The Carcinogenicity of Certain Azo Dyes Related to *p*-Dimethylaminoazobenzene*

J. A. Miller**, Ph.D., and C. A. Baumann, Ph.D.

(From the Department of Biochemistry, College of Agriculture, University of Wisconsin, Madison 6, Wisconsin)

(Received for publication October 10, 1944)

Information on the relation of molecular structure to carcinogenicity is not nearly so extensive for the azo dyes as for the polycyclic hydrocarbons (10). Most of the pertinent publications appeared in Japan shortly after the carcinogenicity of azo dyes was first recognized, but before experimental conditions were established for an adequate survival of animals ingesting these compounds. One of the most active investigators in this field has published in English only his conclusions, without sufficient data for an evaluation of the results (14-16). Virtually all the earlier assays were made with a basal diet consisting of rice and carrot. Although this particular dietary combination was useful in the original discovery of the carcinogens *o*-aminoazotoluene (35) and *p*-dimethylaminoazobenzene (14), and also in the discovery that the development of hepatic tumors varies with diet (27), it may be questioned whether its continued use has not actually hindered further progress. All too frequently the feeding of an azo dye with rice and carrot has resulted in so high a mortality before tumors developed that any conclusion based on the surviving animals was without quantitative significance. The deficiencies in this diet have been discussed elsewhere (23), and the progress made with other diets is a matter of record (4, 8, 21-24, 34). It is now possible to examine the carcinogenicity of a series of closely related derivatives of *p*-dimethylaminoazobenzene under uniform dietary conditions that will permit an adequate survival of the experimental animals. An extension of the older data appeared particularly desirable, because such information is necessary to evaluate an attempt made by Kensler, Dexter, and Rhoads (11) to explain how azo dyes produce tumors.

The essence of their idea is that it is not the azo dye that initiates the carcinogenic process, but an enzyme poison, the split product, formed in the breakdown of the "parent" azo compound in the body. The split product hypothesis is in harmony with the older idea that the tumor problem is essentially one of deranged enzyme systems, and to date it is the only mechanism capable of further experimental test that has been suggested for the action of the azo dyes. The evidence for the hypothesis is as follows: Kensler, Sugiura, and Rhoads (12) noted that the diphosphopyridine nucleotide content of rat livers diminished during the feeding of *p*-dimethylaminoazobenzene, and that the coenzyme was very low in the induced tumors. Kensler, Dexter, and Rhoads then tested the effect of the dye and of its possible metabolites on a yeast zymase system in which the coenzyme was the limiting factor. *p*-Dimethylaminoazobenzene itself, *p*-aminophenol, a known metabolite (31), and aniline, a hypothetical metabolite, did not inhibit this system. However, *p*-phenylenediamine, known to be formed in the body from the azo dye (31), and in particular its possible precursor, N,N-dimethyl-*p*-phenylenediamine, were found to be very toxic to this system at low concentrations. The inhibitory action in the zymase system was then determined for a series of ring- and N-methylated *p*-phenylenediamines that had been employed by Michaelis, Schubert, and Granick (18) in a study of the properties of the free radicals obtained in the one-step oxidation of these diamines. Kensler, Dexter, and Rhoads reached the conclusion that the toxicity of a given diamine paralleled the stability of its free radical and its ease of oxidation (by air and/or the crude zymase preparation).[1] Similar observations were made by Kensler, Young, and Rhoads (13) on a crude yeast carboxylase preparation. The next step (11) was to correlate the carcinogenic potency of a series of azo dyes with the toxicity of the diamine and the stability of the free radicals that were presumed to be formed from the dyes in the animal

* Published with the approval of the Director of the Wisconsin Agricultural Experiment Station. This investigation was aided by the Wisconsin Alumni Research Foundation and the Jonathan Bowman Cancer Fund. We are indebted to Miss Janet Giese for the care of some of the animals.

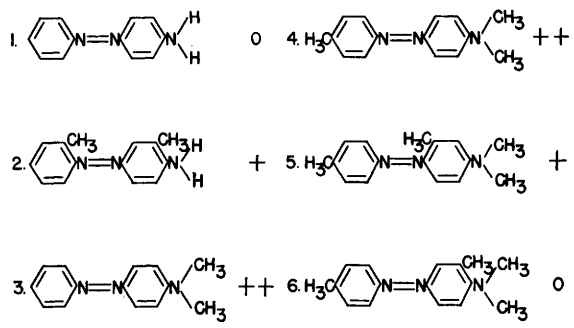
Presented in part at the meetings of the American Association for the Advancement of Science, Gibson Island, August 3, 1944.

