Dyes and Pigments 5 (1984) 65–82

A Comparison of the Predictive Values of the *Salmonella*/Microsome Mutation and BHK21 Cell Transformation Assays in Relation to Dyestuffs and Similar Materials

E. Longstaff

Imperial Chemical Industries PLC, Central Toxicology Laboratory, Alderley Park, Macclesfield, Cheshire, Great Britain

and

D. B. McGregor, W. J. Harris, J. A. Robertson and A Poole

Inveresk Research International, Inveresk Gate, Musselburgh, Scotland, Great Britain

(Received 15 November, 1982)

SUMMARY

A group of 22 dye-related compounds were selected for testing in two short-term predictive tests for carcinogenicity. The group of compounds was made up of nine established animal carcinogens and 13 chemicals for which there was substantial evidence of non-carcinogenicity. The materials were coded and used to assess the predictive value of the Salmonella/*microsome reverse mutation assay and the BHK21 cell transformation test. The overall predictive value with these compounds obtained for the* Salmonella *microsome reverse mutation assay was 86% and it is concluded that because of the good predictive value and the relative ease of experimental procedure, the* Salmonella *mutation assay is a useful first step in any proposed series of toxicological bioassays for the identification of genotoxic agents in the dyestuffs industry.*

The cell transformation test on the other hand was difficult to conduct and interpret. The interpretation of the coded data as judged by the IRI scientists was that of the 22 'unknown' compounds, eight results were judged to be correct, six were wrong and seven were doubtful. However,

when the same data were re-evaluated uncoded by ICI staff, 15 results were judged to be correct and six were wrong. These results serve to exemplify the difficulties encountered with cell transformation assays and it is concluded that the system should not be used as a routine test for dyestuffs and related compounds

1 INTRODUCTION

There is no unequivocal evidence that any dyestuff has caused cancer in man. However, some epidemiological surveys of groups occupationally exposed to dyestuffs and other chemicals have indicated among these workers higher than normal incidences of cancer, notably bladder cancer. These studies have been carefully reviewed by Boeniger.[1]

However, such epidemiological studies are usually fairly insensitive, and only in rare cases can such studies identify individual causative agents because of the mixed exposures normally involved. For these reasons, animal models, in which the exposure can be defined and controlled, are frequently used for detecting carcinogenic potential. Such long-term animal assays are expensive and there are not sufficient laboratory resources available to test all products For the dyestuffs industry with its multitude (*ca* 3000) of different products only a minority have so far been subjected to such testing

In recent years there has been a rapid development of various short-term testing procedures which show some promise as predictive tests for carcinogenicity, thus offering the possibility of more rapidly screening a range of products and identifying those most likely to be carcinogenic. This report describes the results obtained in two such tests—the *Salmonella* reverse mutation assay of Ames *et al.*[2] and the BHK21/C13 cell transformation test of Styles[3]—in a validation study commissioned by the Ecological and Toxicological Association of the Dyestuffs Manufacturing Industry (ETAD), involving control substances (dyestuffs and closely related chemicals), which have been adequately shown to be carcinogenic or non-carcinogenic in animal tests.[4]

Although such short-term tests cannot be considered to provide any conclusive proof of carcinogenicity they may be useful in enabling a prioritisation of products warranting a more detailed study in animal tests to assess carcinogenic potency. The assessment of *potency* is essential if appropriate restrictions of exposure are to be recommended, e.g. safety

precautions in manufacture and processing, or restrictions in use. It must be clearly recognised, therefore, that short-term predictive tests for carcinogenicity could at best answer only half the question. The problems of predicting carcinogenic potency from these tests are at present beyond the limits of scientific practicality

2. MATERIALS AND METHODS

Inveresk Research International (IRI) tested 22 coded compounds using the treat-and-plate version of the *Salmonella* reverse mutation test[2] and the BHK21 cell transformation test.[3] Experimental protocols for these tests have been published, the essential details of each being as detailed below.

Both assays were controlled with carcinogens appropriate to the study. In the case of Ames test, dimethylaminoazobenzene (DAB, CI 11020) was identified as the critical positive control chemical. Previous experience had shown that DAB was difficult to demonstrate as mutagenic in the plate assay and some authors have indicated that a liquid pre-incubation step should be introduced to the protocol to facilitate the assay of azo-dyes.[5] Nevertheless it was decided to evaluate the standard Ames assay as a predictive assay for potential dyestuff carcinogens. Negative results generated for tests which did not identify DAB as positive were ignored and the experiment repeated. A positive response was required to be reproducible and show classical dose–response relationships.

