sults were obtained with about 0.04 mg/kg/D. Regressions of the carcinoma have been obtained with 0.032 to 0.230 mg/kg/D and without toxic manifestations by the hosts.

In general this carcinoma ulcerates in its later stages of growth. However, in the treated animals this occurred less often. In most of the cases where regression started early in the treatment it proceeded to completion without ulceration of the tumor.

It would appear that the Sprague-Dawley rat is less sensitive than the King A rat to TEM. The animals gained weight under treatment.

Summary. 1. The treatment of Sprague-Dawley rats bearing Flexner-Jobling carcinoma with 2,4,6-triethylenimino-s-triazine resulted in complete regression and cures of the tumor in about 70% of the animals treated. There were no spontaneous regressions among the controls. 2. This carcinoma responded to treatment with doses ranging from 0.016 to 0.046 mg/kg, injected twice daily for the entire course of treatment and the rats showed no marked symptoms of toxicity. 3. A short course of treatment with 0.230 mg/kg/D divided in 4 equal doses and given but 4 days, resulted in complete regressions of the tumors in all of the animals treated when the therapy was begun 8 days after the implantation but this method did not give equally good results in another group treated 11 days after implantation. In this case it was necessary to institute a second course of treatment with 0.052 mg/kg, twice daily for 34 days to obtain complete regression of the tumors in 50% of the animals. 4. All of the rats in which the tumor regressed completely were then resistant to further implants of the tumor. 5. Rats that were cured lived for more than a year without the tumor reappearing. In comparison with the controls they were normal. 6. No gross manifestations of toxicity were seen with the therapeutic doses of TEM used. 7. There was no evidence of carcinogenicity of TEM in the therapeutic doses used in the Sprague-Dawley rats up to one year after the end of the period of therapy.

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Received June 13, 1952. P.S.E.B.M., 1952, v80.

A Critical Examination of the Histochemical Staining Technic for Ascorbic Acid. (19655)

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The acid silver nitrate method for the identification and localization of ascorbic acid in tissues has been used extensively since its introduction by Gough and Zilva(1). Some workers(2-5) claim good specificity and precise localization, although they admit that a negative reaction does not exclude the presence of the vitamin. Others(6-8) do not consider precise localization to be possible by this technic, but offer no or inconclusive evidence for their contention.

In the study to be presented, the sensitivity, specificity, and localizability of the histochemical method for ascorbic acid have been investigated by test tube and model experiments and by the use of animal tissues.

Experimental. 1. A solution of acetic acid and silver nitrate was added to the test substances in centrifuge tubes. The precipitate was washed and the unreduced silver removed by thiosulfate or ammoniacal thiosulfate solution. A large number of substances was tested, and positive reactions (a precipitate of metallic silver) were obtained with ascorbic acid, 3,4-dihydroxyphenylalanine, hydroquinone, and a mixture of calcium and magnesium

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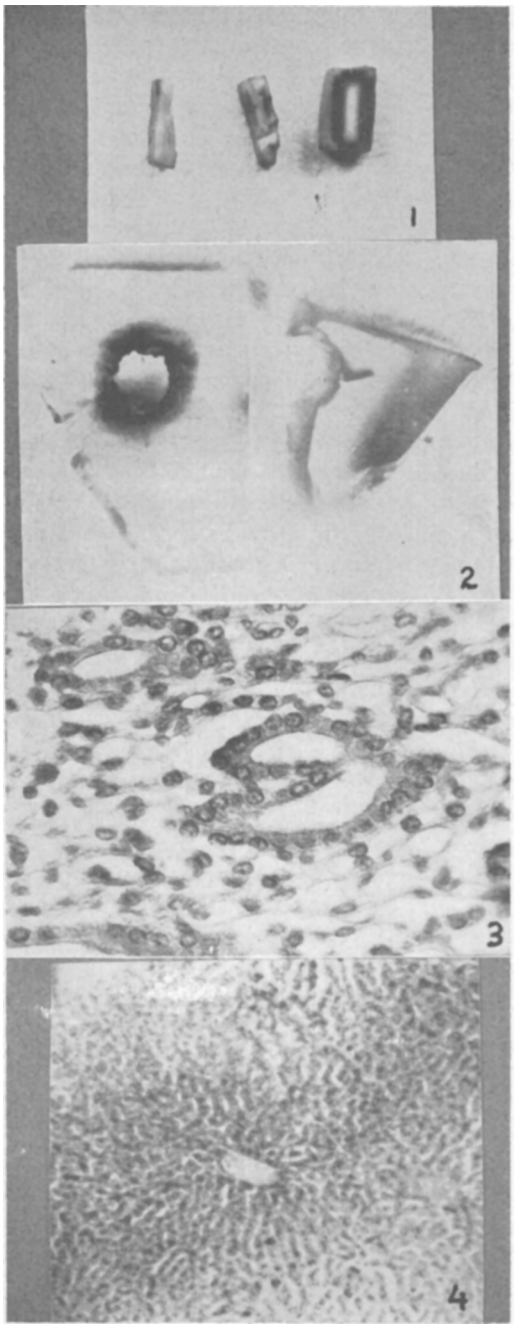

FIG. 1. Ascorbic acid-free gelatin blocks on which the acid silver nitrate histochemical reaction has been performed. Thickness of the blocks and duration of the pre-thiosulfate wash, from left to right, are: 1.5 mm and 30 min, 1.5 mm and 10 min, and 2.5 mm and 10 min. The thiosulfate artifact is demonstrated.

