COMPARISON OF THE TERATOGENIC EFFECTS OF TRYPAN BLUE AND LOW TEMPERATURE IN THE MEDAKA FISH *(ORYZIAS LATIPES)* [1]

JOHN C. BRIGGS [2] and JAMES G. WILSON
University of Florida, Gainesville

The teratogenic activity of the azo dye trypan blue is well established in such animals as the rat, mouse, rabbit, and hamster. The experimental procedure for these forms has usually involved an injection of the dye into the maternal organism during the early stages of pregnancy. Earlier investigators have suggested that the dye produced a change in the maternal metabolism which secondarily affected the embryo or that caused some alteration of placental permeability. However, Ferm (1956) found that in the rabbit trypan blue is demonstrable in the blastocyst fluid after maternal injection on days 7, 8 or 9 of gestation and concluded that the teratogenicity of the dye was due to a direct toxic effect on the embryo. Further evidence for the direct action of trypan blue on the embryo was recently presented by Beaudoin and Wilson (1958) as the result of a series of experiments on the developing chick. Waddington and Perry (1956) claimed a direct teratogenic effect on amphibian embryos but no control animals were referred to in their report. Ingalls, in a popular article (1957), stated that the dye is an effective teratogenic agent upon the developing eggs of the zebra fish *(Brachydanio rerio)* and suggested that the mechanism consists of a reduction in respiratory rate of dividing cells, but no information was given about the experiments which led to these conclusions. No unequivocal evidence of teratogenic action of this dye in a poikilothermic vertebrate has been presented.

The earliest experimental production of abnormalities in fish embryos was that reported by Lereboullet (1864). In one portion of his work he exposed developing European pike *(Esox)* eggs to cold and alternating temperatures (degree and duration not indicated) and reported that this had no effect upon the usual rate of

[1] This investigation was supported by a research grant to the Department of Anatomy, College of Medicine at the University of Florida from the Easter Seal Foundation of the National Society for Crippled Children and Adults.

[2] Present address: Department of Anatomy, University of British Columbia, Vancouver 8, B. C., Canada.

structural abnormality in that species. The next experiment of this type was performed some 50 years later when Jacques Loeb (1915), in attempting to discover the reason why some cave-dwelling fishes possess defective eyes, exposed the eggs of *Fundulus heteroclitus* to unusually low temperatures. He found that when the eggs were newly fertilized or in early cleavage stages, a reduction of the temperature to 0° to 2°C. resulted in the appearance of a "considerable" number of abnormal individuals, many of them possessing eye defects. However, when the temperature was allowed to rise to 7°C. or when embryos of 128 cells or later stages were utilized, the abnormalities did not occur. Kellicott (1916) found that newly fertilized eggs or those in early developmental stages when subjected to temperatures of 8° to 10°C. for a few hours or days either died or developed abnormally. He noted that the circulatory system and the eyes were especially prone to anomaly.

Stockard (1921) subjected 2 to 16 cell cleavage stages of *Fundulus heteroclitus* to 5° to 8°C. for 48 to 69 hours and obtained large numbers of abnormal individuals, included among them several double monsters. He concluded that a connection of primary importance existed between retardation of development by low temperature or other means and the origin of double specimens. Since *Fundulus* and the medaka are members of the same family (Cyprinodontidae) it was decided to use the latter species to test this and others of Stockard's postulates. He maintained, for example, that the time of treatment was the sole determinant of the teratogenic effect and that the nature of the agent was of little consequence so long as it interfered with growth at some "critical moment."

The purpose of this experiment, then, was to compare the effects of a chemical agent, trypan blue, and a physical agent, low temperature, on the developing medaka egg and to examine the results in the light of previous work using these particular teratogens.

Methods

Each pair of breeding adults was isolated in a 10 gallon aquarium. A cluster of fertilized eggs was removed from the female each morning shortly after spawning took place, divided into experimental and control groups of approximately equal size, and

placed in finger bowls in about 150 cc. of water with about 10 to 20 eggs per finger bowl. These eggs were kept well separated from one another. In the trypan blue experiments both experimental and control groups were removed to small stender dishes of 25 cc. capacity. After exposure to either dye or water the eggs were rinsed and returned to the finger bowls until hatching. The dye used was a specially purified sample of trypan blue.[3] Aqueous solutions were freshly made every two weeks and were kept under refrigeration prior to use. In the low temperature experiments the eggs scheduled for treatment were kept in the original finger bowls and placed in a refrigerator for the desired amount of time.

Results

Trypan Blue Experiment. Following the lead of Waddington and Perry (1956), who claimed a significant result in their amphibian work with a concentration of .025%, the initial experiments were carried out using an aqueous solution of trypan blue of this strength. Thirty-eight lots, comprised of 417 eggs, were exposed to the dye for a period of 24 hours beginning at the 15-hour (embryonic shield) stage with an identical number being kept as a control. The dye produced no discernable effect.

