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On the Toxicology and Ecology of Organic Colorants

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The colorants manufacturing industry of western Europe began to investigate toxicological and ecological properties of colorants long before chemical and environmental laws existed. Thus, corresponding studies of colorants have been performed over a period of more than 30 years.

Early understanding of the necessity to gain information on possible hazard potentials led in 1974 to the foundation of ETAD (Ecological and Toxicological Association of the Dyestuffs and Organic Pigments Manufacturing Industry), an international association of now more than 35 member firms, exclusively dealing with toxicological and ecological matters regarding colorants.

Nowadays laws require manufacturers of chemicals to assess the hazard potentials of each of their substances.

1. Definition of Colorants

Solubility is the most important criterion in the classification of organic colorants. According to the German Standard DIN 55943 colorants are divided into dyes which are soluble in the application media, and pigments which are practically insoluble in the application media. Dyes can be subdivided into water soluble and solvent soluble ones.

Another classification follows chemical or applicational point of views; *e.g.* azo colorants, polycyclic colorants, anionic, cationic dyes or direct, reactive, vat, sulphur and disperse dyes, and pigments.

2. Toxicological Investigations

Comprehensive toxicological investigations of colorants are necessary in order to identify possible health hazards of these compounds. Information about the toxicological profile of colorants and exposure minimization permit adequate protection of man and the environment. Toxicological data and knowledge about possible exposure are used in a three-step approach for safe handling of dyes and pigments:

- hazard assessment
- risk assessment
- risk management

Important toxicological and ecological investigations include studies on

- acute toxicity
- toxicity after repeated exposure
- irritation potential
- sensitization potential
- genotoxicity (mutagenicity)
- carcinogenicity
- aquatic toxicity
- biological degradation

All relevant data of these studies and additional information for safe handling of the colorants are summarized in the Material Safety Data Sheets (MSDS) originally developed by ETAD.

2.1. *Acute Toxicity Studies*

ETAD has analysed the Material Safety Data Sheets of more than 4400 organic colorants for which information of single exposure experiments are available. In only 8% of all tested colorants labelling due to acute toxic effects (LD_{50} < 2000 mg/kg b.wt.) was necessary [1]. If not caused by cationic or metal complex dyes, auxiliaries can contribute to the toxic effects since these studies have been performed with trade products (preparations).

2.2. *Studies on Repeated Exposure (28 d/90 d)*

Studies on repeated exposure with non-lethal dose levels are extremely helpful to analyse a potential hazard of prolonged exposure. In many cases where oral dose levels of organic colorants up to 1000 mg/kg b.wt. did not lead to any substantial organ toxicity, no hazard is to be expected from this experimental design [2][3]. In cases of a No-effect-level (NOEL) < 1000 mg/kg b.wt. adequate safety factors have to be considered for non-intended human exposure.

2.3. *Irritation Potential*

Organic colorants are routinely tested for their irritation potential. In a comparative study with 68 colorants, 3 compounds produced skin irritation and 17 were eye irritants [4]. Frequently, additives are responsible for the irritation observed.

2.4. *Sensitization Potential*

There is evidence that a few disperse and reactive dyes can cause allergic reactions such as dermal or respiratory sensitization. Only a small number of the commercially available colorants are proven sensitizers. Substantial reduction in occupational exposure levels have successfully reduced any substantial risk of allergic reactions for many years.

2.5. *Mutagenicity*

Several short-term mutagenicity tests are available to analyze genetic hazards. Among these, the *Ames* test is the one most frequently used. Since its introduction as a routine procedure numerous colorants have been investigated. In a comprehensive study with more than 200 dyes of various structures more than 2/3 of them have been reported to be non-mutagenic [5]. Among 36 organic pigments only one revealed genotoxic effects. More recently re-evaluations have shown that impurities in some cases are responible for the mutagenicity observed.

2.6. *Cancerogenicity*

The carcinogenic hazard of colorants can only be evaluated experimentally by long-term exposure studies in animals. No epidemiological evidence exists indicating carcinogenic activity in man. The limited number of colorants tested in long-term studies indicates that in some cases tumors have been produced. The existing data base, however, needs to be critically validated to understand the significance of these findings. The use of adequate dose levels, the route of applications, the purity of the tested colorant and consideration of species differences are important criteria for the assessment of carcinogenic potential.

In a review of carcinogenicity studies on 87 colorants, only 29 reports were found to be appropriately tested [6]. Of these, 12 compounds were considered to be tumorigenic.

