

Teratogenic Activity of Several Closely Related Disazo Dyes on the Developing Chick Embryo

by ALLAN R. BEAUDOIN¹

From the Department of Anatomy, College of Medicine, University of Florida

THE teratogenic activity of several disazo dyes during rat embryogenesis has been investigated by Wilson (1955). In addition to the marked teratogenicity of trypan blue Wilson also demonstrated that Evans blue was moderately active and that Niagara blue 4B and Niagara sky blue 6B were possibly active. On the other hand, several other closely related disazo dyes such as Congo red and azo blue were found to cause no malformations in the surviving offspring.

Since trypan blue has been shown to be teratogenic to the chick embryo (Beaudoin & Wilson, 1958), it is of interest to determine whether other disazo dyes that have been tested in the rat affect the chick embryo in the same or a similar manner.

MATERIALS AND METHODS

Fertile eggs of the White Leghorn chicken were obtained from a commercial hatchery. The eggs were injected either into the yolk sac or subgerminal cavity at 36 hours' incubation (Hamburger-Hamilton stages 9-11). Fresh 0.1 per cent. saline solutions of the dyes were prepared just prior to injection. Eggs injected into the subgerminal cavity received 0.05 ml. of dye solution and eggs injected into the yolk sac received 0.1 ml. (for a description of injection techniques, see Beaudoin & Wilson, 1958). Control eggs were either injected with saline or incubated without injection. The dyes selected for study were trypan blue, Evans blue, Congo red, azo blue, Niagara blue 2B, and Niagara blue 4B (dyes obtained from Matheson, Coleman, & Bell Co., Norwood, Ohio). In all experiments embryos were recovered on the 10th day of incubation, fixed, weighed, and examined for gross malformations. Control eggs were run with each experimental group and handled in an identical manner. Another series of eggs was injected with trypan blue alone at different times during incubation from 0 to 96 hours to test for the span of time through which trypan blue is effective.

RESULTS

Table 1 summarizes the results of the action of several disazo dyes on the embryonic chick when injected either into the yolk sac or subgerminal cavity.

¹ *Author's address:* Department of Anatomy, College of Medicine, University of Florida, Gainesville, Florida, U.S.A.

When the dyes were injected subgerminally trypan blue and Evans blue were found to be teratogenic while azo blue, Niagara blue 2B, Niagara blue 4B, and Congo red were not significantly more teratogenic to chick development than was the injection of saline. On the other hand, when the dyes were injected into

TABLE 1
Teratogenic activity of several disazo dyes on chick development when injected at the 36th hour of incubation

Dye	Total treated		Percentage mortality		Percentage malformed survivors			
	Sub-germinal	Yolk sac	Sub-germinal	Yolk sac	Sub-germinal	P values*	Yolk sac	P values
Trypan blue . . .	101	94	45.5	56.5	72.8	< 0.001	56.2	< 0.001
Evans blue . . .	78	90	44.8	14.4	51.3	< 0.001	5.2	0.52
Niagara blue 4B . . .	94	93	69.3	29.9	34.5	0.10	33.4	< 0.001
Niagara blue 2B . . .	67	86	23.9	9.3	27.4	0.47	0	—
Azo blue . . .	82	93	18.3	10.7	20.9	0.90	0	—
Congo red . . .	95	90	59.0	3.3	35.9	0.08	3.4	0.98
Saline controls . . .	220	141	20.9	12.0	20.1	—	2.4	—
Untreated controls . . .	—	134	—	11.9	—	—	3.4	—

* P values derived from X^2 test for independence.

the yolk sac the results differed somewhat. Trypan blue was still markedly teratogenic, but Evans blue was not. All other dyes remained non-teratogenic except Niagara blue 4B which when injected into the yolk sac was a teratogen. The reason for this change in behavior of Evans blue and Niagara blue 4B, depending on their route of injection, is not known.

TABLE 2
Frequency of malformations among surviving 10-day chicks (percentage of total survivors)

