Color Yes, Toxicity No: Systematic Approaches to Meeting this Challenge

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While the efficacy of a new chemical substance is critical to its commercial success, few researchers would argue against the proposition that the major barrier to the commercialization of a technically interesting new compound often lies with its toxicological properties. The right to introduce a new chemical product to the marketplace requires its manufacturer to demonstrate minimal human health and environmental risk in its production and intended end use.

With regard to textile dyes, safety is often demonstrated by conducting a battery of tests designed to assess genotoxicity and ecotoxicity. The genotoxicity (adverse interactions between DNA and chemical substances leading to heritable changes in cells of organisms) of dyes used for textile and related applications came to the forefront when it was discovered that certain commonly used azo dye intermediates were either human or animal carcinogens. This recognition led to widespread testing of azo dyes and their intermediates for mutagenicity and/or carcinogenicity and to the assessment of methods used. While these studies were mainly aimed at risk assessment, they were also used to design replacements for certain toxic compounds. The present paper provides an overview of our laboratory studies pertaining to the environmental chemistry of azo dyes, by far the major class of textile dyes.

**ABSTRACT**

This paper provides an overview of studies conducted at the North Carolina State University College of Textiles since the inception of the dye chemistry program in 1984. Design of new textile dyes to replace existing dyes and dye intermediates were studied with the goal of minimizing human health and environmental risks. Initially, correlations between chemical structures and environmental toxicity were established and the resulting information used in design studies. These studies resulted in nonmutagenic replacements for genotoxic aromatic amines used in azo dye development and metal complexed dyes based on benign alternatives to priority pollutants. Key technical properties of newly developed dyes and summaries of certain toxicological test methods and regulatory policies used to ensure dye application safety are presented.

**Test Methods**

**Genotoxicity**

The results described in this paper were obtained mainly from studies using the standard Ames test or Prival modification to assess genotoxicity, or from the use of Ceriodaphnia or Lemna minor to assess aquatic toxicity. The Ames test has been used as a cost effective "first look" at the carcinogenic potential of organic compounds. Specifically, this test is used to determine whether a compound is mutagenic, a property believed to be associated with the early steps of carcinogenesis. The Ames test uses strains of Salmonella bacteria that are sensitive to frameshift and base-pair substitution mutations and unable to grow (replicate) in the absence of histidine. Exposures of Salmonella bacteria to a test compound in which the bacteria replicate to levels at least twice the background count lead to the designation of the test compound as mutagenic. This test is also conducted in the presence of enzyme systems (S9) capable of producing metabolites of the test compound. In this way, an assessment of the potential for the test compound to function as an indirect-acting mutagen is possible.

The development of the Prival modification of the Ames test grew out of a need to enhance the reductive-cleavage of azo dyes during the metabolic process. This was important because azo dyes such as Congo Red (C.I. Direct Red 28; structure 1) are not direct-acting mutagens as Congo Red (C.I. Direct Red 28; structure 1) are not direct-acting mutagens and thus often give a nonmutagenic response in the Ames test, despite being derived from the well-known human carcinogen benzidine. The Prival modification uses hamster liver S9 in lieu of rat liver S9, the former being richer in azo reductase enzymes and demonstrating the importance of azo group reductive cleavage (Fig. 1) in the mutagenesis process.

The choice of test methods for evaluating carcinogenicity is important. Data from a variety of published methods have been assessed for utility. The resulting data for 97 chemicals, mainly azo dyes, associated with the dye industry produced the following conclusions:
Less than one-third of the 97 compounds reported had been adequately tested to permit their carcinogenicity to be judged with a high degree of confidence.

- Test methods involving urinary bladder implantation, repeated injections, or very few test animals are inappropriate for evaluating chemical carcinogenicity.

- The purity of test compounds must be sufficient to attribute the test results to the compound in question and not an impurity.

- The preferred testing involves at least two species of animals and non-conflicting data; a minimum group of 25 animals of each sex, with a sufficient number surviving about two-thirds of their expected life span; and chronic application of test compound at a range of doses up to and including a level giving a biological effect.

Aquatic Toxicity

In keeping with the standard method recommendations for examining water and wastewater, *Lemna minor* (duckweed) was used in our studies to assess the aquatic toxicity of a group of commercially-used metal complex dyes and their iron (Fe) analogs. Under ideal nutrient conditions, duckweed plants can double their mass in less than two days. In solutions containing high levels of nutrients, duckweed is known to be resistant to environmental pollutants, including metal ions. While duckweed appears to have the ability to adapt to sub-lethal concentrations of pollutants over a period of time, it has been shown to be more sensitive to metal toxicity than other aquatic species.