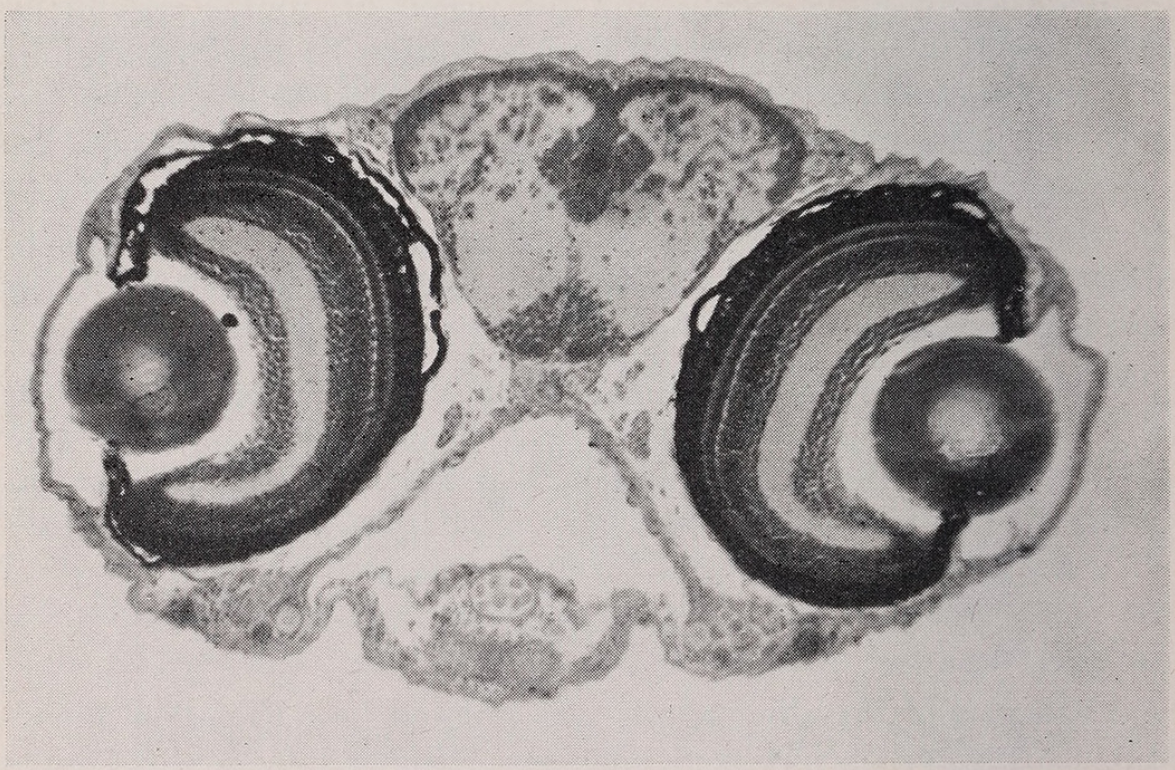

Figure 1.

[3] Procured from Matheson, Coleman and Bell Company, Norwood, Ohio.

TABLE 1

EXPOSURE TO INCREASING CONCENTRATIONS OF TRYPAN BLUE AT THE SIX HOUR (EARLY BLASTULA) STAGE FOR 24 HOURS.

Concentration %	Total Number	Mortality %	Abnormality %
.025	23	30.4	0
Control	22	22.7	0
.050	13	0	0
Control	13	7.7	0
.1	10	60.0	0
Control	10	50.0	0
.2	18	38.9	0
Control	17	17.6	0
.4	26	42.3	0
Control	26	23.0	0
.8	17	5.9	0
Control	17	0	0
1.25	89	38.2	2.2
Control	88	5.7	0
2.50	90	40.0	6.6
Control	90	15.5	1.1

TABLE 2

EXPOSURE TO THE MAXIMUM CONCENTRATION (2.50%) OF TRYPAN BLUE AT PROGRESSIVELY EARLIER STAGES.

Stage	Exposure Hrs.	Total Number	Mortality %	Abnormality %
4-cell	48	44	100.0	0
Control	none	45	8.9	0
4-cell	24	18	94.4	0
Control	none	17	11.8	0
4-cell	4	45	13.3	0
Control	none	46	10.9	0
4-cell	2	124	35.5	0.8
Control	none	122	2.5	0
4-cell	1	67	61.2	1.5
Control	none	66	15.1	0
2-cell	24	171	98.8	0.6
Control	none	169	1.8	0
2-cell	4	131	88.5	1.5
Control	none	132	4.5	0
2-cell	2	138	78.3	0.7
Control	none	134	6.0	2.2
Zygote	.5	26	100.0	0
Control	none	27	0	0

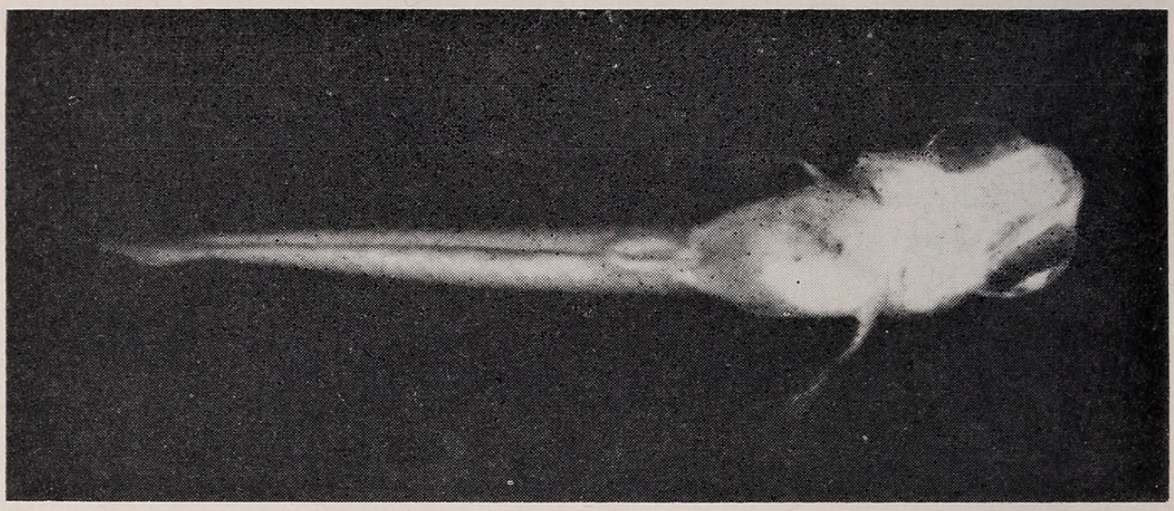

Figure 2.

