected mothers were removed from the uteri with their yolk sacs intact, examined for malformation with the aid of a dissecting microscope and compared with embryos obtained from normal untreated mothers. For histochemical study, malformed embryos were separated from normal littermates. These procedures were carried out in cold 0.9% saline. The embryos were quickly blotted dry, frozen on dry ice, and divided into three groups. Group I consisted of embryos from saline injected control females, Group II consisted of malformed embryos from trypan blue treated females, and Group III consisted of apparently normal embryos from trypan blue treated females. The frozen implants were then processed for histochemistry as described above and enzyme activity was compared among the three groups.

B. Results

1. Description of trypan blue induced malformations

Thirty-one trypan blue treated females sacrificed between the 10th and 13th days of gestation yielded a total

of 349 embryos of which 82 exhibited various degrees of grossly visible malformations (table 8). The abnormalities most commonly included (in order of occurrence) were exencephaly, hematomata, tail kinks and tail shortening, and spina bifida. In addition, the uteri of some females showed resorption sites of which no count was made.

2. Uptake of trypan blue by the yolk sac

Examination of the yolk sacs of embryos which had been exposed to trypan blue revealed that the dye had accumulated within all cells of the visceral endoderm. No trypan blue was ever observed within cells of the parietal endoderm.

When pregnant females were injected with dye on the 7th day of gestation and sacrificed 24 hours later, trypan blue granules were found within both the squamous embryonic and the columnar extraembryonic cells of the proximal endoderm. Thus both the presumptive visceral yolk sac and the presumptive gut epithelial cells took up the dye. The larger cells of the early extraembryonic endoderm were prominently loaded with large numbers of dye granules (fig. 9). In embryonic endoderm, however, because of the flattened and attenuated appearance of the tissue the intracellular dye granules could only be visualized with an oil immersion lens. Thirty-four embryos were observed in this age group and all presented the same appearance.

Yolk sacs from embryos 9 days of age or older injected with trypan blue 24 or 48 hours prior to sacrifice presented a somewhat different picture. By this stage of development the visceral yolk sac completely surrounds the embryo and may be divided into two morphologically distinct regions, a villous area adjacent to the chorioallantoic placenta composed of columnar cells, plus a nonvillous region of cuboidal cells surrounding the remainder of the embryo (fig. 10). Due to their larger size, the columnar cells of the villous portion of the yolk sac may contain more dye per cell than the smaller cuboidal cells of the nonvillous portion of the yolk sac. However, all visceral endoderm cells contained large intracellular deposits of trypan blue within 24 hours of maternal injection. The overall impression was one of uniform dye deposition. This pattern of dye accumulation was identical in yolk sacs of both normal and malformed embryos. Yolk sacs from 17 trypan blue treated embryos were observed in this age group.

3. The histological appearance of mouse embryos (M. musc.) obtained from trypan blue treated mothers on the 8th and 9th days of gestation.

A comparison of the histological appearance of control and trypan blue treated mouse embryos on the 8th day and 9th day of gestation was made. This stage of development is

characterized by the appearance of the primitive streak and of the head process, and is the critical period during which trypan blue induced malformations may be initiated. The 7 to 8 day egg cylinder is a trilaminar structure enclosing a central lumen. The innermost layer is composed of ectoderm, the outermost of endoderm, and the middle layer of proliferating mesoderm and head process. In sections of control embryos, the central layer of mesoderm cells formed a loosely packed tissue which was closely apposed by the inner mass of tightly packed ectoderm cells and an outer ring of proximal endoderm cells (fig. 11a). With respect to the arrangement of the primary germ layers the histological appearance of sections obtained from trypan blue treated egg cylinders was often different from those of controls. In experimental animals, there was a tendency for the proximal endoderm to become pulled away from the mesoderm and for the mesoderm to become more loosely packed. In some instances, blebs or blisters also seemed to be present under the endoderm. Although transverse sections of control embryos were generally round or elliptical in shape, sections of trypan blue treated embryos appeared less symmetrical and often somewhat bent (fig. 11b). This disturbance was noted in 82% (28 of 34) of the egg cylinders observed during the 8th day of gestation, but was no longer visible in the embryos observed during the 9th day of gestation (see table 10).

4. Distribution and increase in acid phosphatase activity in the developing yolk sac endoderm of the mouse.(M. musc.)

The three histochemical methods employed for the study of this enzyme indicated that intensity of staining, an index of acid phosphatase activity (Padykula, 1958), increased in the visceral endoderm as gestation proceeded. Activity increased gradually until at the 12th or 13th day maximum staining was obtained. This level of activity was maintained until the 16th day of gestation when the study was terminated. The Gomori (1950) and Barka (1960) procedures revealed a low level of activity during the 8th and 9th days and a noticeable increase during the 10th and 11th days of gestation reaching maximum intensity by the 12th or 13th day of pregnancy. With the Burstone (1958) technique, no activity was observed until the 12th day of pregnancy.

Acid phosphatase activity was generally restricted to the supranuclear region adjacent to the brush border of the cell. The localization of the enzyme at the apex of the cell was most distinct in the Barka and Burstone preparations. No activity was observed in the mesoderm cells of the visceral yolk sac (figs. 12, 14, and 16).

5. Effect of trypan blue treatment on acid phosphatase activity in the yolk sac endoderm of the mouse.(M. musc.)

In most cases, injection of trypan blue into pregnant females was followed by a depression of acid phosphatase

activity in the visceral endoderm of the yolk sac but not in the parietal endoderm. When females were injected with the dye on the 7th, 8th, or 9th day of pregnancy, depression of enzyme activity in endoderm cells was observed within 24 hours. Enzyme inhibition continued to increase in severity with time and by the 4th or 5th day post-injection, some trypan blue loaded yolk sacs displayed virtually no acid phosphatase activity at all in the visceral endoderm (table 9, figs. 13, 15, and 17).

The time of trypan blue treatment did not influence the pattern of subsequent enzyme depression. Females injected as late as the 10th, 12th, or 14th day of gestation showed as marked a depression of acid phosphatase activity as did those animals which were injected on the 7th, 8th, or 9th days of gestation (figs. 13, 15, and 17).

Trypan blue affects not only the intensity but also the intracellular distribution of acid phosphatase. In untreated embryos, enzyme activity was localized in the supranuclear (apical) region of the endoderm cells, while activity in cells from trypan blue loaded yolk sacs was often evenly dispersed throughout the cytoplasm. This effect was most clearly demonstrated by the azo dye methods. Diffuse staining was usually but not invariably accompanied by depression

in the overall intensity of the reaction (figs. 12 and 13).

No correlation was found between changes in acid phosphatase activity and visible malformations in the trypan blue treated embryos. Almost all embryos obtained from trypan blue treated mothers exhibited depression of acid phosphatase activity, whereas only approximately 25% of all embryos obtained from trypan blue treated mice were visibly malformed (tables 8 and 9). Examination of littermates from trypan blue treated females revealed that grossly normal embryos exhibited as marked a depression in enzyme activity as did their malformed sibs.

Conclusions concerning acid phosphatase activity were based on observations on 63 embryos from 31 saline injected females and 97 embryos from 45 trypan blue injected females (table 9).

