Effect of Sulfonated Azo Dyes on Mouse Tumors

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The possible relationship between deficient reticulo-endothelial function and neoplasia has been investigated by several workers (7). Some investigators utilized the hypothetical relationship between reticulo-endothelial function and tumors for therapeutic approaches; in other words, attempts were made to combat malignant tumors by methods which were expected to increase reticulo-endothelial activity. These mostly unsuccessful endeavors will not be reviewed here; however, it may be remarked that in most instances complex biologic stimuli were employed for the purpose of enhancing reticulo-endothelial function. It was thought possible that stimulation of reticulo-endothelial tissues by means of chemical compounds, defined as to their structure and amenable to exact dosage, might represent a more promising approach (4).

The present study was prompted by a report made by Cestari (1) in 1940 regarding the effect of sulfonated Sudan IV (scarlet red) on the Kupffer cells in the rabbit. According to this author, prolonged intravenous injections of Sudan IV were followed by deposition of the dye in the Kupffer cells; from there it gradually passed into parenchymal cells, causing degenerative changes and proliferation of connective tissue, and this ultimately led to a condition resembling cirrhosis. When the author, however, used a sulfonated derivative of Sudan IV, which was more readily water-soluble, this dye was taken up by the Kupffer cells, caused them to proliferate in response to the storage, and was eliminated without any apparent damage to the liver.

On the basis of this report, it was thought worthwhile to investigate the effect of sulfonated azo dyes on reticulo-endothelial activity and tumors of mice. A number of such compounds were made available by the National Aniline Division, Allied Chemical and Dye Corporation.1 The compounds which have been tested so far are listed in Figure 1. Sudan IV (first horizontal column) was not used in our work but is included in the chart in order to show its structural relationship to the other compounds. All the dyes were readily water-soluble up to concentrations of 1–2 per cent. Subcutaneous injections of the dyes, dissolved in physiologic solution of sodium chloride to the desired concentration and autoclaved before use, were given to the experimental animals.

The first compound tested was cloth red B (CRB). Subcutaneous injections of 0.5 per cent solutions of the dye were given to groups of C57 black and C3H animals for periods ranging from 3 to 20 months. No storage of the dye in reticulo-endothelial cells could be observed. As was briefly reported on a previous occasion (6), moderate proliferation of Kupffer cells in the livers of animals, injected for a period of at least 6 months, could be demonstrated by injecting carmine solution intraperitoneally before sacrificing the animals and by counting the number of storing cells (5). C57 black mice responded to the dye with a more marked proliferation of Kupffer cells than did C3H animals. All the treated female animals of the C3H strain developed spontaneous mammary carcinoma. Likewise, tumor development in C57 blacks injected subcutaneously with methylcholanthrene (0.6 mg.) was not affected by twice-weekly injections of the dye.

In 1 out of 28 C3H mice, and in 1 out of 34 C57 black mice, none of whom had received carcinogen but which had been injected twice weekly with CRB, fibrosarcomas arose at the site of the injection of the dye; this occurred in the C3H animal 15 months and in the C57 black mouse 16 months after the start of the injections. Transplants of the tumors were carried in the respective strains for 21 and 6 passages, respectively. This might indicate a weakly carcinogenic effect of the dye; reference is made to the action of scarlet red itself, which, according to the data summarized by Hartwell (2), has shown carcinogenic activity in isolated instances. More recently, Smith (3) demonstrated its co-carcinogenic properties.

Only brief mention need be made of the results obtained with the compounds wool red 40F, solan-
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None of the dyes, except brilliant scarlet 3R, showed slight storage in reticulo-endothelial cells.

The following data were obtained with Erie Fast Rubine B Conc. (EFR). This compound was readily stored in reticulo-endothelial cells, especially in the liver and spleen. The minimal lethal dose of the dye was approximately 0.6 mg/gm. The dosage actually used was 0.5 cc. of a 0.25 per cent solution for animals averaging 20–25 gm., or approximately 0.06 mg/gm (one-tenth of the

