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Accumulation of DNA, RNA, and Protein by Cultured Rat Embryos Following Maternal Administration of a Teratogenic Dose of Trypan Blue

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ABSTRACT The effect of trypan blue on the rates of DNA, RNA, and protein accumulation in rat embryos was investigated using a system of whole embryo culture. Embryos were isolated on the 10th gestational day (sperm day = Day 0) either 4 or 24 hr after maternal injection. The cultivation period was for 24 or 42 hr. The rate of DNA and RNA accumulation in the embryo following either a 4- or 24-hr previous maternal treatment was significantly decreased at both 24 and 42 hr of cultivation. Protein values were significantly reduced after a 4-hr maternal exposure, but only slightly reduced after a 24-hr maternal exposure. No significant differences in DNA, RNA, or protein content were observed at the beginning of cultivation, following either a 4- or 24-hr previous maternal treatment. The percentage of embryos establishing a visceral yolk sac circulation was greatly reduced, and this supports the conclusion that trypan blue interferes with yolk sac function. The most obvious teratogenic insults include failure of neuropore closure, retention of a dorsiflexed position, and cranial edema.

One of the major unsolved problems in developmental biology remains the elucidation of the causes and physiologic mechanisms responsible for the occurrence of spontanious malformations in vertebrate embryos. An extensive number of physical and chemical agents capable of interfering with normal development have been identified by teratologists, but no single common factor has been found which could give a clue to the basic physiologic mechanisms responsible for developmental errors.

The discovery by Gillman et al. ('48) that the dye, trypan blue, is highly teratogenic in rats has been confirmed by a number of subsequent investigation for a variety of species. Investigations have sparked the hope that the effects of this dye would yield useful information about the mechanisms responsible for abnormal development. Due to its coloration the assumption was that it would then be possible to follow the course of the dye through the embryo, histologically, and ascertain its intracellular localization.

This was not possible, however, since it was also soon discovered that the dye does not enter the embryo proper, but is arrested in the yolk sac epithelium (see review by Lloyd, et al., '68). Beck and Lloyd ('66) found that in vitro incubation of yolk sacs in the presence of trypan blue was followed by a significant depression of several hydrolytic enzymes. This and subsequent investigations have shown the enzymes affected include: acid phophatase, β-glucuronidase, ribonuclease, deoxyribonuclease, and cathepsin-D (Beck et al., '67; Lloyd et al., '68; and Hamburgh, et al., '75). Based on these observations Beck et al. ('67) advanced the hypothesis that the teratogenic action to trypan blue may be mediated through a disturbance in the nutritive function of the yolk sac epithelium.

If this is the case, removal of the embryo from the uterus with the visceral yolk sac covering intact after a very short maternal exposure to the dye should allow observations on immediate disturbance of the accumulation of DNA, RNA, and protein material in the embryo as compared to those embryos left continually exposed in utero for a longer period of time. To test this, embryos were removed from the uterus 4 and 24 hr after matermal injection of the dye and cultured in vitro for 24–42 hr.

Since the best cultivation is made using Day 10 embryos, it is obvious that the manifestation of morphologic and biochemical abnormalities may vary between those embryos injected on Day 9 from those injected on Day 10.

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This requires that concomitant controls be carried with each experiment. Deviation should, therefore, be compared between experimentals and controls and not between experimental groups. However, since abnormalities are seen with either injection time (Beck and Lloyd, 1966), the rate of exposure necessary to detect abnormalities can be tested effectively using this method.

MATERIALS AND METHODS

Morphologic procedures

Wistar-derived rats maintained in our laboratories were fed Teklad Rat Diet (Teklad Mills, Winfield, Iowa) and water ad libitum with lettuce supplemented weekly. Temperature was from 70–72°F and a 14-hr light cycle was maintained throughout. A single male was placed with 3–6 females (~150 grams) in the evening and vaginal sperm was checked the following morning. The discovery of sperm was designated Day 0 of pregnancy.