In the case of the BHK21 test, both DAB and benzidine were used as positive control chemicals.

2.1. *Salmonella*/microsome reverse mutation assay (Ames test)

Salmonella strains TA1535, TA100, TA1537, TA1538 and TA98 were used. Compounds, dissolved in dimethylsulphoxide (DMSO), were tested, at least twice, in the presence and absence of an Aroclor 1254-induced male rat liver post-mitochondrial supernatant (S9-mix). At least four non-toxic concentrations of compound were tested with three plates per dose level. The plates were incubated at 37°C for two days before counting the revertant colonies.

The criteria for a positive, i.e. mutagenic, effect in the test was that the test chemical should induce at least a doubling of the spontaneous

reversion count, together with a clear dose–response, in any tester strain in duplicate experiments

2.2. BHK21 cell transformation assay (Styles test)

Baby Syrian hamster kidney fibroblasts (BHK21/C13) were maintained in the laboratory in Dulbecco's modification of Eagle's minimal essential medium supplemented with 10% newborn calf serum From these stock cultures, 1 ml tester cultures were prepared as 10^6 cells ml^{-1} in medium without serum and treated in triplicate with test compound in the presence and absence of S9-mix. All tests were carried out at least twice

The cultures were shaken for 4 h at 37°C and the cells washed and resuspended in growth medium containing serum Cell survival and growth in semi-solid agar were determined to assess cytotoxicity and transforming ability, respectively, of the chemicals

At the start of the project the aim was to apply the criteria of Styles,[3] viz a five-fold increase in transformation frequency at the LC_{50} as indicative of a positive response. During the project, however, several experiences resulted in IRI extending the criteria. These experiences were:

(1) The insolubility of 12 of the test compounds made it impossible to reduce viability to 50% It was thus impossible to determine the LC_{50}

(2) The structurally related positive controls, DAB and benzidine, did not reproducibly reduce viability to 50% Only about half of the experiments resulted in these positive controls giving greater than 50% lethality at 1 mg/ml The declared positive controls could not therefore be relied upon to meet Styles' criteria

(3) In experiments where DAB and benzidine did not cause 50% lethality, there were, nevertheless, significant and reproducible increases in the absolute numbers of transformants. Thus, a new and valid criterion of transformation in the absence of greater than 50% lethality was taken to be an absolute increase in the number of transformed colonies

(4) Precipitates were often inevitably carried over on to the agar plates growing the transformed colonies The presence of various sizes and random distribution of test compound aggregates in the agar made colony-counting difficult and affected reproducibility. Also, in these cases, it is reasonable to assume the cells were in contact

with the test compound much longer than would otherwise have been planned. The consequences of this on the transformation of BHK21 cells are unknown.

On the basis of these experiences, before decoding IRI applied the following criteria to the test data for the definition of a positive response:

(1) A five-fold increase in transformation frequency at the LC_{50}: or
(2) an absolute increase in transformed colony numbers at two doses. and
(3) reasonable agreement between the conclusions from duplicate experiments

In addition to, and after, decoding the data ICI applied only the first criterion (i e according to Styles[3]), and argued that fulfilment of this criterion, even in one experiment, indicated a positive response. These differences in applied criteria account for the different assessments, displayed in Table 1, and these differences in interpretation of the same data serve to exemplify the difficulty in employing Styles' test to some types of industrial chemicals

3 TEST CHEMICALS

Eight azo-dye carcinogens and one triphenylmethane acid dye carcinogen were used The non-carcinogens comprised eight azo-dyes, three triphenylmethane dyes and two miscellaneous dyes. The structures, source, purity and principal references for biological activity of each compound tested are given in Table 1.

4. RESULTS

The results of the study are summarised in Table 1. The Ames test succeeded in correctly identifying seven of the nine carcinogens and 12 of the 13 non-carcinogens. The test data were found to be reproducible between duplicate experiments and the protocol permitted the regular identification of the positive control DAB.

Dose–response curves from the positive Ames tests were typical, mutation frequency increasing with dose up to toxic dose levels, and in general there was no difficulty in interpretation of the results (see Fig. 1).

