solution of Eastman's bilirubin has been found satisfactory for the depth of readable color.

Bilirubin dissolved in this triple mixture, when exposed to heat, is recovered within the range of experimental error; when previously treated with ether and hydrochloric acid and then exposed to heat, about 70% of the bilirubin is recovered. The loss is chiefly due to the differences in surface tension between the bilirubin and dilute hydrochloric acid. Gallstones do not show this difference, so we feel that little mechanical loss occurs on treating them. Chemical factors may also play a part in the recovery. Figures obtained by this method[4] do not always check within a close range, yet they give a fair idea of the total amount of pigment in stones.

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Anticatalase Activity of Sulfanilamide and Related Compounds. I. Effect of Ultraviolet Irradiation.

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The production of a violet color by irradiation of dilute solutions of sulfanilamide with ultraviolet light has been reported by Ottenberg and Fox.[1] They suggested that this colored substance, possibly produced by oxidative changes in the body, may be responsible for the cyanosis frequently observed in patients under treatment. Whether or not the colored derivative was superior to sulfanilamide in bactericidal power was not determined.

In work to be published[2, 3] it has been suggested, as a possible explanation of the retardation of growth of pneumococci by sulfanilamide, that the bacteriostatic agent involved may be—not sulfanilamide itself—but hydrogen peroxide. The latter substance was presumed to accumulate in the immediate locality of the invading coccus, following oxidation by the coccus of sufficient absorbed sulfanilamide to produce inhibition of catalase as rapidly as the latter principle enters the reaction zone. The oxidation product may be

4 Phemister, D. B., Aronsohn, H. G., and Pepinsky, R., in press.

1 Ottenberg, R., and Fox, C. L., Jr., PROC. SOC. EXP. BIOL. AND MED., 1938, **38**, 479.

2 Locke, A., Main, E. R., and Mellon, R. R., *J. Immunol.*, in press.

3 Mellon, R. R., *Mod. Hosp.*, 1938, **51**, 53.

similar to that produced by irradiation. Qualitative tests showed that irradiated sulfanilamide had a marked inhibitory effect on the activity of serum catalase. This report presents further data, showing that non-irradiated sulfanilamide also has appreciable anticatalase activity, which is increased upon irradiation. The observations of Ottenberg and Fox regarding color production have been extended to a number of related compounds, active and inactive therapeutically, and it has been shown that, in many of these, anticatalase activity either appeared, or was enhanced, after irradiation.

Solutions were irradiated for one minute in thin layers at a distance of 3 inches from a Westinghouse Sterilamp. The results of Ottenberg and Fox, showing that the color produced varied with the concentration of solution used, were confirmed. The color was first detectable at a concentration of about 0.2 mg %. Up to a concentration of 2 mg % the intensity of color was directly proportional to the concentration. Above this concentration the amount of color became increasingly less and at concentrations greater than 16 mg % the color produced was yellow to brownish red. The violet color faded rapidly to a brownish pink on standing. This instability was apparently related to the production of acid during irradiation since the addition of a trace of bicarbonate solution at the time of irradiation stabilized the violet color so that it was maintained for several days. A pink color was produced when the solution irradiated was slightly acid in reaction or when acid was added to the violet-colored solutions.

Attempts to produce a comparable color in sulfanilamide solutions by oxidation with the commonly used oxidizing agents were unsuccessful. The color of irradiated sulfanilamide solution was destroyed by treatment with hydroquinone and could not be restored by hydrogen peroxide.

Substances which produce inactivation of heavy metals[4] modified the effect of ultraviolet irradiation. The addition of diethyl dithiocarbamate, potassium cyanide, or potassium thiocyanate, compounds which act primarily on copper, completely inhibited production of color in irradiated solutions. Sodium pyrophosphate, which inhibits iron, did not interfere with color production. Sodium fluoride, a still more effective inhibitor of iron, caused intensification of color.

The effect of irradiation upon solutions of 17 compounds,* related to sulfanilamide in structure, is summarized in Table I. In 6

[4] Locke, A., and Main, E. R., *J. Infect. Dis.*, 1931, **48**, 419.

* Synthesized and donated to us by the Monsanto Chemical Company, St. Louis, Missouri.

of these, definite colors were produced. The color produced by compounds 11 and 16 resembled that given by sulfanilamide. Compounds 7, 8, and 13 yielded a sky-blue color. No color was produced in solutions of compounds which have substituents in the *p*-amino group or aryl substituents in, or replacing the sulfamido group. In general, color was produced only by those compounds in which the *p*-amino group was free and in which the sulfamido group was either free, replaced by an alkyl group or partly substituted by an alkyl. Thus, the color-producing compounds fell into two groups: (a) Compounds 11, 16 and 18, in which the sulfamido group was free or partly alkylated, produced a violet color, unstable in the presence of acid. (b) Compounds 7, 8, and 13, in which the sulfamido group was replaced by an alkyl group, gave a blue color, unstable in alkaline and neutral solution.

Estimations of anticatalase activity of irradiated and non-irra-

TABLE I.

Color Production by Ultraviolet Irradiation of Solutions of Sulfanilamide and Related Compounds and Anticatalase Activity Before and After Irradiation.