** Finney-Howell Fellow, 1943-44.

[1] However, there appears to be some question whether the free radicals formed from the diamines are the enzyme poisons (11) or whether higher oxidation products such as *p*-quinones are the true inhibitors (17, 28).

body. The conclusion was drawn that if the *p*-diamine split product of a methyl derivative of *p*-dimethylaminoazobenzene inhibits the yeast zymase system employed by at least 65 per cent, and the stability of the free radical is more than 4 hours, the original azo compound can be expected to produce liver cancer in the rat.

The table of correlations published appears on the surface to bear out this statement. The stabilities of the free radicals of the diamines have presumably been determined accurately, and for all practical purposes the toxicities of the diamines toward enzyme systems may also be regarded as factually correct. But a critical examination of the evidence fails to substantiate the carcinogenic activities indicated for several of the azo dyes in the table. Of the 6 crucial compounds tabulated, only the first 3 have been



studied sufficiently to justify the assigned strengths. The latter 3 compounds were not fed at the usual 0.06 to 0.10 per cent levels but at 0.03 per cent for 45 days, then at 0.06 per cent for another 45 days, and finally at 0.2 per cent for the remainder of the experiments, which lasted 402 to 610 days (25). Thus compounds 4 to 6 were fed for long periods of time at levels 2 to 3 times as high as that employed for the first 3 dyes. Compound 4, *p'*-methyl-*p*-dimethylaminoazobenzene, was fed to 31 rats and 15 of these survived for 210 days, when the first tumor was discovered. The experiment was concluded in 402 days, at which time 12 tumors had appeared. This compound was assigned a potency of + +, the same as that given *p*-dimethylaminoazobenzene, compound 3. Actually compound 4 would appear to be considerably less active than *p*-dimethylaminoazobenzene, since 0.06 per cent of the latter dye gave 100 per cent tumors in 120 to 150 days (14, 23). Indeed, Nagao's compound 4 is probably no more active than compound 2, *o*-aminoazotoluene, and may even be less active when fed at comparable levels in the diet. Compound 5, *o,p'*-dimethyl-*p*-dimethylaminoazobenzene, an azo compound which on reduction could yield a *p*-diamine that is a less active enzyme poison than the diamine obtainable from compounds 3 or 4, was fed to 33 rats by Nagao (25). Fourteen survived 240 days on the diet and by 476 days only a few cysts were observed in the livers. At this time *one* liver was found to be tumorous, and the experiment was terminated at 610 days without any more tumors having been noted. Yet this compound was assigned a potency of +, a potency also assigned to the very definitely carcinogenic *o*-aminoazotoluene (10, 35). Compound 6, *m,p'*-dimethyl-*p*-dimethylaminoazobenzene, which on reduction could yield a diamine nontoxic in enzyme experiments (11, 13), was fed to 36 rats. Four survived 352 days, and none of these developed tumors by 610 days. This compound was assigned a potency of 0. However, while 3 references are given in Nagao's paper (25) for the preparation of the azo dyes employed, these references actually refer only to compounds 4 and 5. An examination of the chemical literature has failed to reveal any mention of compound 6. Since a methyl group *ortho* to the dimethylamino group, as in compound 6, strongly inhibits ordinary diazo coupling reactions (5, 7) except when the *p'* position is occupied by a strong negative group (1, 2, 18), it would appear that there is a reasonable doubt concerning the identity of the substance fed as compound 6.

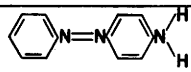
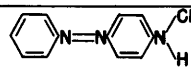
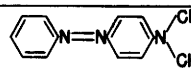
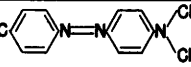
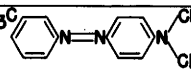
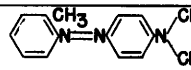
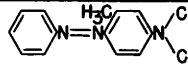
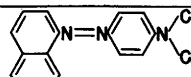
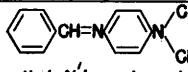
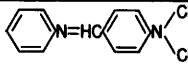
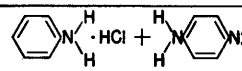
The data on compounds 4 to 6 are thus seen to be inadequate for even a rough comparison of carcinogenic potency with such established properties as the stability of free radicals or the ability of diamines to inhibit enzyme systems. The correlation originally published (11) therefore involves only 2 azo dyes closely related to *p*-dimethylaminoazobenzene whose potencies are known with any degree of certainty: *o*-aminoazotoluene, and *p*-aminoazobenzene. The need for more extensive data thus becomes obvious. Toward this end 11 compounds allied to *p*-dimethylaminoazobenzene were prepared and tested for their carcinogenic activity in rats fed a suitable basal ration. Particular emphasis was placed upon monomethyl derivatives of the dye that could theoretically yield the same diamine as *p*-dimethylaminoazobenzene, but differed in the configuration of the other half of the molecule.

