TETRAZOLIUM CHLORIDE AS A TEST REAGENT FOR FREEZING INJURY OF SEED CORN*

NORAH BENNETT AND W. E. LOOMIS

(WITH ONE FIGURE)

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Rapid methods for estimating viability in seeds have been suggested by a number of workers. Most of these are dependent upon the increased permeability (**6, 10**) or decreased reducing power (**5, 8**) of dead cells or tissues. Staining methods involving the reduction of selenium salts (**4**) or of tetrazolium compounds (**9, 11**) to colored forms by the action of living cells seem to be the most promising quick tests of viability. These tests should be particularly applicable to estimations of freezing injury in seed corn because: (a) corn is easily tested (**11**), (b) immature corn shows a degree of dormancy (**13**) which makes routine laboratory tests of germinability difficult and slow, and (c) speed is always essential to determine whether frozen seed corn is sufficiently viable to justify expensive processing, or whether the crop should be diverted to other uses.

Materials and Methods

Staining tests were limited to the use of 2,3,5-triphenyltetrazolium chloride. The salt was obtained variously from a lot used by PORTER et al. (**11**) and originally obtained from Germany, a lot prepared by the Department of Chemistry, Iowa State College, and several lots supplied by Dr. Nicholas Cheronis of the Synthetical Laboratory, Chicago. Preliminary tests showed that 0.05 per cent. solutions were superior to the 1.0 per cent. used by LAKON (**9**) or the 0.5 per cent. concentration previously used in this laboratory. Corn too dry to cut easily was soaked overnight to 30 or 35 per cent. moisture. Fresh, immature corn was used without soaking. A sample of 50 to 200 kernels was cut carefully with sharp razor blades through the scutellum and embryo axis, and immersed in the tetrazolium solution in petri dishes for two hours at 30° C.

Color readings included records of: (a) intensity of stain, (b) tint, whether purplish, rose or orange red, (c) color texture; viable seeds showed a stippling of deeper staining over the surface of the embryo, (d) the absence, or presence and distribution of unstained areas within the embryo, and (e), in the later readings, the sharpness or disorganization in cellular structure of the stained tissue. In the directions given by LAKON (**9**) and PORTER et al. (**11**) corn was considered viable if the embryo stained pink

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under these conditions. Lakon recognized necrotic (unstaining) areas in old or injured seeds, and attempted to establish the amount of necrosis indicating abnormal or nongerminable seeds.

Preliminary tests were run on several samples of old corn of varying germinability and later on mature grain soaked to the desired moisture percentages and frozen before testing. The main series of experiments was run with corn of varying degrees of maturity, ears of which were given varying freezing treatments in a low temperature chamber. Some of these lots were tested immediately after freezing and some after various holding or drying and resoaking treatments.

Tests of Old Seeds

Three lots of old corn from different sources were used for preliminary tests of the tetrazolium method. Twelve samples of 6-year old corn were

TABLE I

GERMINATION AND STAINING PERCENTAGES OF CORN SEED STORED SIX YEARS IN THE LABORATORY

CLASSIFICATION	SAMPLE 1	SAMPLE 2	SAMPLE 3
Germination in sand			
Normal	36	80	48
Abnormal but not entirely dead	14	2	24
Dead	50	18	28
Tetrazolium tests			
Strong staining	20	74	6
Pale staining	0	6	0
Scutellum weakly stained	0	0	8
Small unstained area at base of scutellum	0	18	64
Few scattered unstained areas in scutellum	22	0	0
Total considered normal	42	98	78
Larger unstained area at base of scutellum	0	0	20
Several scattered unstained areas in scutellum	56	0	0
Shoot apex colorless	0	2	0
Entire embryo colorless	2	0	2

obtained from Dr. E. W. Lindstrom of the Department of Genetics, 16 samples one to four years old were supplied by Northrup, King and Co., and six samples of unknown history were obtained from the College Seed Laboratory.