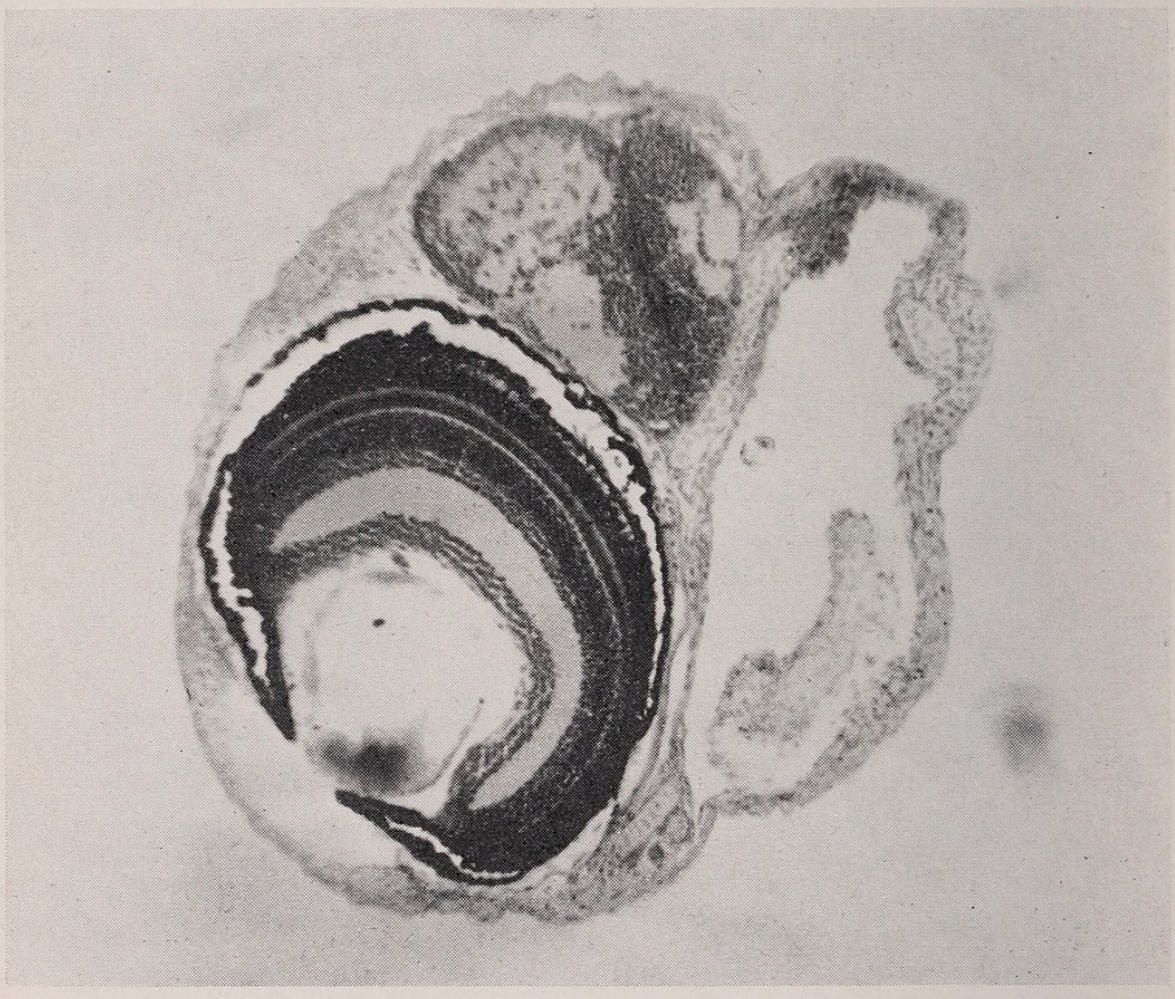

Figure 3.

It was then decided to treat a series of younger embryos at the six-hour (early blastula stage with increasing concentrations of the dye. The results are seen in Table 1. As the concentration increased, mortality in the treated groups exceeded that in the controls, but the rate of malformation still remained very low. Even at the maximal concentration of 2.5%, abnormalities were found in only four lots of the 11 exposed and in only one of these was more than a single-individual affected.

Having ascertained that the majority of the early blastula embryos could survive immersion in a 2.5% solution for several hours, embryos at other stages were exposed to this concentration of dye. Nine lots including 127 eggs were treated for a period of 24 hours beginning at the 25-hour (neural keel) stage, with 122 eggs kept as controls. Mortality was 13.4% and the rate of abnormality 3.9% both considerably lower than was the case with the early blastula embryos. No later stage was treated since the medaka is far advanced in differentiation at 49 hours, the time at which the neural keel lots were removed from the dye.

Finally, a relatively large number of very early embryos was exposed to the maximal dye concentration of 2.5%. These results (Table 2) show a drastic increase in mortality, so much so that in order to obtain any survivors, the period of exposure had to be reduced as younger individuals were treated. The expected increase in numbers of abnormal individuals did not materialize.