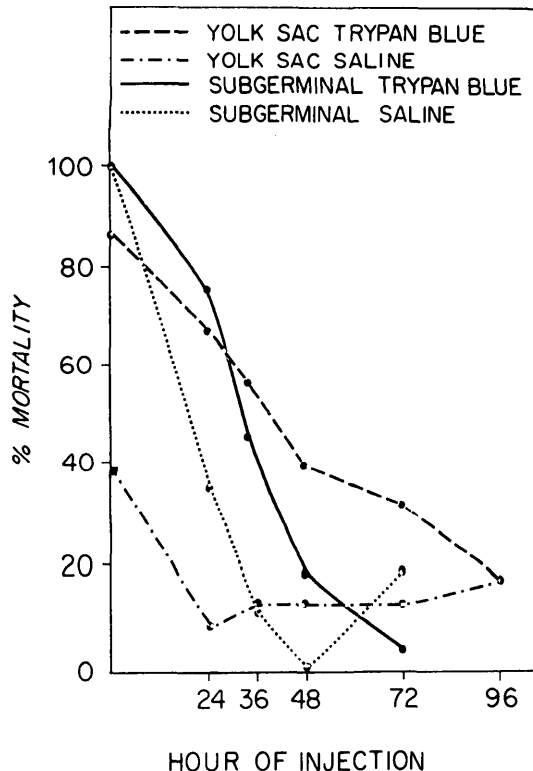
Dye	Rumpless		Eye		Beak		Gastroschisis		Hind limb		Spina bifida		Other	
	SG*	YS†	SG	YS	SG	YS	SG	YS	SG	YS	SG	YS	SG	YS
Trypan blue . . .	69.1	53.6	7.3	2.7	10.9	—	3.6	—	7.3	4.8	—	—	—	2.4
Evans blue . . .	51.2	2.6	7.0	1.3	2.3	—	4.7	—	9.3	1.3	4.7	—	—	6.5
Niagara blue 4B . . .	20.6	31.8	17.2	3.4	17.2	—	3.4	1.5	3.4	—	—	—	—	4.5
Niagara blue 2B . . .	19.6	—	1.9	—	—	—	—	—	—	—	3.9	—	3.9	—
Azo blue . . .	14.9	—	10.4	—	7.5	—	1.5	—	1.5	—	1.5	—	1.5	—
Congo red . . .	33.4	2.3	12.8	2.3	7.7	—	2.6	—	2.6	—	7.7	—	—	—
Saline controls . . .	9.6	2.4	4.6	0.8	1.5	0.8	4.1	—	1.5	—	8.6	0.8	2.5	—
Untreated controls . . .	—	2.5	—	—	—	0.8	—	0.8	—	—	—	—	—	—

* SG = subgerminal.

† YS = yolk sac.

Some general statements can be made regarding the types of malformation observed following treatment with the disazo dyes (Table 2). Rumplessness was the most common single defect observed following treatment with dyes or

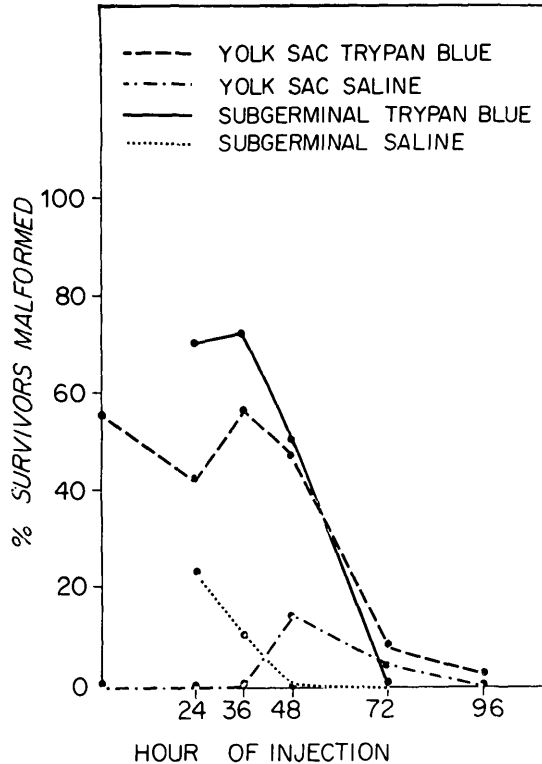
saline, irrespective of the route of injection, and it is noteworthy that rumplessness was also the most common defect among the untreated controls. Two types of eye defects were observed, apparent anophthalmia and various degrees of microphthalmia. Beak defects included cross-beak and deficient beak development. The various other defects were scattered in their occurrence, except spina bifida, which was most common after subgerminal injection, especially following treatment with Congo red and saline. Included in the column headed 'Other' of Table 2 are isolated cases of ectopia cordis, anencephaly, exencephaly, and umbilical hernia. Yolk-sac injections were less traumatic to embryogenesis than injections into the subgerminal cavity. It was found that fewer deaths and abnormalities occurred in embryos from eggs injected into the yolk sac than from eggs injected subgerminally, regardless of the material injected.



TEXT-FIG. 1. The mortality of chick embryos caused by the injection of trypan blue or saline into the yolk sac or subgerminal cavity.