FIG. 2. Gelatin model with ascorbic acid present in the central core (removed) and absent from remainder at start of experiment, demonstrating diffusion artifact (ascorbic acid-free control on right).

FIG. 3. Normal guinea pig kidney, high power (45× oil immersion objective), paraffin section, demonstrating silver impregnation without visible granules.

FIG. 4. Supersaturated guinea pig liver, low power (10× dry objective), paraffin section, showing band of more intense silver deposition (Liesegang ring).

phosphates and carbonates, using neutral thiosulfate to remove unreduced silver. In addition, acid thiosulfate itself, when used as the test substance, was observed to precipitate metallic silver. Using ammoniacal thiosulfate to remove the excess silver nitrate, ascorbic acid, 3,4-dihydroxyphenylalanine, hydroquinone, glucose-6-phosphate, alpha-naphthol, and ribose produced a positive reaction.

2. The addition of gelatin to ascorbic acid solutions had 2 effects: a) the development of color due to reduced silver was greatly delayed; and b) the reduced silver tended to remain in a fine colloidal suspension instead of forming a granular precipitate.

3. The effect of glutathione has again been confirmed by means of spot plate reactions using various mixtures of ascorbic acid, gelatin, and glutathione. In addition to decreasing the sensitivity of the reaction, however, glutathione was observed to delay the appearance of a visible precipitate.

4. Formalin fixed, thoroughly washed gelatin blocks were placed in the acid silver nitrate reagent, washed, and "fixed" in thiosulfate. Unless washings were thorough, acid thiosulfate reduced the silver during the succeeding step (Fig. 1). The addition of ascorbic acid-free serum to the gelatin greatly increased the tendency to diffuse silver impregnation.

5. Holes bored in gelatin blocks were filled with ascorbic acid-carmine red-gelatin solution and the histochemical reaction applied. Diffusion was indicated by the precipitation of silver far beyond the limit of the ascorbic acid-containing central core (Fig. 2).

6. The acid silver nitrate reaction was applied to tissues from normal, scorbutic, and "supersaturated" guinea pigs, along with correlative biochemical studies(9). Fresh frozen, direct mounted sections(10) and tissue blocks

TABLE I. Comparison of Tissue Level of Ascorbic Acid with the Histochemical Reaction.

Organ	Concentration (mg %) Fresh frozen	Paraffin
Histochemically negative		
Adrenal	19	12.5, 9
Testis	16, 29	18
Kidney	15, 34	15, 26, 34
Liver	71, 36, 37	71, 33, 28
Histochemically positive		
Adrenal	127, 61	80, 61
Testis	45	45, 29
Kidney	214	217
Liver	105	109, 37, 33
Bone		2.6, 3, 31, 2.3

were used in the histochemical reaction. The data are listed in Table I. As shown in the table, bone is always silver impregnated when neutral thiosulfate is used following the reaction. Other silver artifacts are seen, including brown to black uniform impregnation of connective tissue of some kidney and testis blocks, the internal elastic lamina of the arteries of one animal, and basement membranes, cell walls, nuclear membranes, and even chromatin of liver, kidney, and testis of some animals (Fig. 3).

Many blocks of tissue showed waves of positive reaction suggesting the Liesegang ring phenomenon (Fig. 4). With fresh frozen sections, the reaction was weaker and more diffuse than with tissue blocks, and was often negative in thinner and positive in thicker areas.

Discussion. The test tube and model experiments and animal observations have been observed to essentially corroborate each other as enumerated below. 1. All bones, regardless of ascorbic acid content, retain silver ions tenaciously for gradual spontaneous and/or photoreduction. The mixture of calcium and magnesium phosphates and carbonates acted similarly in the test tube. 2. High concentrations of ascorbic acid are needed for the formation of a granular precipitate in the presence of tissue proteins as well as in the presence of gelatin. 3. The reduction of silver ions to metallic silver by acid thiosulfate is observed in both tissue and gelatin blocks when inadequately washed following the reaction. 4. Diffusion is prominent as demonstrated by the results with fresh frozen sections, by the waves of positive reaction (Liesegang rings) in tissue blocks, and by the models of Exp. 5. 5. Silver impregnation artifacts occur despite meticulous care in technic. 6. Although the use of ammonium hydroxide (11) to remove the excess silver salts following the reaction minimizes the bone artifacts, it greatly decreases the specificity of the reaction by converting to the more easily reducible alkaline silver nitrate.

Summary. The specificity, interfering artifacts, sensitivity, and localizability of the acid silver nitrate reaction for ascorbic acid were studied by means of test tube and slide reactions, model experiments, and histological methods. Whereas the specificity of the reduction reaction is reasonably good, interfering artifacts are numerous and are difficult to avoid. Protein and glutathione greatly reduce the sensitivity and velocity of the histochemical reaction. Gross diffusion artifacts render the test useless for localization on a cytological level.

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Received June 16, 1952. P.S.E.B.M., 1952, v80.