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Demethylation of Carcinogenic Aminoazo Dyes by Autoxidizing Linoleic Acid.*

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Previous publications from this laboratory have demonstrated that various carcinogens including certain azo dyes inhibit the autoxidation of unsaturated lipids.[1,2] Destruction of the carcinogenic hydrocarbons was shown to occur during the course of the oxidation[3,4] and there were indications that the carcinogenic aminoazo dyes likewise disappeared in the presence of autoxidizing lipids. The present paper describes in more detail the effect of p-dimethylaminoazobenzene and related compounds on the autoxidation of linoleic acid and provides some information on the fate of these dyes during the oxidation.

Methods. Most of the work was done with p-dimethylaminoazobenzene (DAB), p-monomethylaminoazobenzene (MAB), and p-aminoazobenzene (AB); m'-methyl-p-dimethylaminoazobenzene, p'-methyl-p-dimethylaminoazobenzene, p-ethylmethylaminoazobenzene, and p-diethylaminoazobenzene were also tested for their antioxidant activity. These dyes were prepared as described previously[5] and purified by chromatographic adsorption on alumina.[6] We are indebted to Dr. J. B. Brown of the Department of Physiological Chemistry at Ohio State University for the linoleic acid. This preparation was a highly purified product (I.N. = 180.7) obtained by fractional freezing. In general 9.025 mg of linoleic acid and varying levels of dye to give M/200 to M/50 solutions were placed in Warburg flasks and the rate of autoxidation followed manometrically at 37.5°. The various constituents were added to the flasks in petroleum ether and the solvent was then thoroughly removed *in vacuo* at room temperature. The mixture of fatty acid and dye remained on the bottom of the vessels in the form of small droplets. In the studies with DAB, MAB, and AB flasks were removed during the course of the autoxidation and the contents transferred by two washings with 2 ml portions of each of the following solvents in the order named: 11 N KOH, water, and 95% ethanol. Quantitative determinations of the mixtures for DAB, MAB, and AB were done according to the chromatographic method of Miller and Baumann.[6]

Results. Fig. 1 shows the effect of DAB, MAB, AB on the autoxidation of linoleic acid (LA) at a concentration of dye in acid of M/100. DAB and MAB both increased the latent period of autoxidation of linoleic acid, the former being a more effective antioxidant than the latter. The antioxidant effect of each dye was proportional to the concentration employed. Thus, when DAB was used at concentrations of M/200, M/100, and M/50 the oxidation of the linoleic acid at the end of the first 24 hour period had reached only 56, 29, and 0% respectively that of the fatty acid alone. With the same levels of MAB, the amount of oxidation was 73, 45, and 35%

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1 Rusch, H. P., and Kline, B. E., *Cancer Research*, 1941, **1**, 465.

2 Deutsch, H. F., Kline, B. E., and Rusch, H. P., *J. Biol. Chem.*, 1941, **141**, 529.

3 Mueller, G. C., Miller, J. A., and Rusch, H. P., *Cancer Research*, 1945, **5**, 401.

4 Mueller, G. C., and Rusch, H. P., *Cancer Research*, 1945, **5**, 480.

5 Miller, J. A., and Miller, E. C., *J. Exp. Med.*, 1948, **87**, 139.

6 Miller, J. A., and Baumann, C. A., *Cancer Research*, 1945, **5**, 157.

respectively. Repetition of the experiments revealed excellent agreement in the results. Other dyes with approximately the same inhibiting effect as DAB were m'-methyl-p-dimethylaminoazobenzene, p'-methyl-p-dimethylaminoazobenzene, and p-ethylmethylaminoazobenzene. In contrast to the inhibitory effect of the methylated dyes, p-diethylaminoazobenzene had little effect and AB did not alter the latent period. In several experiments, AB actually caused a slight early stimulation of oxidation. However, the total oxygen uptake was decreased with the non-methylated dyes almost as much as it was for the methylated dyes; the chief difference was noted in the length of the latent period.

During the latent period an output of gas was observed with linoleic acid when mixed with all but one of the azo dyes. The exception was AB (Fig. 1). The gas evolved was probably carbon dioxide since the effect could be overcome by the addition of KOH to the center wells of the Warburg flasks. It is of interest that a considerable amount of gas absorbable by KOH was also evolved by the autoxidation of linoleic acid alone; after 96 hours of autoxidation about 200 microliters was evolved from 9.025 mg of linoleic acid. This was only observed toward the end of the period of oxidation, since the total uptake of gas was greater and the plateau was reached later when alkali was present in the wells of the flasks.