The embryos were dissected on Day 10 between 10 AM and 2 PM. Pregnant females had been injected ip with 14 mg/kg of trypan blue either 4 or 24 hr earlier. Concomitantly dissected control animals were either sham injected with the water vehicle or not injected. As no significant differences were noted between the results of the two methods for control embryos, results were pooled.

Dissection was according to the method of New and Coppola ('77). At this time, the embryos normally have 5–9 somites, are dorsiflexed, have neuropores open, have an absence of limb buds, have an allantoic sac separated from the amniotic sac, and do not have a visceral yolk sac circulation evident. Cultivation conditions followed the method of Kochhar ('75) using 50% Waymouth's medium (GIBCO) and 50% fetal calf serum (GIBCO). The Waymouth's medium was supplemented with 5,000 U/liter of penicillin and 5,000 μg/liter of streptomycin. Embryos with the visceral yolk sac intact were individually placed in 10-ml screw-top plastic tubes containing 1.5 ml of medium maintained at 37°C and rotated on a mechanical rotator at 30–40 RPM. The gassing sequence used was that suggested by New and Coppola ('77) beginning with 10% O_2, 5% CO_2, and 85% N_2. The O_2% was increased every 12 hr to 20, 50, and 80%. Nitrogen was decreased concomitantly. The 5% CO_2 was held constant for stabilization of pH. The embryos were either terminated after 24 hr or transferred to fresh medium, and cultivation continued for 42 hr. At least five separate experimental trials for each 4- and 24-hr exposure were made using a total of 20 dams, 150 cultivated embryos, and 232 total embryos. DNA, RNA, and protein assays were made using approximately 26 or more embryos for each time period including a 0 hour cultivation base (see Table 1 for experimental grouping).

Analytic methods

Gross anatomical parameters

At termination of cultivation, at 24 or 42 hr, a number of developmental parameters were analyzed. Growth in size and shape were selectively made by photographic measurements. Heart rate was timed by stopwatch for 15 sec at 37°C. Somite numbers were counted. Over the time period chosen for cultivation, a number of morphologic changes in the embryo occur in vivo. These include: closure of the anterior neuropore; rotation to a ventroflexed position; indication of anterior limb bud swellings; establishment of visceral yolk sac circulation; and fusion of the amniotic and allantoic sacs. Subjective comparisons of treated and nontreated embryos were made for these. The presence or absence of an edematous condition in the pericardial sac or ventricles of the brain was also noted for cultivated embryos.

Protein analysis

Analysis followed the method of Lowry et al. (1951). At 0 and 24 hr, the homogenate of one embryo was used for analysis. At 42 hr the homogenate could be diluted by one-half.

DNA and RNA analysis

Analysis was by the ethidium bromide fluorometric technique of Prasad et al. ('72) as modified by Dr. E. Ritter (personal communication '78) to include the expected DNA and RNA values of 10- to 12-day-old rat embryos for the determined standard curves. The spectrophotofluorometer excitation wave length was 365 nm and emission wave length was 590 nm. Analysis was made at 0, 24, and/or 42 hr for each experiment.

Statistical analysis

A multiple linear regression analysis was used with P values less than 0.05 indicating a level of significance. The Student's t-test was used for comparisons between the treated and nontreated embryos. An analysis of variance and Dunnett's d statistic were also compared.

TABLE 1. Percentage increase of control over experimental cultivated rat embryos dissected on Day 10 of development

Group	Maternal injection day	Dissection interval in hours	Cultivation time in hours	Numbered assayed	DNA accumulation (% Δ)	RNA accumulation (% Δ)	Protein accumulation (% Δ)
1a	9	24	0	44	50 ± 33.1*	34.4 ± 4.1*	2.0 ± 2.3
1b	10	4	0	42	5.6 ± 1.7	108.3 ± 128.9	−1.6 ± 10.4
2a	9	24	24	46	70.8 ± 28.5*	56.5 ± 6.2*	7.8 ± 5.6
2b	10	4	24	43	25.9 ± 6.5*	130.6 ± 36.4*	22.7 ± 9.8*
3a	9	24	42	30	100 ± 72.5*	70.2 ± 10.6*	11.8 ± 15.3
3b	10	4	42	26	44.4 ± 10.3*	146.9 ± 76.7*	44.5 ± 20.8*

* Significant deviation from control levels at $P < 0.05$.