An analysis was made of the duration of effect of trypan blue by injecting this dye at selected intervals during incubation. Text-figs. 1, 2, and 3 show the results of this study. The curves for mortality and numbers of malformed survivors tend to parallel one another. It can be concluded from these figures that trypan blue is an effective teratogen until the 48th or 72nd hour of incubation, after which time little or no teratogenic or lethal effect was seen. If in

addition to expressing the results as the percentage of malformed survivors they are expressed as the percentage of all treated malformed eggs (Text-fig. 3), it is possible to separate the effect of the high mortality from teratogenic activity during the earlier hours of incubation. When this is done it is seen that a peak in susceptibility to the teratogenicity of trypan blue occurred around 36 hours.

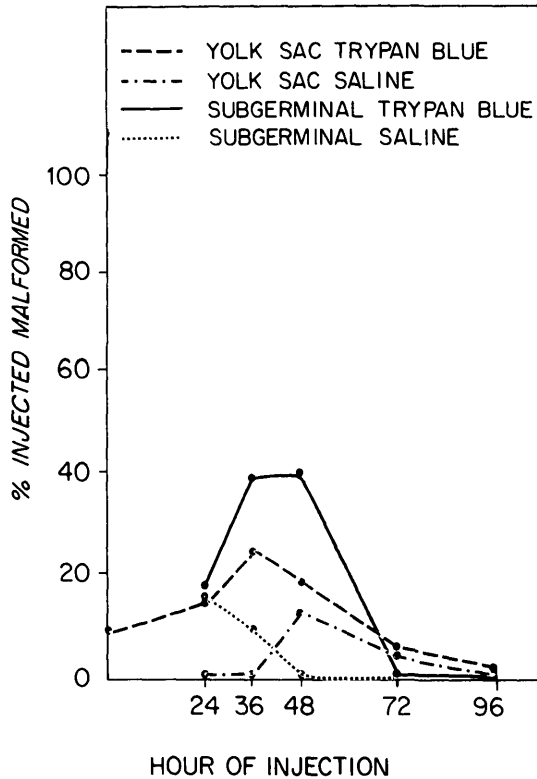


TEXT-FIG. 2. The percentage of 10-day chick embryo survivors malformed following injection of trypan blue or saline into the yolk sac or subgerminal cavity.

DISCUSSION

The results presented in Table 1 for the most part bear out expectations from the work in the rat, in that not all disazo dyes are teratogens. Regardless of the route of injection, trypan blue was markedly teratogenic to the developing chick embryo. However, the teratogenicity of Evans blue and Niagara blue 4B appears to depend upon the route of injection. The remaining dyes tested had no more effect on chick development than did saline. There was always a higher mortality and a higher incidence of malformation among survivors in the subgerminally injected group. This probably is due in part to the mechanical disturbance caused by fluids being forced into the subgerminal cavity under pressure. Landauer & Baumann (1943) have suggested mechanical injury to the blastoderm as a probable cause of rumplessness in chicks when eggs were subjected

to mechanical shaking. In the present study bits of yolk were seen to dislodge and swirl around in the subgerminal cavity when the injection was more forceful than intended. Because of this indication of mechanical disturbance in the original experiment and because of the high incidence of death and abnormality in the saline control series, it was decided to repeat the experiment, but with yolk-sac injections. The results for the yolk-sac injections (Table 1) support the



TEXT-FIG. 3. The percentage of malformed 10-day chick embryos calculated from the total number of eggs injected.

conclusion that the high lethal and teratogenic effect of saline in the subgerminal series may be due in part to mechanical factors, for a significant drop occurred in the mortality and incidence of malformation when the injection was made into the yolk sac. In fact, there were actually fewer malformations in the saline group than in the untreated controls, indicating saline to have no effect as a teratogen in chick development. Nor did such treatment cause any increase in the number of embryonic deaths over the base level established by the untreated controls.

Another factor to be considered is the distribution of the dye following injection. After a subgerminal injection the dye can be observed filling all or a major portion of the subgerminal cavity, thereby presumably coming into direct

contact with cells of the embryo. This is important assuming that there is a direct action of trypan blue on the cells of the embryo. Within one hour after yolk-sac injection the dye had migrated to a point uppermost in the horizontally placed egg, thereby coming to lie directly under the embryo. Regardless of route of injection the dye was never found in any cells of the embryo proper, although cells in the yolk-sac membrane were found to contain particles of the injected dye. The pattern of rise of dye in the egg was the same as that described by Kropp (1957) and Schlesinger (1958). Schlesinger further stated that the migration of substances through the yolk depended upon the relative density of the yolk and of the substance injected. Trypan blue will migrate only very little when injected into yellow yolk, whereas it shows great mobility when injected into the latebra (white yolk). This is due to differences in viscosity, yellow yolk having been estimated to be some 8 times more viscous than the latebral white yolk. Thus, unless dye is injected into the latebra or near enough to it to disrupt the latebra-yellow yolk interface, there will be no migration. Schlesinger has also shown that with an increase in incubation time there is an increase in the speed of migration owing to a decrease in the viscosity of the latebral yolk.

