Survey of Factors Responsible for Reduction of 2,3,5-Triphenyltetrazolium Chloride in Plant Meristems

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Several workers have reported in this journal that dehydrogenase enzyme systems may be responsible for the reduction of 2,3,5-triphenyltetrazolium chloride. Mattson, Jensen, and Dutcher (*1*) suggested that the reduction of tetrazolium is caused by dehydrogenase systems requiring coenzymes I or II. It is possible for this compound to act as an electron acceptor for many pyridine nucleotide dehydrogenases. It was found that one of these holoenzymes, glucose dehydrogenase–coenzyme I, in the presence of its substrate, reduces the salt at pH 6.6 (*1*). It was found that tissues heated to 82° C or higher lose their ability to reduce tetrazolium (*1, 2*). Kun and Abood (*3*) found that tetrazolium is an indicator of succinic dehydrogenase activity of tissue homogenates. Fred and Knight (*4*) reported a lack of specificity for inhibitors. These workers also found that aeration by shaking retarded reduction, possibly because it raised the redox potential over −0.08 v, or because oxygen competed with the indicator. Kretovich (*5*) observed that there is a close correlation between loss of dehydrogenase activity of embryos and loss of viability, and that the dehydrogenase system of wheat embryos is activated by hexose di- and monophosphate, among other hydrogen donors. Other workers have reported that the sites of reduction of tetrazolium were also sites of reactions for phosphate ion (*6*).

In conjunction with a survey of tissues that reduce 2,3,5-triphenyltetrazolium chloride (*7*), the writer conducted experiments to determine the factors and substances responsible for the reduction of the indicator in normal plant tissues. The present study describes the results of the application of a series of enzymatic or metabolic inhibitors on the reduction of the tetrazolium salt to the red insoluble formazan in plant meristems. The effect of buffering these inhibitors was also investigated. Studies were made with 1-(4-chloromercuriphenylazo)-naphthol-2, which has a high specificity for sulfhydryl groups. Comparisons were made between the staining pattern obtained with this reagent and with tetrazolium.

A series of inhibitors (1% aqueous solutions) were used in a study of reactions in embryos of *Zea mays*. *Zea mays* seeds were split longitudinally, and a thin tangential section was cut from one of the exposed surfaces of the embryo. The sections were then placed in a spot plate and the test inhibitor added for 5 min. The sections were washed with water and placed in a 1% aqueous solution of tetrazolium. Readings were taken on the tissue samples after periods varying from a few minutes to several hours. The method of application of the buffer solutions (McIlvaine's standard) was similar to that of the inhibitors. The buffers were added to the tissue samples and allowed to stand for 5 min. The buffers were removed, and a 1% solution of tetrazolium was added to the test materials. The pH determinations were made with a Beckman pH meter.

The inhibition studies revealed four categories of inhibition (strong, medium, weak, and no inhibition). The strong inhibitors were benzaldehyde, ethyl alcohol (80%), iodoacetic acid, pyruvic acid, salicylaldehyde, and thioglycollic acid. The medium inhibitors were 2,6-dinitrophenol, ethyl alcohol (60%), hydroxylamine hydrochloride, and succinic acid. The weak inhibitors were benzidine dihydrochloride, chloroform, ethyl alcohol (40%), phenyl mercuric chloride, and thiourea. Those showing no appreciable effect on the reduction were coumarin, 2,4-dichlorophenoxyacetic acid, ethyl alcohol (20%), ethyl carbamate, potassium cyanide, sodium arsenite, sodium azide, sodium fluoride, and sodium pyrophosphate. Ethyl alcohol inhibited reduction proportionally to the concentration of the alcohol. The aldehydes probably prevented reduction by forming a mechanical barrier to the entrance of tetrazolium. Pyridine is a weak base and may possibly be an enzymatic inhibitor. Although 2,4-dichlorophenoxyacetic acid, in certain concentrations, has an inhibitory effect on respiratory functions, it has no effect in inhibiting the reduction. The reduction was strongly inhibited by 2,6-dinitrophenol, which has been reported to remove the coupling between respiration and phosphorylation. Coumarin, an inhibitor of sulfhydryl groups, had no apparent effect on the reduction. Sodium pyrophosphate, a specific inhibitor for succinic dehydrogenase, had no effect on the reduction. Several of the strong inhibitors are acids, and the pH values are far below the minimum value for normal reduction to take place. The approximate optimum value for the tetrazolium reduction in normal tissues is from pH 6.5 to 7.5. It was found that viable embryos stained intensely from 8.0 to 6.6. From the latter value to 6.0 the staining became progressively paler in color, and little reduction was evident at pH 5.0. On buffering the acid inhibitors at neutrality, reduction was observed in most cases. Acid inhibition probably results from an increased hydrogen ion concentration and not from a selective inhibition of some cellular component.