As the autoxidation proceeded in the acid-dye mixtures the azo dyes disappeared and DAB and MAB were found to be demethylated during the course of their destruction. At the end of 30 hours 90% of the DAB initially added had disappeared but MAB appeared in amounts equal to 85% of the starting level of DAB; almost a quantitative conversion had occurred up to this point (Fig. 2). Thereafter, the amount of MAB gradually decreased and small amounts of AB were also found throughout the period of oxidation. MAB was likewise destroyed when added to linoleic acid, but the rate of disappearance was slower than that with DAB; again demethylation to form AB occurred (Fig. 3). Added AB disappeared very rapidly in autoxidizing linoleic acid and no other basic dye was detected in the mixture (Fig. 4). Demethylation occurred only when the linoleic acid was oxidizing, and no disappearance of dye was observed when the autoxidation was retarded by tocopherol. There was no evidence to indicate that the demethylation

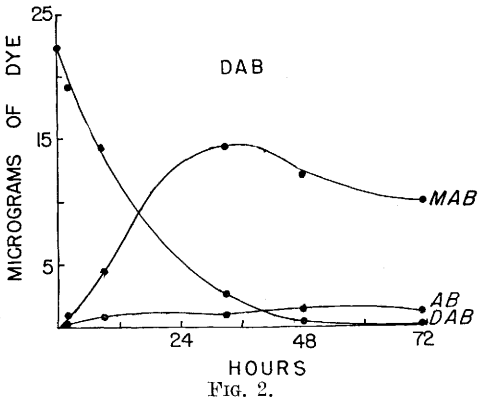

FIG. 2.

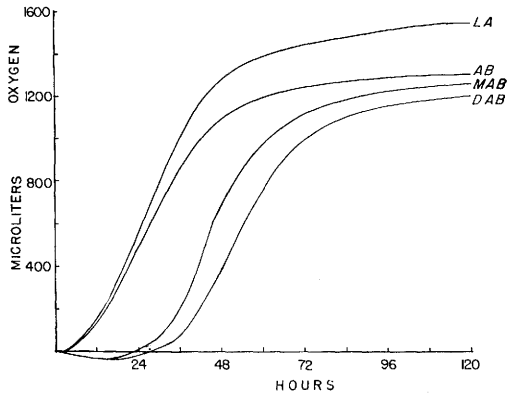

FIG. 1.

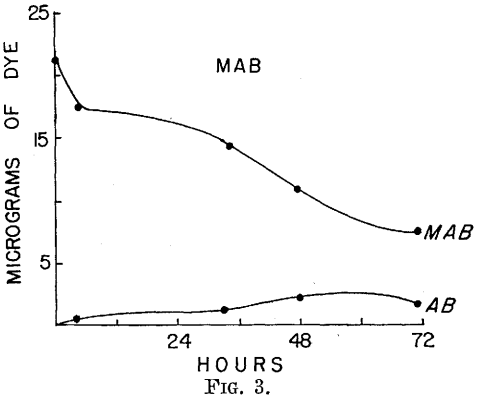

FIG. 3.

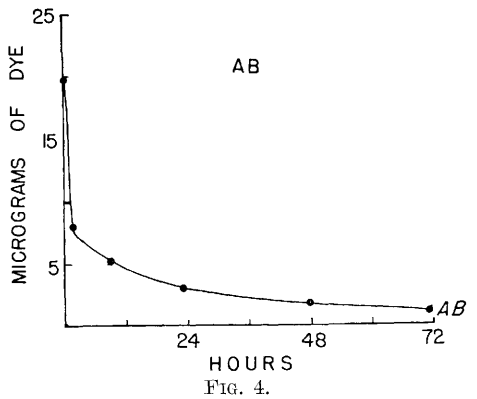

FIG. 4.

was a reversible reaction, since no DAB was detected during the autoxidation of either MAB or AB or a combination of the two.

Discussion. The product of oxidation of the methyl groups removed from the amino nitrogen of these dyes is unknown. However, the fact that they are replaced by hydrogen indicates that oxidation to easily decarboxylated carbamic acids probably occurred. It was not possible to demonstrate the oxidation of the methyl groups to carbon dioxide in this system since even the complete oxidation of the whole dye molecule could account for only a small part of the carbon dioxide evolution that occurred during these autoxidations. Although DAB is stable in the various diets that we have used in studies on tumor induction,[7] Kensler and his associates[8] have found that demethylation of DAB occurs *in vitro* when the dye is dissolved in cottonseed oil and mixed with ground brown rice. Heating the rice largely prevented the destruction of the dye. It seems likely that a coupled oxidation similar to that described in this paper occurred in the rice-cottonseed oil mixture. The loss of methyl groups on the oxidation of other dimethylamino compounds *in vitro* is implicit in the older literature. For example, Bernthsen[9] and Kehrmann[10] obtained demethylated products from the oxidation of methylene blue.

It is interesting that DAB suffers a similar fate *in vivo*. In the rat this dye is reversibly demethylated to form MAB which is then irreversibly demethylated to form AB.[11] The role of oxidizing lipids in the demethylation of this dye *in vivo* has not been ascertained.

