Trypan blue induced teratogenesis of rat embryos cultivated in vitro

By M. M. TURBOW

From the Strangeways Research Laboratory, Cambridge, England

Trypan blue is known to produce embryonic abnormalities in a wide variety of animals, including rats (Gillman, Gilbert, Gillman & Spence, 1948), mice (Waddington & Carter, 1953; Hamburgh, 1954), amphibians (Waddington & Perry, 1956), chickens (Beaudoin & Wilson, 1958; Stéphan & Sutter, 1961), rabbits (Ferm, 1956), and hamsters (Ferm, 1958). Studies on the teratogenic action of this dye have also been made in culture (Mulherkar, 1960).

Since the first observation of the teratogenic action of trypan blue in rat embryos (Gillman et al. 1948), two questions that have remained unanswered are (1) whether the dye acts directly on the embryo or via the maternal system, and (2) what determines the period during which the rat embryo is most sensitive to the dye.

The following account is an extension of a preliminary report (Turbow, 1965) on the direct effect of trypan blue on the isolated rat embryo cultivated in vitro and the role of the yolk-sac placenta in determining the period of sensitivity to the teratogenic action of the dye; the possible mechanisms of the teratogenic effect of the dye have also been investigated, and the results are discussed.

METHODS

The embryos were obtained from rats of an inbred hooded strain, mated 10–11 days previously. Five- to fourteen-somite embryos were explanted and cultured according to the technique of New & Stein (1964).

After the removal of the decidual tissue and Reichert's membrane the embryo, surrounded by the amnion and the yolk sac and immersed in chick embryo extract, was transferred with a large-bore pipette on to clots composed of three parts cock plasma and one part 13-day chick embryo extract. The cultures were incubated at 36.5 °C in a 60% O₂-4% CO₂-36% N₂ atmosphere for 18–48 h. To allow for differences within a litter all embryos from the same mother were incubated for the same period of time; when several of the embryos showed signs of a failing yolk-sac circulation or failing heart beat, the entire litter was removed from culture.

1 Author's address: Department of Pharmacology, University of California Medical Center, San Francisco 22, California, U.S.A.
Before cultivation the embryos were exposed to trypan blue either by immersion in a solution of the dye or by injection of the dye into the 'yolk-sac cavity' (i.e. the extra-embryonic coelom surrounded by the yolk sac) before immersion in a solution of the dye. The trypan blue was dissolved in glucose Tyrode's solution (Tyrode's solution containing an extra 1% glucose) for injection and in a mixture of equal parts of glucose Tyrode's and embryo extract solution for soaking. All embryos were transferred to the culture clots in a drop of the immersion solution containing the dye, so that the yolk sac surrounding the embryo was in contact with trypan blue during the entire culture period. Because of its colloidal nature trypan blue diffused only slightly into the clot, and at the end of incubation only the area immediately surrounding the embryo was stained. Controls were immersed in embryo extract or injected with glucose Tyrode's solution before immersion in embryo extract.

Injections were made with a fine glass needle attached by a silicone tube to an Agla micrometer syringe. While the embryo was held still with a pair of watchmaker's forceps, the glass needle was inserted through the yolk-sac placenta into the yolk-sac cavity and 0.05–0.12 ml of the solution was injected (Text-fig. 1). In some of the embryos injections were made into the amniotic cavity as well as into the yolk sac, but no alteration in teratogenic activity or in the proportion of embryos affected was observed.

In addition to trypan blue, embryos were also exposed to the following agents: 7,2'-methylphenylazo-1-amino-8-naphthol-3,6-disulphonic acid (half-trypan blue), gelatin, and colloidal carbon, which was prepared by suspending lamp-black in a gelatin solution (McClung, 1929). (Trypan blue is a symmetrical molecule, and if it were split into two identical halves at the diphenyl linkage, 7,2'-methylphenylazo-1-amino-8-naphthol-3,6-disulphonic acid, or half the trypan blue molecule, would result.) A 10% solution of the experimental agent in either distilled water or 0.9% NaCl solution was prepared, autoclaved, and then stored under refrigeration until used; no stock solution was kept longer than 3 weeks. The desired concentrations of the agents were obtained by dilution of the 10% stock solution with glucose Tyrode's solution; compounds were