The correlation coefficients of linearized standard curves were all greater than r = 0.98.

RESULTS

Gross anatomical differences

Using a total of 69 control embryos, 41 embryos exposed in utero for 4 hr, and 40 embryos exposed in utero for 24 hr, the effect of trypan blue on the visceral yolk sac circulation was obvious. For the control embryos, 87.2 ± 5% established a visceral yolk sac circulation at 24 hr of cultivation. A significant drop was noted for the embryos exposed in utero for 24 hr to trypan blue with only 17.5 ± 6.1% establishing circulation at 24 hr of cultivation. For those exposed for just 4 hr, there was also a significant drop at 43.9 ± 7.9% establishing circulation at 24 hr of cultivation. The other gross anatomical differences include a significant difference in the number of embryos turning to a ventroflexed position, retention of an open anterior neuropore, and retardation in somite addition. (Other statistics presented elsewhere, Beaudoin and Fisher, '80).

Control increase in somite number for embryos exposed for 24 hr in utero at 0, 24, and 42 hr of cultivation were: 7.5 ± 0.4, 17 ± 0.7, and 22 ± 1.1, respectively. For the respective experimental embryos, increase in somite numbers at 0, 24, and 42 hr of cultivation were: 6.8 ± 0.4, 13.7 ± 0.5,* and 15.8 ± 1.1* (asterisk indicates significant difference). For embryos exposed for just 4 hr to trypan blue in utero, the control increase in somite members for cultivation at 0, 24, and 42 hr were: 7.4 ± 0.6, 17.9 ± 0.3 and 22.2 ± 0.3, respectively. For the respective treated embryos, increase in numbers at 0, 24, and 42 hr cultivation were 6.6 ± 0.4, 15.7 ± 0.8,* and 18.8 ± 1.4* (asterisk indicates significant difference). Along with this, a significant increase in central nervous system and cranial edema was also noted for treated embryos at both 4 and 24 hr in utero exposure with the latter figured (Fig. 1).

Biochemical differences after 24- and 4-hr embryonic exposure to trypan blue

A multiple regression analysis showed that a significant difference for DNA, RNA, and protein accumulation was not detectable at the beginning of cultivation. During the culture period for embryos exposed for both 24 and 4 hr to trypan blue in utero, total embryonic DNA and RNA were significantly reduced at 24 and 42 hr of cultivation (Figs. 2 and 5).