<table>
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<tr>
<th>COMPOUND</th>
<th>COLOR INDEX</th>
<th>STRUCTURE</th>
<th>NUMBER OF SULFO GROUPS</th>
</tr>
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<tbody>
<tr>
<td>SUDAN III</td>
<td>258</td>
<td><img src="image" alt="Structure" /> o-tolueno-azo-o-tolueno-azo-β-saphtol</td>
<td>0</td>
</tr>
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<td>CLOTH RED B</td>
<td>262</td>
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<td>2</td>
</tr>
<tr>
<td>BRILLIANT SCARLET 3R PURIFIED</td>
<td>-</td>
<td><img src="image" alt="Structure" /> sodium salt of 4-sulfo-α-saphtol-azo-β-saphtol-4,6-dinitrosoic acid</td>
<td>3</td>
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<tr>
<td>WOOL RED 40F</td>
<td>184</td>
<td><img src="image" alt="Structure" /> sodium salt of 4-sulfo-α-saphtol-azo-β-saphtol-4,6-dinitrosoic acid</td>
<td>3</td>
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<tr>
<td>SOLANTINE RED 8 BLN</td>
<td>-</td>
<td><img src="image" alt="Structure" /> sodium salt of di-(p-sulfo-o-sulfo-β-saphtol-azo-o-sulfo-β-saphtol-z-sulfoic acid)-urea</td>
<td>6</td>
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<tr>
<td>ERIE FAST RUBINE B Conc.</td>
<td>-</td>
<td><img src="image" alt="Structure" /> sodium salt of di-(6,8-dialso-β-saphtol-azo-β-saphtol-3-sulfoic acid)-urea</td>
<td>6</td>
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</table>

Fig. 1.—Sulfonated azo dyes tested in this study
minimal lethal dose). With a few exceptions, animals tolerated this twice-weekly dosage well and did not lose weight. In some animals ulcerations of the skin, which were probably the result of inadvertent intracutaneous administration of dye solution, occurred after prolonged periods of injections. If such ulcerations occurred near tumors, or near the site of the tumor inoculum or the deposit of carcinogen, the animals were excluded from further experimental observation.

Group II continued to receive the dye injections twice weekly until the end of the experiment. The dye was injected at the farthest possible distance from the carcinogen deposit.

Figure 2 illustrates the delayed development of tumors in the experimental animals, as compared with the controls. Weekly examination included weighing of the animals, searching for tumors, and measuring of tumors present. The weight of the injected animals did not vary from that of the controls by more than 5 per cent—the higher weight of the controls being due to the presence of larger tumors. This was corroborated by establishing the true weights of the animals at death or at the end of the experiment; the average weight of the controls was 17.4 gm., and that of the injected animals was 18.4 gm. A significant difference in the development of tumors was noted up to 17 weeks after administration of the carcinogen; during this period, calculation of p for the difference in the percentage of tumors gave values of less than 0.01, which are considered statistically significant. However, after this period the injected
animals started to develop new tumors, so that the difference between them and the controls became increasingly smaller. No difference in the growth rate of the tumors in, or in the survival time of, injected or control animals was noted.

**Experiment 518-IX.**—Four-month-old C3H males were used. Group I consisted of 15 controls; Group II of 15 experimental animals. Group II received a total of seven subcutaneous injections of 0.5 cc. of 0.25 per cent EFR during a period of 3 weeks. Two days later, animals of both groups were inoculated with a transplantable mammary carcinoma. The dye injections were continued for 2 months after the inoculation. In Figure 3 the tumor development in both groups is recorded. Again there was a marked difference in the time of the appearance of the tumors in the two groups. This difference remained significant for a period of 11 weeks after inoculation, and after this time, just as in the previous experiment, the injected animals caught up with the controls, so that at the end the "takes" reached 100 per cent in both groups.

In addition to these experiments, others were performed with variations in the dosage and number of dye injections and in the timing of the injections, in relation to the administration of carcinogen or to inoculation of the tumor. These experiments showed either similar, or lesser, degrees of tumor inhibition. In none of the experiments was there a complete suppression of the tumor process in injected animals. Negative results were obtained in female mice of the C3H strain, in which injection of the dye was started at the age of 7 months; the incidence of spontaneous mammary carcinoma in this group reached almost 100 per cent, and the time of appearance and growth rate of the tumors was similar in injected and control animals.

Only transient, and statistically not significant, inhibitions of transplantable sarcomas in C57 blacks and transplantable melanomas and carcinomas in dba animals were observed.

In all experiments, similar gross and histologic findings were noted. Liver, spleen, and subcutaneous tissue showed, grossly, reddish-purple staining with the color intensity generally increasing with the length of dye administration. Tumors, when present, did not show staining except for a faint tinge in the adherent connective tissue.

