Synthesis of Substituted 4-Dimethylaminoazobenzenes and a Study of Their Effect on *Lactobacillus casei* and *Escherichia coli*[1]

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A series of 3′,4′-disubstituted 4-dimethylaminoazobenzenes has been synthesized and tested for inhibitory action on microorganisms. No inhibitory activity was found for *Lactobacillus casei*, an organism requiring an exogenous source of riboflavin. The compounds were found to be weak inhibitors for *Escherichia coli*, an organism which is independent of an exogenous source of riboflavin. No support could be found for the belief that the azo dyes function as antagonists of riboflavin.

Kensler and coworkers[3] demonstrated that excess riboflavin provides rats some protection against induction of hepatomas by 4-dimethylaminobenzene (DAB). Griffin and Baumann[4] and Miller and coworkers[5,6] showed that a decrease in riboflavin content of liver occurred when DAB or 3′-methyl-4-dimethylaminoazobenzene (3′-Me-DAB) was fed to rats. These two studies constitute the principal evidence cited in support of the concept that carcinogenicity of azo dyes is related to their ability to inhibit utilization of riboflavin. Supporting evidence for the role of riboflavin as a protective agent against DAB has been presented by Mueller and Miller[7] and others.[8] These investigators have described an azo reductase in rat liver which is believed by them to be a flavoprotein. The *in vitro* activity of this enzyme is enhanced by excess riboflavin or its nucleotide forms.[7,9] It has also been shown that the administration of DAB to rats causes a decrease in the ability of this liver enzyme to cleave DAB.[9,10]

Two studies have been reported in which efforts were made to show a more general existence of an inhibition of riboflavin utilization by azo dyes in the metabolism of microorganisms. The first was a report by Miller and coworkers[11] regarding the inhibition of growth of the riboflavin-dependent bacterium, *Lactobacillus casei*. They concluded that such an inhibition existed. In spite of uncertainty whether DAB was or was not an inhibitor of riboflavin, they did not use it but used 4-monomethylaminoazobenzene (MAB) in its place because it also caused the induction of hepatomas when fed to rats and because it was more soluble in water. No experimental details were given, but it was stated that 1–3 μg/ml of MAB inhibited growth of the organism 60–90% in the presence of riboflavin at concentrations of 0.01–0.15 μg/ml. Reversal of the inhibition by riboflavin was not a certainty on complete, because when the vitamin was increased to the very high concentration of 1.25 μg/ml, the *inhibition* was reduced to 0–40%. The evidence which they presented in support of the claim that *Saccharomyces cerevisiae* was also inhibited was also inadequate and even more difficult to evaluate.

Salzberg[12] studied the effect of azo dyes on two riboflavin-requiring mutants of *Neurospora crassa*. In this case also, while interest was focused on DAB and 3′-Me-DAB, only limited use was made of 3′-Me-DAB; most of the study was devoted to the effects of 3′-methyl-4-monomethylaminoazobenzene (3′-Me-MAB). It was shown that 3′-Me-DAB and 3′-Me-MAB caused inhibition of both strains, but excess additional riboflavin permitted only partial recovery of the growth rate. The 3′-Me-MAB also caused inhibition of an adenine-requiring mutant of *N. crassa*, not in the presence of inadequate levels of adenine, but only in the presence of large excesses of adenine. Large additional quantities of riboflavin were also unable to reverse any part of the inhibition.

The question as to whether carcinogenic 4-dimethylaminoazobenzenes (DABS) are able to show riboflavin antagonism in microorganisms is still largely unanswered. The aim of the study reported here was to determine the effect of a series of ten DABS, of which seven have not been synthesized before, on *L. casei* ATCC 7469, a riboflavin-requiring organism, and on *Escherichia coli* B, an organism which requires no exogenous riboflavin. *L. casei* has been used extensively for the study of riboflavin antagonism.[13,14] *E. coli* was not inhibited by 7-chloro-8-methyl- and 7-methyl-8-chloro-10-(1-D-ribityl)isoalloxazine; these two flavins are the most potent inhibitors of riboflavin in *L. casei*[15,16] to have been reported to date.