6. Distribution of thiamine pyrophosphatase activity in the mouse yolk sac and the effect of trypan blue treatment.

Thiamine pyrophosphatase activity is considered to be a marker of Golgi membranes in all cells (Novikoff and Goldfischer, 1961; Meek and Bradbury, 1963). The visceral yolk sac endoderm and the parietal endoderm cells exhibited strong thiamine pyrophosphatase activity at all stages of gestation between the 8th and 15th days (figs. 18

and 20). Thiamine pyrophosphatase positive structures, linear in appearance, were located adjacent to nuclei. Sections of visceral epithelium obtained from embryos of untreated mothers were easily distinguishable from sections of trypan blue loaded yolk sacs; in contrast to the discrete character of Golgi organelles in the controls, in yolk sacs obtained from embryos of treated mothers they appeared fragmented, thin and occasionally granular. Trypan blue treated embryos displayed these structural alterations whether or not they exhibited gross malformations (figs. 19 and 21, tables 8 and 9). Examination of littermates obtained from trypan blue treated females revealed that alteration of thiamine pyrophosphatase positive structures was as marked in yolk sacs of grossly normal embryos as in their grossly malformed sibs. These results are based on the study of 16 embryos from saline injected females and 21 embryos from 19 trypan blue injected females.

7. The effect of trypan blue treatment on succinic dehydrogenase and glucose-6-phosphate dehydrogenase activity in the yolk sac epithelium.

Both succinic dehydrogenase and glucose-6-phosphate dehydrogenase activity were histochemically demonstrable in the yolk sac placenta of the mouse at all stages examined between the 10th and 16th days of gestation. The accumulation

of trypan blue in the yolk sac endoderm had no effect on the activity of either of these enzymes (table 9). The results on succinic dehydrogenase were based on determinations from 14 embryos obtained from 7 saline injected mothers and 19 embryos obtained from 10 trypan blue injected mothers. Those for glucose-6-phosphate dehydrogenase were based on determinations from 10 embryos obtained from 5 saline injected mothers and 12 embryos obtained from 8 trypan blue injected mothers.

IV. Discussion

A. General Conclusions

In response to the questions raised in the introduction, the results of this investigation yield the following conclusions:

(1) The types of malformations observed in an amphibian embryo following exposure to trypan blue resemble the spectrum of abnormalities reported for mammalian embryos in both appearance and critical period. The mouse embryo responds to injection of appropriate doses of the dye with a variety of malformations affecting the anterior and posterior parts of the chordal axis. The most frequent abnormalities encountered in mice include exencephaly, microcephaly, abnormal closure of the neural folds, aberrant growth of the notochord, kinky tails (Hamburgh, 1952, 1954; Hamburgh and Callahan, 1967; Waddington and Carter, 1952, 1953), and anophthalmia (Beck, 1967). Similar results have been reported for rats (Gillman, et al., 1948; Gunberg, 1956), rabbits (Harm, 1954), guinea pigs (Hoar and Salem, 1961), and hamsters (Ferm, 1958). The abnormalities observed in this study of Rana pipiens embryos include a high incidence of anencephaly, microcephaly, anophthalmia, abnormalities of the notochord and somitic mesoderm, kinky tails, and edema. Edema and notochordal abnormalities were also noted by Waddington and Perry (1956) in

Xenopus laevis. However, on the average, fertility in batches of Xenopus eggs is only about 50% and dejellied eggs of this species are susceptible to exogastrulation when placed into hypertonic solutions (Gurdon, 1967). As these authors did not report controls, their conclusions with respect to trypan blue induced teratogenesis in amphibians have to be disregarded.

(2) A second point of interest that emerged from this study is the finding that the similarity of the response to trypan blue by amphibian and mammalian embryos extends not only to the pattern of abnormality but also to the sensitive periods at which the dye can effectively interfere with development. Exposure to trypan blue for 24 hours, commencing at fertilization and continuing until stage 8 (mid-blastula) had no observable effect upon the embryo. Prolonged exposure initiated after the completion of stage 14 was also without effect on development. On the other hand, brief immersion in the dye beginning at stage 10 when the dorsal lip first appears and terminating at stage 11 (yolk plug), a period of approximately 8 hours (at 18°C), yielded embryos which were as severely malformed as animals that had remained in contact with the dye throughout the entire 8-10 day period of their development. Immersion into the dye at stages 11, 12 or 13 also produced the full spectrum of abnormalities.

It may therefore be concluded that the critical period during which the dye is effective in interfering with the development of Rana pipiens embryos extends from stage 10 through stage 14.

This study also confirms results by Hamburgh (1952, 1954) and Waddington and Carter (1952, 1953) that in mice the critical period of trypan blue teratogenesis extends from the 7th to the 9th day of gestation. This period roughly coincides with the appearance of the mesoderm cells and ends with the closure of the neural folds (Snell and Stevens, 1966), events paralleled in the amphibian embryo during stages 10 through 14.

(3) Beyond this, the present study for the first time demonstrates that the earliest deviation from normal development in trypan blue exposed mouse embryos takes place at the egg cylinder stage (7-1/2 days of gestation). This investigator is not aware of any reports in the voluminous "trypan blue" literature describing similar observations.

(4) In the frog, the dye does not seem to enter the embryonic tissue proper and neither the jelly coats nor the vitelline envelope operate in any way to influence the action of the dye on amphibian morphogenesis. In the histological preparation of amphibian embryos grown

from eggs exposed to the dye, special precautions were taken with regard to the choice of fixative and dehydrating agent so as to prevent leaching-out of trypan blue. No evidence of dye penetration into embryonic tissue was found.

The same conclusion was reached in the study of the mouse embryo. No trace of trypan blue was found within the embryonic tissues proper. Accumulation of dye granules was restricted exclusively to the proximal endoderm of yolk sacs. Similar observations have been reported in other investigations of the uptake and penetration of trypan blue by the mammalian embryo (for review see Beck and Lloyd, 1966).

(5) Using three different histochemical techniques, the present study demonstrates convincingly, depression in vivo of acid phosphatase activity in the yolk sacs of mice treated with teratogenic doses of trypan blue. In trypan blue loaded yolk sacs, the distinct apical localization normally observed in these cells did not occur.

(6) Using thiamine pyrophosphatase activity as a marker of the Golgi, the present study demonstrated alterations of these organelles in the yolk sac epithelium of trypan blue treated embryos. There have been no previous reports describing thiamine pyrophosphatase activity in the mouse yolk sac.

(7) There was no correlation between depression of enzyme activity and the occurrence of grossly observable malformations.

B. Evaluation and implications of enzyme histochemistry.

The circumstance that some enzymes such as succinic dehydrogenase and glucose-6-phosphate dehydrogenase are apparently not inhibited *in vivo* by trypan blue makes it unlikely that those alterations in histochemical reactions that have been observed are merely reflections of a general enzyme inhibition. On the other hand, failure to note differences in histochemical reactions cannot be considered sufficient evidence that no such differences exist since, in most cases, only reactions of considerable magnitude are revealed by histochemical procedures (Hamberger and Sjostrand, 1966).

