Neoplasm Studies

X. The Effects in Tissue Culture of Some Split Products of \textit{p}-Dimethylaminoazobenzene on Rat Liver Tumors* 

M. J. Kopac, Gladys Cameron, and Robert Chambers

(From the Department of Biology, Washington Square College of Arts and Science, New York University, New York, N. Y.)

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Stevenson, Dobriner, and Rhoads (5) reported that \textit{p}-dimethylaminoazobenzene when fed to rats is decomposed and metabolized to several simpler compounds. One of these split products, \textit{N},\textit{N}-dimethyl-\textit{p}-phenylenediamine, has been found by us (i) to be toxic to the glandular epithelium of the rat liver at concentrations of 0.0001 \textit{M}, while equivalent effects on the ductal epithelium were produced only with concentrations of 0.002 to 0.004 \textit{M}. The other compounds that may be derived from \textit{p}-dimethylaminoazobenzene or that have been recovered from the urine of rats fed it are the following: aniline, \textit{p}-amino-phenol, \textit{N}-acetyl-\textit{p}-aminophenol, \textit{p}-phenylenediamine, and \textit{N},\textit{N}'-diacetyl-\textit{p}-phenylenediamine (5).

The tissue culture studies reported in this paper are on the effects of the nonacetylated split products mentioned above on normal rat liver and on liver tumors. The tissue cultures were prepared as described in the preceding report (i), and when good growths were established comparable cultures were selected and exposed to a medium containing one of the split products. This was done by replacing a portion of the serum component in the medium with Tyrode solution containing the split product, the final concentration of the experimental compound being given below.

Neither \textit{aniline hydrochloride}, \textit{aniline sulfate}, nor \textit{p}-aminophenol \textit{hydrochloride} at concentrations of 0.001 \textit{M} produces effects on wandering cells or on gland or duct epithelia, regardless of their source, \textit{i. e.}, normal or neoplastic. This contrasts with our previous finding (i) that \textit{N},\textit{N}-dimethyl-\textit{p}-phenylenediamine at a concentration of 0.001 \textit{M} destroys glandular epithelium but is relatively harmless to ductal epithelium.

\textit{p}-Phenylenediamine or \textit{p}-phenylenediamine \textit{hydrochloride}, at a concentration of 0.001 \textit{M}, produces either one or two general reactions. One concerns the toxicity of \textit{p}-phenylenediamine to some of the wandering cells and the epithelium growing from the explant. Some destruction of the latter was observed, particularly along the margins of the outgrowing epithelial sheets. The cells were disintegrated and became heavily stained a deep brown or a reddish brown. The resulting colored precipitate is presumably an insoluble complex between the oxidation products of \textit{p}-phenylenediamine and the cytoplasmic residue.

The other, a most striking reaction, is the following: Within 24 hours after exposure of normal liver or liver tumor cultures to \textit{p}-phenylenediamine, opaque, blue-black to black, aciform crystals appeared in the surviving epithelial and wandering cells. The crystals were seen in the cytoplasm, never in nuclei or the medium, lying singly or in compact groups forming bundles, rosettes, or fans (Figs. 1 and 2); occasionally their tips seemed to protrude through the cellular surface. All the cells, epithelial or wandering, that contained crystals were in good condition.

After 72 hours' exposure, many epithelial cells with the onset of disintegration had lost their crystals, and none were observed in dying or dead cells, nor were they liberated as such into the medium on the breakdown of the cell. Orange colored vacuoles gradually developed in the wandering cells, and the crystals disappeared before these cells disintegrated. At this time, only the crystal-containing cells were alive.

The epithelial cells that contained crystals were still alive after 96 hours' exposure. Some of the living fibroblasts enclosed a few crystals together with numerous grayish blue cytoplasmic granules that imparted a bluish tint to these cells and stood out in contrast to the colorless nuclei. The macrophages had become progressively moribund with the disappearance of crystals and their replacement by reddish brown granules. These cells soon disintegrated.

The development of crystals in the cytoplasm of surviving cells is apparently an intracellular mechanism for detoxifying the diamine. This follows from

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numerous observations that p-phenylenediamine kills those cells which do not, for some reason, convert the penetrating substance into the insoluble crystalline product.

