Some Properties of 2,3,5-Triphenyltetrazolium Chloride and Several Iodo Derivatives[1]

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The vital staining properties of "tetrazole" (2,3,5-triphenyltetrazolium chloride) (*4*) and of its various derivatives have been under investigation in this laboratory for some time (*1*). An observation of partially selective staining action on sunflower tumor tissue has led to consideration of compounds in this series as neoplasm indicators, of iodo derivatives for quantitative estimation of tumor mass, and as a means of localized application of organically bound radioiodine. Various derivatives are being synthesized for possible extension of their utility in oncology, germinability, enzymology, sperm fertility, and in other applications. Two reports which have appeared from other laboratories concern behavior of 2,3,5-triphenyltetrazolium chloride (*8*) and of the 3-*p*-iodophenyl analogue in animal tumors (*7*). While such compounds serve partially as selective tumor stains, radioiodine injected in this form has not accumulated selectively in animal tumors tested (*6, 7*).

Variations noted in germinability tests (*3*) may be explained in part by the finding that whereas the triphenyltetrazolium chloride is readily prepared by the usual procedure (*9*), purification from HCl solution followed by drying in air yields the di-(tetrazolium chloride) hydrochloride hydrate. Drying at 105°–110° C for 5 hr reconverts the hydrochloride hydrate to the chloride. The added acidity due to the HCl may affect the staining ability of this material, since this property has been found to be pH-sensitive.

The tetrazole employed here has been prepared by the usual procedure (*9*) except that an amount of butyl nitrite equimolar to the isoamyl nitrite previously employed has been used.

When 2,3,5-triphenyltetrazolium chloride of mp 243° C (with decomposition) was dissolved in ten parts of warm water, precipitated with three parts of concentrated HCl solution, and allowed to dry in air, silky needles of mp 180° C with preshrinking were obtained. Some samples exhibited only partial melting. *Analysis.* Calculated for $(C_{19}H_{15}N_4Cl)_2 \cdot HCl \cdot H_2O$: N, 15.48; Cl, 14.69. Found: N (Dumas), 15.70, 15.43; Cl, 14.70, 14.58.

When a sample of this compound was dried at 105°–110° C, crystals of mp 243° C (with decomposition) were recovered. *Analysis.* Calculated for $C_{19}H_{15}N_4Cl$: N, 16.74; Cl, 10.59. Found: N, 16.77, 16.69; Cl, 10.54, 10.60. The hydrochloride hydrate could also be reconverted to the chloride by solution in alcohol and precipitation with ether. Simple washing with ether did not effect the conversion.

More rapid and more intense staining by iodo tetrazoliums (*2*) than by the parent compound was observed in numerous experiments. In corn germinability tests, the times for first visual recognition are noted in Table 1. The solutions employed were 0.05% by weight and were read by an observer unacquainted with the contents of the solutions. The corn was a sample of high germinability. The nitro compound proved not only to be the most rapidly reactive of the group, but also permitted more rapid visual differentiation of the tissues within the embryo.

TABLE 1

TIME FOR REDUCTION OF TETRAZOLIUM SALTS BY CORN KERNELS

Tetrazolium chloride	First perceptible coloration (min)	Coloration equivalent to 30-min staining by 2,3,5-triphenyltetrazolium chloride (min)
2,3,5-Triphenyl	7	30
2,5-Diphenyl-3-(*p*-iodophenyl)	3-4	15
2,3-Di-(*p*-iodophenyl)-5-phenyl	3	10
2-(*p*-Iodophenyl)-3-(*p*-nitrophenyl)-5-phenyl	2	4

Stained sections of sunflower stalk and tumor are shown in Fig. 1. The appearance at 1 hr indicates virtually complete visual specificity in reaction. After 4 hr the appearance of formazan in normal tissue is definitely observable. In color photographs or in actual observation, the contrasts between control and stained sections are more striking. Since these phenomena are based on differential rates of reaction, precise control of test conditions as well as of substituent groups in the compound may be critical in some of the applications of tetrazolium salts.

There were also differences in the actual colors produced. The triphenyl salt upon reduction gave a characteristic pink to carmine color. With the iodo derivative, the red was more pronounced. The diiodo compound gave a more violet-red color, and the color produced by reduction of the iodonitro compound was brownish.

Miscoscopic examination of the stained sections showed considerably less diffusion of color with the three iodine-containing derivatives. More details could be observed in stained parts. This was especially true of the iodonitro compound. Because of this property, as well as the rapidity of reduction, this substance may have special value as a biological stain.

