Effect of Certain Benzimidazoles and Related Compounds Upon Azo Dye Destruction by Liver Homogenates.* (23789)

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Benzimidazole, 2,5-dimethylbenzimidazole and 2-ethyl-5-methylbenzimidazole inhibit formation of liver tumors in rats when fed with the carcinogenic azo dye, 3'-methyl-4-dimethylaminoazobenzene(1). These benzimidazoles also inhibit incorporation of glycine into heme by chicken erythrocytes(2,3), inhibit multiplication of influenza virus(4), and certain benzimidazoles and chemically related substances inhibit firefly luminescent reaction (5). In an attempt to obtain information on the mechanism of action of the benzimidazoles on the azo dye carcinogenic process, the effect of these compounds and certain chemically related compounds has been tested on azo dye destruction by liver homogenates(6,7). Iodoacetate, *p*-aminophenol, hydroxylamine, cyanide, azide and atabrine have previously been reported to inhibit dye destruction when added to the *in vitro* system(8). Of these, atabrine has been shown to affect azo dye carcinogenesis(9). The present study indicates that several compounds chemically related to the benzimidazoles also inhibit the destruction of an azo dye by liver homogenates, and this inhibition can be overcome to some extent by flavin adenine dinucleotide.

Methods. The method of measuring dye destruction by liver homogenates was essentially that described by Mueller and Miller (6,7). Rat liver homogenates (5 to 10%) were prepared in 0.01 M phosphate buffer pH 7.4 and were added last to the ice-cold reaction mixture. The azo dye, 4-dimethylaminoazobenzene, was dissolved in ethyl alcohol, and 60 μg of the dye (0.1 ml of solution) served as the substrate. Incubation was carried out at 37°C for 30 minutes at which time the reaction was stopped by the addition of ethanol-acetone-trichloracetic acid solution, and the red color due to remaining dye was measured (after proper dilution) in the Evelyn photoelectric colorimeter with 515 mμ filter. The benzimidazole derivatives and certain of the other compounds were dissolved in the alcohol solution with the dye to give the desired concentration. This was necessary since it was found that over 0.1 ml of alcohol in the reaction flask was inhibitory. Water soluble materials were dissolved in the phosphate buffer. The final volume in all reaction flasks at incubation was 3 ml. Flasks to which no homogenate had been added served as controls. It was found that the color in these controls was the same as found if the reaction were stopped at zero time.

Results. Of the various types of compounds tested, it was found that certain of the benzimidazole, benzothiazole, indole, and quinoline derivatives were effective inhibitors of azo dye destruction *in vitro* at a level of 1 mg/3 ml in the reaction flask (Table I). The 5,6-dimethyl, 2,5-dimethyl, and the 2-ethyl-5-methylbenzimidazole were the most effective inhibitors of the benzimidazoles. All of the indole derivatives except tryptophan were active inhibitors. Auramine at a level of only 20 μg/reaction flask was very effective in inhibiting the destruction of the dye. Since 2,5-dimethyl and 2-ethyl-5-methylbenzimidazole are very effective in inhibiting tumor formation when fed in conjunction with a carcinogenic azo dye, further studies on the effect of various concentrations and upon the reversal of the inhibition were done with these compounds. At a concentration of 125 μg/flask, 2,5-dimethylbenzimidazole resulted in very little inhibition while the 2-ethyl-5-methyl derivative at this concentration still inhibited dye destruction about 20%. Only slight inhibition was noted when the concentration of the latter compound was reduced to 62 μg/flask.

Addition of 25 μg of riboflavin or 20 μg of pyridoxal phosphate did not affect rate of dye

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TABLE I. Inhibition of Azo Dye Destruction by Various Chemicals.* (1 mg of compound/3 ml of reaction mixture.)