Compound	Color produced	Anticatalase activity		
		Before irrad.	After irrad.	Conc. of soln. mg %
1. Phenyl sulfanilate	None	10	10	2
2. Benzyl p-aminophenylsulfone	"	10	10	2
3. N,N'-diacetylsulfanilamide	"	*	*	*
4. N-(p-aminobenzenesulfonyl)-benzamide	"	17	10	2
5. p-(n-hexylamino)-benzene sulfonamide	"	*	*	*
6. N-(p-aminobenzenesulfonyl)-imino diacetic acid	Trace?	0	57	8
7. Methyl-p-aminophenylsulfone	Blue	0	57	8
8. n-amyl-p- "	"	0	43	8
9. p-acetyl-amino-sulfanilamide	None	0	0	8
10. p-benzyl-amino-benzenesulfonamide	"	0	23	2
11. N,N'-di(p-aminosulfonyl)-ethylene-diamine	Red violet	10	70	2
12. p-(n-amylamino)-benzene-sulfonamide	Trace?	10	53	2
13. β-hydroxyethyl-p-aminophenyl-sulfone	Blue	11	67	8
14. 4,4'-diamino-benzenesulfon-anilide	None	61	58	8
15. 4,4'-di-(acetylamino)-diphenyl-sulfone	"	*	*	*
16. N-(p-aminobenzenesulfonyl)-aminoacetic acid	Red violet	12	69	8
17. Sulfanilic acid	None	9	0	8
18. p-aminobenzenesulfonamide	Violet	17	70	2
18. "	"	17	70	8

* Too insoluble for test.

diated solutions of sulfanilamide showed that a marked increase in such activity appeared simultaneously with the development of color. A 10% dilution of fresh rabbit serum in water was used as a source of catalase and its activity was determined by titrating the unchanged hydrogen peroxide with standard permanganate solution. For the determination of anticatalase activity, a solution of the compound to be tested was used as a serum diluent. After a reaction period which allowed decomposition of about 50% of the added peroxide, the mixture was acidified with *p*-toluene sulfonic acid and titrated immediately.

The amount of active catalase was calculated from the formula: $E = x^2/A$, where x is the amount of peroxide decomposed and A is the amount originally present. The time and temperature factors were considered to be constant. The anticatalase activity or percent suppression of catalase was then expressed as $100—(100\ E/E')$, where E′ is the total catalase value obtained with water. Values of 10 or less were not considered significant. The slight acidity produced in irradiated solutions did not interfere with the determinations. A series of 15 estimations of the anticatalase activity of 8 mg % solutions of sulfanilamide showed that comparable results could be obtained with different sera. The average activity of irradiated solutions was 83, that of non-irradiated, 27.

The effect of the time of irradiation, concentration and aging on the anticatalase activity of sulfanilamide is reported in Table II. With an 8 mg % solution, a maximum activity was reached after irradiation for 45 seconds or less. The activity of irradiated solutions increased with increasing concentration of sulfanilamide up to a concentration of 1-2 mg %, but no further increase in activity was observed at higher concentrations. Conversely, upon dilution

TABLE II.

Effect of Radiation Time, Concentration, and Aging on the Anticatalase Activity of Sulfanilamide Solutions.

Effect of radiation time (8 mg % soln.)		Effect of concentration at time of radiation		Effect of dilution after radiation		Effect of aging in radiated 8 mg % solutions	
Time, sec.	Anticat. activity	Conc., mg %	Anticat. activity	Conc., mg %	Anticat. activity	Days after radiation	Anticat. activity
0	25	8	76	8	87	0	89
5	42	4	73	4	80	1	93
15	67	2	73	2	80	2	73
45	88	1	76	1	73	4	57
120	88	0.5	64	0.5	67		
		0.2	56	0.25	41		
		0.05	36	0.10	37		
		0.025	0?				

of irradiated solutions, no decrease in activity occurred until a concentration of 1-2 mg % was reached. The anticatalase principle was found to be more stable than the substance responsible for the color. Thus solutions showed marked activity after standing for two days at room temperature, whereas the color faded within 2 hours. Although anticatalase activity develops simultaneously with color, the substances responsible for activity and for color are not identical.

Table I shows the relative anticatalase activities of the compounds listed. Compounds 3, 5, and 15 could not be tested because of their extreme insolubilities. Of the 9 compounds which had activities of 40 or more after irradiation, 6 produced definite color and 2 yielded traces of color. Compound 14 was exceptional. It gave no color on irradiation and had a high anticatalase activity both before and after irradiation, possibly due to its sensitiveness to air oxidation.

Three of the compounds listed, 4, 14, and 18, are known to possess therapeutic activity[5] in pneumococcal and some streptococcal infections. It is significant that they showed anticatalase activity without activation by ultraviolet light. A high anticatalase activity, developed as a consequence of irradiation, gave no information as to the therapeutic value of a compound.

Work is now in progress in which an attempt is being made to correlate the anticatalase activity of sulfanilamide with the retardation of growth in pneumococcus cultures.

Conclusions. The ability of sulfanilamide and structurally related compounds to develop color on ultraviolet irradiation appears to be dependent on the presence or absence of substituents in the *p*-amino and sulfamido groups. A study of the anticatalase properties of sulfanilamide and related compounds indicates a possible correlation between therapeutic activity and the intrinsic anticatalase activity associated with the non-irradiated compound. Neither color production nor the high anticatalase activity developed as a consequence of irradiation appear to be related to therapeutic effectiveness.

[5] Mellon, R. R., Gross, P., and Cooper, F. B., *Sulfanilamide Therapy of Bacterial Infections,* Charles C. Thomas, Springfield, Illinois, 1938.