Of the 12 samples obtained from Dr. Lindstrom, only three germinated below 98 per cent. in sand. One of these, designated sample 1, showed scutellar injury in the tetrazolium test, indicated by scattered areas which failed to stain. The other two showed localization of the unstained areas at the base of the scutellum. In all cases the necrotic area was less than the one-third of total area used by LAKON (9) as a measure of loss of viability. The data of table I show the sand test germination of these three lots and their tetrazolium staining reactions. It appeared that the estima-

tion with tetrazolium of seeds considered normal was approximately correct only for sample 1. Germination test for sample 2 indicated that in this lot those seeds with small unstained areas at the base of the scutellum were dead, but in the third sample similarly stained seeds were viable and more than half of them were capable of producing normal seedlings.

The samples obtained from the Northrup, King and Co. were high in germinability, with only one sample going below 90 per cent. This sample was estimated at 100 per cent. by the tetrazolium test. Other estimates agreed within the usual errors of testing. Seeds which germinated abnormally apparently were not separated by the tetrazolium test.

Similar results were obtained with the third set of samples. A reasonably good estimate of normal seedlings was possible, but seeds producing abnormal seedlings were not accurately separated from dead, or sometimes from normal.

Tests of Frozen Seeds

The first tests on frozen seed were with four samples supplied by Mr. M. M. Aboul-Ela from his experiments on chemical changes in frozen corn

TABLE II

GERMINATION AND STAINING PERCENTAGES OF FOUR LOTS OF CORN FROZEN IN THE FALL OF 1946 AND TESTED IN MAY, 1947. (50 TO 100 SEED SAMPLES)

SAMPLE	MOISTURE % WHEN FROZEN	GERMINATION IN SAND			TETRAZOLIUM STAINING			
		NORMAL	ABNORMAL	DEAD	COMPLETE STAIN	PALE STAIN	PARTIAL STAIN	NO STAIN
1	62.5	92	1	7	86	0	14	0
2	41.1	97	1	2	100	0	0	0
3	56.6	3	0	97	4	6	2	88
4	39.9	10	0	90	10	4	4	82

(1). All samples were frozen as snapped ears for six hours at 20° F. and then hung back on the stalks to dry in the field. Samples 1 and 2 were from an early planting which dried rapidly under favorable conditions. Samples 3 and 4 were from later planted corn and were subjected to several weeks of weathering during the drying period. These seeds had either been killed by the freezing treatment or had recovered (**12**) and the time lapse of about six months between the freezing treatment and testing was sufficient for the establishment of clear-cut staining differences (Table II). The variations between complete stain by tetrazolium and normal germination in sand can be ascribed to sample variation.

A second group of samples of frozen seed was obtained from Mr. Elmer C. Rossman. These samples were given varying freezing treatments while still on the ear. After freezing they were allowed to thaw for six to eight hours before being placed in corn-seed drier to dry at approximately 95° F. In the tetrazolium solution all seeds showed staining of the scutellar region.

TABLE III

PERCENTAGE GERMINATION AND STAINING OF SIX LOTS OF FROZEN CORN. GERMINATION BASED ON 35–129 SEEDS; TETRAZOLIUM ON 100

	Germination tests					Tetrazolium tests			
Sample	Normal	No primary root	Total germinable	Abnormal	Dead	Complete staining	Plumule unstained	Radicle unstained	Embryo axis unstained
1	55	6	61	36	3	87	13	0	0
2	5	0	5	15	80	16	0	0	84
3	86	0	86	14	0	85	0	15	0
4	63	0	63	37	0	71	0	29	0
5	45	4	49	49	2	65	0	35	0
6	8	1	9	2	89	8	0	0	92

Those counted non-germinable showed no staining either in the plumule or in the radicle or in both of these areas. Seedlings germinating without the primary root but with well developed seminal roots were included in the total of germinable seeds. Table III shows the comparison of germination and staining tests. The differences in the germination of these samples is credited to differences in the moisture content at the time of freezing. In all but two comparisons the tetrazolium estimation of viable seeds tended to be slightly higher than the germination test, regardless of the type of injury evidenced by lack of staining in certain regions. Partial loss of vitality as a result of freezing seemed to result in an overestimation of germinability when samples were tested by the tetrazolium method.