Low Temperature Experiment. Seven lots, including 69 eggs, were exposed to 6° to 7°C. for 48 hours at the 25-hour (neural keel) stage, with 71 eggs kept as controls. No abnormalities appeared and the mortality of the treated groups did not exceed that of the controls. Early cleavage stages were then exposed to the same temperature for the same period as follows: 140 eggs at the 2-cell stage, 46 at the 4-cell stage, and 21 at the 8-cell stage, with similar numbers at each stage retained as controls. All of the treated groups suffered a 100% mortality.

To reduce the severity of the treatment for the early cleavage stages, an exposure time of 24 hours was utilized and the temperature kept at 6° to 7°C. Forty-eight lots consisting of 641 eggs were exposed at the 2-cell stage, 5 lots totaling 62 eggs at the 4-cell stage, and 5 lots totaling 69 eggs at the 8-cell stage. The results are tabulated in Table 3. The occurrence of 61 abnormal

young in the treated lots, compared to only two in the control lots, leaves little doubt about the teratogenic effectiveness of low-temperature. It seems apparent that the medaka embryo is most sensitive to low temperature effects at the four-cell stage since this group exhibited the highest percentage of abnormality. Also, it is noteworthy that the mortality decreased markedly as treatment was given at later stages, the survival in the 8-cell group being almost three times that of the 2-cell group and that in the neural-keel stage being within normal range. Further reduction of the exposure time to 12 hours but maintaining the temperature at 6° to 7°C. resulted in no abnormalities and a rate of mortality only slightly higher than in controls.

TABLE 3

EXPOSURE OF EARLY CLEAVAGE STAGES TO 6° - 7° C. FOR 24 HOURS.

Stage	Total Number	Mortality %	Abnormality %
2-cell	641	51.9	7.5
Control	639	3.4	0.3
4-cell	62	29.0	12.9
Control	59	8.5	0
8-cell	69	21.7	7.2
Control	71	2.8	0

Additional lots of early cleavage eggs were exposed for 24 hours to 4°C., a lower temperature than had been previously tried (Table 4). Anomalous individuals were again apparent in the treated lots but the incidence was slightly less than that obtained at the 6° to 7°C. level. However, the increased severity of the 4°C. temperature is reflected in the mortality rate which was significantly higher for all three cleavage stages.

Types of Malformations. Too few malformations occurred after trypan blue treatment to permit the establishment of a characteristic pattern of defects. The more prevalent types occurred in both experimental and control groups in almost equal frequency. Among these were general retardation of development and curled body-axis after hatching, with an occasional case of a pug-nosed condition or disproportionately large head. Found only

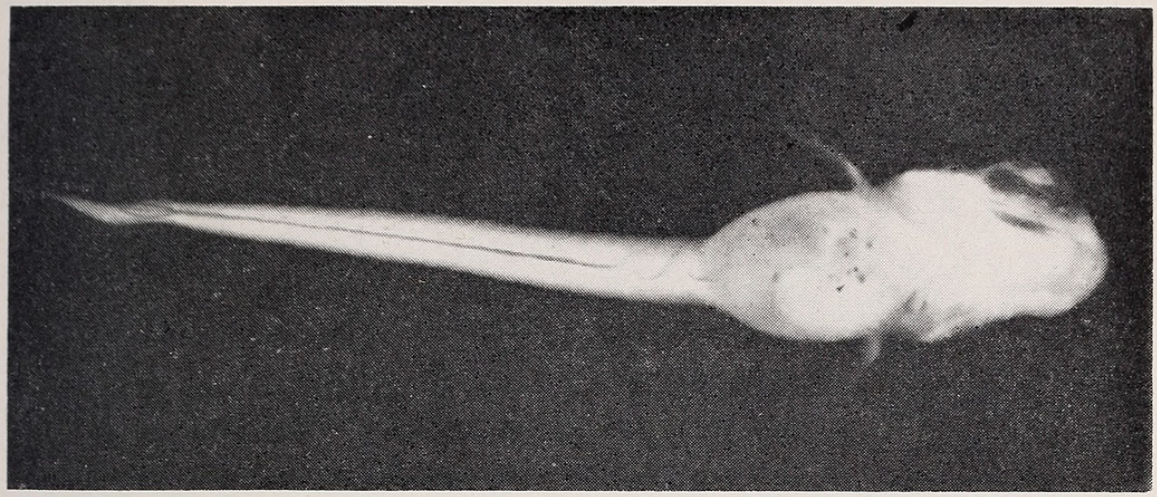

Figure 4.