Basic Research Studies

Structure-Property Relationships

Genotoxicity

Following the recognition that certain aromatic amines used in azo dye synthesis caused bladder cancer, a wide variety of chemicals were evaluated in animal studies. The results showed that aromatic amines and azo compounds of the type shown in Fig. 2 are carcinogenic. In this regard, it is generally agreed that the ultimate carcinogen arises from the metabolic conversion of these compounds to electrophilic species (Fig. 3) that interact with electron-rich sites in DNA to cause adverse effects on protein synthesis. It is also clear that ring substituents that enhance hydrophobic character increase carcinogenic potential. Consequently, adding a methyl group to 2-naphthylamine (structure 2; \( R = H \)) or meta-phenylenediamine (structure 4) enhances carcinogenicity.

As a follow up to the carcinogenicity data assessment study, correlations between dye structure and carcinogenicity data were established. In a comparison of hydrophobic azo dyes containing amino groups in the para- or ortho-position, it was found that the para-isomers were carcinogenic while the ortho-isomers were not. To account for these results, the chemistry in Fig. 4 was proposed. It is believed that the two types of isomers produce nitrenium ions (structure 9) that either interact with DNA (para-isomer) or undergo intramolecular cyclization (ortho-isomer) to produce adducts (structure 10) and benzotriazoles (structure 11) respectively.

A correlation of carcinogenicity data with the structures of hydrophilic azo dyes was also presented. Water-soluble dyes of structure type 12 were carcinogenic, while structure type 13 dyes (Fig. 5) were not. In this case, the nature of the reductive-cleavage prod-
ments has led to the search for alternatives to Cr ions for producing lightfast and washfast acid dyes. In this regard, metals such as Al and Fe have received significant attention, with azo and formazan structures (Fig. 6) serving as the main base colorants (ligands). It is clear from studies pertaining to the aquatic toxicity of the resultant dyes that the Fe complexes are generally less toxic than the Cr counterparts, however, the chemical makeup of the ligand also plays a role in the observed toxicity.

Aquatic Toxicity

The need to address toxicity concerns associated with dyes containing metals designated as priority pollutants was a significant research area. This designation includes Cr⁶⁺, which was commonly used by commercial dyers in the post-metallization of mordant dyes to achieve high fastness properties on wool. Mordant dyes have since been replaced in part by pre-metallized dyes for wool and nylon, placing the metallization process in the hands of dye manufacturers. However, the uncertainty surrounding the fate of metallized dyes in aquatic environments has led to the search for alternatives to Cr ions for producing lightfast and washfast acid dyes. In this regard, metals such as Al and Fe have received significant attention, with azo and formazan structures (Fig. 6) serving as the main base colorants (ligands). It is clear from studies pertaining to the aquatic toxicity of the resultant dyes that the Fe complexes are generally less toxic than the Cr counterparts, however, the chemical makeup of the ligand also plays a role in the observed toxicity.

Molecular Design

Genotoxicity

The focus of studies in this area was the development of approaches to the design of viable alternatives to azo dyes and intermediates determined to be genotoxic. Design considerations emerging from the study of structure-property relationships were implemented. The most important pertain to the properties of the raw materials employed in dye synthesis and the reductive cleavage products of the parent dyes. With regard to both points, it is clear that sulfonated amines such as structures 17-19 (Fig. 7) are nongenotoxic, unlike their sulfonic acid free counterparts. While the design of azo dyes producing sulfonated reductive-cleavage products is often a viable approach to addressing genotoxicity concerns, it is not universally applicable. On the one hand, increasing the number of sulfonic acid groups (–SO₂H) has a deleterious effect on washfastness, and on the other hand, water-soluble dyes are unsuitable for the coloration of hydrophobic fibers. With this in mind, approaches to the design of nongenotoxic hydrophobic dye intermediates were undertaken.

Interesting approaches to developing nongenotoxic hydrophobic azo dye precursors have involved the introduction of heteroatoms, bulky alkyl or alkoxy groups, or bridging groups into the structures of aromatic amines. This led to nongenotoxic dye intermediates such as structures 20-24 (Fig. 8). While the reason for the removal of genotoxicity, especially mutagenicity, is unclear in each case, it is believed that bulky groups placed ortho to the amino groups interfere with nitrenium ion formation. Diamine structures 21, 23, and 24 have been used to make nonmutagenic red to black azo dyes (e., structures 25 and 26; Fig. 9) for cotton, while amine structure 20 and diamine structure 22 have been used as dye precursors for non-textile applications. Diamine structure 21 and related analogs have also been used as alternatives to benzidine and its homologs; e.g., dichlorobenzidine, in organic pigment synthesis (e., structures 27-28). More recently, the mutagenic properties of diaminophenylene homologs (structures 29, Fig. 10) were assessed. While all dyes except diaminouracil (DAOQ) were mutagenic in TA98 with S9 enzyme activation, all four diamines were negative in TA100 with or without S9 activation. It is also clear from Fig. 10 that the mutagenicity of these diamines increased in the order diaminoterphenyl (DATP, n=3) > diaminobiphenyl (DABP, n=2) > diaminodiphenylenyl (DAP, n=1) > DAOQ, at 0-600 μg dose levels. At 700 μg, the mutagenicity of DABP and DATP was essentially the same. S9 activation was not required for DATP to exhibit mutagenicity in TA98, making this diamine a direct-acting mutagen via base-pair substitution. Following the synthesis and testing of Congo Red homologs (structures 30, Fig. 11), mutagenicity data, produced using...
the Prival preincubation assay, for Congo Red (x=2) and the two phenylene homologs (x=3, 4) at low (0–60 μg) and high (0–1,000 μg) dose ranges were studied. At low doses, only Congo Red was mutagenic in both strains, although its activity was most noticeable at the 50 μg dose level. In TA98, the DATP-based Congo Red homolog was more mutagenic than the parent dye, while the DAQP-based homolog was nonmutagenic. Beyond the 50 μg dose level, the mutagenicity of Congo Red increased appreciably beyond that of the DATP homolog, while the DAQP homolog remained nonmutagenic in both strains (Fig. 11).