The foregoing tests indicated that more information on the effect of tetrazolium on frozen seeds was desirable. Because other frozen samples were not available at this time, normally matured and cured seed which had been soaked in water and then frozen was used. Seed from Northrup, King sample 3171A8 was soaked in water at 15° C. to varying moisture contents. The seeds were then drained, blotted to remove excess water and frozen for 24 hours at 20 and at 0° F. After thawing the samples were cut and placed in the tetrazolium solution, with duplicate samples planted in sand. In each case two lots of 100 seeds were used. Table IV contains the data from the two methods.

It was apparent that staining alone was not a criterion of viability in corn seed which had recently been subjected to freezing injury, as there were great differences in the estimation of viability by the two methods in the more severely frozen seeds of higher moisture content. It was discovered that the position of these injured seeds in the tetrazolium solution had a bearing on the intensity of stain produced. However, injured seeds, even though deeply stained, were different from viable seeds. It was noted that: (a) in dead seeds there was much more prominent contrast of color intensity between embryo axis and scutellum, the former being darker; (b) the embryo axis appeared to be blurred without the outlines of the foliage leaves of the plumule being visible; (c) the scutellum had lost the more or less granular appearance characteristic of the living tissues. Retesting with tetrazolium of those samples which showed divergence from the germination test, and using these factors as the basis of separation, gave results closely approximating the sand germination test results.

Tests of Fresh Seeds

The main objective of this study was to evaluate the use of tetrazolium chloride for rapid tests of freshly frozen, immature seed corn. Immature seed required up to eight weeks to germinate in sand, giving a lower average value than dried seed even after this time. On the other hand, Rossman (**12**) found that quick drying, such as would be required for rapid conventional tests of frozen corn, may further reduce viability. We attempted, therefore, to correlate a quick tetrazolium test with sand germin-

TABLE IV

PERCENTAGE GERMINATION AND TETRAZOLIUM STAINING OF CORN FROZEN AT INCREASING MOISTURE CONTENT. (2 LOTS OF 100 SEEDS EACH)

SERIES	MOISTURE (WET WT. BASIS)	FREEZING TEMPERATURE, °F.	GERMINATION IN SAND			TETRAZOLIUM STAINING			REPEATED TETRAZOLIUM
			NORM.	ABN.	DEAD	FULLY COLORED	WHITE SPOT*	COLORLESS	NORMAL
1	18.2	20	99	1	0	87	13	0	
		0	98	2	0	88	12	0	
2	19.6	20	95	4	1	88	10	2	
		0	98	1	1	87	12	1	
3	23.1	20	97	2	1	93	6	1	
		0	68	3	29	59	10	31	
4	25.5	20	98	2	0	87	11	2	
		0	62	4	34	63	26	11	
5	28.1	20	92	0	8	90	0	10	89
		0	11	6	83	32	48	20	10
6	32.0	20	76	8	16	77	5	18	82
		0	2	0	98	74	6	20	1
7	32.9	20	55	15	30	72	19	9	63
		0	0	0	100	47	2	51	0
8	36.1	20	7	0	93	60	26	14	7
		0	0	0	100	.....	.....	.....	0

* In scutellum.

ation after rapid and after slow drying, sometimes with seed from the same ear and sometimes on a composite sample when seed quantities were too small for ear by ear comparisons.

Material for these tests was supplied and freezing treatments made by Mr. M. M. Aboul-Ela. Ear remnants for the sand tests were dried at 95° F., either at once or after 3 or 10 days of slow drying in the field. Our tests did not show any consistent differences in the germination of corn dried rapidly immediately after freezing or ten days after freezing, and these treatments are combined in the data.

Three collections were made, the moisture contents of which were approximately 60, 50 and 40 per cent. Ears were selected from the field at random and divided into three groups for treatment. One group of ears was

TABLE V

COMPARISON OF MEAN PERCENTAGE VIABILITY ESTIMATES OF TETRAZOLIUM TESTS OF UNDRIED EARS, WITH GERMINATION TESTS OF THE SAME EARS AFTER RAPID DRYING.

