# THE USE OF 2,3,5-TRIPHENYL TETRAZOLIUM CHLORIDE IN THE DETECTION OF LOW TEMPERATURE INJURY TO HARD RED WINTER WHEAT DURING DORMANCY AND DURING TRANSITION FROM DORMANCY TO ACTIVE GROWTH

by

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# INTRODUCTION

For centuries, man has observed injury wrought to plants by low temperatures. The conditions causing this injury are usually fall or spring freezes, very low winter temperatures, or, in experimental work, artificially produced low temperatures. It is extremely difficult to evaluate cold injury in most cases, for it is closely associated with such factors as drought injury, insect injury, and plant disease injury, and it is extremely difficult to separate one from another.

Winter wheat is one of the principal crops affected by low temperatures. According to estimates made by the United States Department of Agriculture (40), an average of approximately 4 percent annual reduction of wheat yield occurred as a result of cold injury in the years 1909 to 1923. By the same authority, an average annual reduction in yield of approximately 20 percent occurred during the same 15-year period as a result of all climatic factors combined. From these estimates it may be seen that cold injury is of major importance to the agriculture of our country.

Salmon (34) recognized four general types of injury effected by low temperatures: (1) heaving or exposure of underground portions of plants by alternate freezing and thawing (2) smothering or damage due to oxygen deficiency resulting when ice covers the ground (3) physiological drought, occurring due to inability of roots to take water from frozen soil (4) direct effects of low temperatures on plant tissues.

Although this "direct effect of low temperatures on plant tissues" has been the object of extensive research extending over many years and many theories have been advanced as to the causes, it still remains a controversial issue. Levitt (26), in reviewing pertinent research, other

than Russian, conducted on cold injury, considered that there are three main mechanisms causing cold injury to plants, namely intercellular ice formation, intracellular ice formation, and rapid thawing. Intercellular ice formation is considered by him to be the most common in occurrence of the three. The formation of ice, both intra- and intercellular, appears to result in mechanical injury and/or dehydration of the protoplasm, both of which can lead to death of cells involved. Injury by sudden thawing seems to be due to mechanical damage to the protoplasm resulting when cell walls and protoplasm expand at different rates because of differences in permeability to water. The views of Curtis and Clark (8) are in essential agreement with those of Levitt.

It is often quite difficult to detect cold injury in winter wheat by direct observation of the plants, particularly when the wheat is dormant and temperatures remain below freezing. Reports are annually encountered of wheat that survived the winter and then died soon after the beginning of spring growth. Examination of such wheat plants usually shows damage to inner parts that was not externally apparent in the dormant stage. This type of damage is usually attributed to cold injury. Agronomists engaged in wheat production have long sought a rapid, easily applied test which would indicate cold injury to winter wheat. Such a test would have many advantages. It would eliminate the waiting period now required to determine the extent of cold injury and thus facilitate yield prediction, influence decisions as to abandonment, and accelerate experimental work.

This study was concerned with the development of such a test; i.e., a test for the viability of hard red winter wheat plants that have been subjected at certain developmental stages to abnormally low temperatures. In the strict sense, viability means "the ability to live", but when used

in reference to wheat or other economically important plants it must be qualified to mean the ability to live and produce a profitable yield, for plants may live and still be injured to the point that they do not yield normally. A viability test need not be limited to plant tissues, nor to cases involving cold injury and would obviously be more useful were it not so limited. There are many other uses for viability tests in both plant and animal research. Reference to the Review of Literature, p. 5, or to Smith (37) will illustrate some of the many specific applications of viability tests.

Most attempts to establish viability tests have been dependent upon either the increased permeability or the decreased reducing power of dead cells. The tests that have been developed have been for the most part, however, limited in scope and often not easily applied. The most promising rapid tests of viability have been staining methods involving the reduction of selenium or tetrazolium compounds. Selenium compounds have the disadvantage of being highly toxic and have received rather limited use. The tetrazolium compounds, however, show great promise in this respect.

This group is unique among organic compounds in that its members are colorless or relatively so in the oxidized state, but form brightly colored compounds called formazans upon reduction. This formation of formazans constitutes the basis for the use of tetrazolium compounds in viability testing. In 1941, Kuhn and Jerchel (19) observed that certain tetrazolium compounds were reduced by yeasts and garden cress and that these plants were stained deeply by the formazans produced by the reduction. This finding led to additional research with the tetrazolium compounds and they have proved to be effective as viability indicators in a variety of tissues. In all these tests, viability of the tissue was determined by the nature

of staining in the presence of solutions of tetrazolium salts.

All experimental evidence points to the assumption that reduction of tetrazolium salts by living cells is due to the presence of enzyme systems, namely dehydrogenases requiring di- and triphosphopyridine nucleotide, often designated coenzymes I and II. Thus, tissues having sufficient active dehydrogenase systems will reduce tetrazolium salts and become stained, while tissues not having sufficient dehydrogenases will not reduce tetrazolium salts and will not be stained.

The first workers to experiment with tetrazolium salts believed that they would prove to be a general test for viability; i.e., one that could be used in almost any situation. Lakon (22) in 1943 inferred that coloration of a cell by tetrazolium was a definite indication of its viability because necrotic cells remained uncolored. Other workers (17, 27, 28) considered this a rather strong inference in view of the meager research that had been conducted with tetrazolium salts. Mattson, Jensen, and Dutcher (28) summarize the current view when they state that in all likelihood the reduction of tetrazolium salts by enzymes of living cells cannot be considered a general test for life, but that the compounds will be utilized in many types of research involving differences in tissue viability. A summarization of the views expressed by Jensen and coworkers (17, 27, 28) is that a tissue which normally reduces tetrazolium salts is dead if it does not do so.

As stated earlier, the experiments herein reported had as their objective the establishment of a test to detect cold injury to hard red winter wheat at certain stages of development. Most of the work was conducted with wheat plants in the dormant stage, but limited studies were made on plants during the transition from winter dormancy to active growth.

In the planning and execution of the experimentation, these three criteria were primarily considered:

(1) The tetrazolium staining reaction occurring in normal, non-cold-treated wheat plants.

(2) The tetrazolium staining reaction occurring in wheat plants completely killed by low temperatures and how it differs from the staining reaction of normal, non-cold-treated plants.

(3) The tetrazolium staining reaction occurring in wheat plants which are injured, but not killed, by low temperatures.

## REVIEW OF LITERATURE

Like sulfanilamide and DDT, the tetrazolium compounds were known long before any important use was found for them. Most of the pioneer work was done in Germany. In 1894, von Pechmann and Runge (30) synthesized and described the properties of 2,3,5-triphenyl tetrazolium chloride (which will be called tetrazolium chloride hereafter in this paper except in a few instances). In 1941, Kuhn and Jerchel (19, 20), synthesized a number of tetrazolium salts and discovered that tetrazolium chloride was reduced to a deep red compound, triphenyl formazan, by living yeasts and garden cress. They observed that while reduction of the salt by yeasts and garden cress occurred in neutral solutions, the reduction of glutathione, ascorbic acid, and cysteine took place only above pH 9.0. Jerchel and Mohle (18) determined the reduction potentials of a number of tetrazolium compounds and studied the reduction process of those compounds.

As a result of experimentation with tetrazolium salts, Lakon (22), who had done extensive research with viability tests on the seeds of several

economically important plants, substituted tetrazolium chloride for the highly toxic sodium selenite in his "topographic method" for determining the germinating ability of seeds. This method consisted essentially of the designation of certain areas of the embryo of a given seed that must be stained red in the presence of tetrazolium chloride in order for the seed to be considered germinable. He was able to make a distinction between nonviability, viability, and germinability with this test, using seeds of corn, oats, wheat, rye, and barley.

