Brit. J. Pharmacol. (1962), **19**, 492–497.

EFFECT OF TERATOGENIC AND RELATED DYES ON THE HAEMOGLOBIN NITRITE SENSITIVITY REACTION

BY

W. K. METCALF

From the Department of Anatomy, University of Bristol

(*Received August* 2, 1962)

The teratogenic dyes trypan blue and Evans blue have a marked and immediate action on the oxidation stability of intracellular haemoglobin in rats. Of the proved non-teratogenic substances tested only o-tolidine has an effect on the haemoglobin stability, and this effect is slow. It is suggested that the effect of the teratogens on the haemoglobin oxidation sensitivity is due to interference with the protein component of the flavoprotein enzymes.

The teratogenic effect of trypan blue, described by Gillman, Gilbert, Gillman & Spence in 1948, has been the subject of much recent investigation both in rats and in various other species (Wilson, 1955 ; Beaudoin & Wilson, 1958 ; Wilson, Beaudoin & Free, 1959 ; Beck, Spencer & Baxter, 1960 ; Beck, 1961a, b). The mechanism of this action, exhibited by trypan blue and a few closely related compounds, has not, as yet, been explained. Teratogenic effects can also be produced by radiation and by dietary deficiencies, including that of riboflavine (Warkany & Schraffenberger, 1943 ; Warkany, 1944). The stability of intracellular haemoglobin to oxidation by the nitrite ion appears to be connected with riboflavine metabolism (Metcalf, 1961a, b, c), and this stability is also deranged by radiation (Metcalf, 1961d, 1962). The effect on the haemoglobin nitrite sensitivity reaction of trypan blue and related teratogenic and non-teratogenic compounds has therefore been investigated.

METHODS

The Worcester sub-strain of the Lister hooded rat was used throughout. Only adult animals, in whom the nitrite oxidation reaction is known to be consistent, were employed. They were fed on a diet of Rowett's rat cubes containing approximately 0.25 mg riboflavine/100 g diet (Metcalf, 1961c). Blood samples were obtained from the tail and were taken into excess of isotonic sodium chloride solution. After washing twice, the time taken by 0.01% freshly prepared sodium nitrite in 0.9% sodium chloride solution to convert the haemoglobin of a standard red cell suspension to methaemoglobin was noted (Metcalf, 1961c, e).

The various dyes and other substances were administered to groups of 5 animals subcutaneously into the abdominal wall with the usual aseptic precautions. The single injection dose was 20 mg, that is, that reported to be teratogenic by Wilson (1955) (or an approximately equivalent dose of a non-teratogenic compound). Three such injections on alternate days was the maximum dose given.

RESULTS

Fig. 1 shows the effect of a course of three injections of trypan blue. This effect persisted until the death of the animal some three weeks later. The results for ten other compounds are given in Table 1.

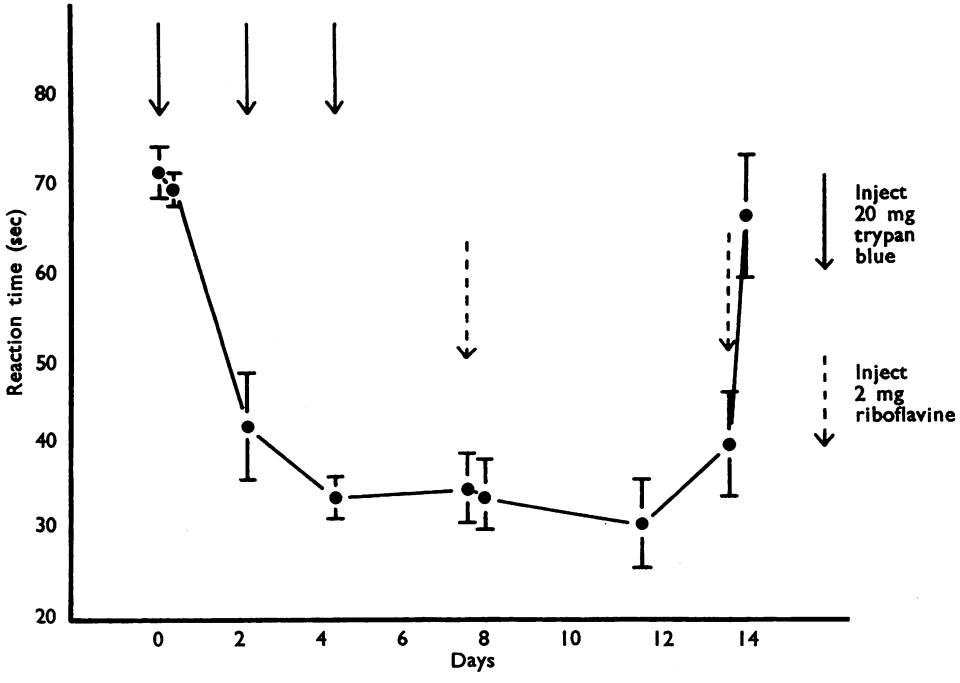

Fig. 1. The effect of trypan blue followed by riboflavine on the haemoglobin oxidation sensitivity in the rat. At the solid arrows 20 mg trypan blue was injected; at the broken arrows 2 mg riboflavine was injected. The experiment was performed in a group of 5 rats. Standard deviations are indicated. Ordinate: reaction time in sec. Abscissa: time in days.