Series	Moisture at freezing	Freezing treatment	Tetrazolium No. of tests	Tetrazolium Mean	Germination No. of tests	Germination Mean	Mean diff.	Stand. dev.	"t"	Freezing temperature
1	58.3	unfrozen	22	99.7	13	95.2	− 4.5	.86	5.23*	20° F.
		8 hours	22	77.1	18	64.5	−12.6	4.98	2.53*	
		16 hours	22	31.8	18	18.4	−13.4	5.50	2.44*	
2	50.3	unfrozen	30	99.8	13	97.2	− 2.6	.77	3.39*	20° F.
		8 hours	30	61.1	10	54.0	− 7.1	6.51	1.09	
		16 hours	30	32.7	9	19.9	−12.8	5.30	2.42	
3	39.4	unfrozen	31	99.7	9	98.9	− .8	1.98	.40	10° F.
		8 hours	25	17.4	10	22.2	− 4.8	4.67	1.02	
		16 hours	25	1.6	9	3.8	− 2.2	4.26	.52	

* Significant at 5% level.

left unfrozen, a second was frozen for eight hours and a third for 16 hours at temperatures of 20 or 10° F. Ears from each treatment were then subdivided into three lots. The first of these was tested immediately, the other two were returned to the field to be dried on racks. One of these lots was tested after three days, the other after ten days in the field. Duplicate samples from each ear were tested by the tetrazolium method and by the germination test. Seed for these tests was removed from the ears in longitudinal rows. The ear remnants were then dried in the corn-seed drier and retested by both methods. Table V presents the summarized data of tetrazolium tests of undried and sand tests of dried samples.

All fresh seeds stained completely when placed in the tetrazolium solution, regardless of freezing treatment. While there was no noticeable difference in the color intensity produced in immature samples, there were differences in the shade of color. The color of the embryo axis was purplish in frozen seeds. Seeds tested three days after freezing could be dis-

tinguished by a white film which masked the staining. This film became more pronounced as the time between freezing and immersion of the cut seeds in the tetrazolium solution was increased. Dead seed tested after ten days exposure in the field was colorless or almost colorless. Color differences were more noticeable in the staining of the more mature fresh seeds. Those assumed to be viable were generally paler and more orange in color. The structure of the viable seed was clearly visible, whereas in frozen seed the outline of the germ was blurred and its internal structure

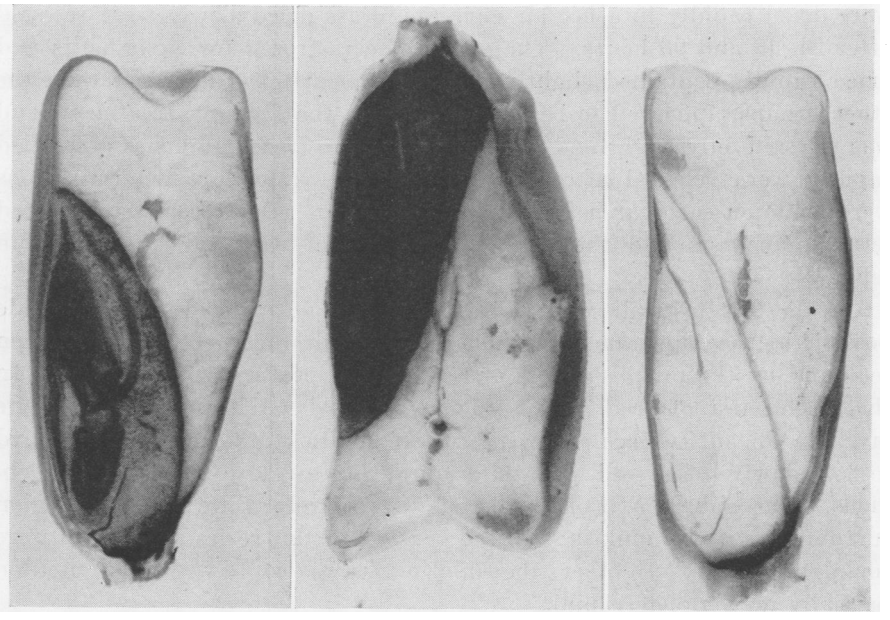

FIG. 1. Staining of maize grain in tetrazolium solution. Left, normal seed; center, freshly frozen seed showing heavy, blurred staining; right, frozen seed 1 week later showing typical Lakon stain for dead grain.

could not be distinguished clearly. Some idea of the differences observed may be obtained from figure 1.