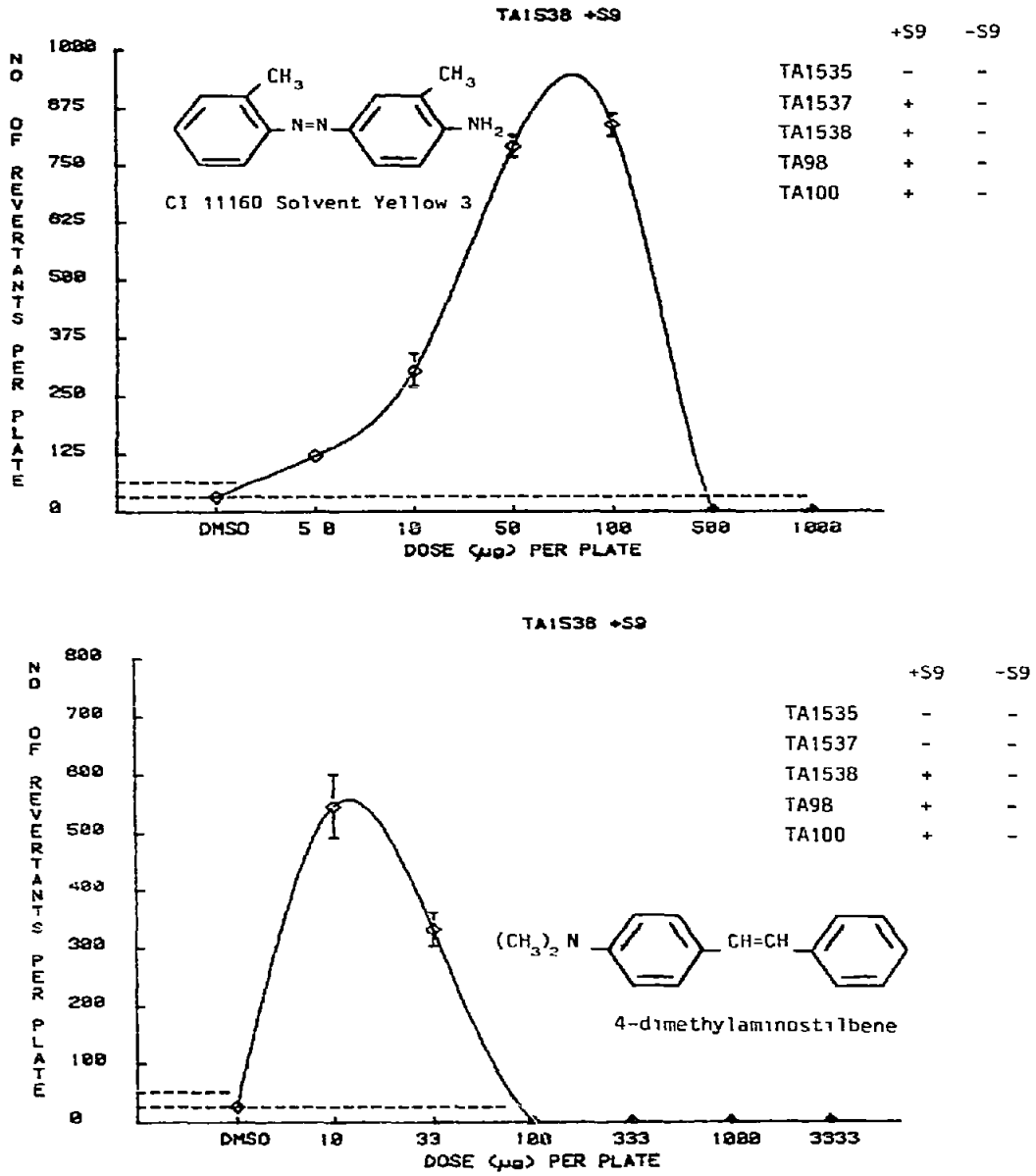


Fig. 1. Computer-drawn dose–response curves for two selected positive results obtained from the Ames treat-and-plate method The two dotted lines associated with each horizontal axis represent the spontaneous background level of that tester strain and twice this level A positive level was exceeded in each case together with a convincing dose–response