In the particular case of benzidine-derived azo dyes, reductive cleavage of the azo bond can liberate the carcinogenic benzidine which obviously is responsible for carcinogenicity. Production and sales of benzidine and benzidine-derived colorants, however, were ceased by the leading colorant manufacturers of western Europe about 20 years ago.

2.7. *Aquatic Toxicity/Biodegradation*

Establishing the toxicological profile of colorants includes testing in aquatic systems, such as fish, daphnia and bacteria toxicity, and biodegradation.

It has been reported by *Anliker* [7] that among 3000 commercial colorants tested 59% were not very toxic to fish (LC_{50} > 100 mg/l). Only 2% have found to be toxic at a level of less than 1 mg/l.

In only 18 of more than 200 commercial

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colorants tested, the bacterial growth inhibition (IC_{50}) was found to be less than 100 mg/l, in three cases IC_{50} was between 1 and 10 mg/l [8].

Two additional validated aquatic test systems (algae growth inhibition test, daphnia acute immobilization test) are also used to determine the influence of colorants on the environment.

The biodegradation of colorants under aerobic conditions is poor, however, anaerobic degradation of azo dyes forming amino derivatives has been shown. Subsequent aerobic degradation of the amino compounds was demonstrated as well.

3. Specific Toxicological Investigations

Five examples are given to illustrate the importance of specific studies on genotoxicity, cancerogenicity, bioavailability, and metabolism in order to assess the hazard potential of selected colorants.

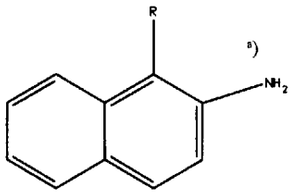
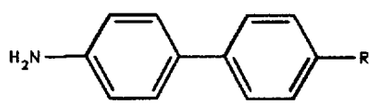
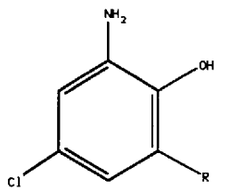
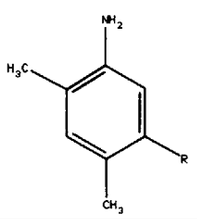
3.1. Ames/Prival *Test*

The *Prival* test, a modification of the *Ames* test, was found to be superior to the routine method for the mutagenicity testing of azo dyes. The reductive enzymatic cleavage of the azo bond occurring in mammals is simulated by this test.

In a comparative evalution, three azo dyes were tested according to *Ames* and the *Prival* modification as well [8].

The results are shown in *Table 1*. Compound **1** and **2** were not mutagenic in the *Ames* test but demonstrate the superiority of the *Prival* test in detecting a mutagenic aromatic amine as cleavage product. The reverse result with **3** explains why the standard *Ames* test cannot be replaced by the *Prival* modification without losing important information on the mutagenicity of azo compounds.

Table 2. Ames *Test Results of Aromatic Amines* vs. *Aromatic Amino-sulfonic Acids*

	$R = SO_3H$	$R = H$
 [a])	–	+
	–	+
	–	+
	–	+

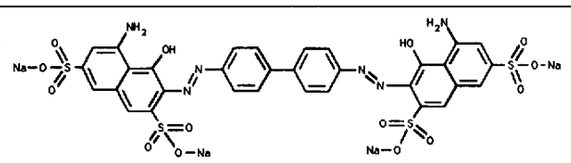
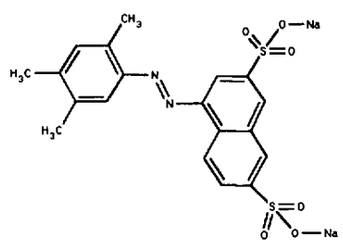
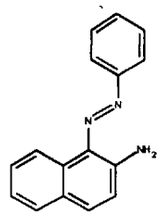
[a]) Applies also for the 5-, 6- or 8-mono-sulfonic acid and the 1,5- and 6,8-disulfonic acid as well [12].

3.2. *Investigation of the Genotoxic Potential of Aromatic Amino-sulfonic Acids (AASS)*

A great variety of water soluble azo dyes can form aromatic phenyl-or naphthylamino-sulfonic acids by enzymatic or by chemical reduction. Due to the fact that many aromatic amines are mutagenic and some of them even carcinogenic, it has been of great importance to understand whether the dyes themselves or their cleavage products (amino sulphonic acids) are genotoxic/carcinogenic or not. A recent compilation of genotoxicity and carcinogenicity data on aromatic amino-sulfonic acids prepared by ETAD [9] has concluded that the amino sulphonic acids ($R=SO_3H$), in contrast to some of their unsulphonated analogues (R=H), generally have no or only a very low genotoxic and tumorigenic potential (see *Table 2*).