Preparation of Compounds [2]

The compounds tested are listed in Table I. The *p*-dimethylaminoazobenzene and the *p*-dimethylaminophenylazo-1-naphthalene were Eastman products. The

[2] Substituents of *p*-dimethylaminoazobenzene have been designated as *o*, and *m* on the ring bearing the $-N(CH_3)_2$ group and as *o'*, *m'*, and *p'* on the other ring, starting in each case from the azo linkage. The corresponding *p*-diamines have been named in a similar fashion.

TABLE I: THE CARCINOGENICITY OF CERTAIN AZO DYES RELATED TO *p*-DIMETHYLAMINOAZOBENZENE

Diet no.	Compound fed	Per cent in diet	Time compound was fed mos.	Average weight increment during feeding of compound gm./rat	Incidence of liver tumors*				
					3 mos.	4 mos.	6 mos.	8 mos.	10 mos.
1	 *p*-Aminoazobenzene	0.053	8	+81				0/9	0/9
2	 *p*-Monomethylaminoazobenzene	0.056	4	+17	not palpable	7/8	8/8		
3	 *p*-Dimethylaminoazobenzene	0.060	4	+5	"	6/8	8/8		
4	 *p'*-Methyl-*p*-dimethylaminoazobenzene	0.064	8	+56				0/10	1/10
5	 *m'*-Methyl-*p*-dimethylaminoazobenzene	0.064	3	−24	5/8	7/8			
6	 *o'*-Methyl-*p*-dimethylaminoazobenzene	0.064	6	+13			2/9	4/9	
7	 *o*-Methyl-*p*-dimethylaminoazobenzene	0.064	8	−10				0/8	0/8
8	 *p*-Dimethylamino-phenylazo-1-naphthalene	0.073	8	+62				0/8	0/8
9	 *N,N*-Dimethyl-*N'*-benzal-*p*-phenylene-diamine	0.060	8	+74				0/10	0/10
10	 *p*-Dimethylaminobenzal-aniline	0.060	8	+104				0/9	0/9
11	 Aniline *N,N*-Dimethyl-*p*-phenylene-diamine	0.035 and 0 056 resp.	8	+92				0/9	0/9
12	*p*-Dimethylaminoazobenzene	0.006	8	+68				0/8	0/8
13	Azo dye as in diet 12 plus split-products as in diet 11	as in diets 11 and 12	8	+74				0/10	0/10

* Incidence = Number with tumors over number surviving when laparotomies were performed.

Schiff bases, N,N-dimethyl-N′-benzal-*p*-phenylenediamine and *p*-dimethylaminobenzal aniline were prepared as directed by Calm (3), and Sachs and Lewin (29) respectively. The split product of *p*-dimethylaminoazobenzene, N,N-dimethyl-*p*-phenylenediamine, was prepared by the reduction of *p*-nitrosodimethylaniline (6). This diamine and aniline were fed as the hydrochlorides. *p*-Aminoazobenzene and *p*-monomethylaminoazobenzene were prepared as described previously (19). The remaining compounds, all monomethyl derivatives of *p*-dimethylaminoazobenzene, were prepared by diazotizing 1 mole of the appropriate amine with 1 mole of sodium nitrite in the presence of 2½ moles of hydrochloric acid at 0° C. and adding the diazo solution to a 70 per cent ethanol solution of 1 mole of the desired tertiary amine in the presence of 2 moles of sodium acetate. The precipitated azo dye was then recrystallized from ethanol-water solution. The melting points of the azo dyes and the Schiff bases agreed with those reported in the literature.

All the 5 possible ring-monomethyl derivatives of *p*-dimethylaminoazobenzene were prepared and tested, with the exception of *m*-methyl-*p*-dimethylaminoazobenzene. This compound is mentioned only once in the literature, by Hantzsch (9), who stated merely that though it had not been described previously he had prepared it ". . . *auf die übliche Weise* . . ." (in the usual way). It is doubtful, however, that the usual direct coupling procedures were meant, since *o*-methyl-N,N-dimethylaniline does not couple appreciably with benzene diazonium salts (5, 7); coupling with this amine occurs only when the diazonium compound is activated by a strong negative group, *e.g.*, $-NO_2$, $-SO_3H$, in the *para-* position (1, 2, 18). Alternative procedures for the preparation of *m*-methyl-*p*-dimethylaminoazobenzene have been attempted in this laboratory without success.