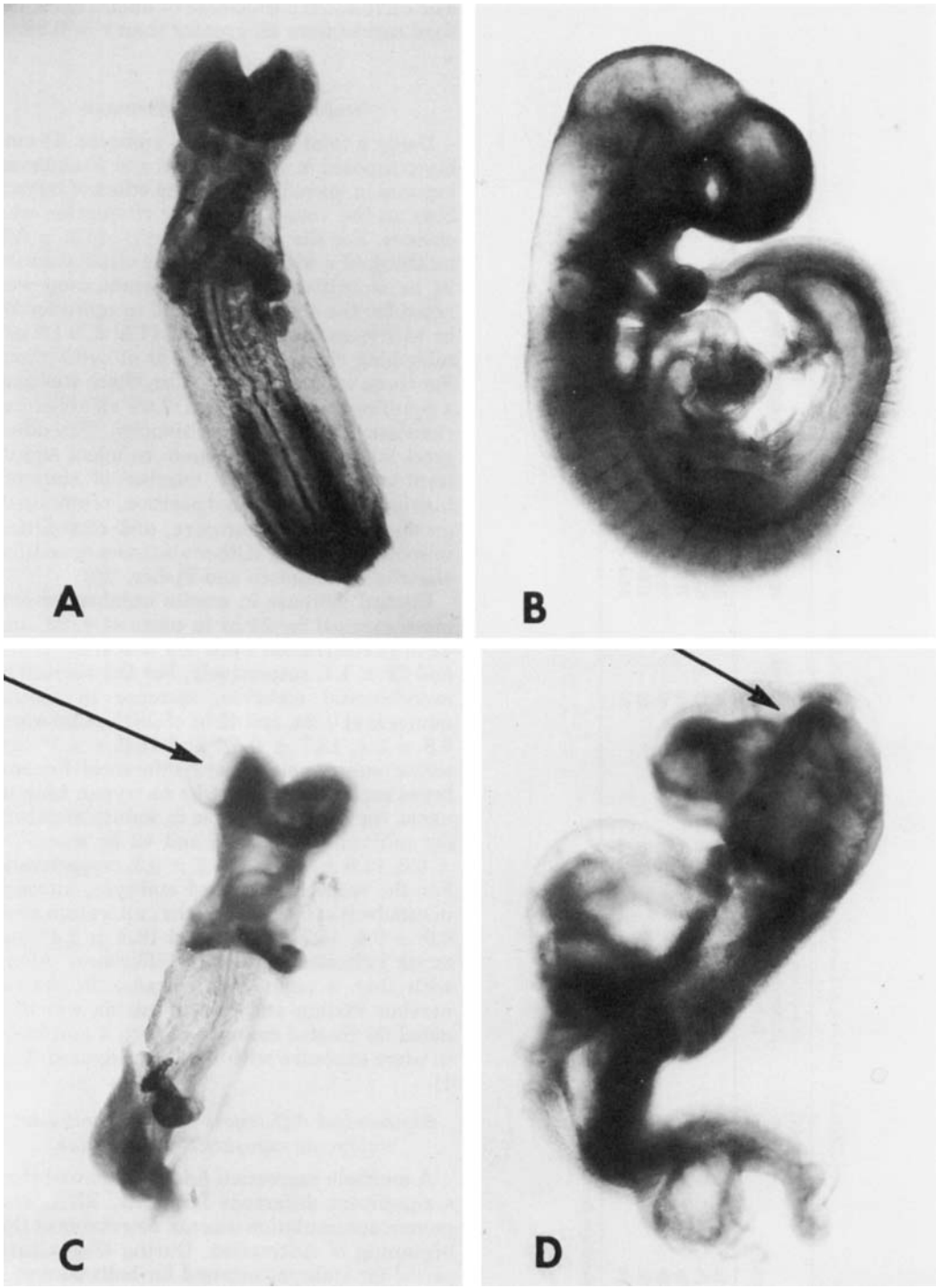

Fig. 1. Development of embryos in vitro following 24 hr of in utero exposure to trypan blue. A, 10-day-old control embryo at beginning of cultivation; B, control embryo after 24 hr of cultivation; C, embryo from trypan blue-treated mother at beginning of cultivation (arrow indicates cranial edema already begun); D, Embryo from trypan blue-treated mother after 24 hr of cultivation (Arrow indicates open anterior neuropore).