Additional studies undertaken were the determination of the ability of these azo dyes to bind to rat liver protein, their potencies as hepatocarcinogens, their dissociation constants, and their absorption spectra. This is the first time that all of these kinds of information have been determined for a large series of closely related azo dyes in the same laboratory, by the same investigators, and by highly standardized procedures.

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(2) This report constitutes part of the thesis submitted by G. M. Bebawi for the Ph.D. degree, University of Nebraska, 1967.

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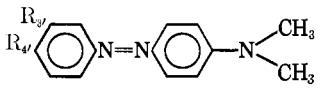
TABLE I

CHEMICAL AND PHYSICAL DATA AND PROPERTIES OF THE 4-DIMETHYLAMINOAZOBENZENES



Substituents			Yield,				pK_a (SE =	Absorption spectra			
								Maxima		Minima	
$R_{3'}$	$R_{4'}$	Procedure	%	Mp, °C	Composition	Analyses	±0.01)	mμ	$\epsilon \times 10^{-3}$	mμ	$\epsilon \times 10^{-3}$
H	H			117–118	$C_{14}H_{15}N_3$		2.28	406	27.60	340	7.00
CH_3	CH_3	A	67	182–183	$C_{16}H_{19}N_3$		2.37	252	11.80	225	8.56
								405	30.60	290	5.69
										335	3.96
CH_3	Cl	A	86	156–157	$C_{15}H_{16}ClN_3$	C, H, N	2.17	256	13.16	242	12.00
								418	30.00	335	2.56
Cl	CH_3	A	62	165–166	$C_{15}H_{16}ClN_3$	C, H, N	2.07	262	10.68	238	7.70
								421	29.40	336	2.30
Cl	Cl	A	79	153–154	$C_{14}H_{13}Cl_2N_3$	C, H, N	1.86	270	11.44	242	7.00
								430	31.60	340	1.58
CH_3	C_2H_5	A	87	95–96	$C_{17}H_{19}N_3$	C, H, N	2.60	252	10.80	220	6.90
								305	5.94	290	5.50
								406	31.00	335	3.55
C_2H_5	CH_3	B	44	94–95	$C_{17}H_{19}N_3$	C, H, N	2.59	252	11.30	222	7.20
								305	5.96	292	5.52
								405	30.40	335	3.66
C_2H_5	C_2H_5	A	69	84–85	$C_{18}H_{21}N_3$	C, H, N	2.58	252	11.40	226	7.36
								305	6.14	292	5.80
								407	31.10	335	3.70
C_2H_5	H	B	92	61–62	$C_{16}H_{19}N_3$	C, H, N	2.16	252	10.40	230	7.20
								410	29.00	335	3.15
CH_3	H	B	73	119–121	$C_{15}H_{17}N_3$		2.33	252	9.00	228	6.40
								410	25.00	335	2.70

As a further effort to standardize procedures, the structures of the DABS studied consisted of a series of compounds all devoid of substituents in the *p*-phenylenediamine ring; the substituents in the aniline ring (prime ring) consisted of a systematic series of changes of the groups in the 3′ and 4′ positions.

Chemistry.—The synthesis of the DABS required a number of specific anilines, only four of which were available commercially; the preparations of the unavailable anilines are outlined in the Experimental Section. The coupling of the diazotized anilines with N,N-dimethylaniline was accomplished by one or the other of two procedures indicated in the Experimental Section. The DABS prepared and synthesized for this study are listed in Table I, along with the maxima and minima of the absorption spectra and the pK_a values for the compounds.

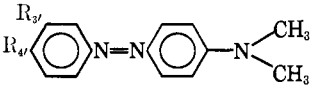
Biological Results.—As indicated above, previous efforts to show antagonism between riboflavin and DABS actually involved the use of one or another MAB, because the latter was ten times more soluble than the former. Quite aside from the fact that DABS and MABS are different compounds, in one case,[12] where 3′-Me-DAB was used, the highest concentration employed was 3 μg/ml; in both cases[11,12] where the MABS were used the maximum concentrations were only 3 and 3.5 μg/ml. We found the DABS sufficiently soluble in the basal medium used for the *L. casei* studies to permit the preparation of stock solutions containing 25 μg/ml. During the actual inhibition studies, the range of concentrations used was from 0 to 10 μg/ml.