Formation of color by the reduction of the tetrazolium solution was not a criterion of viability. The color produced by reduction of the compound accentuated the differences in viable and non-viable seeds, however, so that separation was possible. Our readings with tetrazolium tended to be five or ten per cent. higher than the actual vitality indicated by the germination test. As stated above, we had no evidence that this difference was due to the drying treatment.

Effect of Rate of Drying

Tests were made to determine whether there was an optimum period after freezing for testing with tetrazolium, at which time the separation of

live and dead seed could be made with the greatest accuracy; also to determine whether the rate of drying after freezing would affect this optimum.

Twenty ears of immature corn containing from 40 to 45 per cent. moisture were used in these tests. Ten ears were frozen at 20° F. for eight hours and ten for 16 hours. The first tetrazolium test was made about one hour after removal from the freezing chamber. At the completion of this preliminary test, five ears from each freezing treatment were left to dry at room temperature, while the remaining five ears from each group were dried rapidly in a hot air current. Ears dried slowly were retested after 24, 48 and 96 hours. The group of ears frozen for eight hours and dried rapidly contained slightly (three per cent.) less moisture than the other groups, appeared to be less affected by the freezing treatment, and was retested only at the end of 24 hours. Ears frozen 16 hours and dried rapidly were retested after 24 and 48 hours. Moisture determinations were made on each ear before and after drying. Data from the repeated tetrazolium tests and the germination tests of each ear are shown in table VI.

In the first tests all seeds stained. Separation of dead seed was made possible by the appearance of a whitish film on the cut surface of the frozen seed and by the purplish color and blurred appearance of the germ. In the second test of seeds dried slowly, the seeds all stained. The white layer on the cut surface of dead seeds seemed to be thicker. In the third test of slowly dried seed, the white appearance of dead seed was very obvious. The embryo axis of some seeds was entirely white while the region surrounding it was pinkish. In the last test of this series some seeds were completely white. In others the embryo axis was white with the scutellum partially or completely pink.

In the second test of seed dried rapidly, dead seeds which were placed cut side down in the solution showed deep coloration of the embryo axis. Frozen seeds not in this position were pinkish-white. In the third test of rapidly dried seeds, dead seeds were very pale. In some, the embryo axes were white, while the scutellar region was colored. No increase in the accuracy of the tetrazolium tests, as we read them, resulted from a delay after freezing. The means of the tests for each time interval were very close. Except in the second group, the tetrazolium readings were significantly higher than those obtained from the sand tests.

There appeared to be considerable variation in tetrazolium tests of seed from the same ear. To determine whether this variation was due to misinterpretation of staining or was caused by differences in germinability, all seeds from three frozen ears were planted in sand. Each ear was divided transversely into three sections. Each section was divided longitudinally into four parts for planting. The lowest third of the ear gave the highest germination and the upper third the lowest in each comparison, with differences of more than 30 per cent. on two of the three ears.

TABLE VI

EFFECT OF RATE AND AMOUNT OF DRYING OF FROZEN CORN ON THE TETRAZOLIUM READINGS

TREATMENT	MOISTURE % AS FROZEN		GERMINATION	STAINING 1 HR.	STAINING 24 HRS.	STAINING 48 HRS.	STAINING 96 HRS.	MOISTURE AT END
FROZEN 8 HOURS, FAST DRY.	40.7		95	100	95			20.8
			84	98	97			
			96	92	99			
			95	96	97			
			87	87	86			
		Mean	91.4	94.6	94.8			
FROZEN 16 HOURS, FAST DRY.	43.6		48	48	52	49		17.5
			66	51	59	52		
			81	71	73	79		
			62	53	58	57		
			57	50	50	46		
		Mean	62.8	54.6	58.4	56.6		
FROZEN 8 HOURS, SLOW DRY.	45.7		58	68	62	57	70	36.2
			71	74	75	75	76	
			83	70	80	83	72	
			74	81	82	82	75	
			52	85	83	74	74	
		Mean	67.6	75.6	76.4	74.2	73.4	
FROZEN 16 HOURS, SLOW DRY.	42.2		40	66	71	64	54	34.2
			52	48	51	53	51	
			45	52	52	50	52	
			39	41	49	61	55	
			69	81	73	74	86	
		Mean	49.0	57.6	59.2	60.4	59.2	