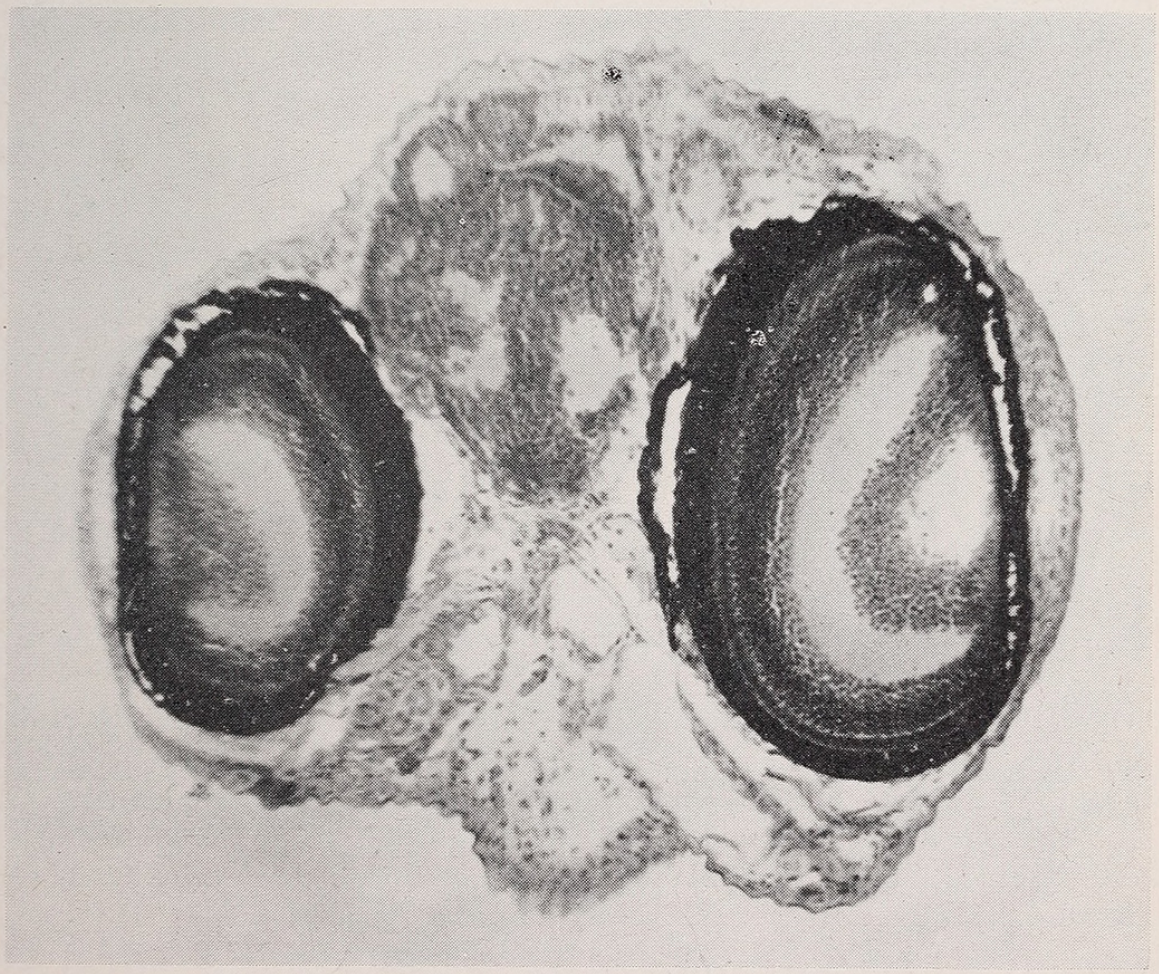

Figure 5.