In the case of the antagonism studies with *E. coli*, we were able to use concentrations of 2.67×10^{-6} moles/ml. In the case of DAB, this was equivalent to 15 mg/25 ml or 600 μg/ml. This high concentration was possible because of the use of a medium as a solvent and because the medium was prepared to contain 10% Tween 80.

Table II shows that there is essentially no inhibition of acid production (growth) of *L. casei* by the azo dyes even at ratios of dye to riboflavin of 333. Table II also shows that the dyes vary considerably in their ability to show a nonspecific inhibition of *E. coli*. The variations in these activities must be due to factors other than antagonism of riboflavin since the medium used contains the vitamin, and the organism is able to synthesize it in excess of its needs.

TABLE II

EFFECT OF 4-DIMETHYLAMINOAZOBENZENES ON *L. casei* AND *E. coli*



Substituents		Inhib of *L. casei*, %		Inhib of
$R_{3'}$	$R_{4'}$	Titratimetric[a]	Turbidimetric[b]	*E. coli*, %[c]
H	H	2	8	24
CH_3	CH_3	9	10	5
CH_3	Cl	10	0	10
Cl	CH_3	4	0	6
Cl	Cl	1	3	16
CH_3	C_2H_5	0	5	30
C_2H_5	CH_3	0	3	28
C_2H_5	C_2H_5	0	5	37
C_2H_5	H			15
CH_3	H			8

[a] Concentration of riboflavin, 0.03 μg/ml; of azo compound, 0–10 μg/ml (maximum). [b] Concentration of riboflavin, 0.03 μg/ml; of azo compound, 10 μg/ml. [c] Concentration of azo compound, 2.67×10^{-6} mole/ml.

The results of these studies do not support the hypothesis that there is an antagonism between the azo dyes and riboflavin as far as the metabolism of a riboflavin-requiring microorganism is concerned. The unimpressive inhibition of cell division of *E. coli* is probably a manifestation of a more general antibacterial activity.[17]

This study does not mean that riboflavin antagonism has no role in the induction of hepatomas by DABS in the rat. If the vitamin plays a major role in protection against hepatoma formation, its inability to prevent dye binding to liver protein[18] and induction of hepatomas by some DABS requires explanation.[19]

All of the DABS used in this study were found to bind to liver protein in the rat, and all have been found to be carcinogenic.[20]

Experimental Section[21]

Chemical Methods.—*m*-Toluidine and 3,4-dimethyl- and 3,4-dichloroaniline were available from commercial sources. The remaining anilines were prepared by the procedures indicated: 3-methyl-4-choloraniline,[22] 3-chloro-4-methylaniline,[22] 3,4-diethylaniline,[23] 3-methyl-4-ethylaniline,[24] and 3-ethyl-4-methylaniline.[24] *p*-Ethylaniline was purified as the acetanilide (75%), mp 92–93°, and the *p*-ethylacetanilide nitrated as described for 3,4-diethylacetanilide[23] to produce 2-nitro-4-ethylacetanilide (48%), mp 50–51°, which was hydrolyzed to 2-nitro-4-ethylaniline[22] (99%), mp 46–47°. The 2-nitro-4-ethylaniline was deaminated by the procedure described for 2-nitro-4-ethyl-5-methylaniline[24] to produce 3-nitroethylbenzene (85%), bp 122–133° (16 mm) (lit.[25] 116° (13 mm)), which was reduced over PtO_2 to yield 3-ethylaniline (95%), bp 106–107° (18 mm), 114–115° (25 mm) (lit.[26] 92–93° (9.5 mm)).