Compound	% inhibition	Compound	% inhibition
Benzimidazoles		*Misc.*	
unsubstituted	0	quinoline	12
2-methyl[1]	15	2-methylquinoline	60
5- "	9	benzotriazole	0
5,6-dimethyl[2]	58	5-methylbenzotriazole	0
2,5- " [1]	54	benzoxazole	5
2-ethyl-5-methyl[1]	61	2-methylbenzoxazole	0
5-chloro[1]	0	2-amino-1,3,4-thiadiazole[3]	0
2-methyl-5-chloro[1]	8	2-acetamido-1,3,4-thiadiazole[3]	0
2-ethyl-5-chloro[1]	4	2-ethylamino-1,3,4-thiadiazole[3]	0
5-nitro[1]	11	dinitrophenol	0
2-methyl-5-nitro[1]	17	methylene blue	0
2-ethyl-5-nitro[1]	18	Na ascorbate	0
2-mercapto	4	cysteine	0
2-hydroxy[2]	0	cystine	0
Benzothiazoles		adenine	0
unsubstituted	15	azaguanine	0
2-methyl	16	6-mercaptopurine	0
2-amino	9	thymine	0
2-phenyl	14	toluene-3,4-diamine	0
2-chloro	80	auramine (20 μg)	62
Indole			
unsubstituted	35		
2-methyl	83		
3- " (skatole)	68		
5- "	72		
tryptophan	0		

* Inhibition varied somewhat with different homogenates. The % inhibition indicated is representative, but small differences between different chemicals may not be significant.

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destruction whether or not an inhibitor was added. However, addition of 9 μg of flavin adenine dinucleotide (FAD), or 10 μg of riboflavin phosphate (RP), was effective in partially counteracting the inhibition. Of the above 2 benzimidazoles, this effect was more noticeable with the less active 2,5-dimethyl derivative, and the effect was more pronounced at lower levels of the inhibitors (Fig. 1). Increased amounts of either riboflavin cofactor had no additional effect. A similar effect of FAD and RP was noted with the other compounds which were active inhibitors.

Discussion. It has been postulated that at least part of the protective effect of dietary riboflavin against carcinogenic azo dyes may be due to its participation in reductive cleavage of the dye forming non-carcinogenic amines(7). The present experiments add additional evidence that FAD is involved in the destruction of dye by liver homogenates. However, contrary to the finding of Mueller and Miller with their system(7), we did find that riboflavin phosphate also was somewhat effective. Our results would indicate that on a molar basis, FAD was approximately twice as active as the mononucleotide.

The rate of dye destruction by liver homogenates or slices can be altered by dietary level of riboflavin(10), methylcholanthrene (11), or iodinated casein(12). These also have an effect upon the incidence of liver tumors resulting from feeding an azo dye(11,12, 13). On the other hand, auramine does not affect development of liver tumors when fed with the dye(9), yet it was found that addition of this compound in small amounts markedly inhibited dye destruction in the present experiments.

The inhibitory effect of certain benzimidazoles on carcinogenesis(1) would not seem to be related to increased dye destruction, since these compounds tend to preserve the intact dye in liver homogenate systems. On the other hand, the process of dye destruction would seem to be associated in some way with carcinogenesis. Of the benzimidazoles studied to date(1), there is good correlation between anti-azo dye carcinogenenic ability and inhibition of azo dye destruction; *i.e.*, the 2-ethyl-5-methyl benzimidazole is more effective than the 2,5-dimethyl-derivative, which is more active than the unsubstituted benzimi-

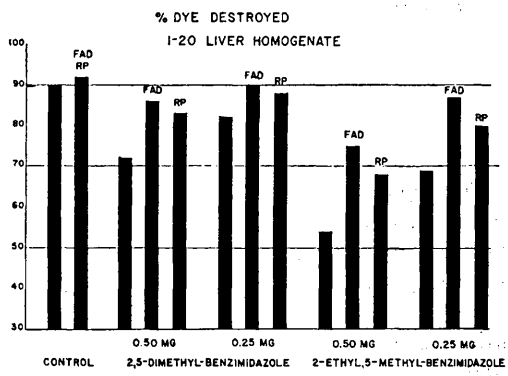

FIG. 1. Effect of flavin adenine dinucleotide (FAD) and riboflavin phosphate (RP) upon destruction of azo dye in the presence of benzimidazoles.