METHODS

Each compound was fed to 10 young adult albino rats 150 to 200 gm. in weight. The rats were kept in groups of 5 in screen-bottomed cages, and food and water were given *ad libitum*. The basal diet was a semisynthetic one of the following composition: crude casein, 120 gm.; salts, 40 gm.; corn oil, 50 gm.; rice bran concentrate, 20 gm.; glucose, 770 gm.; and riboflavin, 0.5 mgm. per kilogram. Each rat received 1 drop of halibut liver oil monthly. This diet produces nearly 100 per cent liver tumors when 0.06 per cent of *p*-dimethylaminoazobenzene is fed for 4 months (23). In the present experiments each compound was fed at molar levels equivalent to the usual carcinogenic level of 0.06 per cent of *p*-dimethylaminoazobenzene. The azo dyes and the Schiff bases were incorporated in the diet by dissolving them with heat in the corn oil. The hydrochlorides of the split products of *p*-dimethylaminoazobenzene were mixed with the dry constituents of the diet. The rats were fed these diets until tumors developed or until 8 months had elapsed. Laparotomies were performed when palpable tumors formed or at 8 months. The rats were then fed the basal diet without the compounds for another 2 months, to allow any latent tumors to develop (23), and a final examination of the livers was made at the end of this period. No attempt was made to ascertain the ultimate carcinogenicity of any of the compounds that proved weakly active or inactive in this study. The arbitrary feeding period of 8 months is twice as long as the period of 4 months generally used with the control compound, *p*-dimethylaminoazobenzene.

RESULTS

The incidence of liver tumors in the rats fed these compounds is presented in Table I. Most of the rats on the various diets gained in weight, their general physical condition remained good, and the survivals ranged from 80 to 100 per cent. Under the conditions of the experiment, 4 compounds other than *p*-dimethylaminoazobenzene were carcinogenic. Together with the high survival in each group this indicates that the inactivity of the remaining compounds is also real under these conditions. The incidence of tumors in the rats fed *p*-dimethylaminoazobenzene (Diet 3) was 88 per cent at 4 months and 100 per cent at 6 months. This incidence is normal, and has been observed many times when the compound was fed in the basal diet employed (23, 24).

The completely demethylated derivative, *p*-aminoazobenzene, was inactive by 10 months (Diet 1). This agrees with the observation of Sasaki and Yoshida (30) that rats fed 0.1 per cent of the dye for 14 months did not develop tumors. The partially demethylated derivative, *p*-monomethylaminoazobenzene, was found to be practically as active as the dimethyl compound (Diets 2 and 3), and its carcinogenic potency has been confirmed by subsequent experiments. The similar carcinogenic potencies of these 2 compounds have counterparts in the known metabolism of the 2 dyes; essentially the same concentrations of these 2 dyes and of *p*-aminoazobenzene occurred in the livers of rats fed either the dimethyl or the monomethyl azo compound (20).

The ring-monomethyl derivatives of *p*-dimethylaminoazobenzene tested ranged in activity from very weak, or moderate, to very high activity. One of the 4, *o*-methyl-*p*-dimethylaminoazobenzene, did not produce any tumors after 10 months (Diet 7). Nagao (26) fed this compound as the hydrochloride at the

very high levels of 0.4 to 2.5 per cent and obtained tumors after nearly 12 months.[3] *p'*-Methyl-*p*-dimethylaminoazobenzene (Diet 4) produced only 1 tumor in 10 rats at 10 months. Nagao (25) fed 0.2 per cent of this dye and obtained liver tumors in 12 of 15 rats by 13 months. *o'*Methyl-*p*-dimethylaminoazobenzene, a compound not tested previously, produced liver tumors in 4 of 9 rats by 8 months (Diet 6).

The activity of the last ring-methyl compound, *m'*-methyl-*p*-dimethylaminoazobenzene, has not been recorded before. Surprisingly this compound proved to be more active (Diet 5) than the parent compound, *p*-dimethylaminoazobenzene. Large tumors were palpated and confirmed by laparotomy in 5 of 8 rats at 3 months, at which time no tumors could be palpated in the rats fed any of the other dyes. By 4 months 7 of the 8 rats had tumors, a negative animal having died in the interim. This dye thus had produced tumors approximately 1 month sooner than *p*-dimethylaminoazobenzene. The high carcinogenicity of the *m'*-methyl derivative, which is also more toxic than the parent dye, has been confirmed in subsequent experiments in this laboratory. The simultaneous substitution of the *o'*- and *m'*- positions by a benzo group, as in *p*-dimethylamino-phenylazo-1-naphthalene, produced an inactive compound (Diet 8). In general, the severity of the toxicity and cirrhosis observed with these dyes paralleled their carcinogenic potencies. Gross cirrhosis was noted only on Diets 2, 3, 5, and 6.

The two Schiff bases (Diets 9 and 10) tested the possibility that the substitution of a $-CH=$ group for the $-N=$ atom in the azo linkage might produce noticeably different results, depending upon the position of the $-CH=$ group; for on cleavage only the first Schiff base (Diet 9) could yield the hypothetical split-product of *p*-dimethylaminoazobenzene. However, both compounds failed to produce liver tumors. But it is doubtful whether more than small amounts of either reached the liver, since Schiff bases are hydrolyzed by weak acids and this may have occurred in the stomachs of the rats. When the first Schiff base (Diet 9) was fed to rats, the diamine portion was found to be excreted in the urine (see below). A benzaldehyde-like odor was evident in diets containing the second Schiff base (Diet 10).