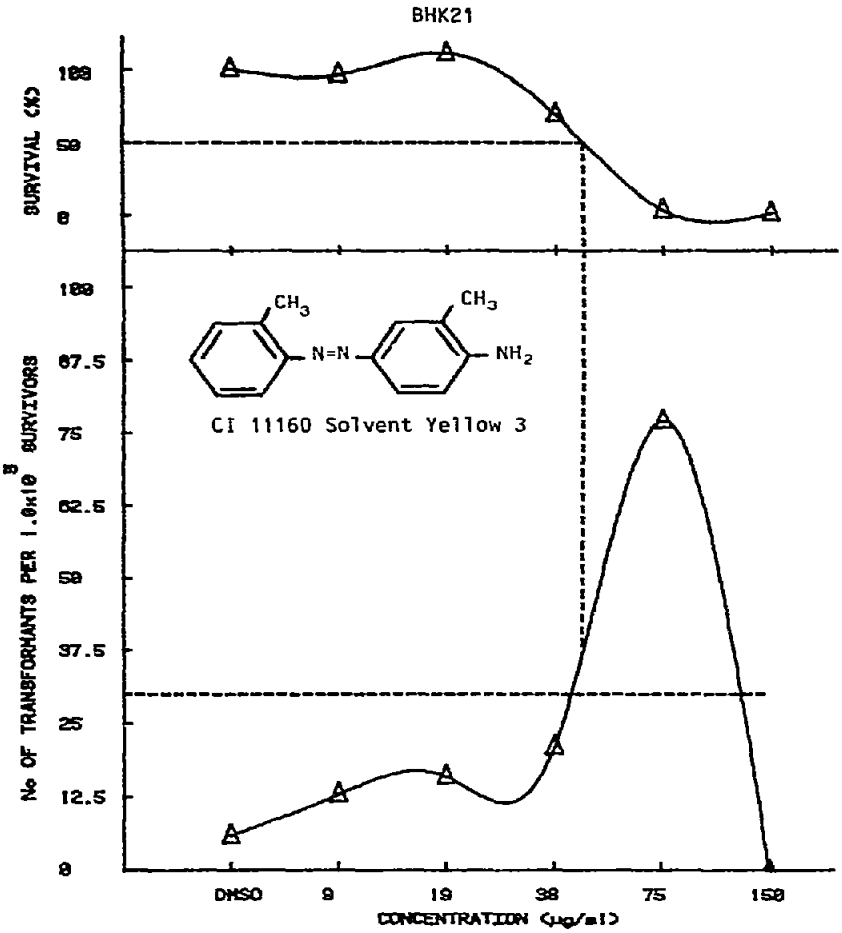


Fig. 2. Cell transformation results Computer-drawn dose–response curves for cytotoxicity (upper plot) and transformation frequency (lower plot) derived from experiments performed with CI 11160 when the concurrent positive controls were correctly identified as positive in the test. The upper horizontal dotted line represents the LC_{50} point and the lower line represents the five-fold increase above the spontaneous transformation frequency required for a positive result at the LC_{50} dose level

The two interpretations derived from the same data from the BHK21 test are also presented in Table 1. It can be seen that applying the IRI and ICI criteria to the data resulted in identical conclusions with 13 compounds, complete disagreement with two compounds, leaving seven compounds about which IRI were unable to derive a conclusive interpretation. Applying the ICI criteria[3] to these seven compounds,

there were four correct and three wrong predictions. Data from the first 'equivocal' experiment are presented in Fig. 2 The IRI criteria for a positive are not met but the curves are sufficiently convincing to ICI to assume a positive transformation.

5. DISCUSSION

5.1. Ames test

The standard *Salmonella*/microsome reverse mutation assay was shown to be good (86 % accurate) at detecting the carcinogenic dyestuffs used However, it failed to detect the carcinogenic mono-azo dye CI 16155 (confirming the finding of Hartman *et al* [6]) when a very similar structure, CI 16150, was correctly identified as previously shown by Matsushima *et al* [7] Also, a bis-azo carcinogen CI 23850 was non-mutagenic (confirming the findings of Brown *et al* [8] and Hartman *et al.*[6]) while its analogue CI 23860 was positive (Brown *et al.*[8] reported CI 23860 Ames-negative) Azo cleavage would release *o*-tolidine from both compounds, but this cleavage would not be expected to occur significantly under the aerobic conditions of the test. The positive result with CI 23860 could have been due to some mutagenic impurity of the commercial sample.

The test was equally successful in the detection of non-carcinogens 12 of the 13 non-carcinogens, i e 92 %, were correctly identified as negative

The only false positive result was from CI 42095, which was uniquely positive amongst three triphenylmethane non-carcinogens, and no explanation of this result can be put forward at this time, there are conflicting reports in the literature on this compound already from Brown *et al* [8] and Bonin *et al.*[9] It should be noted that the cell transformation result for this material was positive also

No carcinogens were detected in this study without S9-mix and all positive compounds could have been identified without base-pair tester strains TA1535 and TA100

In most cases the carcinogens gave a potent dose–response effect in the test and would probably be even more effective in a liquid pre-incubation or suspension-type assay.[7] However, under the conditions of this validation the 'treat-and-plate' method of Ames *et al* would seem to be a useful screening test for identifying possible carcinogenic dyestuffs including azo-dyes. Still, it should be recognised that this study was

limited to a few structural classes and did not include compounds of the anthraquinone or nitro-containing azo-dye classes.