In line with these data is the fact that 28 of the corresponding dyes tested in long-term bioassays were also not carcinogenic.

Table 1. *Mutagenicity Testing in* Ames *and* Prival *Test*

No.	Azo dye		*Ames*	*Prival*
1	Direct Blue 6		–	++
2	Acid Dye		–	+
3	Solvent Yellow 5		+	–

3.3. *Genotoxicity of Nitroazo Compounds*

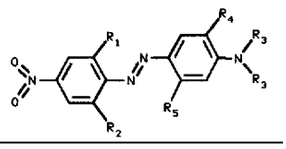
In a study on structure-activity relationship 4'-nitro-4-(diethylamino)azobenzene and seven structurally related analogues were tested in a variety of genotoxicity tests (*Ames* test, point mutation test in mammalian cells in culture, UDS test *in vivo* (rat), micronucleus test *in vivo* (mouse)) [11]. The study was undertaken since a variety of azo dyes have been shown to be mutagenic in the *Ames* test but the significance of these findings for hazard assessment in conjunction with other mutagenicity tests is unknown.

All of the compounds in *Table 3* were mutagenic in the *Ames* test. However, only one nitroazo compound was mutagenic in mammalian cell gene mutation assay (compound **6**). It was the most potent in the *Ames* test. All compounds tested were negative in two animal mutagenicity tests. It is, therefore, concluded that due to non-genotoxicity findings in the animal test systems, these chemicals are unlikely to pose a hazard as genotoxic carcinogens.

3.4. *Substituted Benzidine Structures*

Benzidine and the 3,3'-methyl- and 3,3'-methoxy-substituted derivatives are muta-

Table 3. *Genotoxicity Studies on 4'-Nitro-4-(dialkylamino)azobenzenes* (ETAD-Project T 2017)



No.	R_1	R_2	R_3	R_4	R_5	*Ames* [a] –S9	*Ames* [a] +S9	Mammal. cells [b] (V79/CHO/ML)	UDS[c]	MN[d]
1	H	H	C_2H_5	H	H	–	+	–	–	–
2	Cl	H	C_2H_5	H	H	–	+	–	–	–
3	CN	H	C_2H_5	H	H	+	+	–	–	–
4	Cl	Cl	C_2H_5	H	H	+	+	–	–	–
5	NO_2	Cl	C_2H_5	H	H	–	+	–	–	–
6	NO_2	H	C_2H_4OH	H	H	+	+	(+)	–	[e]
7	CN	H	C_2H_4OH	H	H	+	+	–	–	–
8	H	H	C_2H_5	OCH_3	$NHCOCH_3$	+	+	–	–	–

[a]) *Ames*: Salmonella mikrosome test; –S9: without, +S9: with enzymatic activation.
[b]) Mammal. cells: HGPRT (Hypoxanthin-Guanin-Phosphoribosyl-Transferase) test in V79 or CHO cells, thymidin-kinase test in ML cells.
[c]) UDS: Unscheduled DNA synthesis *in vivo*.
[d]) MN: Micronucleus test (*in vivo*).
[e]) In progress.

genic and carcinogenic. In order to find non-mutagenic 3,3'-substituted derivatives as new intermediate products for improved disazo dyes, a series of alkyl and alkoxy derivatives were studied in the *Ames* test (*Table 4*). In summary it can be stated that a chain length of C_4 resulted in no genotoxicity for the substituted alkylbenzidine derivatives tested. Similarly C_3 or longer substituents in a series of alkoxy-substituted benzidine derivatives were also not mutagenic [12].

3,3'-Dibutoxybenzidine was additionally found non-mutagenic in a second mutation assay in mammalian cells in cultures. It is assumed that steric hindrance will prevent metabolic activation of voluminous 3,3'-substituted benzidine derivatives. Further studies are in process.

3.5. *Non-bioavailability and Non-carcinogenicity of 3,3'-Dichlorobenzidine-Derived Pigments*

In contrast to some soluble azo dyes with carcinogenic potential based on dichlorobenzidine, diarylide azo pigments derived from 3,3'-dichlorobenzidine are not carcinogenic.

In a recent review of the carcinogenic potential of these pigments [3] it is stated that all available experimental data provide no indication of a tumorigenic activity. The mechanistic explanation of the fact is that these pigments are practically insoluble and that no endogenous metabolic cleavage to liberate 3,3'-dichlorobenzidine occurs.

