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Effect of 3'-methyl-4-dimethylaminoazobenzene on Acid Phosphatase of Lysosomes[1),2)]

By

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(With 1 Plate)

While studying histochemical changes induced by 3'-methyl-4-dimethylaminoazobenzene (3'-Me-DAB) in the rat liver, it has been found that acid phosphatase is the first one which undergoes a prominent change after a short term feeding of azo dye among several oxidative and hydrolytic enzymes examined. It has been known that the acid phosphatase exists almost exclusively in lysosomes together with several other hydrolytic enzymes (de Duve, 1959). Novikoff (1961) and Straus (1962, 1964) have reported that lysosomes participate in the intracellular digestion and secretion. Therefore, the above mentioned change in the acid phosphatase activity after azo dye feeding seems to suggest changes in the function and/or the structure of lysosomes, and possibly, modification of the mechanism inherent in the intracellular digestion and secretion.

Here, several questions arise as to (1) whether a noncarcinogenic azo-dye, 2-methyl-4-dimethylaminoazobenzene (2-Me-DAB), possesses an inhibitory effect on the acid phosphatase similarly to 3'-Me-DAB; (2) whether a non-azo-dye carcinogen, N-2-fluorenyl-diacetamide (N-2-FAA), which is less active than 3'-Me-DAB, is similarly inhibitory; (3) whether inhibition of the acid phosphatase can be induced by a hepatic poison, carbon tetrachloride; and (4) whether 3'-Me-DAB is also effective in lowering the acid phosphatase activity of lysosomes in cells *in vitro*. The answers to these questions would probably provide information essential for understanding lysosomal functions under the influence of azo-dye. With the above questions in mind, the present experiments were undertaken on the effect of 3'-Me-DAB on acid phosphatase of lysosomes.

Materials and Methods: Male and female rats of Wistar King/A strain weighing 130–240 g were used for this experiment. The animals were separated into four groups: The first group was maintained on the basal diet containing 0.06% 3'-Me-DAB, the second group on the basal diet containing 0.06% 2-Me-DAB, and the third group on the diet containing 0.04% N-2-FAA. They were sacrificed 7, 14 and 20 days after the onset of feeding of experimental diets. The fourth group was maintained on basal diet, and received subcutaneous injections of CCl_4 at a single dose of 0.05 ml per 100 g of body weight. The injection was made between 10: 00 and 12:30 a.m. to avoid any possible diurnal variation of the hepatic activity. Rats were killed 24, 48, 72, 96 and 120 hours after the injection. None of rats died of this treatment.

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The procedures for the acid phosphatase staining were as follows: pieces of liver tissues less than, 5 mm in thickness were placed on aluminium foil, quenched in acetone chilled by dry ice and then cut at 10μ in a cryostat at −20°C. The sections were frozen-substituted in acetone at dry ice temperature, according to the method of Chang and Hori (1961). They were mounted on coverslips after a brief rinse in 95% ethanol, and fixed in 10% cold formalin for 1 min.. The fixed sections were stained after the method of Gomori (1952). Sections were incubated for 1 hour at 37°C in a freshly prepared substrate medium, rinsed in distilled water, and immersed for a few minutes in a dilute ammonium sulfide solution. They were then dehydrated in graded ethanol, cleared in tetrachloroethylene and mounted in a synthetic resin. General histological observations were made on sections stained with Delafield hematoxylin and eosin.

For studies *in vitro*, Hela cells were adopted as material on account of the failure of hepatic cell culture. They were cultivated in the TC 199 medium containing 10% calf serum. For subculture, the cells were treated 0.2% trypsin, and transferred to culture flasks (17×17×38 mm). A 12×32 mm coverslip was inserted in each flask. After incubation at 37°C for 72 to 96 hours, the medium was changed to that containing 3'-Me-, or 2-Me-DAB at concentrations of 1μg/ml, 10μg/ml, and 50μg/ml. Samplings were made at 24, 48, 72 and 144 hours after treatment. Cultured cells were fixed with 10% formalin for 30 minutes at 0°C to 4°C, and rinsed briefly in distilled water. They were stained for acid phosphatase, and then lightly with Delafield hematoxylin.

Results

Normal Liver: The activity of acid phosphatase in hepatic cells was demonstrated as granules of various sizes lying along bile canaliculi. It was stronger in periportal areas than in centrolobular areas. Kupffer cells in periportal areas richly contained spherical bodies showing the acid phosphatase activity.