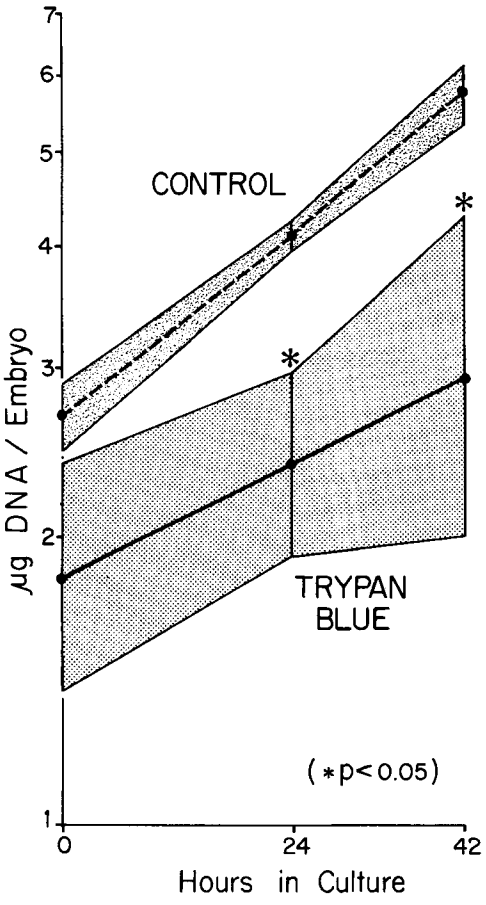

Fig. 2. Effects of trypan blue on DNA accumulation in the embryo over the cultivation period following in utero exposure 24 hr prior to dissection. Changes in value using slope and intercept were determined for each trial of control and experimental embryos over the cultivation period. Data were pooled with a mean and standard error at 0, 24, and 42 hr as indicated. Each point is represented by separate measurements on 15 or more embryos.

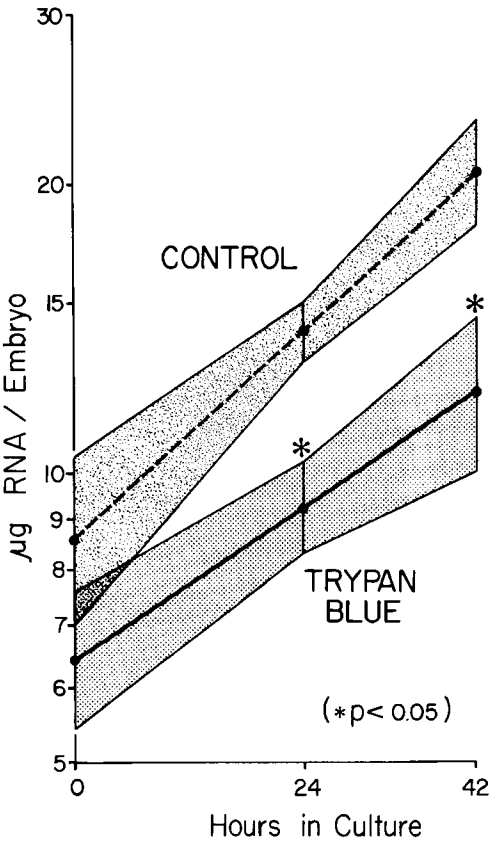

Fig. 3. Effects of trypan blue on DNA accumulation in the embryo over the cultivation period following in utero exposure 24 hr prior to dissection. Changes in value using slope and intercept were determined for each trial of control and experimental embryos over the cultivation period. Data were pooled with a mean and standard error at 0, 24, and 42 hr as indicated. Each point is represented by separate measurements on 15 or more embryos.

Total embryonic protein accumulation during the cultivation period for embryos exposed in utero for 4 hr was significantly reduced over concomitant controls (Fig. 6), but those exposed for 24 hr were not.

If the percentage increase of control over experimental embryos is compared by group for accumulation of DNA, RNA, and protein (Table 1), a significant drop in both DNA and RNA accumulation is noted throughout cultivation for those embryos exposed in utero for 24 hr, but protein accumulation does not differ. For those embryos exposed in utero for just 4 hr, DNA, RNA, and protein accumulation levels were not significantly different at the beginning of cultivation, but all were significantly lower by 24 hr of cultivation and thereafter at 42 hr.

DISCUSSION

Berry ('70) has shown that all embryos from trypan blue-treated mothers are affected by the drug, whether visibly abnormal or not. Our observation on both DNA and RNA accumu-