No tumors resulted by 10 months when the split-products of *p*-dimethylaminoazobenzene, *viz.*, aniline, and N,N-dimethyl-*p*-phenylenediamine (Diet 11), were fed as the hydrochlorides at levels equivalent to 0.06 per cent of the parent azo dye. These amines have been fed previously to rats for long periods of time without the formation of tumors (14, 33). As discussed below, part of the diamine fed was excreted unchanged in the urine. Diets 12 and 13 represent an attempt to discover whether the split products of *p*-dimethylaminoazobenzene possess cocarcinogenic activity. When 0.006 per cent of the azo dye, one-tenth of the usual carcinogenic level, was fed, no tumors resulted after 10 months (Diet 12). Maruya (10) produced 2 tumors in rats fed this level of the dye for 12 months. When 0.006 per cent of *p*-dimethylaminoazobenzene and levels of the split products equivalent to the carcinogenic level of this dye were fed together (Diet 13), the tumor incidence still remained at zero. No toxic symptoms were observed in rats fed the split products, and these rats were among the healthiest throughout the experiment.

An attempt was also made to test the carcinogenicity of *p,p'*-tetramethyldiaminoazobenzene, a compound that on reduction could yield 2 molecules of the hypothetical split product of *p*-dimethylaminoazobenzene. However, this compound, prepared according to Vorländer and Wolferts (32), was so insoluble that difficulty was experienced in dissolving it with heat in corn oil. Furthermore, as evidenced by its red color in strong HCl, considerable amounts of the dye were excreted in the feces. Since the compound thus did not appear to be well absorbed and in addition was difficult to prepare in quantity, feeding was discontinued after only a few weeks.

The "pine chip reaction."—The pine shavings beneath the cages of rats fed N,N-dimethyl-*p*-phenylenediamine as the hydrochloride or as a Schiff base (Diets 9, 11 and 13) were found to be stained purplish-red by the urine shortly after the compounds were fed. When pure water solutions of the diamine hydrochloride were applied to the pine chips, the same color developed. Aqueous solutions of the hydrochlorides of the allied diamines, *p*-phenylenediamine and N-monomethyl-*p*-phenylenediamine produced orange and reddish stains respectively when applied to pine chips. These compounds were also fed to rats and in every case the shade of color observed when the urine came into contact with the pine shavings was the same as that of the compound fed. Preliminary data indicated that the amount excreted was only a small percentage of that ingested; and no similar excretion of chromogen was noted in rats fed the parent azo dye, *p*-dimethylaminoazobenzene. The ingestion of the dye did not interfere with the excretion of the diamine, since rats fed a diet containing 0.06 per cent of the dye plus equivalent levels of the split products excreted urine that gave a typical

[3] No data are given in Nagao's paper (26) on the percentage of survival or on the number of tumors occurring in rats fed these huge doses of dye. The hydrochloride was used because, as Nagao states, the free base underwent chemical changes; in our experiments the pure free base was stable and the dye was fed in this form. We are indebted to Mr. K. Higuchi for the translation of this reference.

reaction with pine chips. Incidentally, the diamines were found to give similar colors with brown paper towelling, and to some extent with alcoholic solutions of vanillin. Filter paper, however, failed to give any color with the diamines. The colors formed in this reaction closely resembled those of the respective free radicals. However, the pigments produced in the wood were not soluble in water.

Apparently, therefore, the rats fed N,N-dimethyl-*p*-phenylenediamine were excreting part of the original diamine as such. This observation is of interest in connection with the failure of tumors to develop when the amines corresponding to the split products of *p*-dimethylaminoazobenzene are fed (14, 33; and Table I, diets 11 and 13), for if N,N-dimethyl-*p*-phenylenediamine were the true carcinogen tumors should develop after the substances reached the susceptible liver cells. It might be postulated that the theoretically active diamine fails to reach the liver when it is fed as the hydrochloride, and that it reaches the proper liver cells only when the parent azo dye is fed. However, the finding of N,N-dimethyl-*p*-phenylenediamine in the urine of rats fed this substance is strong presumptive evidence that the diamine was widely distributed in the body. Indeed, since urine from rats fed *p*-dimethylaminoazobenzene failed to give a pine chip reaction, it would appear that more diamine is present in the tissues after the feeding of the diamine itself than after the feeding of the azo dye.