The present results concerning the localization and increase of acid phosphatase activity in the developing normal mouse yolk sac placenta are in close agreement with previously reported histochemical and biochemical data obtained from the rat yolk sac. Wislocki, Deane, and Dempsey (1946) first reported the histochemical demonstration of acid phosphatase activity in the apical pole of visceral endoderm cells. The work of Padykula (1958) confirmed this

at both the histochemical and biochemical levels. It also indicated that activity increased with fetal age. Johnson and Spinuzzi (1966), also using rat yolk sacs, found electrophoretic evidence for the presence of three isozymic forms of acid phosphatase - i.e., distinct molecular varieties of this enzyme with almost identical substrate specificity. The relative quantities of these different isozymes varied during the course of gestation, and one form did not appear until the latter half of pregnancy. Possibly the variations observed with the three techniques employed in the present study were due to differing selectivities of the isozymes to the different substrates utilized. It is interesting that the Burstone (1958) procedure first gave a positive reaction for acid phosphatase activity at approximately the same stage of pregnancy at which Johnson and Spinuzzi (1966) were first able to detect the third isozymic form of this enzyme. On the basis of experiments on the histochemical localization of acid phosphatase in various tissues of rats, mice, and men Rosenbaum and Rolon (1962) and Maggi, Franks, and Carbonell (1966) also postulated that techniques employing different substrates may measure the activity of different isozymic forms of the same enzyme.

C. Interpretation of Results.

One of the most perplexing problems of teratology

concerns the mechanism of action by which a teratogenic agent interferes with normal development. In the case of trypan blue this problem is further compounded by two facts. (1) The dye does not enter the embryo, and (2) dialysis of the trypan blue sample significantly decreases its teratogenicity.

1. Dialyzed vs. undialyzed samples of trypan blue.

The observation that the exposure of frog eggs to dialyzed trypan blue solution considerably reduced the incidence of malformations (from 100% to about 30%) raises the question whether the dye molecule or some contaminant is responsible for teratogenesis. The results of the present study do not permit one to decide this question. However, the present study clearly indicates that it is not NaCl ions or impurities in the spring water that are responsible for the observed effects. Increasing the concentration of NaCl in the medium in which eggs were raised to an amount equal to that which has been reported to be associated with the dye sample used (Lloyd and Beck, 1963) had no effect on the development of frog eggs (table 6).

Several investigators have tested the effect of purified trypan blue on mammalian embryos using samples which had been subjected to chromatography and dialysis and have concluded that the teratogenic activity resides in the

intact trypan blue molecule rather than in contaminants (Waddington and Carter, 1952, 1953; Beaudoin and Pickering, 1960; Barber and Geer, 1964; Beck and Lloyd, 1966).

2. Evidence for interference with yolk sac function by samples of trypan blue.

It has been suggested that the teratogenic effect of trypan blue on mammalian embryos is due to a disruptive effect on the ability of the yolk sac placenta to provide nourishment to the embryo. The following observations support this assumption: (a) The yolk sac epithelium is the only embryonic tissue which accumulates the trypan blue (Wislocki, 1920, 1921; Everett, 1935; Nebel and Hamburgh, 1966). (b) In rodents, the period during which trypan blue exerts its teratogenic effects occurs at a time when the yolk sac serves as the principal organ of embryonic nutrition (Beck, et al., 1967a, 1967b).

Although Beck, et al. (1967a) have demonstrated trypan blue induced depression of several lysosomal enzymes including acid phosphatase, the relevance of their findings to the teratogenic effects of the dye in the living animal must be questioned. Their experiments consisted of assays of cell free material under in vitro conditions and gave no assurance that such results could be demonstrated in the living animal in situ. The depression of acid phosphatase activity by trypan blue demonstrated by results

presented here indicates that at least one lysosomal enzyme is inhibited *in vivo* if teratogenic dosages of this substance are given to the mother. The diffuse cytoplasmic reaction for this same enzyme in trypan blue loaded yolk sacs is reminiscent of the appearance of the yolk sac just prior to term (Padykula, 1958). Other known terminal regressive changes in the aging yolk sac are: a fall in the height of the epithelial brush border, a decline in the storage of glycogen and lipid (Sorokin and Padykula, 1964), and decreased absorptive capacity by individual cells of the visceral endoderm (Padykula and Wilson, 1960; Padykula, Deren, and Wilson, 1966).

The demonstration of alterations in the Golgi apparatus in response to trypan blue treatment that has been revealed by this study is also of interest, particularly in the light of the important role which this organelle may play in cellular transport. If trypan blue in some way alters intracellular digestion and transport of nutrients as has been postulated (Beck, *et al.*, 1967a) one might indeed expect some alteration in activity or localization of Golgi associated enzymes which are implicated in mechanisms of intracellular transport (Hamberger and Sjostrand, 1966; Torack and Barnett, 1963). Thiamine pyrophosphatase for example, is generally associated with the Golgi complex

and at the light microscope level is considered a marker of this organelle (Novikoff and Goldfischer, 1961; Meek and Bradbury, 1963).

Localization of trypan blue granules was observed in the Golgi region of kidney and liver cells by Ludford (1928) who compared their transport to that of "secretion granules in gland cells." It is also significant to note that in his study on transport of ferritin across the yolk sac placenta of the rat, Lambson (1966) found ferritin particles within Golgi cisternae.

Functions postulated for the Golgi apparatus also include concentration and packaging of hydrolytic enzymes into lysosomes (de Duve, 1968), involvement in lipid transport (Cramer and Ludford, 1925; Weiss, 1955; Palay and Revel, 1963), protein transport (Wellings and Deome, 1961), and participation in the processes of cell secretion and transport (for review see de Robertis, Nowinski, and Saez, 1965). Presumably, nutrition of the embryo prior to the onset of function of the allantoic placenta depends on effective digestion of large molecules by hydrolytic enzymes that are located in the lysosomes of the yolk sac epithelium (Beck, et al., 1967b; Parry, Beck, and Lloyd, 1968). Subsequent transport of nutritive products of intracellular digestion to the embryo probably requires the

participation of Golgi organelles. Thus it seems clear that alterations in lysosomal or Golgi activity in the yolk sac placenta might interfere with the postulated nutritive functions of this organ and therefore lends credence to the theory that this teratogen interferes with yolk sac function.

On the other hand, this theory must be reconciled with the observation that whereas virtually all embryos from trypan blue treated mothers display yolk sac alteration, only approximately 25% of such fetuses studied between the 10th and 13th day of gestation were visibly malformed (tables 8 and 9). The finding that 82% of egg cylinders (8th day of gestation) from trypan blue treated mothers were abnormal in appearance (table 10) suggests the possibility that all dye treated embryos may be malformed initially but that many are able to repair this early damage. Hamburgh and Callahan (1967) have reported evidence indicating that mouse embryos of the Swiss albino strain may have such a repair capacity.