In 1945, R. A. Dutcher, then in Germany as a scientific consultant with the Joint Intelligence Objectives Agency of the United States Armed Forces, observed the use of tetrazolium chloride as a test reagent for seed germinability and obtained publications on the subject that had been unavailable in the United States. Upon his return to this country he, with Mattson and Jensen (28), synthesized two tetrazolium salts and carried out preliminary tests on various types of viable and nonviable tissues. They confirmed the work of Kuhn and Jerchel with yeasts and garden cress and that of Lakon with various seeds, and found many other viable materials which reduced tetrazolium chloride, such as the fleshy parts of apples, oranges, and grapes, the gill area of mushrooms, carrot roots, white and sweet potatoes, young leaves, the stigmas and ovaries of certain pollinated flowers, bull sperm, and the blastoderm of hens' eggs. These tissues all reduced tetrazolium chloride at pH 6.9. They found that tissues heated to 82° C. or higher lose their ability to reduce tetrazolium salts. They further found that reducing sugars will reduce the salt, but only above pH 11.0. They studied the reaction involved in the reduction of tetrazolium chloride and obtained evidence to support their conclusion that reduction of the compound by living tissues is due to the presence of dehydrogenases requiring coenzymes

I and II. Jensen, Sacks, and Baldauski (17) used tetrazolium chloride in studying the dehydrogenases of corn embryos and reported many worthwhile observations on the cause and occurrence of tetrazolium reduction.

Porter, Durrell, and Roan (32) found good agreement between estimates of germinability obtained by the use of tetrazolium salts and standard germination tests with corn, wheat, rice, buckwheat, popcorn, soybean, and Bahia grass.

Cottrell (6) conducted tetrazolium chloride testing on prescaled seeds of cereals and compared the results with standard germination tests. Her results were within British legal requirements for accuracy. Smail (36) reported development of a tetrazolium chloride test for germinability of wheat, oats, and barley in which the seeds being tested were incubated at 43° C. in a 1 percent solution of tetrazolium chloride. His results were accurate with relatively new seed having a germinability above 60 percent. He considered, however, that the tetrazolium test was a "very rough index of germinability" on old seed with low germinability.

In a second paper, Cottrell (7) obtained good results with tetrazolium testing for germinability of wheat, barley, oats, peas, and vetch, but experienced difficulty in testing very small seeds such as those of certain grasses. Goodsell (14) developed a tetrazolium chloride test for detection of cold injury to seed corn. He found high correlation between the tetrazolium test and standard germination tests.

Flemion and Poole (10) tested seeds of seventeen families and fifty-eight species with tetrazolium chloride. They experienced difficulty in interpreting the staining in seeds of many species because of size and other factors. No cereals were included in their testing. Flink and Schweiger (9) discussed chemical tests for viability and compared them with

germination tests. They concluded that while the chemical methods are rapid, they fail to differentiate between "germinating energy" and "germinating power". Detailed applications of the tetrazolium test to barley, rye, and wheat were given.

Bennett and Loomis (3) conducted considerable experimentation with various concentrations of tetrazolium chloride solutions in testing for viability of cold-injured seed corn. They obtained highest accuracy with corn that had been stored for a time after freezing and that was fairly high in germinability. A consideration of the pH ranges of the tetrazolium reaction was included in their paper.

Lakon (23) published a paper in 1949 in which he corrected some erroneous interpretations of his work. He restated his "topographic method" for determining the germinability of seeds with tetrazolium chloride and included many worthwhile suggestions on the practical execution of that test. Brewer (5) reported the use of tetrazolium chloride as a test for heat damage in artificially cured peanuts. He found correlation between damage and loss of viability as determined by tetrazolium testing. Lederberg (24), a bacteriologist, while attempting to develop methods for finding dehydrogenase mutants, found tetrazolium chloride useful for detecting fermentative variants. The basis of this detection was differential staining of cultures grown on agar containing tetrazolium chloride. Fults, Schaal, and Michaelson (12), Huddelson and Baltzer (16), Fred and Knight (11), Harahara, Quittner, Goldman, and Antopol (29), and Wood (43) have also used tetrazolium chloride in bacteriological research. They have conducted studies on species differentiation, metabolism, parasitism, and various other aspects of that field.

Waugh (41) found that tetrazolium reduction occurred in the cambium layers of woody plants. No staining took place in boiled portions of these

tissues. Roberts (33) conducted a survey of the factors responsible for the reduction of tetrazolium chloride in plant meristems and concluded that this reduction is probably due to several physiologically active enzyme systems.

Considerable research has been conducted on the differentiation of normal and neoplastic animal tissues with tetrazolium salts. Some of the workers in this field were Straus, Cheronis, and Straus (39), Black and Kleiner (4), and Antopol, Glaubach, and Goldman (1). Gall (13) used tetrazolium chloride to demonstrate oxidation-reduction activity of bean tissue cultured in 2,4-dichlorophenoxyacetic acid. He observed that the concentrations of triphenyl formazan appeared to be at the mitochondria in the cells of the bean tissue. This observation has been reported by several workers.

Weiner (42), in a study of the effect of a large number of chemicals on tetrazolium chloride, reported development of a qualitative test for reducing compounds. This has proven to be a promising application of tetrazolium compounds, and tests of this type have been reported by Mattson and Jensen (27), Kun and Abood (21), and Seligman and Rutenburg (35).

Although tetrazolium chloride has proved to be a highly useful compound in many ways, it has disadvantages in certain types of work, particularly work involving animal tissues. Consequently, several new tetrazolium compounds have been developed in the past few years. These compounds have properties similar to tetrazolium chloride, but differ with respect to solubility, color of formazan produced, toxicity, and other properties. These new compounds have made possible a wider application of tetrazolium tests for reducing agents and tissue viability. Three of the newly developed compounds, blue tetrazolium, neotetrazolium chloride, and

neotetrazolium phosphate, have received appreciable use in bacteriological and medical research.

The latest development in the way of tetrazolium compounds is the use of these salts labelled with radioactive compounds. Atkinson, Malvin, and Fox (2) studied the action of tetrazolium chloride and several derivatives labelled with radioactive iodine in normal and neoplastic sunflower tissues and in seed corn.

From this review it may be seen that while the tetrazolium compounds have been used in scientific experimentation for only a few years, they have proven to be versatile and of great value, both in basic research and practical problems. Indications are that they will become more important.

## GROSS MORPHOLOGY OF THE DORMANT WINTER WHEAT PLANT

A general discussion of the gross morphology of the dormant winter wheat plant is pertinent to this study. The following descriptions are adapted from Percival (31) and Hayward (15) and are presented in terms of the dormant plants used in the experimentation. Brief mention will be made of the changes occurring in the spring transition from dormancy to active growth, as limited testing was carried out with plants during this phase of development. Plate I, Fig. 1 shows a diagrammatic representation of a dormant winter wheat plant.

The root system consists of a primary seminal system and a permanent adventitious one. The primary system consists potentially of five roots, a tap root and two pairs of laterals which arise in the region of the vascular plate of the scutellar node (a sixth may develop behind the epiblast). The usual number of primary roots to develop is three, but

the development of four or five is not uncommon. The primary system makes up a large part of the total root system of the dormant plant. The permanent root system consists of whorls of adventitious roots which arise from the lower nodal regions of the main stem and its branches near the soil level. These roots may begin to develop in the fall, but do not reach an appreciable length until growth resumes in the spring.