Analysis of the types of defects found in all groups, including untreated controls, showed rumplessness to be the most common single defect observed (Table 2). It is of interest to note that Landauer (1945) reported rumplessness in 2.6 per cent. of his untreated controls and in 2.7 per cent. of his saline yolk-sac injected eggs. This compares with 2.4 per cent. in the untreated controls and 2.5 per cent. in saline-injected eggs of the present study. Only two cases of defects other than rumplessness occurred in the untreated controls, gastroschisis in one rumpless chick, and in another embryo a poorly formed beak as its only defect. Thus the rumpless condition not only occurs spontaneously in the chicks used in these experiments but is the most prevalent of the spontaneous abnormalities. A comparison of the defects found in the chicks with those found in rats showed that many of the same general type of abnormality were present in both. However, the most common defect present in the rat, hydrocephalus, was not observed in any chick embryo. The most susceptible period for the rat is just before organogenesis begins, whereas it appears that the dye can be injected somewhat later in the chick, since organogenesis is well advanced at 36 hours' incubation.

Trypan blue was found to be an effective teratogen until some time between the 48th and 72nd hour of incubation. At this time a marked decrease in its activity occurred. Because the chick embryo develops in a cleidoic system, dye injected during the first hours of incubation will be present when the embryo passes through later susceptible periods. This accounts for the fact that a high percentage of malformations resulted from treatment at any time during the first 48 hours of incubation (Text-fig. 1). There is, however, a high mortality among eggs injected during the first 24 hours, and when allowance was made for this (Text-fig. 3) it was found that a peak in the incidence of rumplessness

apparently occurred at about 36 hours of incubation. This agrees rather closely with 31 hours as observed by Landauer & Bliss (1946) in insulin-induced rumplessness. All major organogenesis in the chick has begun by the 48th hour of incubation, but it is not until the 51st–56th hour that the tail-bud first appears from the primitive knot. Thus any injection prior to the 48th hour would allow dye to be present at the time of formation of the tail. The reason that the 36th hour is more critical may be that the teratogenic effect is on the ‘chemical differentiation’ of the tail tissue before the time that any morphogenetic change is noticeable. It is not known why the formation of the tail is so susceptible while at the same time other organs are undergoing much more pronounced changes. It is quite possible that trypan blue has a specific rumpless-inducing effect in the chick.

SUMMARY

1. The teratogenic activity of trypan blue, Evans blue, azo blue, Congo red, and Niagara blue 2B and 4B was investigated in the chick embryo, using subgerminal or yolk-sac injections at various times during early incubation.

2. In addition to trypan blue it was found that Evans blue was teratogenic when injected into the subgerminal cavity but was not when injected into the yolk sac. The converse was true for Niagara blue 4B. The remaining dyes tested were no more teratogenic than saline.

3. Rumplessness was the most common type of defect observed following injections with a teratogenic dye, regardless of the route of injection. The most susceptible period for the production of rumplessness was during the first 48 hours of incubation, with a peak at 36 hours.

RÉSUMÉ

L'activité tératogène de quelques colorants ‘diazo’ de constitutions chimiques très voisines sur le développement de l'embryon de Poulet

1. L'activité tératogène du bleu trypan, du bleu Evans, de l'azoblu et du bleu Niagara 2B et 4B a été étudiée sur l'embryon de poulet, par la technique des injections dans le sac vitellin ou dans la cavité sous-germinale à différents stades du début de l'incubation.

2. On a trouvé qu'en plus du bleu trypan, le bleu Evans est tératogène quand il est injecté dans la cavité sous-germinale; mais il ne l'est pas quand il est injecté dans le sac vitellin. L'inverse est vrai pour le bleu Niagara 4B. Les autres colorants éprouvés ne sont pas plus tératogènes que les solutions salines.

3. L'absence de croupion est l'anomalie la plus commune que l'on observe après les injections d'un colorant tératogène, quelle que soit la voie d'injection. La phase de plus grande sensibilité pour la production de cette malformation se place dans les 48 premières heures d'incubation, avec un maximum à 36 heures.

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