TABLE 1

THE EFFECT OF VARIOUS DYES ON THE TIME TAKEN TO CONVERT INTRACELLULAR HAEMOGLOBIN TO METHAEMOGLOBIN BY THE ACTION OF NITRITE *IN VITRO*

Standard deviations are given. Five animals were used in each experiment.
* Suspended in 1 ml. 0·5% carboxymethyl cellulose

Dye	Dose/ injection	Haemoglobin/nitrite reaction time: Initial time	After 2 days sec	After 4 days sec	After 6 days sec
Trypan blue	20 mg	71±3	42±6	34±2	35±4
Evans blue	20	71±4	45±5	31±3	33±4
Chlorazol black	20	66±8	64±4	68±7	72±5
Congo red	20	68±7	69±6	65±7	64±9
H-acid	20	70±5	68±3	65±2	65±4
*o-Tolidine	5	69±5	53±6	49±8	42±4
Carboxymethyl cellulose	5	68±5	66±3	66±5	69±5
Indian ink	1 ml.	70±5	66±4	72±8	71±4
Mepacrine	12 mg	71±5	39±5	49±8	46±6
Acridine orange	20	70±5	67±4	60±7	62±7

In an attempt to neutralize the effect of the trypan blue, 2 mg of riboflavine was given three days after the completion of the course of trypan blue injections but was found to be without effect on the haemoglobin oxidation time (Fig. 1). The administration of 5 mg of adenosine triphosphate with the 2 mg of riboflavine was also without effect at that time. Six days later, however, 2 mg of riboflavine alone did cause the reaction to revert rapidly to normal (Fig. 1).

Even when 5 mg of riboflavine was given simultaneously with each trypan blue injection, no protective effect could be detected. Increasing this dose to 25 mg (flavine mononucleotide, the phosphate ester, was used because of its greater solubility), and giving it every day throughout the trypan blue course, still had no protective effect. It was not until five days after the last trypan blue injection that even this dose of flavine mononucleotide restored the haemoglobin stability to normal (Fig. 2). However, 2 days after only one injection of trypan blue, the normal

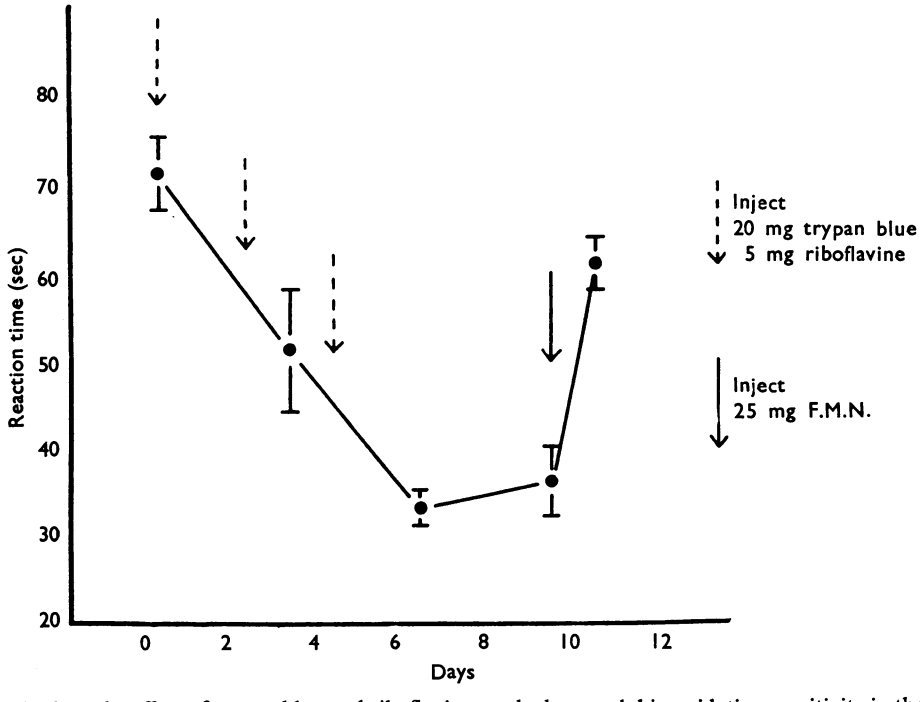

Fig. 2. The effect of trypan blue and riboflavine on the haemoglobin oxidation sensitivity in the rat. At the broken arrows 20 mg trypan blue and 5 mg riboflavine were injected in each of 5 rats. At the solid arrow 25 mg flavine mononucleotide was injected. Ordinate: reaction time in sec. Abscissa: time in days.

teratogenic dose, 25 mg of flavine mononucleotide was completely successful in restoring the haemoglobin stability to normal levels.