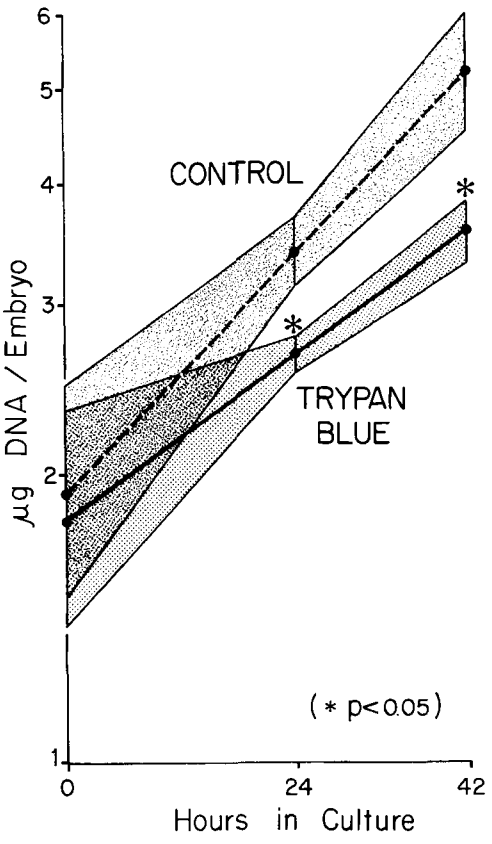

Fig. 4. Effects of trypan blue on DNA accumulation in the embryo over the cultivation period following in utero exposure 4 hr prior to dissection. Changes in value using slope and intercept were determined for each trial of control and experimental embryos over the cultivation period. Data were pooled with a mean and standard error at 0, 24, and 42 hr as indicated. Each point is represented by separate measurements on 15 or more embryos.

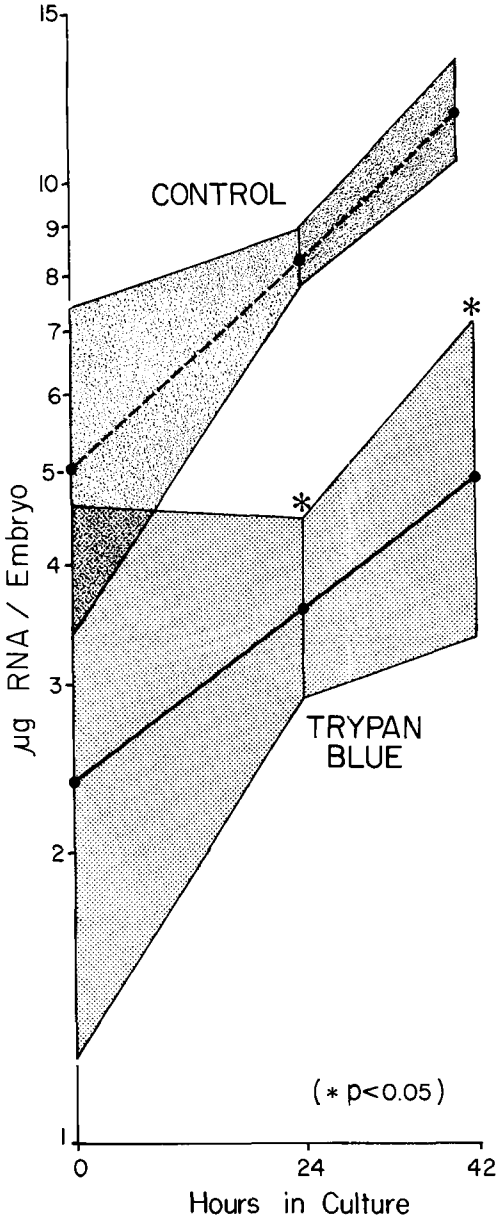

Fig. 5. Effects of trypan blue on RNA accumulation in the embryo over the cultivation period following in utero exposure 4 hr prior to dissection. Changes in value using slope and intercept were determined for each trial of control and experimental embryos over the cultivation period. Data were pooled with a mean and standard error at 0, 24, and 42 hr as indicated. Each point is represented by separate measurements on 15 or more embryos.

lation in the embryo would tend to support this. It was evident that within 24 hr after isolation from the uterus, a drastic alteration appears in the total embryonic content of both DNA and RNA following the embryonic exposure to trypan blue of both 4 and 24 hr (group 2 of Table 1). In both cases the visceral yolk sac on isolation had a visible blue tint to it. An impairment in functional development of the visceral yolk sac by reduction of the percentage of embryos developing a normally evident circulation may account for the total cellular reduction. From the data, the differences be-