The basal internode (the hypocotyl) of the wheat plant is very short. The second internode varies in length with the depth of planting. This second internode is in the form of a thin rhizome. It terminates at a point approximately .5 inch below the soil surface. The plants used in this study developed from seed planted at depths of .5 to 1 inch below the soil surface and thus had rhizomes varying from .1 to .5 inch in length. The node immediately above the rhizome is known as the "tillering node". It gives rise to numerous adventitious roots.

Immediately above the tillering node are a number of very short internodes. The nodes in this region give rise to adventitious roots, leaves, and axillary buds. These axillary buds are capable of developing into short lateral stems. The short stems in turn bear axillary buds which are capable of developing into lateral branches of the third order, etc. By this process, known as "stooling" or "tillering", a large number of stems or "tillers" may develop on a single plant. Percival (31) states that tillering occurs when the temperature is above the minimum for growth. Thus it occurs at the highest rate in the fall and spring and almost ceases in midwinter. The wheat plants used in these studies varied considerably in extent of tillering, the number of tillers varying from three to sixteen. Most of the plants had from six to eight tillers.

In the dormant wheat plant, the terminal buds of the main axis and the

tillers are located approximately .25 inch above the tillering node and are thus below the soil surface. They remain below the soil surface during the dormant period. The region of short internodes immediately above the tillering node is commonly called the "crown" region and included the short, thick, main axis with its terminal and lateral buds and the axes of the tillers with their terminal and lateral buds (Plate I, Fig. 2).

The leaves of the wheat plant are alternate and two-ranked. Percival (31) lists four types of leaves in the dormant wheat plant: (1) The scutellum, which is present but nonfunctional, having been utilized as food early in development of the plant. (2) The coleoptile, which sheaths the epicotyl during its early development, and is present in the dormant plant, but is nonfunctional. (3) The first leaf of each lateral shoot, which is a prophyll somewhat resembling the coleoptile. It sheaths the lateral shoot until the second leaf emerges, then gradually dries and becomes nonfunctional. (4) The ordinary green foliage leaf, the main parts of which are the sheath and the blade. The sheath encloses the axis of the shoot and the younger leaves, being entire near the base, but open at its upper end. The blade develops faster than the sheath and is longer than the sheath in the dormant wheat plant.

In the dormant plants used in this experimentation, two to three leaf blades had emerged. As temperatures began to rise in the spring more blades emerged. Percival (31) states that when six leaf blades are visible on a shoot, both the stem and the head can be recognized within the plant. On this basis, the heads of the field wheat used in this study were formed around the middle to the last of March. About the first of April, appreciable elongation of stems began to occur. They elongated about .75 inch between April 1, 1952 and April 17, 1952. No testing was conducted after this date.

EXPLANATION OF PLATE I

Fig. 1. Diagrammatic representation of a dormant winter wheat plant.

a. Blade of foliage leaf
b. Prophyll
c. Sheath of foliage leaf
d. Adventitious root
e. Rhizome
f. Scutellum
g. Primary roots

Fig. 2. Face view of the portion of the dormant winter wheat plant used in tetrazolium chloride testing (designated "test section").

a. Leaves
b. Terminal bud
c. Main axis
d. Lateral axis (tiller)
e. Lateral bud
f. Adventitious root
g. Rhizome
h. Crown region

PLATE I

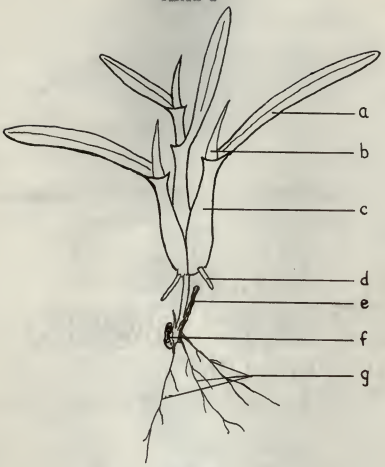



Fig. 1

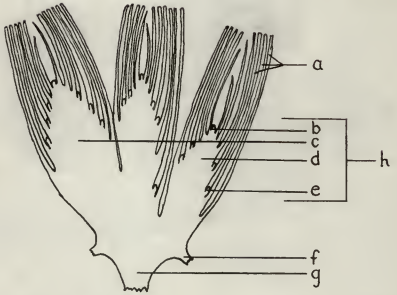



Fig. 2

Plate I, Fig. 2 represents the portion of the wheat plant utilized in all viability testing in this study. This section will be designated hereafter as the "test section". The rhizome, roots, and green, aboveground tissues were not included in the test section. Portions used included only white, underground tissues in which a stain could be readily seen, and included the main axis and lateral axes with their terminal and lateral buds, partial or entire sheaths of emerged leaves, and partial or entire unemerged leaves.

It should be emphasized that these plant portions contained both meristematic and permanent tissues, thus differentiating the testing reported in this study from that conducted by other workers with wheat embryos, which, with the possible exception of the scutellum, consist wholly of meristematic tissues.

## MATERIALS AND METHODS

### Tetrazolium Compounds and Solutions

Because several tetrazolium compounds were available, it was necessary to conduct preliminary testing to determine which compound was the most suitable for a viability test to detect cold injury to winter wheat plants. Such criteria as availability, cost, solubility, and staining properties were considered. The choice of a compound was made only upon the basis of the specific problem under consideration.

The compounds used in preliminary experimentation were 2,3,5-triphenyl tetrazolium chloride (tetrazolium chloride or TTC), neotetrazolium chloride (NTC), neotetrazolium phosphate (NTP), and blue tetrazolium (BT). Definite

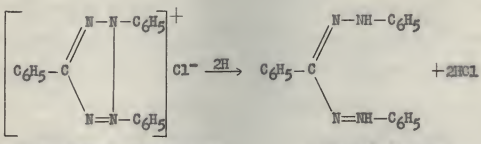
disadvantages were encountered with three of these compounds. Neotetrazolium chloride is only slightly soluble in water. Aqueous solutions were desired for simplicity in testing, and because water had no deleterious effect on the tissues being tested; hence, neotetrazolium chloride was eliminated from the study. Neotetrazolium phosphate and blue tetrazolium were eliminated because their staining reactions did not prove suitable.

Tetrazolium chloride proved to be acceptable with regard to the criteria considered in the choice of a testing compound and was used in all testing reported. Quantities of tetrazolium chloride were obtained from the Parmons Chemical Company, Farmington, Connecticut (no longer in existence), The Synthetical Laboratories, Chicago, Illinois, and the Montclair Research Corporation, Montclair, New Jersey. The commercial grade proved to be acceptable for the work herein and cost 30 to 50 cents per gram in the spring of 1952.

Tetrazolium chloride is obtained commercially as a fine, white, crystalline powder. This powder is light sensitive, turning yellow upon exposure to light. Samples were found to become bright yellow upon 48 to 72 hours exposure to full sunlight. If the compound is kept in a dark situation at room temperature, yellowing proceeds very slowly. This yellowing was not observed to interfere with the staining properties of the compound.