**Aquatic Toxicity**

Except for dye structure 32, the unmetallized forms of eachazo dye had EC50 > 1,000 mg/L. This low level of toxicity is probably due to the low water solubility of dyes structures 14, 15, and 31. The Fe complexes of structures 14, 15 (R=NHAc), and 31 had lower aquatic toxicity than the corresponding commercial Cr and Co complexes. On the other hand, there was no apparent benefit arising from substituting Fe for Co in metalizing dye structure 15 (R=H). In these studies, an EC50 > 300 mg/L was regarded as good and EC50 > 1,000 mg/L as excellent.

An example of the data generated is provided in Fig. 12. The graph shows the frond count (number of "leaves" on the duckweed plant) at the end of three, six, and seven days as the dye concentration was varied. Fresh dye solutions were introduced on days three and six to simulate the periodic release of effluents from a manufacturing plant. It is clear from the graph that frond counts above day one levels were produced at the end of day seven indicating plant growth. Although not shown in the graph, aquatic toxicity testing of ozone-decolorized dye solutions showed no change in the pattern observed with colored solutions, in that the Fe complexes were generally less toxic than their commercial prototypes following ozone treatment.

In the case of formazan dyes, the unmetallized dyes had HC50 > 300 mg/L and only one Fe-complexed dye gave an unacceptably low HC50. This dye, structure 16 where Z = SO2NH2 and Y = H, is the only dye in the group that lacks a substituent in the para-position of the diazo component. The presence of a -Cl, -NO2, or -SO2NH2 group in the para-position led to a significant increase in HC50. In contrast to its adverse effect on mutagenicity, the -NO2 group did not have a similar effect on aquatic toxicity. In fact, the 1:2 Fe-complex having Z = SO2NH2 and Y = NO2 had HC50 > 400 mg/L before ozone treatment and > 200 mg/L following ozone treatment. While ozone-decolorizing experiments did not involve dye concentrations above 200 mg/L, our calculations projected an HC50 > 600 mg/L for this NO2-substituted dye. Interestingly, our studies revealed a correlation between the pH of azo dye solutions following ozone treatment and aquatic toxicity, with those solutions producing a pH below 3.3 exhibiting higher aquatic toxicity (HC50 < 200 mg/L). A similar correlation between the aquatic toxicity of the decolorized formazan dye solutions and pH was not observed, even though the pH dropped as low as 3.0 in one case.

**THE ROAD AHEAD**

In the absence of a crystal ball to help point the way forward in the field of color chemistry, one can simply note and take advantage of a few clues provided in the
form of national and international regulatory policies.

First, it is clear that regulations, such as the German Ordinance that bans the sale of goods containing dyes derived from a specific group of genotoxic aromatic amines, are here to stay. Consequently, attention must be given to the selection and safe handling of intermediates used in dye manufacturing and to the nature of potential metabolites from enzyme interactions with synthetic dyes. It is evident that the entire global community will soon be within reach of such policies.

Secondly, restrictions on the number and quantities of chemicals permitted in R&D laboratories require strategies that minimize the impact of reduced chemical inventories. The answer lies in the judicious use of computer-aided design (molecular modeling) methods that permit the prediction of technical properties of contemplated dye structures, thus reducing the number of compounds requiring synthesis before a viable product is obtained.

The search for metal-free lightfast dyes must continue, as those based on priority pollutants will eventually fade into the sunset. Finally, we must find a way to attract the brightest available students back to color chemistry and train them to tackle the challenging opportunities we face. Without them, the suggestion that we are part of a sunset industry will become a reality faster than we would like to think possible. It would be a tragedy for this to happen in an environment such as ours, where leading-edge technologies in the hands of talented researchers could help to open new frontiers in dye chemistry and applications.

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