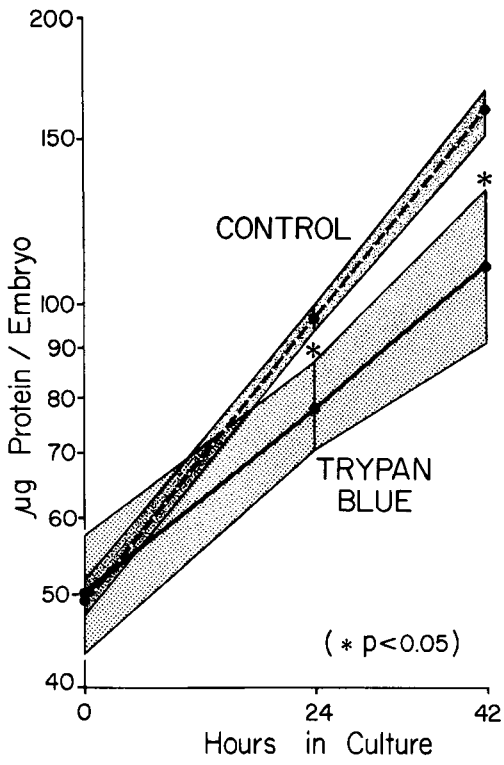

Fig. 6. Effects of trypan blue on protein accumulation in the embryo over the cultivation period following in utero exposure 4 hr prior to dissection. Changes in value using slope and intercept were determined for each trial of control and experimental embryos over the cultivation period. Data were pooled with a mean and standard error at 0, 24, and 42 hr as indicated. Each point is represented by separate measurements on 15 or more embryos.

tween the two control levels for either 4- or 24-hr treatment groups for DNA and protein accumulation as well as increase in somite number in the embryos were not significant. The significant differences in RNA values for the two control groups indicates the necessity for concomitant controls to be run with each experiment. Although the time between injection and dissection were closely controlled, final dissection age varied ±4 hr and may account for the difference noted in control embryo groups for total RNA accumulation.

It can be concluded that as little as 4 hr of exposure in utero is enough to impair visceral yolk sac function (group 2b and 3b of Table 1). The decrease in somite number due to trypan blue treatment for 4 hr in utero would also support this conclusion. The perplexing problems deal with total protein content of the embryo. Those embryos treated for 4 hr showed a significant decrease in total protein content over the cultivation period while those treated for 24 hr did not.

Berry ('70) determined that when protein content for a given somite number was measured, the reduction of somites does not permanently affect the capacity of embryos to grow. He suggested that after the chorioallantoic placenta is established the protein deficit is repaired. The suggestion that embryos can repair damage sustained during early morphogenesis has been advanced in the literature. Hamburgh and Callahan ('67) and Hamburgh et al. ('75) indicated it is possible that mouse embryos possess repair capacity after treatment with trypan blue. From our observation it is evident that cellular damage due to trypan blue treatment does occur, but that recovery, as far as protein synthesis capacity is concerned, is possible for rat embryos also, but only those left in vivo for 24 hr after exposure on Day 9. A 4-hr exposure on Day 10 is adequate to allow damage to the visceral yolk sac of the embryo to occur, but may not be sufficient time to allow for protein synthesis repair capacities to be initiated. In both cases of a 4- and 24-hr exposure, the dye was administered and isolation occurred before the functional chorioallantoic placenta was established with a visceral yolk sac circulation. Repair mechanisms, thus, appear to have occurred earlier than that suggested by Berry ('70).

It is of course conceivable that the transitory effects on total embryonic proteins may be irrelevant to the actual malformations that follows trypan blue treatment. If it were possible to extend the cultivation period, one would also like to know whether there is eventually any recovery in DNA or RNA content. The embryos treated 4 hr before dissection also were older at treatment than those treated on Day 9 of development, and initiation of protein accumulation restoration may take more than 24 hr to repair at this age. It can also be postulated that recovery of protein synthesis capabilities for the embryo may be maternally mediated and required the in utero presence of the embryo for between 4 and 24 hr. Further experimentation is necessary before a definitive conclusion can be made.

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