Bennett (*8*) synthesized a sulfhydryl reagent, 1-(4-chloromercuriphenylazo)-naphthol-2, in the hope that it would retain the high specificity for sulfhydryl possessed by phenyl mercuric chloride, and that the sites at which it combines with sulfhydryl protein in the tissue might be visualized directly under the microscope. The sulfhydryl reagent was used in a study of the sulfhydryl protein pattern in *Zea mays* embryos. A solution of the reagent in toluene was added dropwise to embryo slices that previously had been killed and dehydrated in an alcohol series. Within an hour the vascular traces of the embryo slices were stained, and 24 hr later the embryo slices were stained throughout. The sulfhydryl pattern obtained was identical

with that of the tetrazolium pattern of *Zea mays* embryo slices. Attempts were made to inhibit the sulfhydryl reaction with the tetrazolium reduction inhibitors. It was found that several of the tetrazolium reduction inhibitors also blocked the sulfhydryl groups. Embryo slices treated with thioglycollic acid gave no sulfhydryl pattern, iodoacetic acid resulted in a weak reaction, and 2,4-dinitrophenol in a medium reaction.

It appears highly probable that dehydrogenase enzyme systems are responsible for the oxidation of various substrates and the concomitant reduction of tetrazolium to formazan. The reducing agent is heat-labile, but remains undamaged by freezing. It has also been observed by the author that homogenized tissues give a much weaker reaction. The classic experiments of Thunberg demonstrated that dehydrogenases are responsible for reduction of reversible redox dyes. However, because of the lack of specificity for the reaction, as shown by inhibition studies, it is probable that no one reductase system is responsible for the characteristic reduction in plant tissues. It seems more likely that a general redox potential level, maintained by the operation of several physiologically active systems, brings about the reduction of tetrazolium.

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The Relationship of Acoustical Energy to the Lethal Effects of Ultrasonic Vibrations on *E. coli*[1]

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In a previous paper (*1*) it was shown that the rate of destruction of *E. coli* by ultrasonic vibrations at 400 kc was influenced significantly by the environmental temperature. Many other factors undoubtedly influence the germicidal properties of these vibrations, one of which should be the energy input to the sample under treatment. Accordingly, it was desirable to investigate this factor and to determine its significance.

Using the apparatus previously described (*1*), a series of tests was conducted wherein the energy input to a sample containing an aqueous suspension of *E. coli* was varied by controlling the variable transformer in the electronic driving circuit.

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TABLE 1

PERCENTAGE OF *E. coli* SURVIVING ULTRASONIC VIBRATIONS AFTER VARIOUS EXPOSURE TIMES AND AT VARIOUS ENERGY INTENSITIES AT 15.5° C

Exposure duration (min)	Energy intensity, acoustical w/cm²								
	4.8	5.8	7.2	9.1	11.5	14.4	18.6	24.0	31.3
3	97.5	100.1	88.6	83.3	88.6	74.7	71.5	73.6	76.9
6	85.4	85.0	75.7	73.3	71.8	56.4	52.8	58.1	59.4
15	65.1	66.5	54.5	50.5	40.2	22.4	15.9	17.9	26.4
25	53.2	46.1	35.3	36.3	21.5	13.2	10.5	7.1	9.9
40	39.1	34.1	20.9	15.9	9.24	5.04	3.41	1.77	2.69
60	21.0	18.0	9.87	4.11	0.10	0.42	0.08	0.34	0.62

In order to determine the amount of acoustical energy that reached the sample, a Siemens power meter was suspended in the oil bath at the same position with regard to the crystal that the sample normally occupied. Varying amounts of acoustical energy were beamed to the meter by changing the setting of the variable transformer in the driving circuit. The readings of the power meter were correlated with readings obtained simultaneously from a voltmeter inserted in the electronic circuit across the crystal. In this manner the voltmeter was calibrated to read in terms of the acoustical energy applied to the sample. Since the power meter used did not cover the entire range of energy intensities available from the generator, it was necessary to extrapolate the calibration curve for high intensity energy inputs.

Altogether, sixty-seven 1-hr runs were made on *E. coli* suspended in buffered water. The suspension was prepared by introducing 1 ml of a 24-hr broth culture of *E. coli* into 100 ml of sterile buffered water. The environmental temperature for all the observations was maintained at 15.5° C. The initial concentration of viable cells in all cases was approximately 80,000/ml. Statistical analyses of the results obtained were made and are presented herewith (Table 1 and Fig. 1).

It is apparent from Fig. 1 that the killing curve is essentially logarithmic at all energy intensities. In some cases, toward the end of the run, the curves tend to level out somewhat, but generally speaking the straight-line relationship applies. In those cases where the rate of killing showed a slight curvature, the initial killing rate, obtained during the first 30 min, was chosen as the characteristic rate for that particular energy intensity. It may also be concluded from Fig. 1 that, although an increase in energy intensity (within limits) results in an increase in the killing rate, an intensity is finally reached which yields the maximum killing rate; and that further increases in energy intensity result merely in reduced lethal effects. This is clearly shown in Table 2 and Fig. 2.

Fig. 2 was obtained by plotting the killing rate constant, as determined by the slope of the killing curve,