![Text-fig. 1. Injection of material by fine glass needle into ‘yolk-sac cavity’ (i.e. the extra-embryonic coelom surrounded by the yolk sac).]
administered to the embryo in the following concentrations: trypan blue, 0.05%, 0.1%, 0.2%, 0.4%; half-trypan blue, 0.05%, 0.1%; colloidal carbon 5.0%, 10.0%; gelatin, 0.4%. As determined spectrophotometrically the commercial sample of trypan blue (purchased from George T. Gurr) used in these experiments contained approximately 20% dyestuff, the remainder probably being water and NaCl. As Barber & Geer (1964) demonstrated, only the blue fraction of the commercial dyestuff is teratogenically active. Since only 20% of the trypan blue solution administered to the embryo was active, related compounds (e.g. half-trypan blue) which were at least 85% pure contained approximately 4-5 times more potentially active teratogen than the trypan blue solution.

The embryos were examined as living mounts at intervals during and at the end of the incubation period. Cardiovascular function was assessed by observing the heart beat and the yolk-sac circulation 18-24 h after the beginning of incubation. The culture dish containing 2-7 embryos was removed from the incubator, and the activity of the heart and yolk-sac circulation of all the embryos was examined within 1-2 min; the cultures do not cool sufficiently during this period to affect cardiovascular activity. The embryos were fixed in Bouin’s fluid, stained with eosin or borax carmine, cleared with cedarwood oil or di-n-butyl phthalate, and examined as whole mounts; all measurements and final assessment of gross abnormalities were made on the stained whole mounts. After examination as whole mounts, selected specimens were sectioned, stained with haematoxylin and eosin, and examined histologically.

**RESULTS**

As can be seen in Table 1, all concentrations of trypan blue injected into the yolk sac produced a significant number of abnormalities. Soaking in trypan blue at the lowest concentration produced only an occasional abnormal embryo. Increasing concentrations of dye produced an increasing proportion of abnormal embryos. At most concentrations used the injected embryos showed a higher proportion of abnormalities than did those immersed, but at the highest concentration, 0.4%, the immersed embryos showed more abnormalities than did the injected.

Regardless of the route of administration, the types of abnormalities produced by trypan blue were similar: oedema, swelling of the head or head folds, enlarged pericardium, subectodermal blisters, distension of blood vessels, enlargement of the heart, and a decreased rate of development. The most common and conspicuous effect was the enlargement of the head (Plate 1, figs. B, E), and approximately 60% of the affected embryos exhibited this malformation. Generalized oedema and enlargement of the body were often associated with distended heads. Subectodermal blisters (Plate 1, fig. C; Plate 2, fig. C) and enlarged pericardium (Plate 2, fig. E) and/or heart appeared in approximately 20% of the malformed embryos. Histological examination confirmed observa-
tions made on whole mounts and revealed further pathological changes such as
distended blood vessels (Plate 2, figs. B, E) and haematomas.

Growth and development were much affected by the dye. Regardless of the
route of administration, as the concentration of trypan blue increased, growth,
as measured by the increase in the number of somites during the incubation
period, was proportionately less (Text-fig. 2). The functional activity of the
cardiovascular system also was severely disturbed after 18–24 h incubation.
Rising doses of trypan blue resulted in progressively lower percentages of
embryos with beating hearts (Text-fig. 3), and there was a similar inverse
relationship between the concentration of dye and the presence of an active
circulation in the yolk sac placenta.