Whatever the subsequent treatment, three injections of trypan or Evans blue proved fatal in approximately 3 weeks. No ill effects were observed from any of the other compounds tested. Post-mortem examinations were carried out on all the

injected animals. The teratogenic dyes, trypan and Evans blue, produced a generalized and permanent staining of all the connective tissues, whilst the non-teratogenic dyes produced, apart from local staining at the injection site, only very transient general staining. Both groups of dyes, as would be expected, caused intense staining of the local lymphatic glands. The tissue staining following the injection of acridine orange disappeared almost immediately, whilst that due to mepacrine, although not completely permanent, persisted for a considerable period.

DISCUSSION

Of the various compounds that have been tested both for teratogenic action and for their action on the haemoglobin stability to oxidation, only the two teratogens, trypan blue and Evans blue, produce a large and rapid increase in the haemoglobin oxidation sensitivity.

Of the substances proved to be non-teratogenic when given on the 8th day of pregnancy in the rat (Wilson, 1955 ; Beck, 1961a, b), chlorazole black, congo red, indian ink, carboxymethyl cellulose and H-acid are without effect on the haemoglobin sensitivity ; o-tolidine does have a slow effect on the haemoglobin stability but requires six days to develop the full effect. As the timing of the administration of the teratogen during pregnancy is of paramount importance (Wilson, 1955), the slow action of o-tolidine may account for its failure to cause teratogenesis.

Trypan blue is a combination of two molecules of H-acid and one molecule of di-azotized o-tolidine. By testing these separately it was hoped to determine which part of the trypan blue molecule was responsible for the effect. The results seem to indicate that the o-tolidine section is implicated. The slowness of its action when isolated could well be due to its insolubility in water contrasting with the relatively water-soluble trypan blue.

The experiments in which both trypan blue and riboflavine, or its phosphate, were given simultaneously are interesting in that they shed some light on the possible site of their antagonism. As even a very large dose of flavine mononucleotide (25 mg) competely failed to prevent the increased haemoglobin sensitivity caused by trypan blue, it would seem unlikely that the effect is a direct one on riboflavine or its mononucleotide. The simultaneous administration of riboflavine and adenosine triphosphate, a procedure successful in correcting the increased sensitivity following cobalt radiation (Metcalf, 1962), is ineffective following trypan blue. It would seem, therefore, that the mode of action of trypan blue on the haemoglobin sensitivity is not the same as that of radiation—a block in the manufacture of the complete enzyme by interference with phosphorylation. The timing of the change in haemoglobin stability after trypan blue is similar to that in acute riboflavine deprivation by starvation (Metcalf, 1961c). This suggests that the production of the complete holoenzyme is halted at some other point or that the action of trypan blue is a slow but direct one on the completed enzyme.

The permanency of the tissue staining with the teratogenic dyes suggests that they form stable compounds with the proteins of the connective tissues whilst no such compounds are formed by the non-teratogenic dyes. Haas, Horecker & Hogness

(1940) have shown that the flavoprotein enzymes are dissociable, and it may be that the active dyes form relatively stable compounds with the protein part of these molecules, that is, the apoenzyme, thus competing with the isoalloxazine prosthetic group. The five days needed after the last injection of trypan blue before even a large dose of flavine mononucleotide can restore the nitrite sensitivity to normal could then be attributed either to the necessity for the animal to excrete excess dye (its urine continues to be stained throughout this period), or for the necessity to manufacture further specific protein before the complete enzyme can be reconstituted.

The experiments with mepacrine and acridine orange were carried out to test this hypothesis. These two compounds have quite dissimilar formulae from the trypan blue group of di-azo dyes, but are closely related to one another. As can be seen from Table 1, acridine orange (which causes transient tissue staining only) is completely without effect on the haemoglobin sensitivity, whilst mepacrine (which causes semi-permanent tissue staining) produces the same effect as trypan blue. In preliminary experiments, Beck (1961c) has found acridine orange to be ineffective as a teratogenic agent. It would be interesting to know the effect of mepacrine in this respect.