3. Evidence for a direct action of trypan blue on embryonic tissues.

An alternative hypothesis to explain the mechanism by which trypan blue causes congenital malformations is that the dye has a direct effect upon the cells of the embryo. In mammals, the critical period of trypan

blue teratogenesis extends from the 7th through the 9th day of gestation. This period starts with the appearance of the primitive streak and ends with the closure of the neural folds (Snell and Stevens, 1966; Rugh, 1968). The present study demonstrates that in amphibians (which develops without benefit of a protective yolk sac barrier) the critical period of sensitivity to trypan blue coincides with the stage of development generally referred to as gastrulation and early neurulation and is therefore comparable to that of the mouse. It was also shown that the spectrum of malformations induced in amphibian embryos exposed to dye is similar to that obtained in fetuses from trypan blue treated mothers. It should be noted that in both the mammalian and amphibian embryos, prospective notochord and mesoderm cells are located at the surface of the embryo in contact with dye during early embryogenesis. In amphibians, the presumptive mesoderm and chord tissues are exposed to the dye until involution has been completed at the end of gastrulation. In the mouse, the cells of the head process, early notochord (Snell and Stevens, 1966; Rugh, 1968) and possibly the primitive streak (Bonnevie, 1950) are exposed to fluids in the yolk sac cavity prior to the complete envelopment of the embryo by the yolk sac. The presence of trypan blue in the yolk sac fluid of rabbit

embryos between the 7th and 9th days of gestation was demonstrated by Ferm (1956). Once the yolk sac placenta effectively surrounds the early embryo on the 9th day of gestation, maternally injected trypan blue is no longer teratogenic (Wilson, Beaudoin, and Free, 1959) and can no longer be found within the yolk sac fluid (Ferm, 1956). However, injection of the dye directly into the yolk sac cavity of 9 or 10 day old embryos still results in characteristic trypan blue induced abnormalities (Turbow, 1966). Thus the possibility exists that the rodent yolk sac may actually serve to protect the embryo from the effects of trypan blue. The report by Davis (1967) that litters possessing the higher percentages of apparently normal fetuses contained more trypan blue in their visceral yolk sacs than did litters containing higher incidences of malformations supports this hypothesis. Additional evidence that trypan blue may exert a direct effect on the cell surface comes from the report of Kochhar, Bostrom, Larsson, and Reio (1967) that this dye inhibits uptake of ^{35}S-sulfate by calf costal cartilage *in vitro* without entering the cells.

One possible unifying mechanism by which trypan blue may cause abnormalities in both amphibian and mammalian embryos may be through an alteration in ionic and/or fluid balance brought about by direct contact of the cell

surface to the dye solution. In the chick embryo, trypan blue induced caudal hematomata and blisters have been shown to give rise to rumplessness (Kaplan and Grabowski, 1967). In mice, hematomata along the caudal axis very likely lead to spina bifida, tail kinks, and tail shortening (Hamburgh, 1954). Excess fluid within the interstitial spaces of egg cylinders may also be responsible for the disrupted appearance of trypan blue treated mouse embryos at this early stage. A large number of trypan blue treated Rana pipiens embryos exhibit edema at stage 15 or 16. Also suggestive is the fact that the abnormalities present in trypan blue treated Rana pipiens embryos so closely resemble the abnormalities caused by subjecting amphibian eggs to an excess of lithium ions (Hall, 1942) or to hypertonic or hypotonic salt solution (Holtfreter and Hamburger, 1955) during early development.

Although the observations reported here do not lead with certainty to an explanation of a common mechanism by which trypan blue causes malformations in rodent and amphibian embryos, they do suggest working hypotheses for future investigation. It might be suggested that during gastrulation, the mechanism in both groups of animals is similar. During gastrulation cells of both types of embryos are directly exposed to the dye. There is consider-

able evidence indicating that the dye does not enter the cells of affected tissues and therefore the hypothesis that trypan blue induced teratogenesis operates at the cell surface may merit further study. Though it has been demonstrated that trypan blue alters those organelles in the yolk sac epithelium which are implicated in nutritive functions of that organ, i.e., lysosomes and Golgi, it remains to be demonstrated that there is an actual change in the transport of metabolites. Thus it is possible that the enzymic changes observed in the yolk sacs of trypan blue treated embryos are not directly involved in the etiology of other dye induced malformations but merely represent a class of malformations not subject to repair.

V. Summary

Rana pipiens eggs raised in trypan blue solution developed abnormally. Malformations included anencephaly, microcephaly, anophthalmia, abnormalities of the notochord and somitic mesoderm, kinky tails, and edema. The critical period during which exposure to trypan blue resulted in abnormalities extended from late blastula (Shumway stage 9) through closure of the neural folds (Shumway stage 14). The presence or absence of the jelly coats and/or vitelline envelope had no effect on trypan blue induced teratogenesis in this species. Abnormalities already in the process of formation as a consequence of exposure to trypan blue could not be reversed when such embryos were subsequently removed from dye solution to fresh spring water.

Examination of the yolk sacs of mouse embryos which had been exposed to trypan blue revealed that the dye had accumulated within all cells of the visceral endoderm. No trypan blue was ever observed within cells of the parietal endoderm. When trypan blue treated 7-1/2 day old egg cylinders were observed, dye was found in the extraembryonic proximal endoderm as well as the squamous endoderm of the embryonic region.

A comparison of the histological appearance of control and trypan blue treated mouse embryos on the 8th

and 9th days of gestation revealed that dye treated embryos exhibited abnormalities during the 8th day of gestation which were no longer apparent during the 9th day of gestation.

Four histochemically demonstrable enzymes were compared in trypan blue loaded and untreated yolk sacs at different stages of gestation. Acid phosphatase, a lysosomal constituent, thiamine pyrophosphatase, an enzyme associated with the Golgi apparatus, succinic dehydrogenase, a mitochondrial enzyme, and glucose-6-phosphate dehydrogenase, a cytoplasmic enzyme, were chosen for study. The three histochemical methods employed for the study of acid phosphatase indicated that intensity of staining, an index of enzyme activity, increased as gestation proceeded. Acid phosphatase was generally restricted to the supranuclear region adjacent to the brush border of the cells. No acid phosphatase activity was observed in the mesoderm cells of the visceral yolk sac. In most cases injection of trypan blue into pregnant females was followed by a depression of acid phosphatase activity in the visceral endoderm of the yolk sac but not in the parietal endoderm. Trypan blue injection also caused the acid phosphatase activity to be diffused throughout the cytoplasm.

The visceral yolk sac endoderm and the parietal

endoderm cells exhibited strong thiamine pyrophosphatase activity at all stages of gestation between the 8th and 15th day. Thiamine pyrophosphatase positive structures, linear in appearance, were located adjacent to nuclei. In yolk sacs from trypan blue treated embryos these structures appeared thin, fragmented, and occasionally granular.

Whereas almost all trypan blue treated embryos exhibited alterations in acid phosphatase and thiamine pyrophosphatase activity, only about 25% of such embryos are visibly malformed.

Both succinic dehydrogenase and glucose-6-phosphate dehydrogenase activity were histochemically demonstrable in the yolk sac placenta of the mouse at all stages examined between the 10th and 16th days of gestation. The accumulation of trypan blue in the yolk sac endoderm had no effect on the activity of either of these enzymes.

The results presented here support either of two hypotheses. Trypan blue treatment may cause malformations by altering nutritive and transport functions of the yolk sac placenta or the dye may exert a direct effect upon the cells of the embryo.