Table 1. Abnormalities produced by trypan blue and other agents

<table>
<thead>
<tr>
<th>Agent and concentration</th>
<th>Route of administration</th>
<th>No. of embryos treated</th>
<th>No. of normal embryos</th>
<th>No. of abnormal embryos</th>
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<tr>
<td>Controls</td>
<td>Soak</td>
<td>12</td>
<td>12</td>
<td>0</td>
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<td></td>
<td>Inject</td>
<td>59</td>
<td>56</td>
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<td>17</td>
<td>15</td>
<td>2 (12%)</td>
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<td>0.05%</td>
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<td>37</td>
<td>15</td>
<td>22 (60%)</td>
</tr>
<tr>
<td>0.1%</td>
<td>Soak</td>
<td>9</td>
<td>7</td>
<td>2 (22%)</td>
</tr>
<tr>
<td></td>
<td>Inject</td>
<td>15</td>
<td>8</td>
<td>7 (47%)</td>
</tr>
<tr>
<td>0.2%</td>
<td>Soak</td>
<td>11</td>
<td>6</td>
<td>5 (45%)</td>
</tr>
<tr>
<td></td>
<td>Inject</td>
<td>11</td>
<td>3</td>
<td>8 (73%)</td>
</tr>
<tr>
<td>0.4%</td>
<td>Soak</td>
<td>11</td>
<td>5</td>
<td>6 (54%)</td>
</tr>
<tr>
<td></td>
<td>Inject</td>
<td>11</td>
<td>9</td>
<td>2 (18%)</td>
</tr>
<tr>
<td>Half-trypan blue</td>
<td>Soak</td>
<td>11</td>
<td>5</td>
<td>6 (54%)</td>
</tr>
<tr>
<td>0.05%</td>
<td>Inject</td>
<td>15</td>
<td>14</td>
<td>1 (6%)</td>
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<td>0.1%</td>
<td>Inject</td>
<td>10</td>
<td>10</td>
<td>0</td>
</tr>
<tr>
<td>Gelatine</td>
<td>Soak</td>
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<td>7</td>
<td>2 (22%)</td>
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<tr>
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<td>Inject</td>
<td>11</td>
<td>11</td>
<td>0</td>
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<tr>
<td>Carbon</td>
<td>Soak</td>
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<td>13</td>
<td>1 (7%)</td>
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<tr>
<td>5–10%</td>
<td>Inject</td>
<td>14</td>
<td>13</td>
<td>1 (7%)</td>
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</table>

Plate 1

All embryos cultured for 20–30 h.

Fig. A. Control embryo (eighteen somites). × 35.
Fig. B. Embryo after treatment with trypan blue (sixteen somites) showing enlarged head and
generalized oedema of the trunk. × 40.
Fig. C. Embryo after treatment with trypan blue (fifteen somites). Note the blister over optic
vesicle (marked by arrow) and enlarged head. × 50.
Fig. D. Control embryo sectioned transversely through head (eighteen somites). × 80.
Fig. E. Embryo after treatment with trypan blue (seventeen somites), sectioned as in Fig. D.
Note enormous, oedematous head with general sparseness of the tissue. × 80.
Trypan blue induced teratogenesis

Text-fig. 2. The effect of trypan blue on embryonic growth as measured by the increase in the number of somites during the incubation period: ■, 0–6 somite increase; □, 7–10 somite increase; □□ > 10 somite increase. The figures represent both injected and soaked treatment for each concentration.

Text-fig. 3. The effect of trypan blue on the development of the heart beat of embryos cultivated in vitro. The figures represent both injected and soaked treatment for each concentration.

PLATE 2

All embryos cultured for 20–22 h.

Fig. A. Control embryo sectioned transversely at level of the heart. ×185.
Fig. B. Trypan blue treated embryo sectioned as in fig. A, showing distended blood vessel over the neural tube. ×185.
Fig. C. Trypan blue treated embryo sectioned at level of heart, showing subectodermal blister on pericardial sac. ×160.
Fig. D. Control embryo sectioned at level of the heart. ×95.
Fig. E. Trypan blue treated embryo sectioned as in fig. D. Note the enlarged pericardium and dilated blood vessels (dorsal aorta). ×95.
In contrast to the oedemic abnormalities observed in embryos treated with trypan blue, two of the control and two of the carbon-treated embryos showed severe twisting and distortion of the body axis. The injection of colloidal carbon did not cause a significant increase in number of abnormalities or disturbed cardiovascular function, but it retarded growth as indicated by a lower increase in the number of somites during incubation. The growth of the embryos treated with 5 and 10% carbon was about the same as that of embryos treated with 0.05% trypan blue. The injection of half-trypan blue had no adverse effect and produced no malformation, retardation of growth, or decline in cardiovascular activity.

DISCUSSION

The results of the present study show that trypan blue has a direct teratogenic effect on the rat embryo cultivated in vitro.

The critical period of sensitivity of the rat embryo in utero to the teratogenic action of trypan blue injected into the maternal circulation is from the 7th–9th day of gestation (Gillman et al. 1948). Wilson, Beaudoin & Free (1959) correlated the ending of the period of sensitivity with the closure of the yolk-sac placenta on day 9 and first suggested that the closure of the yolk sac was a possible mechanism terminating the critical period. The work reported here supports this suggestion and shows that in vitro the embryo is still sensitive to trypan blue after day 9. That the yolk sac protects the embryos of 10–11 days’ gestation in utero against trypan blue is shown by the fact that most embryos with the yolk sac intact develop normally in culture media containing low concentrations of the dye, but the same concentrations injected into the yolk sac cause a high proportion of abnormalities. At the highest concentration, 0.4%, the immersed embryos show more abnormalities than the injected because the latter die before abnormalities can develop.