Demethylation of p-dimethylaminoazo dyes in vivo.—Preliminary analyses were made to determine whether the *p*-dimethylaminoazo dyes other than *p*-dimethylaminoazobenzene listed in Table I also undergo demethylation *in vivo* prior to cleavage at the azo linkage. The methods employed have been described previously (19, 20). Three separate azo dyes were found to be present in the livers of rats fed each dye, and these corresponded closely in their adsorption characteristics to the derivatives obtained from *p*-dimethylaminoazobenzene (20). The blood in each case contained only one compound which, from its chromatographic behavior, appeared to be the completely demethylated derivative. When *o*-aminoazotoluene was fed, only one compound, presumably the original dye, could be found in the liver and blood. Apparently, therefore, demethylation prior to fission of the azo group is a characteristic common to the metabolism of many *p*-dimethylaminoazo dyes.

DISCUSSION

The carcinogenic potencies found for the *o'*-, *m'*-, and *p'*- methyl derivatives of *p*-dimethylaminoazobenzene support the split-product theory of carcinogenesis (11) only in so far as each compound in the series caused some tumors to develop. But the wide quantitative variations in carcinogenicity observed among the members of this group, each of which could theoretically yield the same diamine on cleavage, indicate that the other non-diamine half of the parent molecule also exerts an important effect on the carcinogenicity of an azo dye. The *m'*- methyl compound was definitely more active than *p*-dimethylaminoazobenzene, the *o'*- methyl compound was roughly half as active, while the *p'*- methyl derivative was only very weakly carcinogenic.

Other examples of a lack of correlation between carcinogenicity and the toxicity of the corresponding diamine are as follows: *o*-methyl-*p*-dimethylaminoazobenzene is only weakly carcinogenic (26; and Table I, Diet 7), much less so than might be expected from the toxicity and stability of the free radical of the corresponding diamine, *o*-methyl-N,N-dimethyl-*p*-phenylenediamine [2] (11, 18). Furthermore, the carcinogenicity of *p*-monomethylaminoazobenzene is equal to that of *p*-dimethylaminoazobenzene, although the toxicity and free radical stability of the corresponding diamine (11) would lead one to predict a lower degree of carcinogenicity for the monomethyl compound, if the stability of the free radical were the determining factor. It would have been desirable to test the carcinogenicity of *m*-methyl-*p*-dimethylaminoazobenzene for on reduction this substance could yield a nontoxic diamine, *m*-methyl-N,N-dimethyl-*p*-phenylenediamine (11), and hence, according to the criteria of Kensler and his group, the parent azo dye should be noncarcinogenic.

The discrepancies enumerated are probably best judged in the light of analytical results on the metabolic fate of azo dyes in the body of the rat. Both *p*-dimethyl- and *p*-monomethylaminoazobenzene are demethylated in the animal prior to cleavage at the azo linkage (20). The monomethyl derivative can also be methylated to yield the dimethyl compound, and the quantitative distribution of azo dyes in the tissues, and especially in the liver, is essentially the same whether *p*-monomethyl- or *p*-dimethylaminoazobenzene is given (20). Since the rate of tumor formation is also essentially the same when either dye is fed, it would appear that the concentration of azo dye in the liver, rather than the stability of a hypothetical cleavage product, is the determining factor in carcinogenesis with these azo dyes. Indeed, the concentration of the completely demethylated product, *p*-aminoazobenzene, in the tissues is essentially the same whether this dye or either of its two carcinogenic N-methyl derivatives is fed, suggesting that most of the carcinogen given may be demethylated prior to cleavage in the animal body, thus precluding the formation of appreciable amounts of the hypothetical N,N-dimethyl-*p*-phenylenediamine.

Hence neither of the two new lines of evidence, the analytical data or the carcinogenicity of related azo dyes, supports the split-product theory of carcinogenesis. It must also be stated, however, that these results do not completely rule out the possibility of N,N-dimethyl-*p*-phenylenediamine functioning as originally postulated (11). For even if 99 per cent of *p*-dimethylaminoazobenzene should be demethylated prior to cleavage, it would still be possible that the remaining fraction could yield the cleavage product postulated (although proof to that effect ought to be furnished). The demonstration of N,N-dimethyl-*p*-phenylenediamine in the urine of rats fed this substance suggests its presence in the body fluids, but does not prove it to be within the liver cells. The lack of correlation between the postulated carcinogenicity of the various methyl derivatives of *p*-dimethylaminoazobenzene and that observed could have been due to secondary complications, such as, *e.g.*, unequal efficiencies of transportation from the gut to the liver depending upon other groupings in the molecule. Indeed it might even be argued that if carcinogens can undergo metabolic reactions unrelated to the carcinogenic process, the number of tumors obtained is not necessarily an index of the carcinogenicity of the molecule tested. This would, however, add still another difficulty to attempts at establishing a table of correlations.