Rats fed 3'-Me-DAB: No change was observed in the liver after 7 days of treatment. A slight decrease in the activity of acid phosphatase occurred in the liver tissue after 14 days. By the end of 20 days treatment the pronouncing decrease was noted. The decrease of acid phosphatase activity occurred first in the cells surrounding central veins, and then became remarkable in those lying the periphery of hepatic lobules. The treated liver, showed often the diffusion of the reaction product. This seems to be an indication of the change in nature of the lysosomal membrane. No sex difference was observed with respect to the change of the acid phosphatase activity in the liver. Histologically, the appearance of oval cells as well as of hyperplastic cells in the periportal zone characterized the livers of rats treated for 20 days, in agreement with the finding of Farber (1956). This change was more prominent in the liver of males than in that of females. Many Kupffer cells were found around the hyperplastic cells. The oval cells showed no granules of the acid phosphatase activity. The enzyme activity was stronger in hyperplastic cells than in other hepatic cells.

Rats fed 2-Me-DAB: The changes in the liver of rats fed 2-Me-DAB were histochemically the same as those in rats fed 3'-Me-DAB.

Rats fed N-2-FAA: The acid phosphatase activity exhibited no visible change in the hepatic cells of experimental animals of both sexes. In males oval cells were observed in the liver on the 14th day, and their increase in number was noted on the 20th days. Histological features of the liver were generally similar to those described by Farber (1956).

Rats received injection of CCl_4: Twenty-four hours after injection a number of damaged cells appeared in centrolobular areas of the liver. Necrotic changes took place from centrolobular areas towards periportal areas. No acid phosphatase activity was demonstrated in the necrotic cells. By 72 hours the restitution was taken place. There was an appearance of Kupffer cells with intense acid phosphatase activity around inflammatory cells. By 120 hours damaged cells were almost completely replaced by healthy cells. In the course of the destruction and restitution, the hepatic cells showed mostly a normal activity for acid phosphatase.

In vitro experiment: Non-treated Hela cells showed lysosomes scattering throughout the cytoplasm or locating in the nuclear hof. In some of the mitotic cells were observed the mass of lysosomes in the peripheral area of the cytoplasm. A similar feature was described by Yamamoto (1965) in cultured cells and ascites tumor cells. Damaging cells showed an intense activity of the enzyme.

Treatment of cells with 3'-Me-DAB at various concentrations from 1 to 50μg per ml of the culture medium showed no evidence for the inhibition of the enzyme activity, as well as for the change of cell proliferation. A number of mitotic figures were observed in affected cells. On the other hand, 2-Me-DAB exerted a severe damaging effect on cells; all the affected cells were died within 24 hours at a concentration of 50μg per ml of the medium. After 6 days at a concentration of 10μg per ml many picnotic cells appeared, and at 1μg per ml mitotic figures were observed.

Discussion

The present histochemical study with rat livers has shown that the acid phosphatase activity of hepatic lysosomes apparently decreases following the administration of 3'-Me-DAB. The decrease in the enzyme activity occurred more prominently in cells of centrolobular areas than in those of periportal areas of the liver. A similar change was also noted in the livers of rats fed 2-Me-DAB, though not carcinogenic. This seems to indicate that the decrease of the acid phosphatase activity of lysosomes after feeding of 3'-Me-DAB may not be attributable to the carcinogenicity of the dye. In view of the fact that a non-azo-dye carcinogen, N-2-FAA, exerted no inhibitory action on the lysosomal acid phosphatase activity, a speculation is possible that the inhibition of the acid phosphatase under the influence of 3'-Me-DAB and 2-Me-DAB may be the result of a direct or otherwise indirect action of Me-DAB. The data provided by the present experiments accord well with those presented by Allard *et al.* (1957). Based on the present finding that

the degeneration of hepatic cells induced by CCl_4 did not cause the decrease in the activity of lysosomal acid phosphatase, it is possible to assume that degenerative changes of hepatic cells taken place after azo-dye feeding do not account for the azo-dye induced decrease of the acid phosphatase activity. Futher, the results of the *in vitro* study with Hela cells indicate that 3'-Me-DAB itself exerts no direct pathological effect on the lysosomes as well as on the acid phosphatase. On the other hand, 2-Me-DAB showed a pronounced damaging action on the *in vitro* cells, in a striking contrast to the data from *in vivo* experiment. The difference of the results between the *in vivo* and *in vitro* experiments remains unanswered at the present status of knowledge. The reason why 3'-Me-, and 2-Me-DAB inhibit the acid phosphatase can not be fully explained by the present study. However, the evidence suggesting the change in nature of the lysosomal membrane in azo-fed rats may be useful as an important clue for understanding the above features.