The precise nature of the crystals is unknown, although these may consist of polymeric derivatives of oxidized p-phenylenediamine. Many such polymers are known in the field of dye chemistry (4). In this connection, several oxidizing agents were added to test tubes containing a solution of p-phenylenediamine. Almost immediately after the oxidant had been added a precipitate would form associated with a change in color of the solution. With hydrogen peroxide, the reddish brown precipitate was an amorphous, jelly-like mass. With potassium ferricyanide, the dark blue precipitate consisted of crystals of various shapes including the aciform type. When a drop of solution from the latter preparation was evaporated, aciform and other crystalline patterns of microscopic dimensions would develop. The dry crystals dissolved on addition of water. It is entirely possible that the crystals appeared in the cytoplasm because the oxidized product of p-phenylenediamine had a low solubility in cytoplasm. Likewise, the failure to find crystals in the medium following the disintegration of cells that produced them may have been the result of an apparent higher solubility of the oxidized crystalline product in the culture medium.

Kensler, Dexter, and Rhoads (2) found that cysteine, 3 mgm. per 4 ml., suppresses the inhibition of the diphosphopyridine nucleotide system by p-phenylenediamine. Potter (3) reported that cysteine, 0.001 M, likewise suppresses the inhibition of urease by p-phenylenediamine. On the other hand, cysteine hydrochloride in concentrations as high as 0.005 M does not detoxify p-phenylenediamine to permit the survival of liver cells grown in tissue culture. Following 24 hours' exposure to cysteine (0.005 M) + p-phenylenediamine (0.001 M) glandular and ductal epithelia were dead and stained a deep reddish brown. The fibroblasts, however, had begun to develop crystals and were therefore able to survive.

The observations just cited may be significant. They might indicate that, in liver cells grown in tissue culture, systems other than diphosphopyridine nucleotide or urease, or other sulfhydryl-containing enzymes, are involved, an idea previously suggested by Potter. Another interpretation is that cysteine in the tissue culture medium, pH = 7.6, inter alia, enhances the penetrability of p-phenylenediamine into liver cells. This matter is considered in greater detail below.

The addition of 0.001 M sodium sulfite, an agent commonly used for preventing autoxidation, to the culture medium inactivated p-phenylenediamine by preventing both the destruction of epithelial cells and the formation of crystals in the cytoplasm. None of
the cells showed any kind of crystal development during the first 48 hours' exposure and still the cultures were in good condition and compared favorably with the controls. After 72 hours' exposure crystals began to develop in some of the wandering cells. All cultures containing sodium sulfite were in better condition than those grown in media with p-phenylenediamine alone.

The oxidation of p-phenylenediamine is retarded by sodium sulfite. The survival of cells without crystal formation indicates that the reduced p-phenylenediamine (diamine stage) penetrates very slowly, if at all; the molecules presumably penetrate the cells in the form of the free radical, its dimere, or the diimine. The oxidation of the diamine to free radicals and beyond is retarded or inhibited by sodium sulfite, depending on the concentration of the latter.

Similar results were previously obtained with culture media containing sodium sulfite, > 0.0025 M, and N,N-dimethyl-p-phenylenediamine (1). Although the latter compound does not form intracellular crystals, its penetration into glandular and ductal epithelia is inferred from its toxic action. The toxic effect can be completely blocked by sodium sulfite. Apparently, the penetrating form of N,N-dimethyl-p-phenylenediamine is likewise the free radical, its dimere, or the diimine.

SUMMARY

Several split products of p-dimethylaminoazobenzene were tested on normal liver and rat liver tumors grown in tissue culture. Aniline hydrochloride or aniline sulfate, also p-aminophenol hydrochloride at concentrations of 0.001 M, were nontoxic to both glandular and ductal epithelia. Either p-phenylenediamine or p-phenylenediamine hydrochloride, under autoxidizable conditions, at 0.001 M was toxic to all cells except those that form intracellular crystals (possible oxidation polymers of the diamine). Sodium sulfite blocked intracellular crystal formation and, in such cases, no toxicity of p-phenylenediamine could be demonstrated.

None of the split products mentioned above was found to be so toxic as N,N-dimethyl-p-phenylenediamine, which destroyed normal or neoplastic glandular-cell epithelium at concentrations of 0.001 M (1). On the other hand, ductal epithelium (normal or neoplastic) at this concentration survived and grew.

REFERENCES

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