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Table 1

Physical Agents That Cause Malformations

Agent	Species	Malformation or Structure Affected	Reference
X-ray	human rat mouse	cleft palate limbs vertebrae & ribs tail	Murphy and Goldstein (1929) Hanson (1923) Russell (1950)
Hypothermia	hamster	hydrocephalus exencephaly anophthalmia cleft palate limbs	Smith (1957)
Hyperthermia	guinea pig rat	anencephaly microphthalmia limb, tail, palate	Edwards (1967) Edwards (1968)
Hypoxia	mouse	exencephaly cleft palate vertebrae & ribs	Ingalls, Curley, and Prindle (1950, 1952)
Elevated CO_2	rat	heart	Haring and Polli (1957)
Maternal audio-visual stress	rat	spina bifida eye defects abdominal hernia hematomata	Geber (1966)

Table 2

Maternal Nutritional Deficiencies That Cause Malformations

Deficiency	Species	Malformation or Structure Affected	Reference
Fasting 24 hours	mouse	exencephaly vertebrae & ribs	Runner (1954)
Vitamin A	pig rat	anophthalmia	Hale (1933) Warkany and Schraffenberger (1944)
Riboflavin	rat mouse	skeletal abnormalities, heart, urogenital system	Warkany and Nelson (1941) Kalter and Warkany (1957)
Folic Acid	rat mouse	hydrocephalus	Hogan, O'Dell, and Whitley (1950); Runner (1954)
Pantothenic Acid	rat	Exencephaly microphthalmia edema & hemorrage	Boisselot (1948)
Vitamin B_{12}	rat	hydrocephalus	O'Dell, Whitley, and Hogan (1951)
Vitamin E	rat	hydrocephalus exencephaly microphthalmia skeletal defects	Cheng and Thomas (1953)
Vitamin D	rat	skeletal defects	Warkany (1943)

Table 3

Growth Inhibitors and Specific Antagonists That Cause Malformations

Agent	Species	Malformation or Specific Structure Affected	Reference
Nitrogen Mustard	rat mouse	cleft palate limbs	Haskins (1948) Danforth and Center (1954)
Alkylating Agents	rat	edema limbs, tail palate, skeleton	Murphy (1959)
6-Aminonicotinamide	rat	cleft palate vertebrae, sternum limbs, feet	Murphy, Dagg, and Karnofsky (1957)
8-Azoguanine	mouse	cleft palate feet	Nishimura and Nimura (1958)
5-Flourouracil	rat	feet tail cleft palate	Dagg (1960)
Galactoflavin	mouse	cleft palate limbs	Kalter and Warkany (1957)
2-6-Diaminopurine 6-Chloropurine Thioguanine	rat rat rat	not described	Thiersch (1957) Thiersch (1957) Thiersch (1957)

Table 4

Hormone Injections and Endocrine Conditions That Cause Malformations

Hormone	Species	Malformation or Structure Affected	Reference
Androgen	rat	intersexes	Greene, Burrill, and Ivy (1939)
Estrogen	rat	intersexes	Greene, Burrill, and Ivy (1940)
Insulin	rat	exencephaly vertebrae & ribs	Lichentenstein, Guest, and Warkany (1951)
	mouse		Smithberg and Runner (1963)
Cortisone	mouse	cleft palate	Fraser and Fainstat (1951)
Hydrocortisone	mouse	cleft palate	Ingalls and Curley (1957)
ACTH	mouse	cleft palate	Heiberg, Kalter, and Fraser (1959)
Vasopressin	rat	limb degeneration	Jost (1951)
Adrenalin	rat	limb degeneration	Jost (1953)
Thyroid Stimulating Hormone	rat	anophthalmia hydrocephalus	Beaudoin and Roberts (1966)
Alloxan diabetes	mouse	cleft palate	Ross and Spector (1952)

Table 5

Miscellaneous Drugs and Chemicals That Cause Malformations

Agent	Species	Malformation or Structure Affected	Reference
Trypan Blue	rat mouse	Exencephaly spina bifida hydrocephaly tail	Gillman, Gilbert, Gillman and Spence (1948) Hamburgh (1952, 1954)
Vitamin A Excess	rat	cleft palate exencephaly feet	Cohlan (1954)
Nicotine	mouse	feet	Nishimura and Nakai (1958)
Salicylates	rat	hydrocephalus exencephaly feet, eye vertebrae & ribs	Warkany and Takacs (1959)
Thalidomide	human mouse	limbs vertebrae hydrocephaly	Spiers (1962) DiPaolo (1963)
Sulphamerizine	rat	not described	Speert (1940)
Dimethylsulphoxide	hamster	exencephaly rib fusions microphthalmia cleft lip limb deformities	Ferm (1966)

Table 5 (continued)

Agent	Species	Malformation or Structure Affected	Reference
Actinomycin D	rat	most systems of the body	Tuchman-Duplessis and Mercier-Parot (1960)
LSD	rat	growth retardation	Alexander, Miles, Gold, and Alexander (1967)
Tolbutamide	mouse	exencephaly vertebrae & ribs	Smithberg and Runner (1963)
Caffeine	mouse	cleft palate foot abnormalities	Nishimura and Nakai (1960)
Mitomycin C	mouse	cleft palate foot abnormalities tail abnormalities	Nishimura (1964)
Tetracycline Penicillin-streptomycin	rat	not described	Filippi (1967)
Anti-rat-kidney antiserum	rat	cleft palate face limbs brain kidneys genitalia viscera	Brent (1966)

Table 6

Trypan Blue Induced Teratogenesis in Rana pipiens Embryos Using Dialyzed and Undialyzed Samples of Trypan Blue

Treatment	Number of Exp. Embryos Used	Number of Embryos Malformed At Stage 20	Percent of Embryos Malformed
Embryos raised in spring water (controls)	210	14	7%
Embryos raised in distilled water	49	1	2%
Embryos raised in 0.25% NaCl solution	81	5	6%
Embryos raised in undialyzed trypan blue (0.1% solution)	125	125	100%
Embryos raised in dialyzed 0.1% trypan blue, then transferred to distilled water at stage 14	125	37	30%
Embryos raised in dialyzed 0.1% trypan blue, then transferred to spring water at stage 14	69	18	26%
Embryos raised in dialyzed 0.5% trypan blue diluted to 0.1% with spring water	39	15	39%
Embryos raised in dialyzed 0.1% trypan blue which was made 0.35% with respect to NaCl after dialyzation	38	13	34%

Table 7

Differential Treatment of Rana pipiens Embryos with Undialyzed Trypan Blue

	Number of Embryos	Stage at Which Embryos Were Placed In Dye Solution	Stage Reached by Paired Controls When Experimental Embryos Were Removed from Dye Solution to Spring Water	Number of Embryos Exhibiting Malformations	Number of Untreated Paired Controls
Series 1*	180	3	21	180	100
Series 2**	4	3	8	2	209
	10	3	9	10	
	25	5	8	5	
	15	3	10	15	
	28	3	11	28	
	5	3	13	5	
	60	3	14	60	
	3	3	15	3	
	3	3	18	3	
Series 3**	10	8	18	10	150
	15	10	18	15	
	23	10	11	23	
	10	8	21	10	
	20	9	21	20	
	225	10	21	225	
	10	11	21	10	
	10	13	21	10	
	10	14	21	5***	
	25	21	25	0	
Totals	691				459

* Series 1 was carried out to determine the total frequency of malformations that can be induced by an undialyzed sample of trypan blue in eggs of Rana pipiens.

** Series 2 and 3 were carried out to determine the onset and duration of the critical period of response to trypan blue.

*** Only tail malformations were observed in this group.

Table 8

Incidence of Gross Malformations in Embryos from Swiss Albino CD1 Mice

	Number of females used	Number of Embryos Recovered	Number of Embryos Malformed	Percent Embryos Malformed
Controls	30	282	2	0.71
Experimentals*	31	349	82	23.5

* Female mice were injected with 0.25 ml of 1% trypan blue on the 7th or 8th day of gestation. Controls received an equivalent volume of 0.9% physiological saline.