5.2. Styles test

As mentioned previously, the Styles test was not easy to conduct or interpret. The IRI team identified and recorded a positive response with the test in only four out of the nine carcinogens, i.e. 44 % correct, and five out of 13 non-carcinogens, i.e 39 % correct However, a re-evaluation of the uncoded data by ICI which recognised as valid only those experiments which yielded adequate control data gave more favourable results. Under these conditions seven out of the nine carcinogens, i.e. 78 %, and eight out of 13 non-carcinogens, i e. 62 %, were correctly identified.

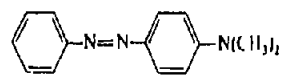
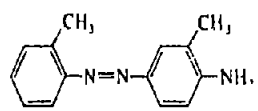
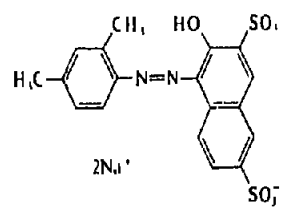
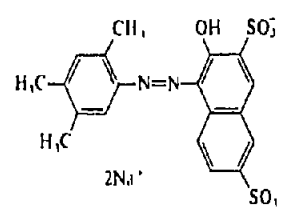
The differences between the IRI and ICI interpretations are recorded in Table 1.

The current validation study indicates that the Ames 'treat-and-plate' technique provides a suitable preliminary test for assessing the potential carcinogenicity of dyestuffs. Ideally this screen should be supplemented by another validated test to improve the predictive value of the screen. However, because of the reported practical difficulties encountered in obtaining an adequate cell transformation assay, this test cannot be recommended as the second test of choice at this time. It should be noted, however, that in the two cases of the Ames false negatives discussed above, the Styles test was able to pick these up as positive. (It may be significant that CI 42095 returned a double 'false' positive, supporting the finding of Bonin *et al.*[9] that the material is genotoxic and therefore potentially carcinogenic.)

In contrast to the IRI experience with the Styles test, Strobel and Greb[10] demonstrated good reproducibility within their laboratory for this test. Daniel and Dehnal[11] were able to reproduce the technique with confidence when they modified Styles' original protocol sufficiently to suit their own needs. A review by Brookes[12] of the value of *in vitro* cell transformation tests concludes: 'with the appropriate attention to detail, the system can be reliable and it remains the only *in vitro* transformation system whose performance in a "blind-trial" has been published (de Serres and Ashby 1981)'. It follows, therefore, that in order to improve the predictive value of a screen for potential carcinogens of the azo-dye class, and to satisfy the EEC 6th Amendment (79/831/ECC) to the Directive on classification, packaging and labelling, either the practical problems of

TABLE 1

Structures, Source, Purity and Principal References for Biological Activity for the Compounds Used in this Validation Study and the Published Short-Term *in vitro* Test Results

Compound and source	*Structure*	*Purity*	*Principal animal bioassay reference*	*Previous in vitro tests* — *Results*	*References*	Salmonella *results*[a]	*BHK21 results*[a,b] — *IRI*	*ICI*
(a) *Carcinogens*								
CI 11020 (uncoded +ve control),		Analytical	13	*Salm* + Cell trans +	5 14	+	+	+
CI 11160, ex ACNA		88%	15	*Salm.* + Cell trans +	16 17	+	−	+
CI 16150, ex PCUK		Commercial (2 bands t l c)	18	*Salm* +	19	+	−	−
CI 16155, ex May and Baker		Commercial	20	*Salm* −	21	−	+	+