Tetrazolium chloride is an organic compound with a molecular weight of 334.8 and melts with decomposition at 243-245° C. It is readily soluble in water, chloroform, and alcohol but insoluble in ether. It forms a clear, colorless aqueous solution that is, according to Weiner (42), stable chemically. Tetrazolium chloride in the presence of a suitable reducing agent is reduced to the deep red compound, triphenyl formazan. Triphenyl formazan is insoluble in water and is chemically stable. Cottrell (6) reports the

following reaction for the reduction of tetrazolium chloride:



2,3,5-triphenyl tetrazolium chloride

triphenyl formazan

Tetrazolium chloride is reduced by a variety of agents. According to Weiner (42), the reduction is effected in acid solution by active metals and other strong reducing agents and in alkaline solution by certain sugars and other moderate reducing agents. The same author reports that in reactivity and selectivity in alkaline solutions, tetrazolium chloride is similar to Fehling's reagent, but the onset of reaction is more easily visible and requires no heating. Reducing sugars have been shown by Mattson, Jensen, and Dutcher (28) to reduce tetrazolium chloride only above pH 11.0. Kuhn and Jerchel (19) reported that cysteine, glutathione, and ascorbic acid reduce the compound above pH 9.0.

In contrast to these common non-living agents which reduce tetrazolium chloride only in alkaline solution, the salt is reduced by living tissues below pH 7.0. Considerable research has been conducted on the theory of tetrazolium reduction in living tissues. The salt readily penetrates living tissues, for crystals of the red formazan are formed inside the cells of tissues which reduce tetrazolium chloride. Mattson, Jensen, and Dutcher (28) found that tissues heated at 82° C. or higher lost the ability to reduce tetrazolium chloride, and expressed the view that tetrazolium reduction in living tissues is due to the presence of enzyme systems (which are inactivated by application of sufficient heat). Jerchel and Mohle (18) reported

the apparent reduction potential of tetrazolium chloride as -0.08 v. Mattson, Jensen, and Dutcher reasoned that since the reduction potentials of certain pyridine nucleotide dehydrogenases fall below -0.08 v., tetrazolium chloride could act as an electron acceptor for these enzyme systems and thus be reduced to triphenyl formazan. Reduction of tetrazolium chloride in living tissues is not thought to be accomplished by any one enzyme for Weiner (42) and Fred and Knight (11), in studies of agents inhibiting the reduction of this compound, reported lack of specific inhibitors for the reaction, and presented this as evidence against reduction by a specific enzyme.

Jensen, Sacks, and Baldauski (17) recently reported experiments in which reduction of tetrazolium chloride was effected by isolated dehydrogenases in the presence of their specific substrates at pH 6.6. They were prepared from corn embryos. The enzymes effecting reduction were glucose dehydrogenase, alcohol dehydrogenase, malic dehydrogenase, $\beta$-hydroxybutyric dehydrogenase, lactic acid dehydrogenase, 3-phosphoglyceraldehyde dehydrogenase, and $\alpha$-glycerophosphate dehydrogenase. They are all pyridine nucleotide dehydrogenases requiring DPN as a coenzyme. Isocitric dehydrogenase, a pyridine nucleotide dehydrogenase requiring TPN as a coenzyme, also reduced tetrazolium chloride, but did so very slowly. No reduction occurred upon omission of apoenzyme, coenzyme, or substrate. These findings strongly indicate that certain pyridine nucleotide dehydrogenases are an important factor in the reduction of tetrazolium chloride by living tissues. These workers consider that any tissue which normally reduces tetrazolium chloride may be considered dead when it no longer does so. This view forms the basis for most of the research conducted with tetrazolium chloride.

Various methods of preparing biological tissues for tetrazolium chloride

testing have been reported. In this study, satisfactory distribution of the compound for testing purposes required that dormant wheat plants be cut longitudinally through the center of the axes (Plate I, Fig. 2). These free-hand cuts were made with a safety razor blade. Some work was also done with thin sections cut from the face of the plant halves prepared in the above manner, but these proved unsatisfactory for macroscopic examination because of the lack of depth of color in staining.

Aqueous solutions of 0.5 percent, 1 percent, and 2 percent concentrations of tetrazolium chloride were used in the determination of the concentration most suitable for testing. The 0.5 percent solution did not give a suitable staining reaction and the 2 percent gave excessive precipitation of formasan. The 1 percent concentration was considered the most satisfactory and was used exclusively in this study.

Satisfactory staining of wheat plants occurred in solutions prepared from commercial tetrazolium chloride without adjustment of pH. The pH values of the solutions were measured with a Leeds and Northrup Universal pH Indicator. Freshly prepared solutions of tetrazolium chloride had pH values of 3.8 to 4.2. Immersion of wheat plants in these solutions changed the pH values to 6.6 to 7.0. Because reduction of tetrazolium chloride by reducing sugars and other non-living agents occurs only in alkaline solutions and since satisfactory staining of wheat plants occurred at the pH levels of solutions prepared from commercial tetrazolium chloride, no adjustment of pH levels was considered necessary.

Solutions of tetrazolium chloride were stored in the refrigerator in clear glass bottles until used. It was found impractical to keep solutions under these conditions for periods exceeding four to five weeks because of the growth of bacteria and fungi. Solutions used in testing were ordinarily

not kept for more than two to three weeks before use.

Tetrazolium chloride solutions could not be used a second time in the testing because of the continued precipitation of formazan from the solution after the plants were removed. Microscopic examination of the solution revealed heavy bacterial contamination, likely introduced during testing. Presumably, the continued reduction of tetrazolium chloride was caused by enzymes produced by these bacteria.

Aluminum caps used on certain of the vials reduced tetrazolium chloride and were replaced with corks.

## Cold Treatment

Artificially-produced low temperatures for cold treatments of wheat plants were obtained in the cold chamber at the Kansas State College Plant Research Laboratory and in the quick-freeze chamber at the Manhattan Ice and Cold Storage Company. Complete thermograph records were taken in the cold chamber at the plant research laboratory. Temperature readings were taken in the quick-freeze chamber at the Manhattan Ice and Cold Storage Company at the beginning and end of each cold treatment.

The first consideration in the cold studies was to determine the tetrazolium chloride staining reaction occurring in wheat plants killed by low temperatures. Killing was found to be accomplished in the freezing chamber by temperatures of -5° F. or below. There was no recovery among plants treated for twelve hours or more at these temperatures. Plants that had broken winter dormancy which were studied late in this investigation were killed by temperatures of 0° to 5° F. In the limited studies conducted with temperatures which injured, but did not kill, winter wheat plants, the

cold chamber at the plant research laboratory was used exclusively, because temperatures could be accurately controlled and recorded. Temperatures used in this phase of the study were 4° to 10° F.

Levitt (26) reports that in research dealing with cold injury, artificially-produced low temperatures have consistently given results comparable to those induced by low temperatures under natural conditions. In the cold treatments conducted in this investigation, wheat plants studied in the dormant stage were placed in the low temperature situations directly from the field where temperatures were near or below freezing. Following cold treatment, the plants were allowed to thaw slowly. According to Levitt (25), it is agreed by workers engaged in cold studies that rapid thawing causes more injury in plants than slow thawing.

Most of the plants tested for viability either as experimental materials subjected to low temperatures or as control materials not so treated were freed of soil when dug in the field. Such plants were cold treated in a cloth-wrapped bundle and were assumed to be killed by a specific low temperature when comparable plants of the same variety growing in soil in 6-inch pots were killed by that temperature.

In a limited number of tests, dormant plants and their surrounding soil were lifted from the drill row and placed in large, wooden flats. Although the longer roots were severed, the plants were essentially undisturbed in the soil. It was possible to place from 100 to 150 plants in a flat.

## Plants Used in Testing

The wheat plants used in these studies were obtained for the most part from a field of Pawnee wheat in the winter dormant condition grown at the

Agronomy Farm. For special phases of the study some plants were used from that field after the plants had broken winter dormancy. For other phases, potted plants of dormant Pawnee wheat were used. All plants studied were planted the fall of 1951 and were grown out-of-doors.