Although the yolk-sac placenta accumulates trypan blue, the dye cannot be detected within the embryo by either macroscopic or microscopic examination. The apparent absence of dye in the embryo may be explained by the absence of reticulo-endothelial cells at this stage of development; as pointed out by Ferm & Beaudoin (1965), only in such cells would one expect accumulation of the dye. Since such exceedingly low doses are sufficient to produce abnormalities, malformations might result from undetectable amounts of the dye within the embryo.

Since malformations were not found in embryos treated with half-trypan blue, two structural requirements related to the teratogenic activity of trypan blue are apparent: the diphenyl linkage must be intact and a single substituted naphthol group is not adequate to cause teratogenesis. Since chick embryos treated with particulate material develop abnormally (Williamson, Blattner & Lutz, 1963), the possibility that the trypan-blue-induced malformations were a result of mechanical forces was investigated by injecting carbon into the yolk
Trypan blue induced teratogenesis

sac of the embryo. Since the carbon did not produce oedemic malformations, although it retarded growth, the oedemic syndrome produced by trypan blue cannot be due to the presence of particulate material next to the embryo.

The oedemic abnormalities caused by trypan blue are similar to changes observed in 9½- to 11½-day-old mice treated with the dye in utero (Waddington & Carter, 1953). Oedemic abnormalities have also been described in chick embryos treated with trypan blue (Stéphan & Sutter, 1961), in chick embryos exposed to mild hypoxia (Grabowski, 1964), and in rat embryos of pregnant mothers subjected to deficiencies of pantothenic acid (Giroud, Lefebores, Prost & Dupuis, 1955) and linoleic acid (Martinet, 1952). The abnormalities such as eye defects, visceral eventuration, hydrocephalus and vertebral defects found in full-term embryos subjected to these agents are probably secondary developments resulting from interruption by oedema of the normal tissue and induction relationships, the migration of cells, or growth of nerve.

A possible cause for oedema is alteration of the permeability of cell membranes. However, when concentrations of 0·01–1·0 % trypan blue were incubated with erythrocytes (method of Dingle & Lucy, 1962), permeability, as measured by the release of potassium ions from the erythrocytes, was not affected.

SUMMARY

1. Five to fourteen somite rat embryos cultivated in vitro were treated by immersion in trypan blue or by injection of the dye into the yolk-sac cavity (i.e. extra-embryonic coelom).

2. Trypan blue has a direct teratogenic effect on the rat embryo cultivated in vitro.

3. The administration of trypan blue caused malformations described as the oedemic syndrome: oedema, swelling of the head, subectodermal blisters, enlargement of the pericardium, distension of blood vessels, and enlargement of the heart; it also retarded growth and disturbed cardiovascular function.

4. The yolk-sac placenta protected the embryo against the teratogenic and toxic effects of trypan blue.

5. Compounds related to trypan blue (i.e. half-trypan blue) have no teratogenic or toxic effects; colloidal carbon did not cause a significant increase in abnormalities, although it retarded growth.

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RÉSUMÉ
Teratogenèse induite par le bleu trypan sur des embryons de rat cultivés in vitro

1. Des embryons de rat, aux stades de cinq à quatorze somites, cultivés in vitro, ont été traités par immersion dans du bleu trypan ou par injection du colorant dans la cavité du sac vitellin (c'est-à-dire dans le coelome extra-embryonnaire).

2. Le bleu trypan a un effet tératogène direct sur l'embryon de rat cultivé in vitro.

3. L'administration de bleu trypan a provoqué des malformations décrites sous le nom de syndrome d'œdème: œdème, gonflement de la tête, vésicules sous-ectodermiques, dilatation du péricarde, distension des vaisseaux sanguins, dilatation du cœur; elle a aussi retardé la croissance et perturbé le fonctionnement cardiovasculaire.

4. Le placenta du sac vitellin a protégé l'embryon contre les effets tératogènes et toxiques du bleu trypan.

5. Des composés apparentés au bleu trypan n'ont pas d'action tératogène ou toxique; du carbone colloidal n'a pas provoqué d'accroissement significatif des anomalies, bien qu'il ait retardé la croissance.

REFERENCES
Trypan blue induced teratogenesis


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