dazole. This same order of activity also exists for inhibition of heme synthesis from glycine by chicken erythrocytes(2,3). The correlation between effect upon heme synthesis and dye destruction, however, is not as good with some of the other benzimidazoles. Certain of the 5-nitro and 5-chloro derivatives are inhibitory in the erythrocyte system(14) while they had little effect upon azo dye destruction. Additional experiments are needed to determine whether anti-cancer properties may be correlated with inhibition of dye destruction or with inhibition in the chicken erythrocyte system. Studies on metabolism of azo dyes with relation to the genesis of liver neoplasia as yet have not revealed the mechanism of the process. Further studies of possible interrelationships between azo dye carcinogenesis, the mechanism of dye destruction in liver homogenates and glycine incorporation in nucleated erythrocytes, might provide some basic information.

Summary. Certain benzimidazole, benzothiazole, indole, and quinoline derivatives, and auramine, inhibit destruction of the azo dye, 4-dimethylaminoazobenzene, by liver homogenates. This inhibition can be partially overcome by addition of riboflavin adenine dinucleotide and to a lesser extent by riboflavin phosphate. Correlation of inhibition of *in vitro* dye destruction and of heme synthesis by avian erythrocytes with the anti-azo dye carcinogenic effect of certain benzimidazoles is discussed.

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1. Clayton, C. C., Abbott, L. D., Jr., *Cancer Research,* 1958, v18, 94.

2. Abbott, L. D., Jr., Dodson, M. J., *J. Biol. Chem.,* 1954, v211, 845.

3. ———, PROC. SOC. EXP. BIOL. AND MED., 1954, v86, 475.

4. Tamm, I., Folkers, K., Shunk, C. H., Heyl, D., Horsfall, F. L., Jr., *J. Exp. Med.,* 1953, v98, 245.

5. McElroy, W. D., Hastings, J. W. in Johnson, F. H., (ed.), *The Luminescence of Biological Systems,* Washington; A.A.A.S., 1955, 169.

6. Mueller, G. C., Miller, J. A., *J. Biol. Chem.,* 1949, v180, 1125.

7. ———, *ibid.,* 1950, v185, 145.

8. ———, *ibid.,* 1949, v176, 535.

9. Rumsfeld, H. W., Clayton, C. C., Baumann, C. A., *Cancer Research,* 1952, v12, 671.

10. Kensler, C. J., *J. Biol. Chem.,* 1949, v179, 1079.

11. Conney, A. H., Miller, E. C., Miller, J. A., *Cancer Research,* 1956, v16, 450.

12. Miller, W. L., Jr., Baumann, C. A., *ibid.,* 1951, v11, 634.

13. Rusch, H. P., Baumann, C. A., Miller, J. A., Kline, B. E., in *A.A.A.S. Res. Conf. on Cancer, Lancaster;* Science Press, 1945, 267.

14. Abbott, L. D., Jr., Gindin, R. A., PROC. SOC. EXP. BIOL. AND MED., 1957, v96, 846.

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Renal Function in Human Pregnancy. I. Changes in Glomerular Filtration Rate and Renal Plasma Flow* (23790)

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The reports on the effects of pregnancy on renal functions have been controversial. Earlier investigators(1,2,3) have asserted that pregnancy does not alter renal functions while more recent studies(4,5) have shown a progressive increase in renal plasma flow (RPF) and glomerular filtration rate (GFR) which reaches a maximum around the eighth month of gestation and returns to near normal non-pregnant values after gestation. This divergency of opinion is probably due to the fact that the data reported by various authors were obtained from different groups of patients studied at different periods of gestation and were compared to still another group of non-pregnant individuals.

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