## Testing Procedure

Cold-treated plants were thawed before testing. It was found during preliminary testing that incompletely thawed plants gave a tetrazolium staining reaction which was difficult to interpret. In most cases, it was possible to apply the test to plants within three to four hours following cold treatment.

In the preliminary testing, certain portions of the wheat plant proved unsuitable and unnecessary for testing. While reduction of tetrazolium chloride and subsequent staining occurred in the terminal one or two millimeters of the wheat root tip, other parts of the root stained irregularly, if at all. All roots were therefore removed before testing. It was also found that while green portions of the wheat leaves gave a weak color reaction with tetrazolium chloride solutions, it did not prove of value in a macroscopic test of this type. Consequently, all green portions of the leaves were excised.

Upon the removal of roots and green tissues, the white underground portion of the plant remained. This portion, described in detail on p. 15, contained the crown region with its main axis and lateral buds, and varying portions of the leaves of the plant, depending upon their age and degree of emergence. This portion of the plant was sectioned longitudinally through the center of as many tillers (axes) as possible. Half of each

plant was then used in testing. Plate I, Fig. 2 shows a face view of the test section used in the tests.

The test sections were immersed in a 1 percent solution of tetrazolium chloride in a vial. Vials containing tetrazolium chloride solutions and wheat plant sections were placed in the dark for twelve hours at room temperature. Best results were obtained when the testing solution containing plant parts was stored in the dark during the testing period. In light, triphenyl formazan precipitated in the solution at such a rapid rate that it masked the staining reaction in the plant tissues. There was little excess precipitation of formazan in solutions in the dark for at least twelve hours. If plants were allowed to remain in tetrazolium chloride solutions in the dark for more than thirteen or fourteen hours, heavy precipitation of formazan occurred. Maximum staining, both in depth and extent, occurred in ten to twelve hours. Thus, maximum staining of the test section and minimum precipitation of formazans outside the plant tissue occurred in twelve hours.

Following the twelve-hour staining period, test sections were removed from the solution and washed thoroughly with distilled water. The staining of the sections, both as to depth and extent, was observed.

The procedure outlined above proved sufficient to demonstrate differences in staining reaction between normal plants and plants subjected to conditions sufficiently abnormal to cause injury. It was found that the application of heat to wheat plants immediately preceding tetrazolium chloride testing served to accentuate the staining differential between normal and cold-injured plants. This was apparently due to the fact that in the presence of heat, cold-injured plants lost the ability to reduce tetrazolium chloride more rapidly than uninjured plants. A temperature

of 50° C. was found most suitable for this effect. The extent to which cold-injured plants could reduce tetrazolium chloride was greatly decreased after three hours of heating in air at 50° C., whereas eight to ten hours of heating were required to reduce appreciably the staining reaction of normal, non-cold-treated plants. Although this heating procedure accentuated quantitative staining differentials between normal and cold-injured plants, it was less exact than the staining procedure not involving heating. The staining of unheated plants gave a definite indication of the specific tissues damaged by low temperatures, while the heating procedure caused a general decrease of staining of injured plants and a decided loss of specific indication of tissues injured.

It is possible that situations requiring storage of tested plants could arise, particularly in experimental work where large numbers of tests are being run or photographs of materials are desired. For this reason, experimentation was conducted on the storage of plants following testing. Storage of plants in distilled water without refrigeration was totally unsuccessful because of the development of bacteria, resulting in rapid deterioration of the plants. Plants were stored in distilled water in the refrigerator at 38° F. for periods of four to five weeks in some cases, but this method was inconsistent and marked bacterial growth usually developed in a relatively short time. Lakon (23) suggested the use of a 1/1000 aqueous solution of mercuric chloride for the storage of plant materials stained with tetrazolium salts, and this proved successful. In this investigation, bacterial development did not occur in vials of plants containing mercuric chloride solutions, either in the refrigerator or at room temperature. Plants stored in mercuric chloride solutions in the refrigerator for six months remained unchanged in appearance and coloration.

Although no bacterial growth developed in vials of stained plants stored in mercuric chloride solutions at room temperature in the light, the color of the plants faded.

Antopol, Glaubach, and Goldman (1) reported the use of 10 percent formalin for preservation of tissues by means of tetrazolium salts. Plants stored in 10 percent formalin in the dark in this study were found to remain firm and to retain their color for two months, but this method was not investigated further.

## Stage and Treatment of Plants Tested

In these investigations, the reaction of wheat plants to tetrazolium chloride was determined following exposure to various abnormal conditions. In order to establish a basis for all comparisons, it was necessary to determine the staining occurring in normal, uninjured wheat plants in tetrazolium chloride solutions. This was facilitated by the fact that natural cold injury to winter wheat plants used in the testing was negligible during the winter of 1951-1952. Normal, uninjured plants were removed from the soil and tested with tetrazolium chloride regularly from November 16 through April 15. These tests were usually made two to three times a week, and in no case did more than ten days elapse between tests. Thus, a constant check was maintained on the staining reaction of normal wheat plants and this formed the basis of comparison for the tests conducted upon wheat plants exposed to abnormal conditions.

A second important point necessary to establish was the staining reaction occurring in plants which were known to be dead. Several tests using wheat plants boiled for various periods of time were made with

tetrazolium chloride, and their staining reaction was observed. A number of such tests were also conducted upon plants which had been killed by exposure to temperatures of 100° to 105° C. in a hot air oven. In a third type of testing, plants were removed from the soil and allowed to dry at room temperature. Periodic tetrazolium chloride tests were made upon these plants as they died. A series of tests was then conducted upon plants subjected to temperatures sufficiently low to kill them. Temperatures of -5° to -9° F. were employed and the death of all check plants treated at these temperatures established the fact that killing occurred. Both dormant plants and plants in the spring transition from dormancy to active growth were included in this phase of testing.

In the limited amount to testing that was conducted upon plants which were injured, but not killed, by low temperatures both dormant plants and plants in the transition from dormancy to active growth were also used. Plants transplanted into flats from the field were cold treated, allowed to thaw, and a random sample from each group was tested with tetrazolium chloride. The remaining plants in each group were left in the soil and their subsequent development was observed. Several flats of plants not subjected to low temperatures served as controls.

## EXPERIMENTAL RESULTS

The results reported in this study are based upon the staining reaction occurring in test sections of hard red winter wheat plants during immersion in a 1 percent aqueous solution of tetrazolium chloride for twelve hours at room temperature. Tests on cold-treated wheat plants were made within 24 hours following cold treatment. The tetrazolium chloride staining reaction

of normal, uninjured wheat plants served in all cases as a basis for comparison and evaluation of the staining reactions of plants subjected to abnormal conditions.

The staining reaction of normal winter wheat plants was determined by testing such plants at two stages of development: (1) winter dormant stage, and (2) spring transition from dormancy to active growth. The staining reaction of normal plants was found to be the same at both stages. When test sections of normal wheat plants at these stages were immersed in tetrazolium chloride solutions, a faint red color became visible in fifteen to thirty minutes in the youngest leaves and the terminal and lateral buds. The red color rapidly became deeper and more widespread, progressing from younger to older tissues. Maximum staining, both in depth and extent, usually occurred in ten to twelve hours; hence, twelve hours was adopted as the testing time. Control plants shown in Plates II, III, and V illustrate the staining of test sections of normal, uninjured wheat plants. Microscopic examination of thin sections cut from the face of the test section showed that the staining was due to the formation of small, irregular crystals of triphenyl formazan within the cells. Plants not split longitudinally did not give a satisfactory test because of the slow rate of penetration of the reagent. The splitting procedure was considered essential for accurate testing.