Another observation of interest was the photoreducibility of tetrazolium salts by strong reflected sunlight in the presence of some media and the inhibition of such photoreduction in other media. This was first noted in a search for growth inhibitors. Bits of sunflower tumor

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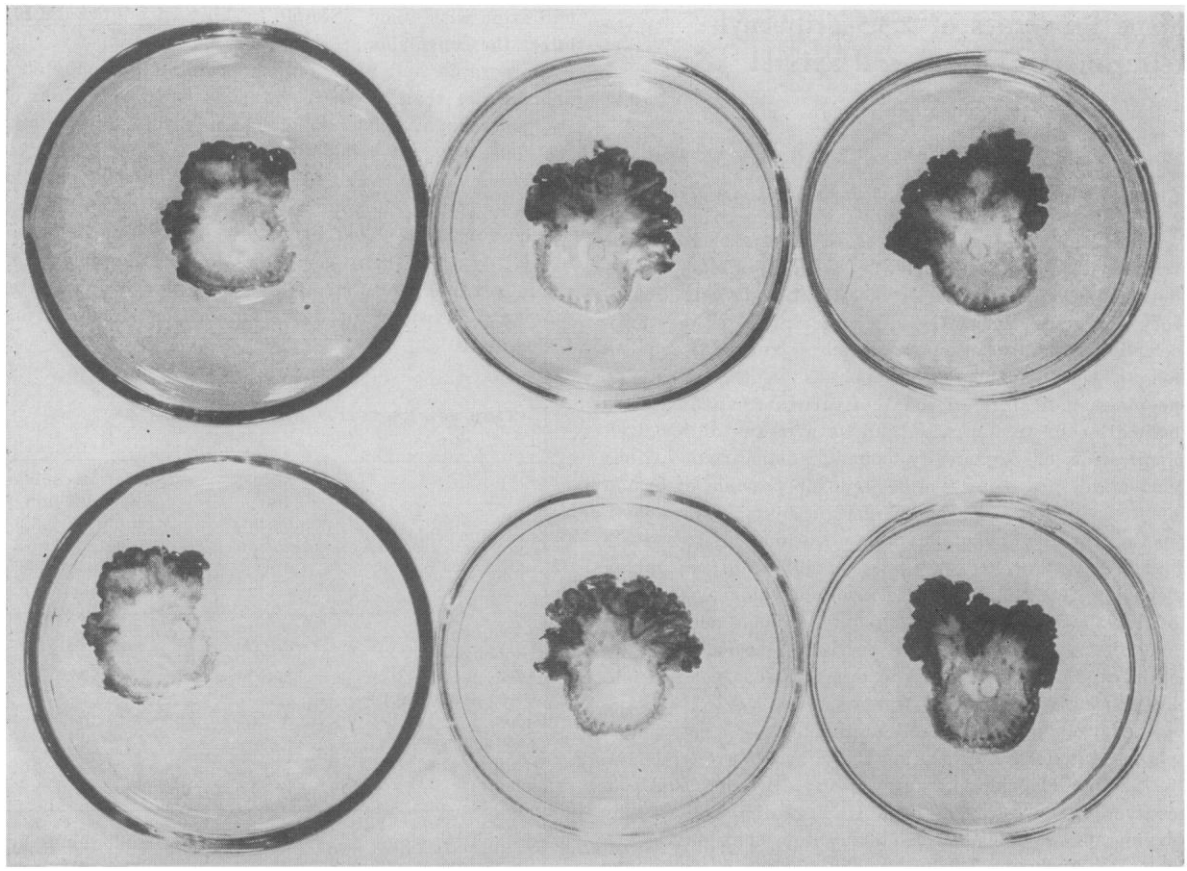

FIG. 1. Photographs of stained sections of sunflower stalk and attached tumor (induced by *Phytomonas tumefaciens*). Sections at the left are controls; those in the middle were stained with tetrazole and those at the right with monoiodotetrazole. The three top sections were stained for 1 hr and the three at the bottom for 4 hr.

tissue were nourished on an agar medium containing 2,3,5-triphenyltetrazolium chloride. The visually colorless medium turned carmine with photographic speed when exposed to sunlight. Similar results were observed in the bacteriological medium of Kuiken *et al.* (*5*); testing various portions of it revealed that the phosphates mixture possessed this property especially (Table 2), and the effect may therefore be one that is particularly pH-sensitive. The presence of small amounts of riboflavin were found to inhibit the reduction.

TABLE 2

EFFECTS OF DIRECT SUNLIGHT AND OF RIBOFLAVIN UPON THE PHOTOREDUCTION OF 2,3,5-TRIPHENYLTETRAZOLIUM CHLORIDE

Solution	Appearance after 5-min exposure
Tetrazolium solution*	Faint pink
Tetrazolium solution + phosphates†	Dark red
Tetrazolium-phosphates + 0.05 μg of riboflavin	Pink
Tetrazolium-phosphates + 0.5 μg of riboflavin	Faint pink
Tetrazolium-phosphates + 5.0 μg of riboflavin	Colorless
Tetrazolium-phosphates + 25 μg of riboflavin	Colorless

* Fifty mg of 2,3,5-triphenyltetrazolium chloride in 5 ml of water.

† Five hundred μg each of KH_2PO_4 and K_2HPO_4 added.

As a means of determining the approximate photochemical threshold, plastic boxes of four different colors (red, green, yellow, blue) were made. These were placed over the Petri dishes containing the tetrazolium solution to which the phosphate solution was added. At the end of 10 min the solution under the blue box was almost as red as the control which was covered with glass. The solutions under the yellow, green, and red boxes were not appreciably affected. At the end of 1 hr the control and the solution under the blue box were very dark red. The solutions under the green and yellow boxes were uncolored, whereas that under the red box was a very faint pink.