Several metabolism studies with oral or inhalation exposure illustrate that 3,3'-dichlorobenzidine is not released and, therefore, not bioavailable.

3.6. *Study of the Toxicological and Ecological Profile of* Remazol Black B®

Representing an important type of reactive dyes a comprehensive study was performed with *Remazol Black B* (R = SO_3 Na):

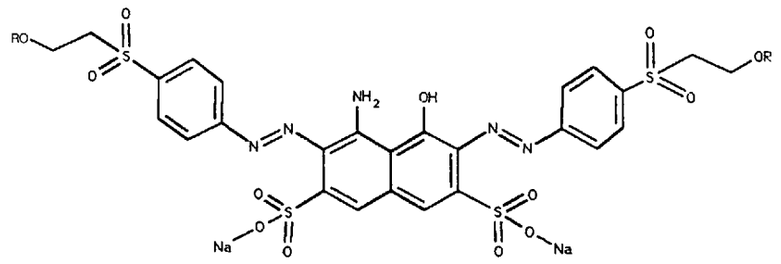
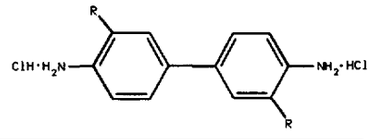


Table 4. Ames *Test of 3,3'-Substituted Benzidine Derivatives*



[+]) S-9; TA98, TA 1538: frame shift.

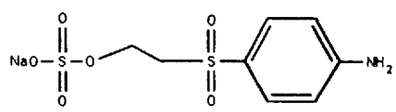
R	OR	
CH_3	+	+
C_2H_5	+	+
C_3H_7	(+)[a])	–
i-C_3H_7	(+)[a])	–
C_4H_9	–	–
i-C_4H_9	–	–
$-\underset{CH_3}{\overset{}{C}}HC_2H_5$	–	–
	$-CH_2CH_2OCH_3$	–
		–

[a]) TA 1538, weak effect.

The dye was found to be non-acute toxic (LD_{50} > 14 000 mg/kg), neither skin nor eye irritant, the NOEL of a 21-day feeding study was 500 mg/kg b.wt. In a study on the sensitization potential in animals *Remazol Black B* was found not to be a dermal sensitizer. However, in man a few cases of allergic reaction are known.

The reactive dye itself showed a low toxic potential in aquatic organisms (fish LC_{50} 100–500 mg/l; bacteria EC_{50} ~ 2 000 mg/l) as well as the hydrolysed dye (hydroxy dye, R = H; fish LC_{50} > 500 mg/l).

Neither *in-vitro* tests on mutagenicity such as *Ames*, *Prival* and the cytogenetic-*in-vitro* test nor *in-vivo* tests such as the micronucleus and the cytogenetic *in-vivo* tests did indicate any genotoxic effect. Kinetic studies in rats with the radioactive-labelled dye revealed the formation of the 'p-base ester' as the major metabolite (84%):



This compound being the result of the reductive cleavage of the azo bonds proved also to be non-mutagenic in the *Ames* test [10].

It can be concluded that *Remazol Black B* is considered to be of low acute toxicity, non-irritant, a weak sensitizer and has no

genotoxic potential. Even in its hydrolysed form it is not hazardous to the effluent water.

4. Summary

Comprehensive information on toxicological and ecological properties is available for the majority of commercial colorants. With increasing information it became evident that many of the colorants do not pose a pronounced toxicological hazard. It is now possible to focus further attention to those groups of compounds which possess a specific toxicological behavior. Beside adequate labelling and exposure minimization, attempts are made to understand and study their modes of action in more detail.

General considerations on colorants:

- Evaluation in the event of azo dyes possibly being reduced to mutagenic or cancerogenic aromatic amines.
- Azo dyes which could form amino sulphonic acids as hypothetic metabolites are not carcinogenic.
- Reactive dyes and disperse dyes should be evaluated with regard to a possible sensitizing potential.
- Azo pigments based on 3,3'-dichlorobenzidine have proved to be not toxic after acute or repeated exposure and proved to be not mutagenic and not cancerogenic due to their non-bioavailability.
- Organic pigments in general, and in particular when extremely insoluble due to their chemical structure are of very little toxicological and ecological relevance.
- To a certain extent auxiliaries must be made responsible for toxic effects in the commercial colorants.
- Hazard assessment of the mutagenicity with aromatic nitro and amino compounds (colorants) cannot rely on *Ames* test data only.