Approximately 2500 untreated wheat plants were tested with tetrazolium chloride during the months of November through April. The tests were conducted periodically, with no more than ten days elapsing between tests. Uniform, deep red staining of all tissues of the test section occurred in all normal, uninjured plants.

Winter wheat plants boiled in water for two minutes or more were not

stained when immersed in a 1 percent tetrazolium chloride solution. Approximately 700 plants were utilized in the determination of the effect of boiling in water upon this staining reaction in wheat plants.

Winter wheat plants heated in air at 100-105° C. for ten minutes or more also completely lost the ability to reduce tetrazolium chloride. Approximately 500 plants were used in this determination.

Winter wheat plants which were removed from the soil and allowed to stand in the air at room temperature until they were completely dry showed a gradual decrease in depth of color and extent of area stained when tested periodically with tetrazolium chloride. Tests were conducted every day for fourteen days, at which time the plants were completely dried and were obviously dead. After two days of drying, the first reduction in area stained was apparent in the outer leaf sheaths. This decrease in area stained progressed inward and in twelve to fourteen days, no staining occurred. Approximately 500 plants were tested in this manner.

The tetrazolium chloride staining of plants killed by temperatures of -5° F. or below proved to be consistent. The typical staining reaction of these plants, which is illustrated by the upper right specimens of Plate II, consisted of a faint, purplish-red coloration in the sheaths of the three or four oldest leaves. The younger leaves and entire crown region showed complete or nearly complete lack of staining. In a few cases, a faint, purplish-red stain developed in the lateral buds, while all surrounding tissues remained unstained. Approximately 2000 plants were killed by temperatures of -5° F. or below and tested and in no case could the resulting stain be confused with that of a normal, uninjured wheat plant. Plate II shows the contrast between these two staining reactions. The results of this phase of testing are given in Table 1.

The results described thus far established the fact that a marked difference existed between the tetrazolium chloride staining reaction of normal, uninjured wheat plants and wheat plants killed by low temperatures and that the same difference in staining reaction existed in both dormant wheat plants and wheat plants in the transition from dormancy to active spring growth.

Exploratory testing of cold-injured plants indicated that there was a relationship between the severity of cold treatment and the tetrazolium chloride staining reaction of test sections, both as to depth and extent of coloration. Additional experiments were conducted in which winter wheat plants in the spring transition from dormancy to active growth were killed by exposure to temperatures of 0° to 5° F. The cold treatment was applied to plants which had been transplanted into flats from the field. Following cold treatment, some of the plants were tested, while others were left in the soil as checks on mortality. None of the check plants survived. While test sections of the tested plants did not lose the ability to reduce tetrazolium chloride as completely as did plants killed by temperatures of -5° F. or below, they did exhibit a consistent staining reaction which differed markedly from that of normal, uninjured plants. In every case, the apical meristematic regions of the axes exhibited no staining and the crown region at the base of the plant either remained completely white, or developed a mottled, red staining. The lack of staining or the mottled staining in the crown region and the lack of staining in the apical meristematic regions were found in all tests of plants killed by low temperatures. This staining reaction is therefore considered typical of all plants killed by low temperatures, and is shown in Plates II, III, and IV. Table 2 compares the results of the tetrazolium chloride test in this phase of study with the recovery shown

by comparable plants, comparably treated.

A limited number of tests were conducted with temperatures which injured wheat plants, but did not produce 100 percent mortality. Tests of this type were conducted upon seven groups of wheat plants in the transition from dormancy to active spring growth. The results of tetrazolium chloride tests made upon samples from these groups of plants were compared with the observed effect of identical low temperatures upon comparable plants and in this manner a basis of evaluation of the tetrazolium chloride staining reaction for this type of cold treatment was established. Table 3 compares the results of the tetrazolium chloride tests in this phase of the study with the recovery shown by comparable plants, comparably treated. It was found that a decrease in depth of color and extent of staining in certain tissues of test sections of wheat plants was directly related to the degree of injury to those plants by low temperatures.

Because of the limited number of tests conducted in this phase, the results are not conclusive. Results were markedly consistent, however, and indicated that the tetrazolium chloride test is applicable to situations of this type. The wheat plants which were in the transition from dormancy to active growth were rapidly changing in development and weather conditions, both temperature and moisture, at the time of testing fluctuated widely from day to day. For these reasons, the tests in this phase were considered comparable only in a general manner.

Wheat plants in which slight cold injury was apparent to the aboveground leaves, but which developed almost as well as control plants, gave a tetrazolium chloride staining reaction identical with that of normal, uninjured wheat plants; i.e., a uniform, deep red staining in all tissues of the test section. In all cases where test sections showed a reduction in staining in

comparison with that of normal plants, comparable plants exposed to the same temperatures showed reduction in number of heads produced. Groups of plants in which the number of heads produced was only slightly reduced in comparison to controls exhibited a lack of staining in the apical meristematic regions and very young leaves of one or two tillers per plant, the other tillers being stained a uniformly deep red as in plants not cold treated. Plants which recovered from cold treatment, but developed very weakly and produced no heads, stained red in the crown region, but exhibited a lack of staining in the apical meristematic regions and young leaves of all tillers (Plates V and VI).

These results demonstrate that cold injury to hard red winter wheat plants causes a definite reduction in the ability of certain tissues of those plants to reduce tetrazolium chloride and become stained. Also, the depth of color and extent of staining decreases as the degree of cold injury increases. Control plants were tested periodically with tetrazolium chloride throughout the period of experimentation, November, 1951 through April, 1952, and were found without exception to acquire a deep red stain in all tissues of the portion used as the test section. Since cold-injured plants consistently exhibited certain tissues of this section which did not stain, the reduction of staining in these tissues following cold treatment was considered a definite indication of cold-injury.

In the section entitled Materials and Methods a heating process was described by which differences in tetrazolium chloride staining between uninjured wheat plants and cold-injured wheat plants could be accentuated. In this procedure, heating at 50° C. for three hours immediately prior to tetrazolium chloride testing greatly reduced the staining of test sections of cold-injured wheat plants in the presence of tetrazolium chloride

solutions, but did not appreciably affect the staining reaction of test sections of uninjured plants. As emphasized earlier, this procedure was only employed as a means of accentuating a staining differential already present between normal and cold-injured plants. Plates II, III, and V illustrate the effect of the heating procedure by comparison of tetrazolium chloride tests on plants not heated with tests on comparable plants which were heated.

Complete thawing of wheat plants prior to tetrazolium chloride testing was necessary to insure a definite test. The staining of plants tested while frozen was indeterminate and did not indicate the specific tissues affected by low temperatures.

Plants killed by low temperatures gave, under one condition, a positive reaction with the tetrazolium test which could be misleading. Dead plants kept sufficiently moist were decomposed rapidly by bacterial action. Such dead tissue, when immersed in a tetrazolium chloride solution, acquired a red stain. Presumably, this coloration resulted from bacterial reduction of tetrazolium chloride.

The preservation and maintenance of color in stored, tested plant sections presented a problem. Such test sections were retained for later examination and for photographing. A mercuric chloride solution of 1/1000 concentration prevented their decomposition by bacteria, while storage in the dark at 40° F. or below prevented a loss of coloration.