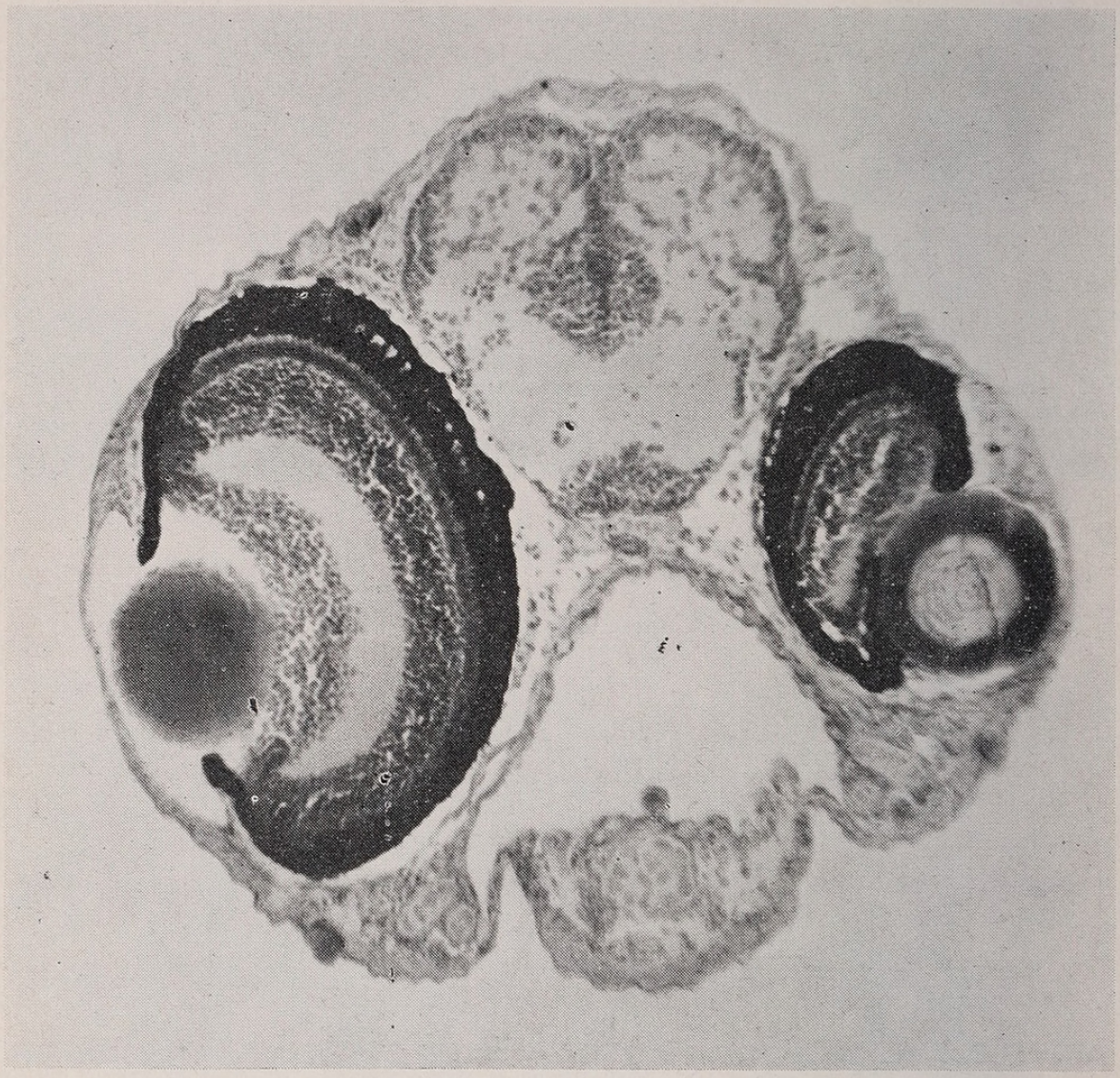

Figure 6.

TABLE 4

EXPOSURE OF EARLY CLEAVAGE STAGES TO 4° C. FOR 24 HOURS.

Stage	Total Number	Mortality %	Abnormality %
2-cell	91	75.8	5.5
Control	89	14.6	0
4-cell	66	89.4	6.1
Control	65	3.1	0
8-cell	38	47.4	7.9
Control	39	0	0

among treated embryos were 4 instances of monophthalmia, one of bilateral microphthalmia and one of complete anophthalmia. Two fishes, one of which also had an eye defect, had conspicuously reduced tail development. Thus, of 1594 treated embryos only 7 had types of developmental abnormalities not observed in equivalent frequency among the controls.

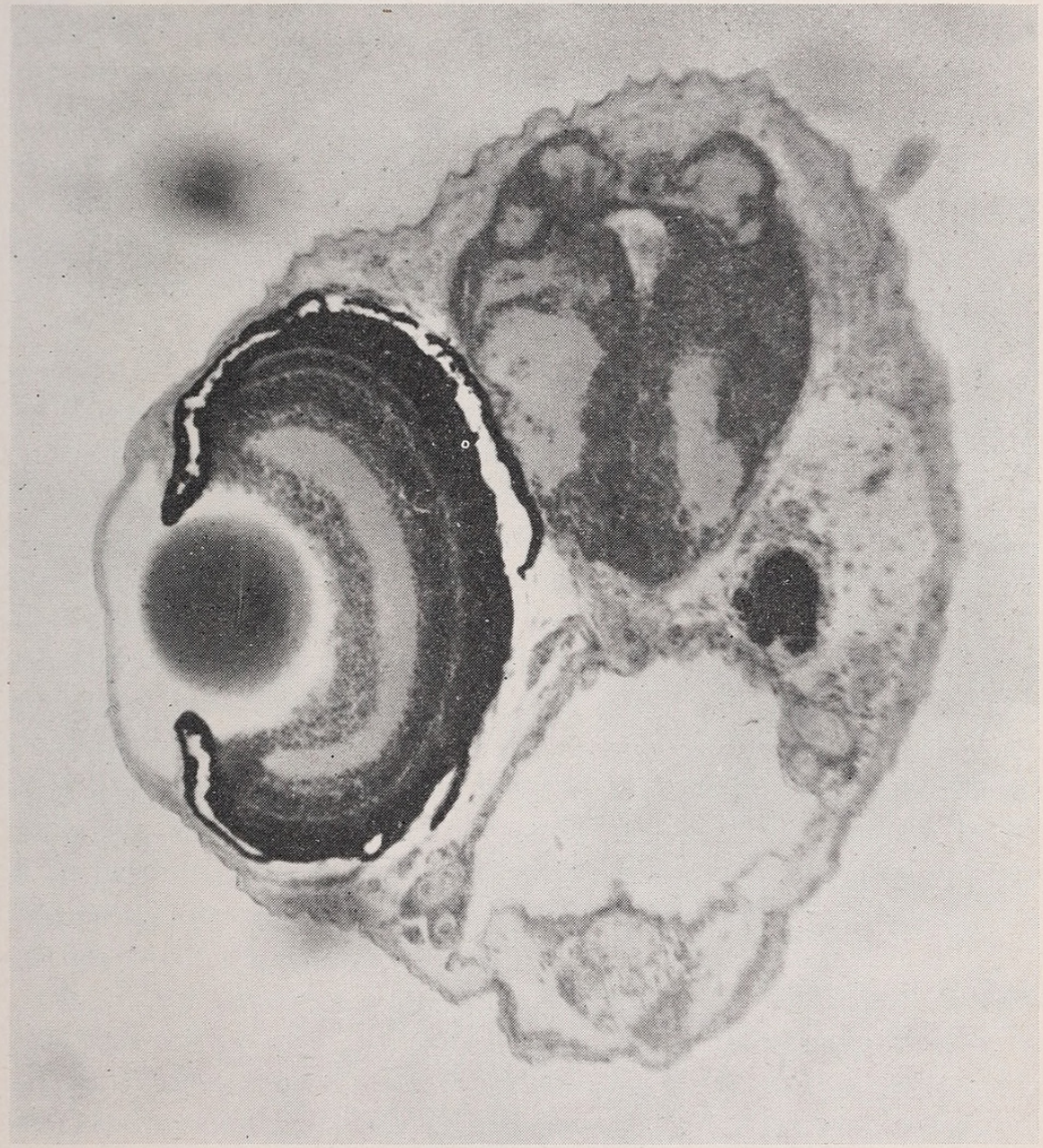

Figure 7.