Table 9

Histochemistry of the yolk sac placenta

Enzyme	Number Control Females Supplying Litters	Number Control Embryos Examined	Number Exp. Females Supplying Litters	Number Exp. Embryos Selected for Study	Number of Yolk Sacs Showing Enzyme Depression****
Acid phosphatase*	31	63	45	97	83/97
Thiamine pyrophosphatase**	15	16	19	21	21/21
Succinic dehydrogenase***	7	14	10	19	0/19
Glucose-6-phosphate dehydrogenase***	5	10	8	12	0/12

* Acid phosphatase activity studied by the techniques of Gomori (1950), Barka (1960), and Burstone (1958).

** Thiamine pyrophosphatase activity studied by the method of Novikoff and Goldfischer (1961).

*** Succinic dehydrogenase and glucose-6-phosphate dehydrogenase activity studied using techniques suggested by Barka and Anderson (1963).

**** This column refers to the number of yolk sacs from experimental embryos (column 4) in which enzyme alteration was clearly demonstrated.

Table 10

The Histological Appearance of Mouse Embryos Obtained from Trypan Blue Treated Mothers on the 8th and 9th Day of Gestation

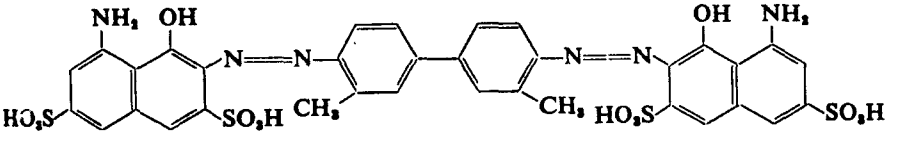
	Age of Embryos in Days	Number of Females Supplying Litters	Number of Embryos Selected For Study*	Number of Embryos Exhibiting Abnormality
Controls	8	9	28	3**
Experimentals***	8	8	34	28
Controls	9	3	11	0
Experimentals***	9	3	10	0

* Embryos for histology were selected at random.

** Control embryos of questionable appearance were scored as abnormal.

*** Females were injected with 0.25 ml of 1.0% trypan blue on the 7th day of pregnancy.

Figure 1. The structural formula of trypan blue.



1

Figure 2. On the left, a control Rana pipiens embryo at approximately stage 20. Embryos to the right of the control were placed into trypan blue solution at stage 10, and remained in trypan blue until control embryos from the same clutch of eggs had reached stage 20. Note the complete absence of the head on the central embryo and the microcephalic appearance and abnormal tail of the embryo to the right. X5.

Figure 3. Cross section through the level of the foregut. A. Control embryo at stage 15-16. B. Rana pipiens embryo placed into trypan blue at stage 3 and fixed when controls from the same clutch of eggs reached stage 15-16. Note absence of neural tube, lack of clearly distinguishable notochord, and failure of somite formation in experimental embryo. H & E. X56.

2

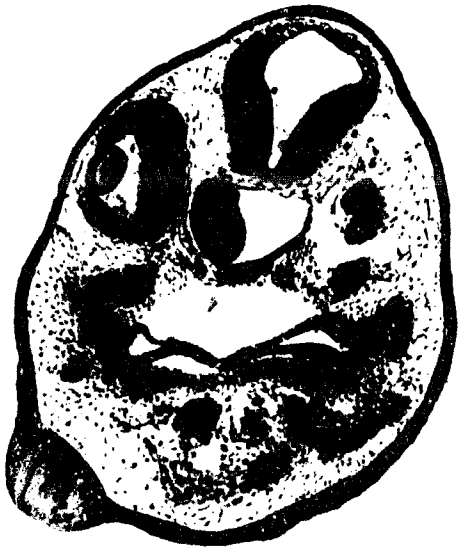
3A 3B

Figure 4. Cross section through the level of the liver diverticulum. A. Control embryo at stage 20. B. Embryo placed into trypan blue solution at stage 10 and fixed when controls from the same clutch of eggs reached stage 20. Note lack of notochord and neural tube and failure of mesodermal differentiation in the experimental embryo. H & E. X56.

Figure 5. Cross section through the level of the diencephalon. A. Control embryo at stage 22. B. Embryo placed into solution of trypan blue at stage 10 and fixed when controls from the same clutch of eggs reached stage 22. Note microcephalic brain and failure of optic cup to form in the trypan blue treated embryo. H & E. X56.

4A

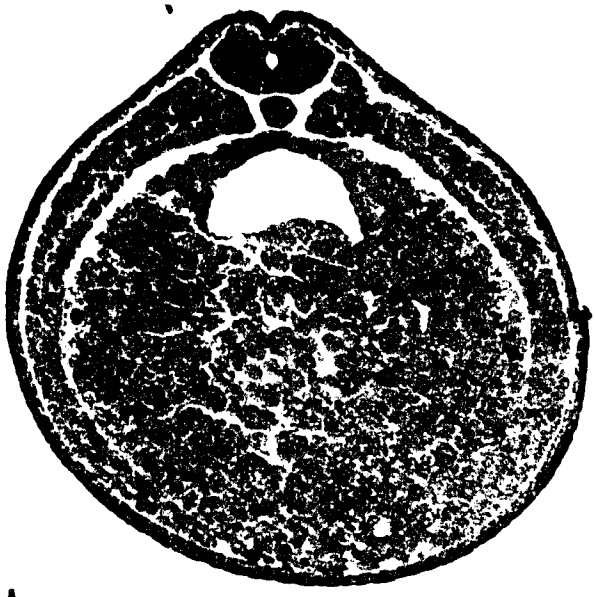
4B



5A

5B

Figure 6. Cross section through the level of the hind gut. A. Control embryo at stage 15-16. B. Embryo placed into solution of trypan blue at stage 3 and fixed when controls from the same clutch of eggs reached stage 15-16. Note the absence of notochord and the solid neural tube in the experimental specimen. H & E. X56.



6A

6B

Figure 7. On the left, a control Rana pipiens embryo at stage 21. In the center, an embryo placed into trypan blue solution at stage 14 and fixed at the time when controls reached stage 21. Note that tail outgrowth is retarded. On the right, an embryo placed into solution of trypan blue at stage 10 and remained in trypan blue until controls from the same clutch of eggs reached stage 21. Note complete suppression of development. X5.

Figure 8. On the left, a control Rana pipiens embryo at stage 23. In the center, an embryo placed into solution of trypan blue at stage 3 and removed to fresh spring water at stage 13. The embryo was fixed 7 days later at a time when controls from the same clutch of eggs had reached stage 23. Note microcephalic appearance and general retardation of growth. On the right, an embryo which remained in trypan blue solution from stage 3 until the time of fixation at which time controls from the same clutch of eggs had reached stage 23. Note complete suppression of development. X5.