Histologically, the most intensive storage of dye was found in the liver, which contained large numbers of dye-laden macrophages (Kupffer cells). Many of them were distorted or swollen, with displaced pyknotic nuclei (Figs. 4, 5). Occasionally, dye was found in accumulations of histiocytes in the vicinity of larger vessels. In some animals, the parenchymatous liver cells showed hardly any changes; in others, degenerative changes appeared with a moderate increase of binucleated cells. In later stages focal areas of liver

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**Fig. 3.**—Effect of EFR on the development of a transplanted adenocarcinoma in C3H male mice.

**Fig. 4.**—Liver of C37 black mouse. Storage of Erie Fast Rubine in Kupffer cells. No change apparent in hepatic parenchyma. Photomicrograph, X150.

**Fig. 5.**—Higher magnification of Figure 4 showing macrophages laden with dye. Photomicrograph, X300.

**Fig. 6.**—Liver of C37 black mouse injected with dye for 5 months. Note disorganization of hepatic structure and proliferation of connective tissue in central portion of field. Photomicrograph, X150.

**Fig. 7.**—Higher magnification of central portion of Figure 6 discloses presence of dye-storing macrophages in the proliferating connective tissue. Note degenerated liver cells in lower margin. Photomicrograph, X375.

**Fig. 8.**—Fibrosarcoma, induced by methylcholanthrene, in C37 black mouse injected with dye. Photomicrograph, X375.

**Fig. 9.**—Transplanted adenocarcinoma, growing in C3H mouse injected with dye. Histologic features of this tumor and of tumor in Figure 8 were indistinguishable from those in tumors of control animals. Photomicrograph, X375.
necrosis were noted, with dye accumulating in these areas. In some mice which had been injected for many months, severe alterations in the liver structure occurred, with large foci of proliferating histiocytes separating distorted liver cords (Figs. 6, 7).

It is noteworthy that, even 3 months after cessation of injections, dye was found stored in the liver and spleen. The latter organ presented variable numbers of storing macrophages within the red pulp, and dye was also found in the endothelial lining of sinusoids. The follicles contained no dye and appeared as white spaces in unstained sections; in some instances the peripheral follicular portion was replaced in hematoxylin-eosin-stained sections by homogeneous pink staining material, which, in unstained sections, contained dye in diffuse form.

In accord with the gross findings, neither carcinomas nor sarcomas showed dye storage in the tumor proper; dye-laden histiocytes were found in the peritumoral connective tissue. In none of the experiments, even when inhibition of tumor growth had been previously noted, were there any structural or cytologic differences between tumors of injected animals and those of controls (Figs. 8, 9).

After prolonged dye administration, delicate dye granules were also found in the tubular epithelium of the kidneys. No urinary excretion of the dye was noted at any time. After dye administration had continued for many weeks, dye was found in the blood serum of the mice.

**DISCUSSION**

The above experiments were carried out for the purpose of finding out whether it is possible to increase the resistance of mice to neoplasia by means of administration of dyes which are stored in reticulo-endothelial tissue. A moderate transient inhibition of certain types of mouse tumors was observed with the sulfonated azo dye Erie Fast Rubine Conc. B.

In connection with these findings the following points may be considered. Significant inhibition of tumors was found only in two types of tumors: (a) sarcoma induced by methylcholanthrene and (b) a transplanted mammary carcinoma, both of which showed a rather long latent period of induction or of "taking," respectively. This might indicate that resistance brought about by the dye is operative only in the presence of slow neoplastic processes.

The absence of histologic changes in tumors of treated animals is evidence against a direct action of the dye on tumor cells; it is possible that it acts indirectly by way of increasing some defensive mechanisms in the host, which we hypothetically connect with the reticulo-endothelial proliferation in the treated animals.

While the results of the present work have failed to demonstrate any remarkable effects of the administration of sulfonated azo dyes, they might have some value since they show an instance in which proliferation of reticulo-endothelial tissue was accompanied by a delay in carcinogenesis.

**SUMMARY**

Erie Fast Rubine Conc. B., one out of five sulfonated azo dyes tested, was found to be readily stored in reticulo-endothelial cells of mice. Injections of this dye into mice caused a temporary inhibition of the development of sarcoma induced by methylcholanthrene in C57 blacks and of a transplanted mammary carcinoma in C3H mice. An interpretation of these findings was presented.

**REFERENCES**

2. HARTWELL, J. L. Survey of Compounds Which Have Been Tested for Carcinogenic Activity, FSA, USPHS, pp. 215-17, 1941.
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Kurt Stern


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