In the low temperature experiments there was no obvious difference in the type of malformation produced at either of the two temperature levels or among the three cleavage stages. In almost all cases, the most striking result was the relatively large

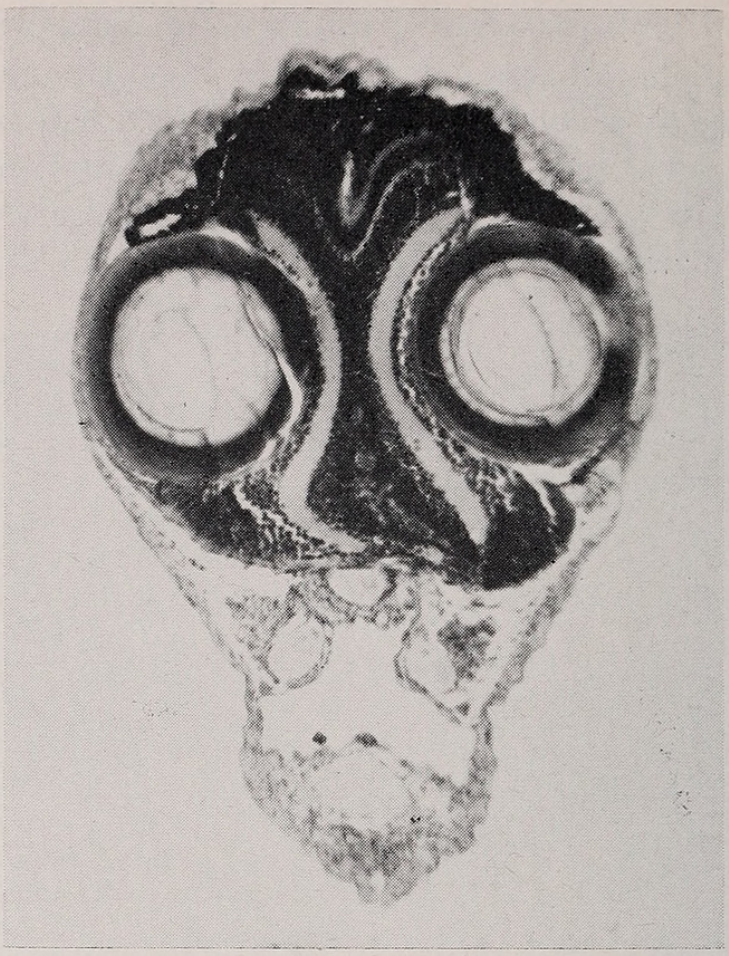

Figure 8.

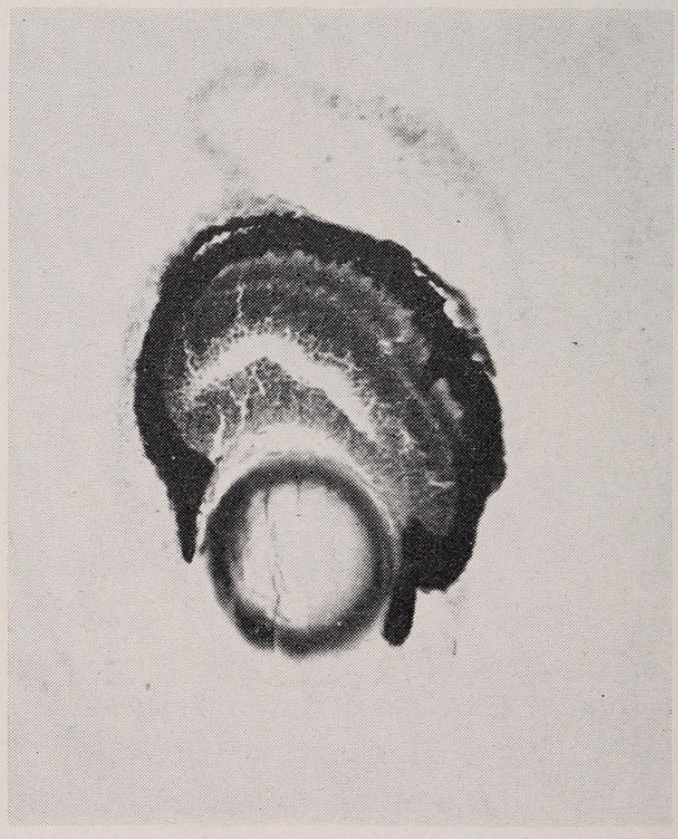

Figure 9.

number of eye defects. Of the total of 73 anomalous individuals produced by all the low temperature experiments, 46 showed some type of eye malformation of which monophthalmia occurred 15 times (Figs. 3 and 4), unilateral microphthalmia 14 times (Figs. 5, 6 and 7), anophthalmia 6 times, bilateral microphthalmia 5 times, various degrees of synophthalmia 5 times (Fig. 8) and cyclopia once (Fig. 9). The majority of these individuals also demonstrated some degree of general retardation and a few possessed other anomalies such as a short, deformed tail or a swollen pericardial region.

Thirteen animals showed general retardation of development with which was associated little visable blood, a small heart and a feeble pulse. Seven of these also had very short and tightly curled tails and the remaining six showed a large, swollen head and/or pericardial region.

Discussion

These results have demonstrated that the medaka fish reacts quite differently to two agents, one chemical and the other physical. Both have been shown to be effective in other species. On the one hand, trypan blue in suitable concentration caused a high mortality rate but a very low rate of malformation when applied to the early embryo for a 24-hour period. On the other hand, reduced temperature caused comparatively less mortality and considerably more malformation during the same period. The full meaning of these quantitative differences is not clear but at least they seem to indicate that in this species trypan blue tends to act upon the embryo in an "all or none" fashion, that is, either killing it or permitting normal development, while reduced temperature frequently does neither but produces what might be regarded as an intermediate result, that is, defective development.