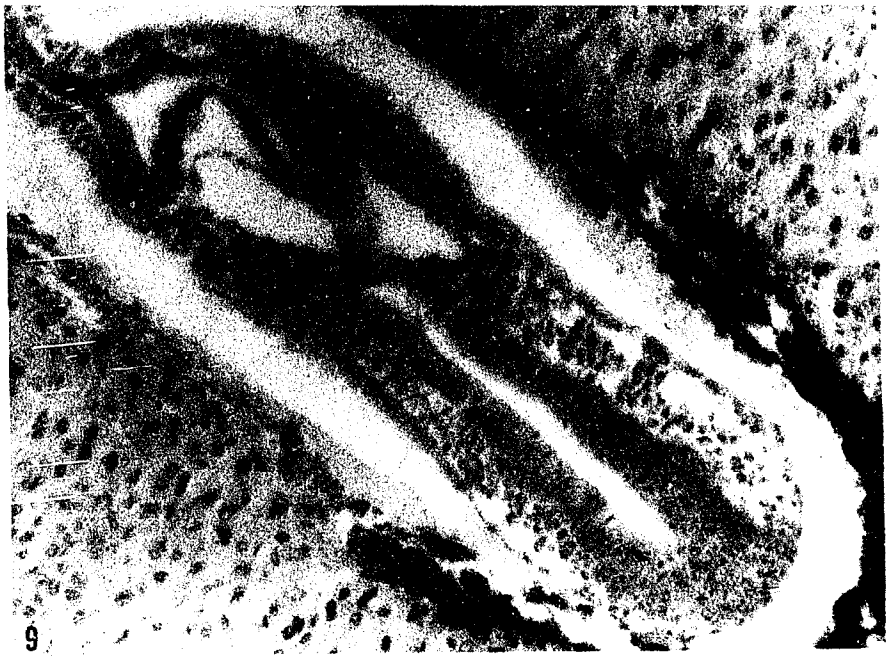
7

8

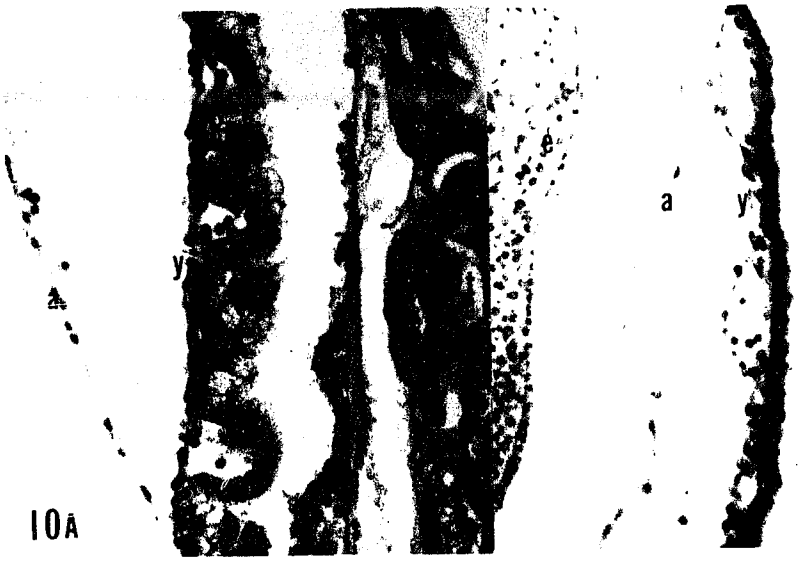
Figure 9. Semisagittal section through an egg cylinder stage embryo on the 8th day of gestation after injection of the mother on the 7th day of gestation. Note trypan blue granules in visceral endoderm cells. X160.

Figure 10. Sections through a yolk sac from a 10-1/2 day old embryo. A. Section through the villous portion of the yolk sac adjacent the chorioallantoic placenta. B. Section through the non-villous portion of the yolk sac. X160.

a = amnion
e = embryo
t = trophoblast
y = yolk sac



9



10A

10B

Figure 11. Transverse sections through the embryonic portion of 7-1/2 day old egg cylinders. A. A section from a control embryo. B. Section from trypan blue treated embryo. Note the disruption of the embryonic endoderm and the shape of the embryos. X160.

Ec = Ectoderm
M = Mesoderm
En = Endoderm

Figure 12. Acid phosphatase activity in yolk sac epithelium of a normal mouse embryo on the 10th day of gestation. Barka's (1960) simultaneous coupling azo dye technique. Note the intense apical reaction. The reaction product is red. X450.

Figure 13. Acid phosphatase activity in yolk sac epithelium of a mouse embryo on the 10th day of gestation following injection of the mother with trypan blue on the 8th day of gestation. Incubated "back to back" with section displayed in Figure 12. Note depression of activity. X450.

12

13

Figure 14. Acid phosphatase activity in yolk sac epithelium of a normal mouse embryo on the 14th day of gestation. Burstone's (1958) simultaneous coupling azo dye technique. Note intense apical reaction. The reaction product is red. X410.

Figure 15. Acid phosphatase activity in yolk sac epithelium of a mouse embryo on the 14th day of gestation following injection of the mother with trypan blue on the 12th day of gestation. Incubated "back to back" with section shown in figure 14. X410.

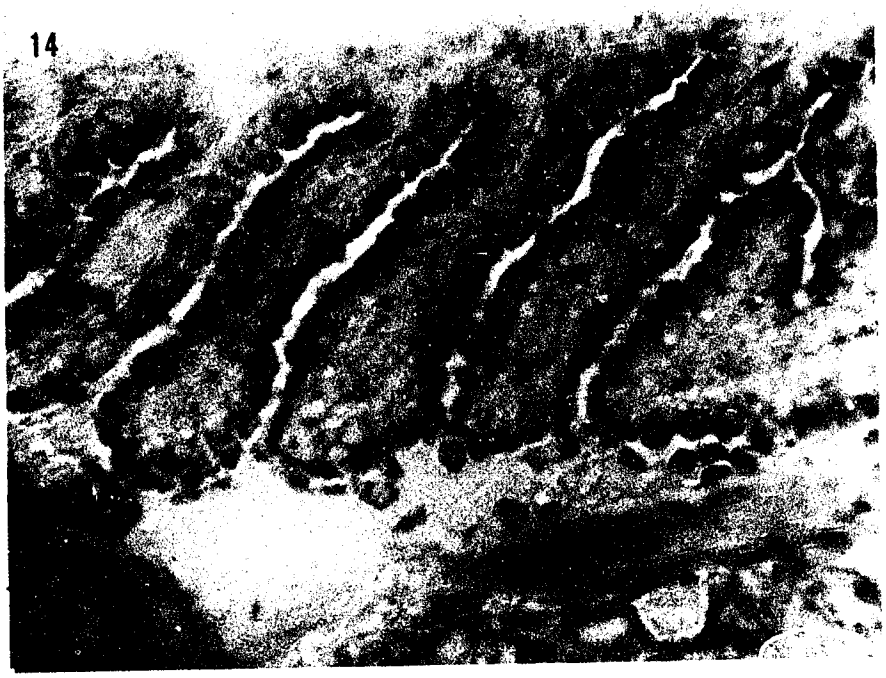


Figure 16. Acid phosphatase activity in yolk sac epithelium of the same embryo shown in figure 14 (normal embryo on 14th day of gestation). Gomori's (1950) lead salt technique. X 425.

Figure 17. Acid phosphatase in yolk sac epithelium of the same embryo shown in figure 15 (embryo sacrificed on 14th day of gestation after injection of the mother with trypan blue on the 12th day of gestation). Incubated "back to back" with section shown in figure 16. Gomori's lead salt technique. X 425.

Figure 18. Thiamine pyrophosphatase activity in yolk sac epithelium of a normal mouse embryo on the 11th day of gestation. Technique of Novikoff and Goldfischer (1961). Note the elongate TPPase positive perinuclear arrays. X650.