Only rough correlations can be made between the antioxidant effect of these dyes and their carcinogenic activity.[5] Thus DAB and MAB are of equal carcinogenic activity but DAB retards oxidation more effectively than MAB. Similarly AB and p-diethylaminoazobenzene each lack carcinogenic and antioxidant capacity. On the other hand m'-methyl-p-dimethylaminoazobenzene, p'-methyl-p-dimethylaminoazobenzene, and p-ethylmethylaminoazobenzene are antioxidants of approximately equal activity. Yet the first of these three dyes is more active as a hepatic carcinogen than DAB, and the last compound is equal in its carcinogenic activity to that of DAB, whereas the p'-methyl-derivative is a very weak carcinogen.

Summary. p-Dimethylaminoazobenzene and p-monomethylaminoazobenzene both increased the latent period of autoxidation of linoleic acid, the former being a more effective antioxidant than the latter. The antioxidant effect of each dye was proportional to the concentration employed. Other dyes with approximately the same inhibiting effect as p-dimethylaminoazobenzene were m'-methyl-p-dimethylaminoazobenzene, p'-methyl-p-dimethylaminoazobenzene, and p-ethylmethylaminoazobenzene. In contrast p-diethylaminoazobenzene had only a slight effect and p-aminoazobenzene had no inhibitory influence. During the latent period an output of gas was observed when any of the methylated dyes were mixed with the linoleic acid; a gas output was also found with autoxidizing linoleic acid alone at the end of the oxidation period. The gas evolved was probably carbon dioxide.

As autoxidation of the linoleic acid-dye mixtures proceeded demethylation of p-dimethylaminoazobenzene and p-monomethyl-

[7] Miller, J. A., Kline, B. E., Rusch, H. P., and Baumann, C. A., *Cancer Research*, 1944, **4**, 158.

[8] Kensler, C. J., Magill, J. W., and Sugiura, K., *Cancer Research*, 1947, **7**, 95.

[9] Bernthsen, A., *Ber. Deut. Chem. Ges.*, 1906, **39**, 1804.

[10] Kehrmann, F., *Ber. Deut. Chem. Ges.*, 1906, **39**, 1403.

[11] Miller, J. A., Miller, E. C., and Baumann, C. A., *Cancer Research*, 1945, **5**, 162.

aminoazobenzene occurred. At the end of 30 hours, 90% of the p-dimethylaminoazobenzene initially added had disappeared and as much as 85% was accounted for in the form of the monomethyl derivative. Thereafter, the amount of p-monomethylaminoazobenzene decreased. Small amounts of p-aminoazobenzene also were formed. The demethylation occurred only during autoxidation; none was found when the oxidation was inhibited with tocopherol.

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Ergotoxine Hyper- and Hypothermia in Albino Rats.*

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In 1906 an alkaloid was isolated from ergot of rye by Barger and Carr[1] and called ergotoxine. A year later Barger and Dale[2] reported that "the high temperature at and after death seemed to be a characteristic effect of fatal doses of ergotoxine." Since these fundamental studies and throughout the succeeding 40 years, other alkaloids have been isolated and identified until at present we have to deal with 6 natural alkaloids of different composition, each with 2 isomers. Stoll and Hofmann[3] have shown that the compound called ergotoxine is not a uniform chemical substance, but a molecular complex of 3 alkaloids: ergocristine, ergocornine, and ergokryptine.

Sollmann,[4] in his discussion of the central nervous system effects of ergotoxine as shown by several investigators, stated that the body temperatures of rabbits are raised from 3 to 4.5° C by the intravenous injection of .5 to 1.5 mg per kg of ergotoxine. Since there was no rise in decapitated animals, it was assumed that the action must be central, and that it probably involved both increased heat production and diminished heat loss.

Goodman and Gilman,[5] on the other hand, considered the action of ergotoxine on the central nervous system, when administered in moderate amounts, to be variable and, as a rule, unimportant. They noted that large doses of ergotoxine, given intravenously in certain laboratory animals, produced marked excitement, sham rage, and "other evidence of stimulation of central sympathetic centers."

Githens[6] found that cats responded to ergotoxine by a rise of temperature, but that rats, mice and pigs exhibited falls in temperature. Youmans and Trimble[7] reported that ergotoxine produced practically no effect on body temperature in dogs. Rothlin,[8] in discussing the effect of the ergot alkaloids on temperature regulation, maintained that the action was of a central nature because it was suppressed by general anesthesia. Ergotamine, which according to Sollmann,[4] and Goodman and Gilman,[5] gives essentially the

* Supported by a grant from the Office of Naval Research.

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2 Barger, G., and Dale, H. H., *Biochem. J.*, 1907, **2**, 240.

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4 Sollmann, T., *A Manual of Pharmacology*, 7th ed., W. B. Saunders Co., Philadelphia, 1948. 403.

5 Goodman, L., and Gilman, A., *The Pharmacological Basis of Therapeutics*, The Macmillan Co., New York, 1941, 658.

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7 Youmans, J. B., and Trimble, W. H., *J. Pharm. and Exp. Therap.*, 1930, **39**, 201.

8 Rothlin, E., *Bull. de l'Acad. Suisse des Sci. Med.*, 1946-47, **2**, 249.