In recent years considerable evidence has accumulated to suggest that different teratogenic agents act in characteristic ways on a given animal species (Wilson, 1955). This has been particularly well demonstrated in rodents and chicks where on several occasions more than one agent has been carefully studied in the same animal by one group of investigators. In most of these instances qualitative as well as quantitative differences in the types of malformations were clearly shown. This has been designated as "agent specificity," implying that each agent acts on the embryo in a specific way, for example, upsets a particular enzyme system, enters

into competitive antagonism for a needed metabolite or in some other way interferes with growth or developmental processes. If two different agents happen to interfere with the same process at the same stage of development, the types of malformations produced should be identical. If they acted on different processes at the same stage or perhaps the same process at different stages, the malformations would not be alike and it should be possible to identify certain patterns as specific to the agent which produced them.

From his extensive experiments on *Fundulus*, Stockard (1921) concluded that all teratogenic agents act in a basically similar way by arresting development at some "critical moment." When development was later resumed the original delicately integrated schedule of organogenesis was never restored and the disproportionate growth that followed resulted in malformation. According to this hypothesis, interruption of growth becomes the only adequate stimulus for teratogenesis and the only other requirement is that growth be interrupted at a critical time in differentiation or organ formation. It would follow that all effective agents should act alike so long as they act during comparable periods in development. This was shown not to be the case in the present experiment. Trypan blue was effective to the extent that it caused a high rate of mortality but it caused only negligible numbers of malformations. Low temperature applied to the same animal at the same stages of development resulted in appreciably less mortality but somewhat more malformation. The specificity of the agent, therefore, seems to be operative in teratogenesis in this lower form as well as in the chick and rodents. It is worthy of incidental note that the medaka did not respond to either of these agents by forming double embryos, a defect which Stockard felt was directly related to treatment of the *Fundulus* embryo with low temperature or hypotonic solutions at a particular time in early development.

We have concluded that trypan blue cannot be considered an effective teratogenic agent upon the medaka embryo. If any such action exists at all, it is certainly to a minor degree and not comparable to the effects which have been demonstrated for several mammalian species and the chick. The findings of Waddington and Perry (1956) in regard to certain amphibian embryos and the conclusions of Ingalls (1957) concerning effects upon the zebra

fish are, therefore, not substantiated by our work on the medaka. In fact, it must be said that a clear-cut teratogenic action by trypan blue upon any poikilothermic vertebrate has yet to be proved.

Summary

Trypan blue in sufficient concentration causes a high rate of mortality among developing medaka embryos. This lethal effect is more pronounced in early than in later stages. At all stages of development and with all concentrations of dye used very few or no malformations were produced, and it is concluded that trypan blue is not an effective teratogenic agent in the medaka.

Exposure of medaka embryos at the 2-, 4- and 8-cell cleavage stages to temperatures of 6 to 7°C. for 24 hours resulted in maldevelopment in an appreciable percentage of the hatchling fish. Such treatment also raised mortality to 51.9% at the 2-cell stage and to more moderate levels at later stages. Doubling the time of exposure caused 100% mortality in all early cleavage stages but caused neither anomalies nor increased mortality at the 25-hour (neural keel) stage. Reducing the exposure to 12 hours resulted in no malformations and very little rise in mortality above control levels. When early cleavage stages were treated at 4°C. for 24 hours both malformation and mortality rose to levels comparable to those obtained with 6 to 7°C. Ocular defects were the predominant type of malformations, although short tail, curled body axis and generally retarded development were observed with some frequency.

Acknowledgments

We wish to thank Mrs. Elenor Parets for her technical assistance and Mr. Robert L. Hay and the Department of Medical Illustrations for the photography.

Literature Cited

BEAUDOIN, A. R., and J. G. WILSON

1958. Teratogenic effect of trypan blue on the developing chick. *Proc. Soc. Exp. Med. and Bio.*, 97:85-90, 8 figs.

FERM, V. H.

1956. Permeability of the rabbit blastocyst to trypan blue. *Anat. Rec.*, 125:745-759, 1 pl.

INGALLS, T. H.
1957. Congenital deformities. *Sci. American,* 197:109-116, 17 figs.

KELLICOTT, W. E.
1916. The effects of low temperature on the development of Fundulus. *Am. Journ. Anat.,* 20:449-482.

LEREBOULLET, A.
1864. Recherches sur les monstrousités du Brochet observées dans l'oeuf et sur leur mode de production. Resumé des expériences précédences. *Ann. Sci. Nat. (Zool. et Paleo),* Ser. 5, 1:257-320.

LOEB, J.
1915. The blindness of the cave fauna and the artificial production of blind fish embryos by heterogeneous hybridization and by low temperatures. *Biol. Bull. Wood's Hole,* 29:50-67, 13 figs.

STOCKARD, C. R.
1921. Developmental rate and structural expression, etc. *Am. Journ. Anat.,* 28:115-266, 32 figs., 6 pls.

WADDINGTON, C. H., and M. M. PERRY
1956. Teratogenic effects of trypan blue upon amphibian embryos. *Journ. Embry. and Exp. Morph.,* 4:110-119, 1 pl.

WILSON, J. G.
1957. Is there specificity of action in experimental teratogenesis. *Pediatrics,* 19:755-763.