Once the toxicological and ecological profile has been established (hazard assessment) reliable risk assessment must include the exposure situation. Control and minimization of non-intended exposure and knowledge of the chemical characteristics will guarantee a good hygiene standard and safe handling of dyes and pigments at the work place (risk management).

Improvement in recycling processes and attempts to decrease waste burden, in combination with knowledge of ecological behavior, will further reduce adverse effects on the environment.

[1] R. Anliker, *J. Soc. Dyers Col.* **1979**, *95*, 317.
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Methämoglobin-Bildung und Hämoglobin-Bindung bei aromatischen Aminen

Hans-Günter Neumann*

Die Geschichte der synthetischen Farbmittel ist wesentlich mit der Chemie des Anilins, des Nitrobenzols und der davon abgeleiteten Arylamine verknüpft. Der Apotheker *Unverdorben* stellte 1826 Anilin erstmals durch Kalkdestillation aus natürlichem Indigo dar. Synthetisch wird es durch Reduktion von Nitrobenzol auf einem Weg gewonnen, auf dem auch Hydrazobenzol und aus diesem durch Umlagerung Benzidin erhalten wird. Diazotieren eröffnet den Weg zu den Azofarbmitteln. Damit ist eine Entwicklung skizziert, die immer neue Anwendungen erschloss und so stürmisch verlief, dass die damit verbundenen Gefahren für die Gesundheit bei Herstellung und Anwendung nur wenig beachtet wurden. Die Geschichte der synthetischen Farbmittel ist damit auch eine Geschichte der Verdrängungen und sie zeigt, wie die Beschäftigung mit den Gesundheitsgefahren und den daraus zu ziehenden Schlussfolgerungen bisher immer hinter der Entwicklung zurückgeblieben ist.

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Die Erzeugung von Methämoglobin

Bereits 1863 wurde die akut toxische Wirkung von Anilin und Nitrobenzol festgestellt [1]: Beide erzeugen Methämoglobin, jene Form des Hämoglobins, in der aufgrund der Oxidation des zweiwertigen zum dreiwertigen Eisen in der Hämgruppe die Fähigkeit zur Bindung molekularen O_2 verlorengegangen ist. Auch die Farbe des roten Blutfarbstoffs verändert sich dabei, und erste Vergiftungserscheinungen sind besonders an Lippen und unter den Fingernägeln erkennbar. Daher stammt der Begriff der Blausucht (Zyanose). Die sogenannten «Blue boys» waren in der Anilin-Fabrikation keine Seltenheit und eine Art von Gesundheitsschutz bestand lediglich darin, besonders gesunde Arbeiter für diese Tätigkeit auszusuchen.

Nach 1886 wurde wiederholt über die Vergiftung von Säuglingen berichtet, deren Windeln mit Anilin-haltiger Wäschetinte markiert waren [2]. Dies zeigt einerseits die gute Aufnahme über die Haut, andererseits die besondere Empfindlichkeit in diesem Alter, in dem die Rückreduktion des Methämoglobins zu Hämoglobin, eine physiologische Schutzreaktion, noch nicht voll ausgebildet ist.

Bereits 1891 wurde 4-Aminobiphenyl als stark Methämoglobin-erzeugend beschrieben [3], und bald danach diese akut toxische Wirkung als allgemeine Eigenschaft der Substanzklasse erkannt. Weitere Marksteine sind die Aufdeckung dieser Eigenschaft 1935 für Prontosil (Chrysoidin sulfonamid) [4], das ebenso wie das zur Schönung von Pflanzenfetten verwendete Buttergelb (*N,N*-Dimethylaminoazobenzol) [5] reduktiv zu Arylamin-Komponenten gespalten wird. Für die antibakterielle Wirkung von Prontosil ist das freigesetzte Sulfanilamid, die Muttersubstanz der Sulfonamide, verantwortlich. Bei seiner therapeutischen Anwendung tritt Methämoglobinämie nicht selten als Nebenwirkung auf. Durch den Verzehr Buttergelb-gefärbter Margarine wurden zwar beim Menschen keine Cyanosen ausgelöst, aber im Tierversuch konnte die Methämoglobin-Bildung durch zahlreiche Azo-Verbindungen gezeigt werden [6]. Die vorangehende Azo-Spaltung wurde zunächst als NADPH-abhängige Reaktion mit Rattenleberhomogenaten nachgewiesen [7]. Erst 1962 wurde erkannt, dass bestimmte Azofarbmittel (FD and C Red No. 2 und 4; FD and C Yellow No 6) auch durch Darmbakterien reduktiv zu den Kupplungskomponenten gespalten werden [8]. *In vivo* spielt