The substituted DABS were prepared by modifications of convenience of procedure A, used by Shunk, *et al.*,[27] and others[21] for the preparation of azo compounds or by procedure B used by Miller and Miller[28] for the preparation of several DABS. DAB was purchased and purified by recrystallization from C_6H_6. The other DABS were recrystallized from C_6H_6, C_6H_6–hexane, EtOH, or 80% EtOH.

Physical Methods.—The absorption spectra were determined on solutions with concentrations of 5.0×10^{-5} *M* in 1-cm quartz cells using a Beckman Model DU spectrophotometer. The molar extinction coefficients for the maxima and minima for each compound are given in Table I.[29]

The dissociation constants for the compounds were determined by measuring the optical density of 5×10^{-5} *M* solutions at different wavelengths from 320 to 600 mμ in 0.01 *N* NaOH, 0.005 *N*, 0.01 *N*, and 2 *N* HCl at 22–26°. The pK_a of a compound was determined as the average value of those calculated from the optical densities of the base form, salt form, and mixtures of base and salt forms at different pH values over a 60-mμ wavelength range at 10-mμ intervals between the isosbestic points. An excellent example of this procedure has been published by Sawicki and Ray.[30] The pK_a values are listed in Table I.[29]

Biological Methods. *Lactobacillus casei.*[31]—The tubes used for the lactic acid production by the organism in the presence of riboflavin alone were prepared by the usual procedure.[32] To duplicate tubes were added graded increments of riboflavin (USP Reference Standard) from 0 to 0.3 μg/tube, 5 ml of basal medium, and sufficient water to make a total volume of 10 ml. The maximum concentration of riboflavin in the tubes for the standard curve was, therefore, 0.03 μg/ml. A series of tubes was prepared for each of the azo dyes studied. To a series of tubes (18 tubes for each dye) were added 5 ml of H_2O containing 0.06 μg of riboflavin/ml and, in duplicate, graded quantities from 0.0 to 4.0 ml in 0.5-ml increments of a solution of basal medium containing 25 μg of the azo compound/ml. To each tube was then added sufficient basal medium to complete the volume of the contents to 10 ml. The series of tubes for each dye contained, therefore, 0.03 μg of riboflavin and from 0.0 to 10.0 μg of the particular dye/ml; the ratios of dye to riboflavin ranged from 0 to 333. The tubes were incubated at 37° for 5 days after which time the lactic acid produced was determined by titration with 0.1 *N* NaOH. The inhibition was determined as the difference in acid production in the tube containing the azo dye and riboflavin and the acid production in the tube containing riboflavin alone, divided by the latter. The results are summarized in Table II.

In another study, duplicate tubes were prepared to contain per 10 ml of the final medium, 100 μg of the azo dye and 0.3 μg of riboflavin, with one pair of tubes containing riboflavin alone. The tubes were incubated at 37° and the turbidity was determined at 640 mμ in a Spectronic 20 spectrophotometer every 3 hr for 24 hr. The results are summarized in Table II.

Escherichia coli **B.**[33]—A series of 50-ml culture flasks were prepared by adding to individual flasks none or approximately 0.067 mmole of each of the azo compounds. (These additions ranged from 15 mg for DAB to 19.4 mg for 3′,4′-Cl_2-DAB.) To each flask was added 2.5 ml of Tween 80, and all flasks were warmed gently on the steam bath until the azo compounds were in solution. To each flask was then added 10 ml of nutrient broth–NaCl medium and the contents were heated to 37° in a Gyrotory water bath shaker.[34] At this time, 12.5 ml of a culture of *E. coli* B containing 1×10^8 viable cells/ml was added to each flask. This provided an intial cell count of approximately 5×10^7 and a concentration of azo compound of 2.67×10^{-6} mole/ml for each completed flask. A sample was removed from the flask containing no azo compound and the viable cell count was determined for zero time by the usual plate out procedure. The flasks were incubated for a period of 4 hr, samples being removed from each flask at 1-hr intervals for viable cell count determinations. The inhibition was determined at the 4-hr interval as the difference between the viable cell counts of the control flask and an experimental flask divided by that of the control flask. A summary of the results is shown in Table II.

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(21) All melting points were determined on calibrated thermometers.

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