Spectral transmission curves were determined for the plastics used by inserting samples into the light path of a Coleman Model II spectrophotometer. From the curves it was apparent that the threshold must lie near 450 mμ.

Tests made simultaneously on solutions of triphenyltetrazolium chloride, and on the iodo, diiodo, and iodo-

nitro derivatives showed that the triphenyltetrazolium chloride is most subject to photoreduction in sunlight. A solution of the latter compound was definitely pink after 10 min in direct sunlight. At the end of 1 hr, the iodo derivative was barely pink, whereas the other two compounds showed no pink coloration.

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Use of Dyestuffs for Determining the Activity of Proteolytic Enzymes

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Numerous investigators (*1*) have shown that native albumin has an affinity for simple dye anions, whether the protein is on the acid or alkaline side of its isoelectric point. This is usually indicated by a spectral change when the protein is added to an aqueous solution of the dye. Pepsin, as well as other proteolytic enzymes, shows practically no combining capacity for these dyes. This fact suggests a new method of determining activity of proteolytic enzymes which may offer advantages.

Klotz (*2*) has shown that common denaturants, such as sodium hydroxide and heat, cause bovine serum albumin to lose its binding ability for anionic dyes. We have found that the action of proteolytic enzymes upon this native protein produces the same effect. Thus a change in the structure of the albumin molecule due to denaturation or fission of its peptide linkages can be detected in the presence of an enzyme. Quantitative activity measurements may be made by estimating the concentration of the native protein in the presence of the proteolytic enzyme as a function of the time, using an empirical curve as is usually done in spectrophotometric analysis. It should be emphasized that this procedure alone will not indicate the state of the protein that has been acted upon by the enzyme. The method is based on measuring the quantity of native protein remaining in solution, whereas the usual activity determination is based on an analysis of the products of hydrolysis.

Fig. 1 shows the effect of a 0.1% pepsin solution upon a 0.2% bovine albumin[2] solution at pH 5.4 in the presence of a constant concentration of the dye, orange I. The extinction is plotted as a function of the wavelength at various time intervals. The cuvettes containing the reaction mixture were kept in the housing of the Beckman quartz spectrophotometer throughout the experiment. No attempt was made to regulate the temperature of the solution, which was about 37° ± 2° C. It will be seen that addition of bovine albumin to the dye causes a depression of about 20% in the absorption maximum, which is in keeping with the data reported by Klotz. Upon addition of crystallized pepsin, the optical density of the reaction mixture in the presence of the dye approaches that for the dye alone.

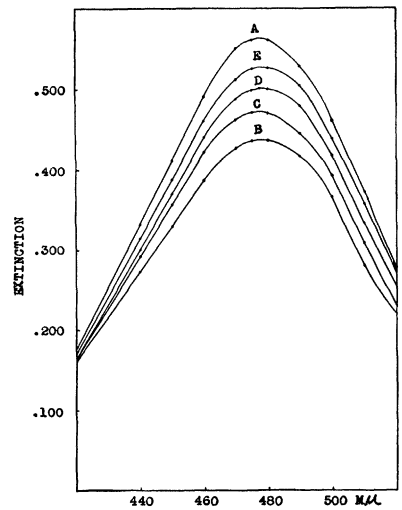

FIG. 1. Effect of 0.1% pepsin on 0.2% albumin in 2.0×10^{-5}M orange I at pH 5.4 (phosphate buffer). A—Dye alone. B—Dye + albumin. C—Dye + albumin + pepsin after 25 min. D—Same as C after 62 min. E—Same as C after 195 min.

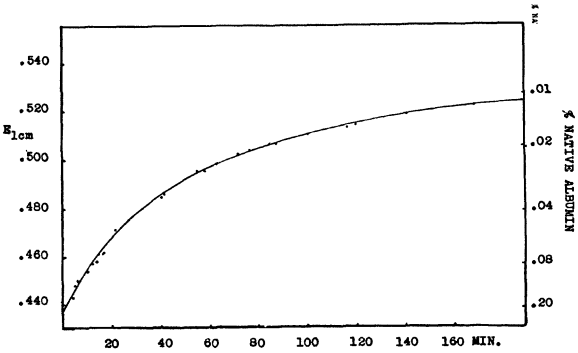

FIG. 2. Optical density at 475 mμ for orange I (2.0×10^{-5}M) in presence of 0.2% albumin and 0.1% pepsin at pH 5.4.

The values of the optical density of the reaction mixture at 475 mμ are plotted as a function of the time in Fig. 2. The values for the concentration scale are on

[1] The interest and suggestions of Prof. A. F. Thomas are gratefully acknowledged.

[2] Crystallized bovine albumin and crystallized pepsin (porcine mucosa) were used in preparation of solutions. These were purchased from the Armour Laboratories, Chicago, Illinois.