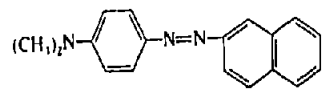
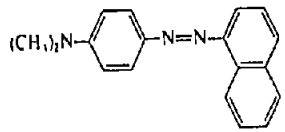
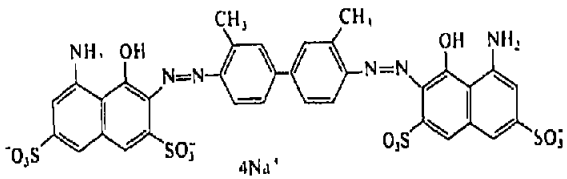
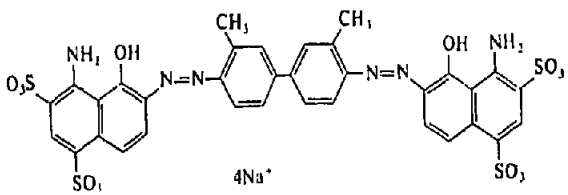
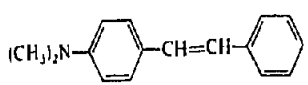
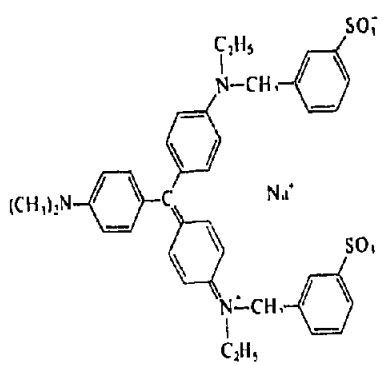
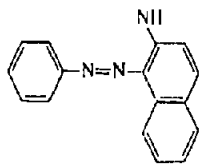
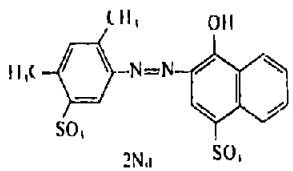
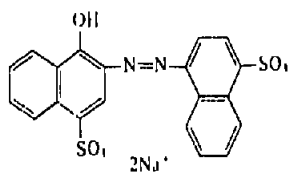
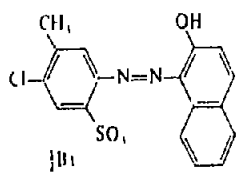
4-(Dimethylamino)-benzeneazo-2-naphthalene, ex Sandoz		Analytical	22	No previous data		+	+	+
4-(Dimethylamino)-benzeneazo-1-naphthalene, ex Sandoz		Analytical	13			+	−	−
CI 23850, ex. Ciba–Geigy		Analytical	23	*Salm* −	8, 6[c]	−	+	+
CI 23860, ex PCUK		Commercial	23	*Salm* −	8	+	±	+
4-Dimethylaminostilbene, ex BASF		Unknown	24	No previous data		+	±	+
CI 42640, ex Bayer		52%	25	*Salm* + *Salm* −	9 8	−	+	+

TABLE 1—*Contd*

Compound and source	*Structure*	*Purity*	*Principal animal bioassay reference*	*Previous in vitro tests*		*Salmonella results*[a]	*BHK21 results*[a,b]	
				Results	*References*		*IRI*	*ICI*
(b) *Non-carcinogens* CI 11380, ex ICI		Analytical	26	*Salm* –	8	–	±	+
CI 14700, ex ICI		Analytical	27	*Salm* –	19, 28	–	–	–
CI 14720, ex Bayer		85%	29	*Salm* –	8	–	±	–
CI 15585 1, ex Montedison		95%	30	*Salm* –	31	–	+	+

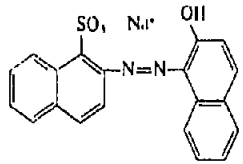
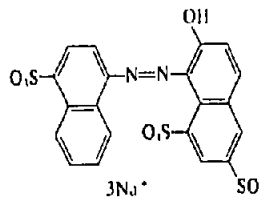
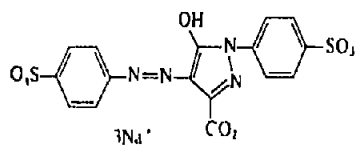
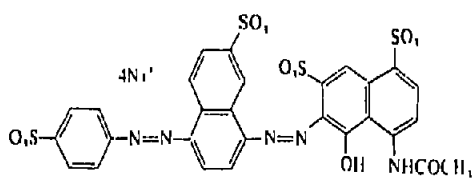
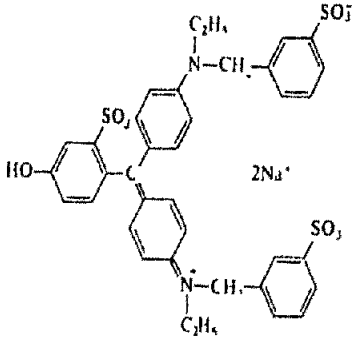
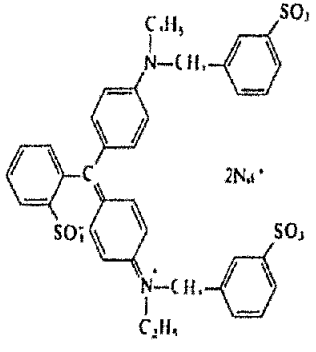
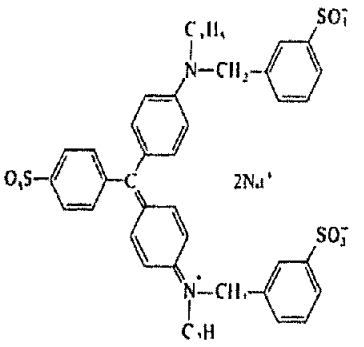
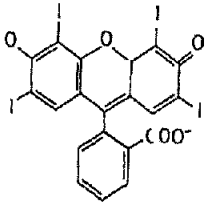
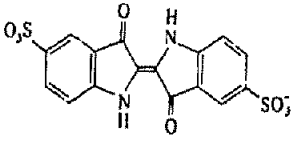
CI 15630, ex ACNA		95%	32	*Salm* –	31	–	±	+
CI 16255, ex Ciba-Geigy		Analytical	29	*Salm* –	33	–	–	–
CI 19140, ex Hoechst		88 7%	34	*Salm* –	35, 28	–	–	–
CI 28440, ex Bayer		79%	36	No previous data		–	+	–