Summary

Effects of 3'-Me-DAB on the acid phosphatase activity of lysosomes were studied in the livers of rats, use being made of the Gomori's method. The enzyme activity decreased markedly after 14–20 days of feeding of 3'-Me- or 2-Me-DAB, but not of N-2-FAA. No inhibition of lysosomal acid phosphatase occurred after the treatment of Hela cells for 6 days with 3'-Me-DAB. The findings suggest that the inhibition of acid phosphatase by 3'-Me-DAB may not be attributable to the direct action of DAB on the protein. In contrast, 2-Me-DAB affected severely cells *in vitro*.

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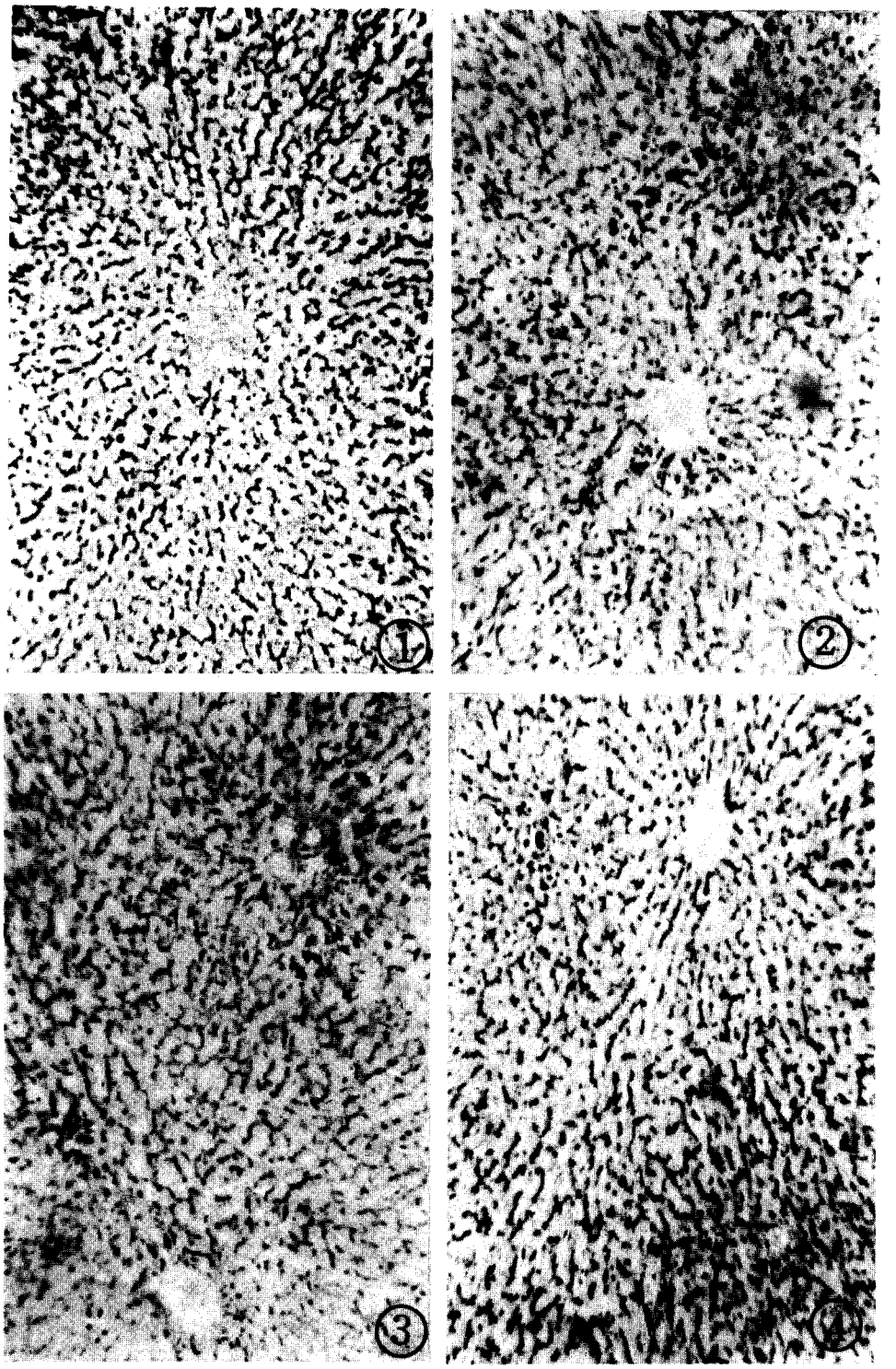
Explanation of plate III

Fig. 1. Acid phosphatase in normal liver cells. ×400.
Fig. 2. Liver tissue of a male rat fed 3'-Me-DAB for 20 days.
Fig. 3. Liver tissue of a male rat fed 2-Me-DAB for 20 days.
Fig. 4. Liver tissue of a male rat fed N-2-FAA for 20 days.



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