On the basis of their similar formula, it has been suggested that mepacrine is a specific riboflavine antagonist. I would, however, agree with Wright & Sabine (1944), Haas (1944), Hellerman, Lindsay & Bovarnick (1946) and with Hemker & Hulsmann (1960) that the effect is a non-specific one on the protein component of the enzyme. On the basis of the present work, I would suggest that trypan blue and Evans blue owe their action on the haemoglobin stability and perhaps their teratogenic effect to the same cause.

The failure of riboflavine or flavine mononucleotide to prevent the ultimate death of the animals receiving the full course of the trypan blue injections can, perhaps, be explained on the basis of very high local concentrations of the dye in a vital organ. Beck (1961b) has shown necrotic changes in the renal tubules following trypan blue and a severe anaemia and liver damage also occurs (Gillman *et al.*, 1948). The systemic administration of riboflavine could not be expected to protect a tissue from such high local concentrations of the dye.

I would like to thank Dr F. Beck, of Cardiff, for supplying samples of assayed trypan blue; Miss B. Woodham for her invaluable technical assistance; and Professor J. M. Yoffey for his encouragement and the very generous laboratory facilities he always so willingly makes available.

REFERENCES

BEAUDOIN, A. R. & WILSON, J. G. (1958). Teratogenic effect of trypan blue on the developing chick. *Proc. Soc. exp. Biol. (N.Y.)*, **97**, 85–90.

BECK, F. (1961a). Comparison of the different teratogenic effects of two commercial samples of trypan blue. *J. Anat.*, **95**, 452*P*.

BECK, F. (1961b). Observations on trypan blue toxicity. *J. Anat.*, **95**, 614*P*.

BECK, F. (1961c). Personal communication.

BECK, F., SPENCER, B. & BAXTER, J. S. (1960). Effect of trypan blue on rat embryos. *Nature (Lond.)*, **187**, 605–607.

GILLMAN, J., GILBERT, C., GILLMAN, T. & SPENCE, I. (1948). A preliminary report on hydrocephalus, spina bifida and other congenital anomalies in the rat produced by trypan blue. *S. Afr. J. med. Sci.*, **13**, 47–90.

HAAS, E. (1944). The effect of atabrine and quinine on isolated respiratory enzymes. *J. biol. Chem.*, **155**, 321–331.

HAAS, E., HORECKER, B. L. & HOGNESS, T. D. (1940). The enzymatic reduction of cytochrome C reductase. *J. biol. Chem.*, **136**, 747–774.

HELLERMAN, L., LINDSAY, A. & BOVARNICK, M. R. (1946). Flavoenzyme catalysis. Inhibition of d-amino acid oxidase by competition with flavin-adenine-dinucleotide of atabrine, quinine and certain other compounds. *J. biol. Chem.*, **163**, 553–570.

HEMKER, H. C. & HULSMANN, W. C. (1960). Inhibition of enzymes by atebrin. *Biochim. biophys. Acta*, **44**, 175–177.

METCALF, W. K. (1961a). The effect of diet on the oxidation sensitivity of rat haemoglobin. *J. Anat.*, **95**, 452*P*.

METCALF, W. K. (1961b). Riboflavine and the haemoglobin oxidation stability of rats. *J. Physiol. (Lond.)*, **157**, 51*P*.

METCALF, W. K. (1961c). The sensitivity of intracorpuscular haemoglobin to oxidation by nitrite ions. (i) The effect of growth, starvation and diet. *Phys. in Med. Biol.*, **6**, 427–435.

METCALF, W. K. (1961d). Radiation, growth rate and the haemoglobin oxidation sensitivity of rats. *J. Anat.*, **95**, 607*P*.

METCALF, W. K. (1961e). A biochemical change in the blood in pregnancy and malignant disease. *Phys. in Med. Biol.*, **5**, 259–269.

METCALF, W. K. (1962). Experimental oxidation of haemoglobin: the effects of irradiation in rats. *Nature (Lond.)*, **194**, 783.

WARKANY, J. (1944). Congenital malformations induced by maternal nutritional deficiency. *J. Paediat.*, **25**, 476–480.

WARKANY, J. & SCHRAFFENBERGER, E. (1943). Congenital malformations induced in rats by maternal nutritional deficiency. V. Effects of a purified diet lacking riboflavine. *Proc. Soc. exp. Biol. (N.Y.)*, **54**, 92–94.

WILSON, J. G. (1955). The teratogenic activity of several azo dyes chemically related to trypan blue. *Anat. Rec.*, **123**, 313–334.

WILSON, J. G., BEAUDOIN, A. R. & FREE, H. J. (1959). Studies on the mechanism of teratogenic action of trypan blue. *Anat. Rec.*, **133**, 115–128.

WRIGHT, C. I. & SABINE, J. C. (1944). The effect of atabrine on the oxygen consumption of tissues. *J. biol. Chem.*, **155**, 315–320.