TABLE 1—*Contd*

Compound and source	*Structure*	*Purity*	*Principal animal bioassay reference*	*Previous in vitro tests*		Salmonella *results*[a]	*BHK21 results*[a,b]	
				Results	*References*		*IRI*	*ICI*
CI 42053, ex Ansted		Unknown	37	*Salm* –	8	–	±	–
CI 42090 ex Hoechst		77%	37	*Salm* – Cell –	8 38	–	–	–

CI 42095, ex Ciba-Geigy		Analytical	39	*Salm* – *Salm* +	8 9	+	±	+
CI 45430, ex PCUK		90 5%	40	*Salm* –	35	–	+	+
CI 73015, ex Sumitomo		Commercial	40	*Salm.* –	35	–	–	–

[a] The current validation test data for each compound are also shown for comparison

[b] The two columns (IRI and ICI) are the original interpretations of the IRI scientists, and the re-evaluated but uncoded interpretations by ICI staff.

[c] After reduction with cell-free extract of *Fusobacterium* sp

the Styles cell transformation assay need to be resolved or a second predictive test should be identified and validated. Tests such as the C3H mouse transformation test, mouse lymphoma point mutation test, *in vivo* micronucleus or *in vitro* human lymphocyte cytogenic analysis tests would seem to be potential candidates for validation

6 CONCLUSION

The 'treat-and-plate' variant of Ames' *Salmonella* reverse mutation test[2] is an adequate predictive test for the identification of potential carcinogenic agents in the dyestuffs and related chemical industries.

The BHK21 cell transformation test is unreliable in this regard. Great difficulty was experienced in reproducing the test as described by Styles,[3] and also, because of the additional practical problems experienced with insoluble or non-toxic dyes, the criteria for a positive response in the test as described by Styles could not be achieved. It is concluded, therefore, on the basis of these data, that a more practical second predictive short-term test should be identified and validated

ACKNOWLEDGEMENTS

The authors acknowledge the valuable advice and assistance of Dr A. S. Martin, ICI Organics Division, and Drs J. Ashby and J Styles, ICI Central Toxicology Laboratory, given during the course of this work.

The work was sponsored by the Ecological and Toxicological Association of the Dyestuffs Manufacturing Industry, Clarastrasse 4/6, CH-4058, Basel, Switzerland

REFERENCES

1 M Boeniger, *Carcinogenicity and metabolism of azo dyes, especially those derived from benzidine* NIOSH Technical Report, DHHS (NIOSH) Publication No 80-119 (July 1980)
2 B N Ames, J McCann and E Yamasaki, *Mutat Res*, **31**, 347 (1975).
3 J A Styles, *Brit J. Cancer*, **36**, 558 (1977)
4 E Longstaff and J A Ashby, ETAD Project T2002 *Assessment and categorisation of the animal carcinogenicity data on selected dyestuffs and*