## CONCLUSIONS

The red staining which occurred in certain tissues of hard red winter wheat plants in the presence of tetrazolium chloride solutions was due to the intracellular formation of small, irregular, red crystals of triphenyl

formazan. Triphenyl formazan is formed by the reduction of tetrazolium chloride. The tetrazolium chloride test for the viability of plant tissues is based upon differences in reducing power between normal and injured tissues.

Exposure to aqueous solutions of tetrazolium chloride consistently produced a deep red stain in the crowns and non-green leaf tissues, designated the "test section", of normal, uninjured, hard red winter wheat plants during dormancy and during transition from dormancy to active spring growth. Maximum staining of these tissues required a period of twelve hours.

The following treatments rendered the tissues of test sections of winter wheat plants totally unable to reduce tetrazolium chloride: (1) boiling for two minutes or more (2) heating in air at 100-105° C. for ten minutes or more (3) killing by drying at room temperature.

Exposure of winter wheat plants during dormancy and during transition from dormancy to active spring growth to temperatures of -5° F. or below for twelve hours or more brings about a marked decrease of tetrazolium chloride reduction in the tissues of the test sections of those plants. Plants cold treated in this manner and tested with tetrazolium chloride exhibit the following stains in the tissues of the test sections: (1) a faint purplish-red coloration in the sheaths of the older leaves and (2) no coloration or at most a faint pink staining in the tissues of the crown region and younger leaves. Experimentation with temperatures in the range of -9° F. to 5° F. has shown that wheat plants killed by low temperatures consistently exhibit either no staining or a faint mottled, red staining in the tissues of the crown region and unemerged leaves when tested with tetrazolium chloride. Tetrazolium chloride testing of plants which were appreciably injured, but not killed, by low temperatures produced a uniform, deep red stain in most of the crown region, but revealed various degrees of staining in the apical

meristematic regions (terminal and lateral buds) and the unemerged leaves of the axes, depending upon the degree of injury. The reduction of tetrazolium chloride by wheat plant tissues in the test section decreased as amount of cold injury increased. The tetrazolium chloride test indicated that meristematic tissues and newly differentiated tissues of wheat plants are more easily injured by low temperatures than are more mature tissues.

The most satisfactory procedure found for the detection of cold injury to hard red winter wheat plants is as follows: (1) Remove wheat plants from the soil. (2) Thaw plants slowly. (3) Remove rhizome, roots, and green portions of leaves. (4) Section the remaining portion of the plant longitudinally through the center of as many tillers as possible to obtain the test section (which consists of the main and lateral stems with their terminal and lateral buds and various portions of foliage leaves, depending upon their age and degree of emergence). (5) Immerse the test section in a 1 percent aqueous solution of tetrazolium chloride (pH 4.0 to 7.0) and allow it to remain in this solution in the dark for twelve hours at room temperature. (6) Remove the section from the solution, wash thoughly with distilled water and observe the staining reaction. A uniform, deep red stain in all tissues of the test section indicates that a plant has not been injured appreciably. A lack of staining or irregularity of staining of any part of the test section indicates injury to that part. This study indicated that plant parts reacting in this manner are dead or will die. Upon this basis, the tetrazolium chloride test gives promise in indicating whether plants injured, but not killed, by low temperatures will produce a satisfactory stand and yield.

Plants that have been tested may be stored without decomposition or loss of coloration for as long as six months if kept in the dark in a 1/1000

solution of mercuric chloride at temperatures of 40° F. or below. Decomposed plant tissues should not be tested because enzymatic activity of bacteria in these tissues will cause reduction of tetrazolium chloride and subsequent staining of the tissue. This staining could cause misinterpretations of the tetrazolium chloride test.

Results of this study establish the validity of the tetrazolium chloride test as an indicator of the death of hard red winter wheat plants through exposure to low temperatures and indicate that the test will demonstrate accurately the degree of cold injury to hard red winter wheat plants when the injury is sufficient to decrease appreciably the normal stand and yield of the plants.

The test requires no special equipment and results are available within 24 to 36 hours after exposure of the wheat plants to low temperatures. Tetrazolium chloride is available from a number of chemical supply houses. No special training or experience is required to conduct the test.

Table 1. Mortality in cold-injured, dormant, Pawnee wheat as indicated by the tetrazolium chloride test compared with recovery shown by comparable plants, comparably treated. Manhattan, Kansas, 1952.

| Date | Artificial cold treatment | | Tetrazolium chloride test | | Effect of cold treatment on check plants | |
|---|---|---|---|---|---|---|
| | Temp. (F.) | Time (hr.) | Number of plants | Estimated mortality (%) | Number of plants | Mortality (%) |
| 1951 | | | | | | |
| 11/16 | -5° | 18 | 100 | 100 | 20 | 100 |
| 11/21 | -9° | 48 | 32 | 100 | 16 | 100 |
| 11/23 | -9° | 48 | 48 | 100 | 20 | 100 |
| 11/27 | -9° | 48 | 56 | 100 | 20 | 100 |
| 12/3 | -9° | 24 | 123 | 100 | 38 | 100 |
| 12/4 | -9° | 48 | 135 | 100 | 17 | 100 |
| 12/6 | -9° | 24 | 96 | 100 | - | - |
| 12/7 | -9° | 48 | 75 | 100 | - | - |
| 12/10 | -9° | 24 | 140 | 100 | 43 | 100 |
| 12/10 | -9° | 48 | 112 | 100 | - | - |
| 12/11 | -9° | 48 | 70 | 100 | 26 | 100 |
| 12/12 | -9° | 72 | 110 | 100 | - | - |
| 12/13 | -9° | 24 | 88 | 100 | 35 | 100 |
| 12/14 | -9° | 36 | 68 | 100 | 32 | 100 |
| 12/17 | -9° | 26 | 74 | 100 | - | - |
| 12/18 | -9° | 48 | 60 | 100 | 27 | 100 |
| 1952 | | | | | | |
| 2/19 | -7° | 20 | 110 | 100 | 34 | 100 |
| 2/20 | -7° | 24 | 130 | 100 | - | - |
| 2/25 | -8° | 20 | 95 | 100 | 19 | 100 |
| 3/21 | -8.5° | 16 | 120 | 100 | - | - |
| 3/25 | -9° | 19 | 155 | 100 | 38 | 100 |

Table 2. Mortality in cold-injured Pawnee wheat in the transition from dormancy to active spring growth, as indicated by the tetrazolium chloride test compared with recovery shown by comparable plants, comparably treated. Manhattan, Kansas, 1952.

| Date | Artificial cold treatment | | Tetrazolium chloride test | | Effect of cold treatment on check plants | |
|---|---|---|---|---|---|---|
| | Temp. (F.) | Time (hr.) | Number of plants | Estimated mortality (%) | Number of plants | Mortality (%) |
| 4/3/52 | 5° | 24 | 60 | 100 | 165 | 100 |
| 4/4/52 | 4.5° | 24 | 50 | 100 | 173 | 100 |
| 4/10/52 | 0° | 24 | 70 | 100 | 210 | 100 |
| 4/11/52 | 0° | 24 | 60 | 100 | 225 | 100 |
| 4/12/52 | 4° | 24 | 60 | 100 | 233 | 100 |
| 4/13/52 | 4° | 24 | 60 | 100 | 198 | 100 |

Table 3. Mortality in cold-injured Pawnee wheat plants in the transition from dormancy to active spring growth as indicated by the tetrazolium chloride test compared with recovery shown by comparable plants, comparably treated. Manhattan, Kansas, 1952.