The present status of the split-product theory, therefore, appears to be that there is enough circumstantial evidence against the theory to raise the question of its validity. But whether or not the original theory ultimately prevails is probably of secondary consequence; it has already furnished a stimulus for much experimental work, and at least one concrete result is the discovery of the most carcinogenic azo dye known, *m'*-methyl-*p*-dimethylaminoazobenzene. We are unaware of any evidence against the view that the intact azo molecule is the true carcinogen. In contrast to this, however, an extreme opposite view should also be stated: Kuhn and Beinert (17) believe that the carcinogenicity of these azo dyes depends on the ultimate oxidation product, benzoquinone, or its methyl derivative, rather than upon the diamine or its free radical.

SUMMARY

1. Eleven compounds closely related to *p*-dimethylaminoazobenzene were fed to rats at levels equivalent to 0.06 per cent of this dye in an adequate semisynthetic diet for periods up to 240 days, and their potencies as liver carcinogens noted up to 300 days.

2. *m'*-Methyl-*p*-dimethylaminoazobenzene proved to be more active than *p*-dimethylaminoazobenzene. *p*-Monomethylaminoazobenzene was practically as active as the parent compound. *o'*-Methyl-*p*-dimethylaminoazobenzene and *p'*-methyl-*p*-dimethylaminoazobenzene in that order were less active than *p*-dimethylaminoazobenzene.

3. The remaining 7 compounds, *p*-aminoazobenzene, *o*-methyl-*p*-dimethylaminoazobenzene, *p*-dimethylamino-phenylazo-1-naphthalene, N,N-dimethyl-N'-benzal-*p*-phenylenediamine, *p*-dimethylaminobenzal aniline, N,N-dimethyl-*p*-phenylenediamine, and aniline were inactive under the conditions employed.

4. Aqueous solutions of the hydrochlorides of *p*-phenylenediamine and its N-monomethyl and N,N-dimethyl derivatives were found to give characteristic stains on pine chips. By this test rats fed the split product of *p*-dimethylaminoazobenzene, N,N-dimethyl-*p*-phenylenediamine, were found to excrete some of this diamine as such in the urine. No similar excretion of chromogen was noted in rats fed the azo dye.

5. Each of the *p*-dimethylaminoazo dyes tested underwent a stepwise demethylation *in vivo* prior to cleavage at the azo linkage.

Our data are not in accord with the theory of Kensler, Dexter, and Rhoads on the means by which azo dyes produce liver cancer.

REFERENCES

1. Bamberger, E. Isomerieerscheinungen auf dem Gebiet der Azokörper. Ber. d. deutsch. chem. Ges., **28**:837-854. 1895.
2. Bamberger, E., and Meimberg, F. Einige weitere Beobachtungen über Azofarbstoffe. Ber. d. deutsch. chem. Ges., **28**:1887-1897. 1895.
3. Calm, A. Ueber eine Reaktion der Aldehyde. Ber. d. deutsch. chem. Ges., **17**:2938-2941. 1884.
4. du Vigneaud, V., Spangler, J. M., Burk, D., Kensler, C. J., Sugiura, K., and Rhoads, C. P. The Procarcinogenic Effect of Biotin in Butter Yellow Tumor Formation. Science, **95**:174-176. 1942.
5. Friedlaender, P. Über *o*-substituirte Alkylaniline. Monatsh. f. Chemie, **19**:627-646. 1898.
6. Gattermann, L., and Wieland, H. Laboratory Methods of Organic Chemistry. Translation of 24th German edition. New York: The Macmillan Company. 1937, pp. 317-318.
7. Gnehm, R., and Blumer, E. Ueber alkylirte *o*-Toluidine. Ann. der Chemie, **304**:87-115. 1899.
8. György, P., Poling, E. C., and Goldblatt, H. Necrosis, Cirrhosis, and Cancer of Liver in Rats Fed a Diet Containing Dimethylaminoazobenzene. Proc. Soc. Exper. Biol. & Med., **47**:41-44. 1941.
9. Hantzsch, A. Weiteres über die Isomerien der Helianthine und der Amino-azobenzol-Salze. Ber. d. deutsch. chem. Ges., **48**:167-180. 1915.
10. Hartwell, J. L. Survey of Compounds Which Have Been Tested for Carcinogenic Activity. Washington, D. C. Federal Security Agency. U. S. Pub. Health Service. 1941, pp. 195-221.
11. Kensler, C. J., Dexter, S. O., and Rhoads, C. P. The Inhibition of a Diphosphopyridine Nucleotide System by Split Products of Dimethylaminoazobenzene. Cancer Research, **2**:1-10. 1942.