an extrapolation of that data to an evaluation of the relative carcinogenic risk to man (1980)
5 Y Yahagi, M. Degawa, Y Seino, T Matsushima, M Nagao, T Sugimura and Y. Hashimoto, *Cancer Letts.*, **1**, 91 (1975)
6 C P. Hartman, G E Fulk and A. W Andrews, *Mut Res.*, **58**, 125 (1968)
7 T. Matsushima, T. Sugimura, M. Nago, T Yahagi, A. Shirai and M Sawamura, in *Short-term test systems for detecting carcinogens*, eds K. H Norpoth and R C. Garner. p 273. Berlin, Springer-Verlag (1980)
8. J P Brown, G. W Roehm and R. J. Brown, *Mutat Res.*, **56**, 249 (1978).
9 A M. Bonin, J B. Farquharson and R S U Baker, *Mutat. Res* , **89**, 21 (1981).
10 R Strobel and W Greb, *Toxicology*, **19**, 55 (1981).
11. M R Daniel and J M Dehnel, *Carcinogenesis*, **1**, 657 (1980)
12 P Brookes, *Mutat Res.*, **86**, 231 (1981)
13 A S Mulay and H I Firminger, *J. Nat Cancer Inst* , **13**, 35 (1952)
14 J Ashby, *Brit J Cancer*, **38**, 34 (1978)
15 L Tomatis, G Della Porta and P. Shubik, *Cancer Res* , **21**, 1513 (1961)
16 J McCann, E Choi, E. Yamasaki and B Ames, *Proc Nat Acad Sci USA*, **72**, 5135 (1975)
17 J Pienta, in *Carcinogens identification and mechanisms of action*, ed A C Griffin and C.R. Shaw, p 121 New York, Raven Press (1978)
18 Y Ikeda, S Horiuchi, K Kobayashi, T Furuya and K Kohyo, *Food Cosmet Toxicol* , **6**, 591 (1968)
19 T Matsushima, B Teichmann, M Sawamura and T Sugimura, *Mutat Res* , **54**, 220 (1978)
20 H C Grice, W A Mannell and M G Allmark, *Toxicol Appl. Pharmacol* , **3**, 509 (1961)
21 C P Hartman, A W Andrews and K T Chung, *Infection and Immunity*, **23**, 686 (1979)
22 A S Mulay and C C Congdon, *J Nat Cancer Inst* , **14**, 571 (1953).
23 A H E. Marshall, *Acta Path Microbiol Scand* , **33**, 1 (1953)
24 L A Elson, *Brit. J Cancer*, **6**, 392 (1952)
25 K Uematsu and T Miyaji, *J Nat Cancer Inst* , **51**, 1337 (1973)
26 M G Allmark, H C Grice and F C Lu, *J Pharm Pharmacol* , **7**, 591 (1955)
27 K J Davis, A. A Nelson, R E Zwickey, W. H Hanson and O G Fitzhugh, *Toxicol Appl Pharmacol* , **8**, 306 (1966)
28 A E. Auletta, L M Kuzava and A S. Darmar, *Mutat Res* , **56**, 203 (1977)
29 P L Mason, I F Gaunt, K R Butterworth, J Hardy, I S Kiss and P Grasso, *Food Cosmet Toxicol* , **12**, 601 (1974)
30 K J Davis and O G. Fitzhugh, *Toxicol Appl Pharmacol* , **4**, 200 (1962)
31 J P Brown, P S Dietrich and C M Bakner, *Mutat. Res* , **65**, 181 (1979)
32 K J. Davis and O. G. Fitzhugh, *Toxicol. Appl Pharmacol* , **5**, 728 (1963)
33 M Viola and A Nosotti, *Boll Chim. Farm.*, **117**, 402 (1978)
34 K. J Davis, O G Fitzhugh and A A Nelson, *Toxicol Appl. Pharmacol.*, **6**, 621 (1964)

35. A. M. Bonin and R S U. Baker, *Food Technology in Australia*, **32**, 608 (1980)
36 I F. Gaunt, F. M B. Carpanini, P. Grasso, I.S. Kiss and D.S. Gangolli, *Food Cosmet Toxicol*, **19**, 17 (1972)
37 W H Hansen, E L. Long, K. J. Davis, A A Nelson and O G Fitzhugh, *Food Cosmet. Toxicol*, **3**, 389 (1966).
38 P. J. Price, W A Suk, A E Freeman, W. T. Lane, R. L Peters, M L. Vernon and R J. Hulbner, *Int J. Cancer*, **21**, 361 (1978).
39. W. H. Hansen, K J. Davis, A A Nelson and O. G Fitzhugh, *Toxicol. Appl. Pharmacol.*, **8**, 29 (1966).
40 W H Hansen. H J Davis, S L. Graham, C H. Parry and K. H. Jacobson, *Food Cosmet Toxicol*, **11**, 535 (1973)