Figure 19. Thiamine pyrophosphatase activity in yolk sac epithelium of a mouse embryo on the 11th day of gestation after injection of the mother with trypan blue on the 9th day of gestation. Incubated "back to back" with section shown in figure 18. Note organelles are disrupted, fragmented and scattered. X650.

Figure 20. Same section as figure 18. X1000.

Figure 21. Same section as figure 19. X1000.

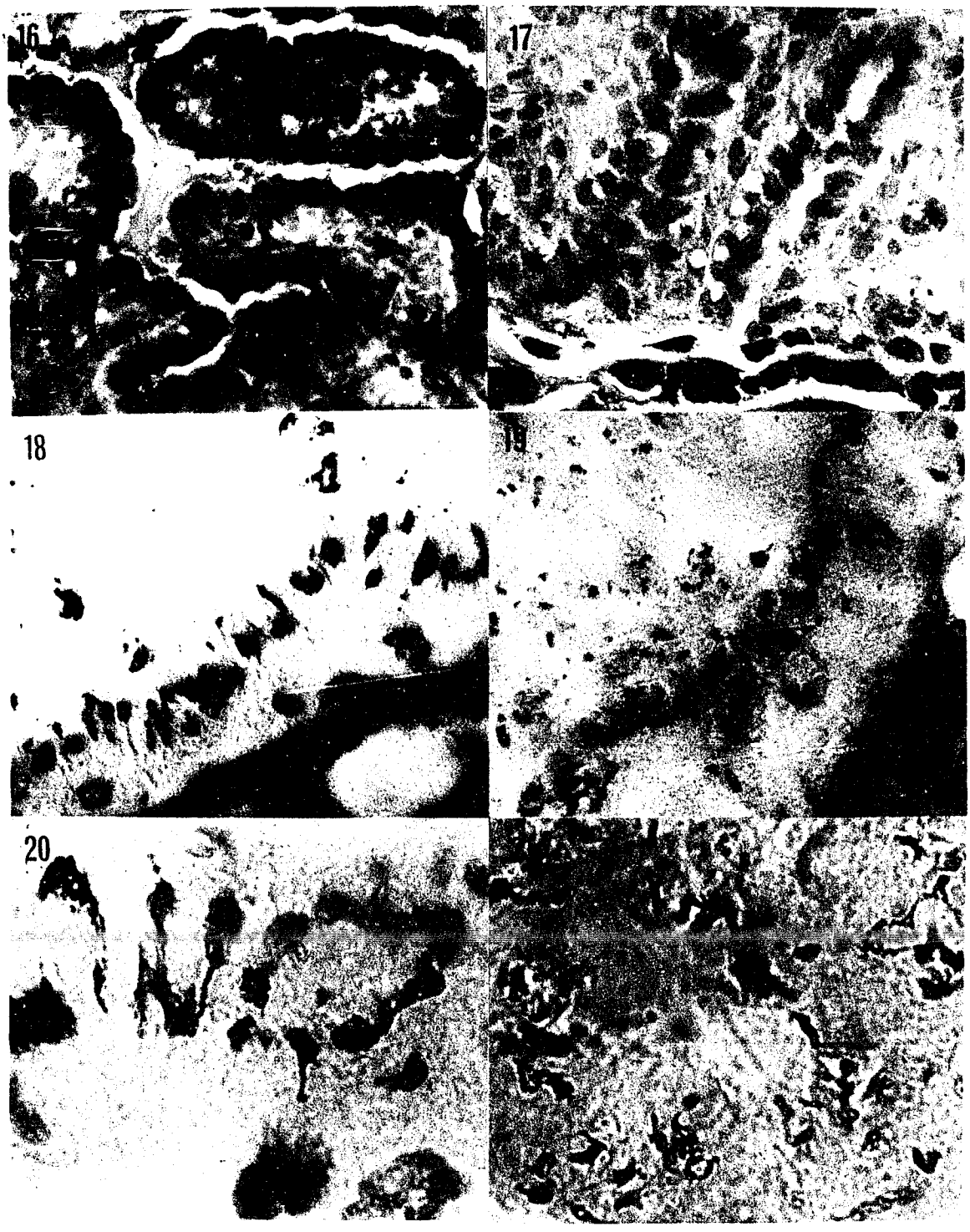
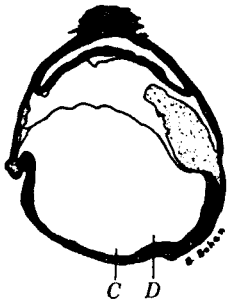
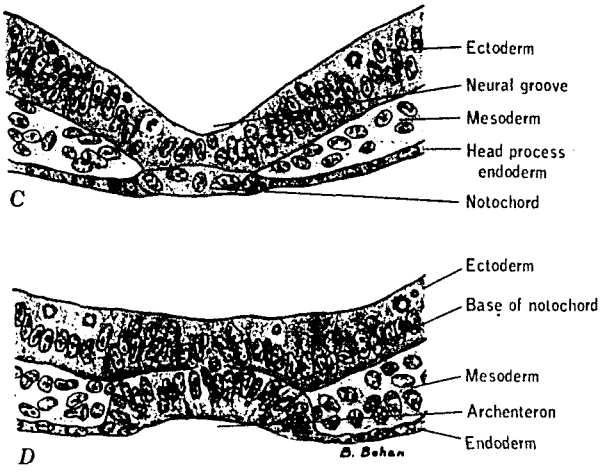

16
17
18
19
20

Figure 22. Projection drawings of 7 day 10 hour mouse embryo showing the location of notochord cells at the outer surface of the egg cylinder (From Snell and Stevens, 1966).


C
D


Ectoderm
Neural groove
Mesoderm
Head process
endoderm
Notochord
C
Ectoderm
Base of notochord
Mesoderm
Archenteron
Endoderm
D
B. Behan

Autobiographical Statement

I was born on October 18, 1942, in Brooklyn, New York, where I lived until the age of nine. In 1952 my family moved to Queens, New York, where I attended P.S. 115, Bellerose Junior High School, and Martin Van Buren High School. After graduating from Martin Van Buren High School in 1960, I received a New York State Regents Scholarship and attended Queens College of the City University of New York. I was an active participant in intramural and intercollegiate athletics during my undergraduate years and earned several varsity letters in track and field. In 1964 I was awarded a Bachelor of Arts degree in biology and entered the masters program in biology at Queens College. I transferred to the Ph.D. program at the City College in 1966 and have been supported by a stipend from a training grant awarded to Dr. Max Hamburgh from that time to the present. I have had experience teaching several biology courses at Queens College, including general biology and embryology. Upon completing the requirements for the degree of Doctor of Philosophy I will begin my tenure as a National Institutes of Health postdoctoral fellow at the Department of Biology at Massachusetts Institute of Technology.

Publications

Greenhouse, G. and Hamburgh, M. (1968). Analysis of trypan blue induced teratogenesis in Rana pipiens embryos. Teratology 1:61-74.

Greenhouse, G. and Hamburgh, M. (1967). Analysis of embryogenesis of Rana pipiens eggs treated with trypan blue. Amer. Zool. 7:745 (abstract).

Greenhouse, G. (1969). Trypan blue induced teratogenesis and enzymes in the mouse yolk sac. Anat. Rec. 163:191 (abstract).

Hamburgh, M., Nebel, L., and Greenhouse, G. (1966). Penetration and uptake of trypan blue in the yolk sac placenta of the mouse. Amer. Zool. 6:581-582 (abstract).