| Date | Artificial cold treatment: Temp. (F.) | Time (hr.) | Tetrazolium chloride test: Number of plants | Estimations (%): No injury | Moderate injury | Survived - no heads | Killed | Effect of cold treatment on check plants: Number of plants | Observations (%): No injury | Moderate injury | Survived - no heads | Killed |
|---|---|---|---|---|---|---|---|---|---|---|---|---|
| 4/1 | 9.5° | 24 | 60 | 13 | 17 | 50 | 20 | 139 | 11 | 16 | 55 | 18 |
| 4/2 | 7° | 24 | 70 | - | - | 26 | 74 | 155 | - | - | 30 | 70 |
| 4/5 | 7.5° | 24 | 80 | 5 | 9 | 53 | 33 | 179 | 7 | 3 | 62 | 28 |
| 4/6 | 7° | 24 | 50 | - | 8 | 32 | 60 | 170 | - | 10 | 25 | 65 |
| 4/7 | 10° | 24 | 40 | 23 | 60 | 17 | - | 173 | 26 | 54 | 20 | - |
| 4/8 | 6° | 24 | 50 | - | - | 8 | 92 | 160 | - | - | 4 | 96 |
| 4/9 | 7.5° | 24 | 50 | 8 | 10 | 44 | 38 | 154 | 4 | 19 | 46 | 31 |

EXPLANATION OF PLATE II

Tetrazolium chloride staining reaction of test sections of Pawnee wheat plants in transition from dormancy to active growth. April, 1952.

Left-Controls; i.e., not cold treated
Lower-Heated at 50° C. for 3 hours prior to testing
Upper-Unheated prior to testing

Right-Killed by a 24 hour exposure to a temperature of -9° F.
Lower-Heated at 50° C. for 3 hours prior to testing
Upper-Unheated prior to testing

PLATE II

EXPLANATION OF PLATE III

Tetrazolium chloride staining reactions of test sections of Pawnee wheat plants in transition from dormancy to active growth. April, 1952.

Left-Controls; i.e., not cold treated
Lower-Heated at 50° C. for 3 hours prior to testing
Upper-Unheated prior to testing

Right-Killed by a 24 hour exposure to a temperature of 0° F.
Lower-Heated at 50° C. for 3 hours prior to testing
Upper-Unheated prior to testing

PLATE III

## EXPLANATION OF PLATE IV

Tetrazolium chloride staining reactions of test sections of Pawnee wheat plants in transition from dormancy to active growth which were killed by a 24 hour exposure to a temperature of 0° F. April, 1952. Note mottling in crown region of upper left specimen and absence of color in crown region of upper middle specimen.

PLATE IV

EXPLANATION OF PLATE V

Tetrazolium chloride staining reactions of test sections of Pawnee wheat plants in transition from dormancy to active growth. April, 1952.

Left-Controls; i.e., not cold treated
Lower-Heated at 50° C. for 3 hours prior to testing
Upper-Unheated prior to testing

Right-Plants which survived cold treatment at 8.5° F. for 24 hours, but produced no heads.
Lower-Heated at 50° C. for 3 hours prior to testing
Upper-Unheated prior to testing

PLATE V

EXPLANATION OF PLATE VI

Tetrazolium chloride staining reactions of test sections of Pawnee wheat plants in transition from dormancy to active growth following a 24 hour exposure to a temperature of 7.5° F. Plants survived cold treatment, but produced no heads. April, 1952.

PLATE VI

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# THE USE OF 2,3,5-TRIPHENYL TETRAZOLIUM CHLORIDE IN THE DETECTION OF LOW TEMPERATURE INJURY TO HARD RED WINTER WHEAT DURING DORMANCY AND DURING TRANSITION FROM DORMANCY TO ACTIVE GROWTH

by

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AN ABSTRACT OF A THESIS

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## Introduction

Numerous workers have used 2,3,5-triphenyl tetrazolium chloride (tetrazolium chloride hereafter) to demonstrate differences in reducing power between normal and injured plant cells and have developed successful viability tests upon this basis. Tetrazolium chloride forms a clear, colorless, aqueous solution and upon reduction is converted to triphenyl formazan, a deep red compound insoluble in water. Reduction of tetrazolium chloride occurs within living cells of a variety of plant tissues. Research has established that this reduction is accomplished by certain pyridine nucleotide dehydrogenases requiring coenzymes I and II. Accumulation of triphenyl formazan within the cells of a tissue causes that tissue to stain red. The present view on the use of tetrazolium chloride as a test for plant tissue viability is that a plant tissue which normally reduces the compound may be considered dead when it no longer does so.

## Purpose

This study has as its purpose the establishment of a rapid, simple test to detect cold injury to hard red winter wheat plants during dormancy and during transition from dormancy to active growth.

## Materials and Methods

Experimentation was conducted in the months of November, 1951, through April, 1952. Pawnee wheat, planted in the fall of 1951 and grown out-of-doors, was used in the testing. Cold injury to this wheat by natural causes was negligible.

Preliminary testing was conducted to determine the most suitable method of application of tetrazolium chloride to the wheat plants to be tested. The method developed is as follows: (1) remove plants from soil (2) if plants are frozen, thaw slowly (3) remove rhizome, roots, and green leaf portions from

plants (4) split the remaining portion longitudinally through the center of as many tillers as possible. One of the resulting halves is designated the "test section". (5) immerse test section in a 1 percent aqueous solution of commercial tetrazolium chloride, pH 4.0 to 7.0 (6) allow test section to remain in solution in the dark for twelve hours (7) observe staining as to both extent and depth of coloration.

The test section of the wheat plant included the crown region and varying portions of the foliage leaves, depending upon their age and degree of emergence. The crown region contained the main and lateral axes and their buds.

Throughout the period of experimentation, tests were made upon control plants, and their staining reaction served as a basis of comparison for the staining reaction of plants subjected to low temperatures.

Wheat plants were subjected to artificially-produced low temperatures ranging from -9° F. to 15° F. for periods of 12 to 72 hours. Tetrazolium chloride tests were made upon a random sample from each group of cold-treated plants, and the remaining plants of the group served as a check on the effect of the cold treatment.

Most of the work dealt with temperatures which were known to kill wheat plants. Limited studies were conducted with temperatures which injured, but did not kill, wheat plants.

## Results

The following results were obtained in tetrazolium chloride testing:

(1) All tissues of the test section of control plants consistently exhibited a uniform, deep red coloration.

(2) A decrease in staining (in comparison to controls) was consistently encountered in test sections of plants injured by low temperatures.

(3) Depth of coloration and extent of staining of test sections decreased as cold injury increased.

(4) Test sections of plants killed by low temperatures invariably exhibited either a lack of staining or mottled staining in the crown region and the unemerged leaves.

(5) Test sections of plants which were appreciably injured, but not killed, by low temperatures stained a uniform, deep red in the major portion of the crown region, but exhibited various degrees of non-staining in the unemerged leaves and apical meristematic regions.

(6) Comparison of the staining reaction of test sections and development of check plants showed a direct relation between number of apical meristematic regions lacking stain and reduction in the number of heads produced.

(7) It was possible to apply the test within 24 hours following cold treatment.

## Conclusions

Results of this study establish the validity of the tetrazolium chloride test as an indicator of the death of winter wheat plants through exposure to low temperatures. Results obtained with plants injured, but not killed, by low temperatures indicate that the test will demonstrate degree and location of cold injury to winter wheat plants when that injury is sufficient to decrease appreciably the stand and yield. The test requires no special equipment or training and may be applied within a few hours following exposure of wheat plants to low temperatures.