12. Kensler, C. J., Sugiura, K., and Rhoads, C. P. Coenzyme I and Riboflavin Content of Livers of Rats Fed Butter Yellow. Science, **91**:623. 1940.
13. Kensler, C. J., Young, N. F., and Rhoads, C. P. The Inhibition of Yeast Carboxylase by Split-Products of N,N-Dimethylaminoazobenzene. J. Biol. Chem., **143**:465-472. 1942.
14. Kinosita, R. Special Report. Studies on the Carcinogenic Chemical Substances. Tr. Soc. path. jap., **27**:665-727. 1937.
15. Kinosita, R. Studies on the Cancerogenic Azo and Related Compounds. Yale J. Biol. & Med., **12**:287-300. 1940.
16. Kinosita, R. The Changes of the Liver of the Rat Caused by 4-Dimethylaminoazobenzene-1-azo-1-naphthalene, 4-Dimethylaminobenzene-1-azo-2-naphthalene, etc. Gann, **34**:165-167. 1940.
17. Kuhn, R., and Beinert, H. Über das aus Krebserregenden Azo-Farbstoffen entstehende Fermentgift. Ber. d. deutsch. chem. Ges., **76**:904-909. 1943.
18. Michaelis, L., Schubert, M. P., and Granick, S. The Free Radicals of the Type of Wurster's Salts. J. Am. Chem. Soc., **61**:1981-1992. 1939.
19. Miller, J. A., and Baumann, C. A. The Determination of *p*-Dimethylaminoazobenzene, *p*-Monomethylaminoazobenzene, and *p*-Aminoazobenzene in Tissue. Cancer Research, **5**:157-161. 1945.
20. Miller, J. A., Miller, E. C., and Baumann, C. A. On the Methylation and Demethylation of Certain Carcinogenic Azo Dyes in the Rat. Cancer Research, **5**:162-168. 1945.
21. Miller, J. A., Kline, B. E., Rusch, H. P., and Baumann, C. A. The Carcinogenicity of *p*-Dimethylaminoazobenzene in Diets Containing Hydrogenated Coconut Oil. Cancer Research, **4**:153-158. 1944.
22. Miller, J. A., Kline, B. E., Rusch, H. P., and Baumann, C. A. The Effect of Certain Lipids on the Carcinogenicity of *p*-Dimethylaminoazobenzene. Cancer Research, **4**:756-761. 1945.
23. Miller, J. A., Miner, D. L., Rusch, H. P., and Baumann, C. A. Diet and Hepatic Tumor Formation. Cancer Research, **1**:699-708. 1941.
24. Miner, D. L., Miller, J. A., Baumann, C. A., and Rusch, H. P. The Effect of Pyridoxin and Other B Vitamins on the Production of Liver Cancer with *p*-Dimethylaminoazobenzene. Cancer Research, **3**:296-302. 1943.
25. Nagao, N. Über die Fütterungsversuche mit 4'-Methyl-4-N-Dimethylaminoazobenzol und weiteren Polymethylderivaten bei weissen Ratten. Gann, **35**:8-20. 1941.
26. Nagao, N. Über die Fütterungsversuche mit 2-Methyl-4-N-dimethylaminoazobenzol chlorhydrat und anderen azoverbindungen bei weissen Ratten. Gann, **35**:280-282. 1941.
27. Nakahara, W., Mori, K., and Fujiwara, T. Inhibition of Experimental Production of Liver Cancer by Liver Feeding. A Study in Nutrition. Gann, **33**:406-428. 1939.
28. Potter, V. R. The Inhibition of Sulfhydryl-Containing Enzymes by Split Products of p-Dimethylaminoazobenzene. Cancer Research, **2**:688-693. 1942.
29. Sachs, F., and Lewin, W. Zur Kenntniss des *p*-Dimethylamidobenzaldehydes. Ber. d. deutsch. chem. Ges., **35**: 3569-3578. 1902.
30. Sasaki, T., and Yoshida, T. Experimentelle Erzeugung des Lebercarcinoms durch Fütterung mit o-Amidoazotoluol. Virchow's Arch. f. path. Anat., **295**:175-200. 1935.
31. Stevenson, E. S., Dobriner, K., and Rhoads, C. P. The Metabolism of Dimethylaminoazobenzene (Butter Yellow) in Rats. Cancer Research, **2**:160-167. 1942.
32. Vorländer, D., and Wolferts, E. Über Hydrochloride der *p*-Aminoazo-verbindungen. Ber. d. deutsch. chem. Ges., **56**:1229-1239. 1923.
33. White, J., and Edwards, J. E. Effect of Oral Administration of Aniline and *p*-Aminodimethylaniline on the Growth of the Rat. J. Nat. Cancer Inst., **2**:531-533. 1942.
34. White, J., and Edwards, J. E. Effect of Dietary Cystine on the Development of Hepatic Tumors in Rats Fed *p*-Dimethylaminoazobenzene (Butter Yellow). J. Nat. Cancer Inst., **2**:535-538. 1942.
35. Yoshida, T. Experimenteller Beitrag zur Frage der Epithelmetaplasie. Virchow's Arch. f. path. Anat., **283**:29-40. 1932.