Effect of pH

The preparation of 2,3,5-triphenyltetrazolium chloride involves purification of the salt from hydrochloric acid, and the acidity of the resulting product varies with the method of preparation. To determine whether such variations in acidity might have an effect on the staining qualities of the compound, buffer solutions were made with mixtures of citric acid and disodium phosphate (McIlvaine's standard). The pH determinations were made on a Leeds and Northrup glass electrode pH meter. The sample of corn used was one which had been frozen while immature. It

had a germination of 70 per cent. and an additional 12 per cent. of seeds which developed abnormally.

In the first test the seeds were soaked overnight, then cut and placed in the buffer solutions with 0.5 per cent. tetrazolium chloride. Dishes containing the samples were kept at 30° C. for two and one half hours, and at the end of that time the staining in the different solutions was compared. It was found that viable seeds stained intensely from pH 8.0 to 6.8. From 6.6 to 6.0 the staining became progressively paler although it was sufficient to distinguish viable from dead seeds. Below 5.0 the staining was too pale for satisfactory readings. All viable seed appeared to stain comparably at each pH level, with no differentiation of seeds which might produce abnormal seedlings.

In a second experiment, uncut seeds were soaked overnight in the buffer solutions, after which they were cut and treated in the usual manner. At the conclusion of the tests it was found that all viable seeds stained adequately, although staining was paler in seeds presoaked in buffers with pH less than 5.0. Again abnormal seeds could not be distinguished by their staining differences.

Determinations of pH were made on solutions of different lots of tetrazolium chloride. The pH range was from 9.5 to 3.3. Solutions of all these samples showed no differences in the staining of viable seeds.

When the pH of the buffered tetrazolium solutions was below 5.0 the efficiency of the reducing agent was decreased so that only a pale stain resulted. In unbuffered solutions, however, the pH of the solution was unimportant. Staining was affected only slightly by soaking the seed in the buffered solutions before testing. It is probable that the cells of the tissue have a strong buffering action. It would appear, therefore, that variations in the pH of the tetrazolium solutions normally used in testing have little effect on staining.

Discussion

The tetrazolium test is a test of reducing enzymes or substances within the seed rather than of viability. One of the authors (**2**) has been unable to show a satisfactory correlation between peroxidase activity, used as an index of enzyme action, and tetrazolium reduction in frozen corn. Peroxidase remained constant while staining first increased and then decreased to near zero. It seems probable, therefore, that labile reducing substances normally maintained, or produced in the viable seed during soaking, are the basis of the Lakon test. The first increase of staining characteristic of freshly frozen seed is probably due to increased permeability and more rapid absorption of stain. The decreased tendency to stain with tetrazolium developed most rapidly when the frozen seeds were dried slowly at room temperatures, but it was usually several days before the presence or absence of stain in the embryo could be used as an index of viability. On the other hand it was not difficult to measure other differences in the

staining and appearance of frozen embryos which made it possible to estimate their viability immediately after freezing. While the criteria used here, tint, mottling, texture, etc., are difficult to describe, they can be read by an experienced observer. (Cf. fig. 1.)

Since increased permeability is a characteristic (**3**) and immediate response of cells to freezing injury, tests of permeability with various dyes suggest themselves for quick measurements of freezing injury to corn. The tetrazolium test also is affected by permeability, however, as indicated above, and so is useful in early as well as in later tests of injury.

The uniformly high estimates of viability obtained with the tetrazolium seem to be largely the result of partial injury in seeds which are actually "viable" but not "germinable" in sand in the sense of being capable of producing a normal seedling.

Since this manuscript was prepared Goodsell has published a study of the staining of frozen maize with tetrazolium (**7**). His results agree with ours in showing that the Lakon test of pink staining is not applicable to freshly frozen, immature corn. Goodsell's recommended method is to dry the frozen corn rapidly and resoak before staining. He notes that even this procedure was not successful with a sample frozen at 51 per cent. moisture. By changing the basis of reading the tests, we have been able to estimate freezing injury immediately in corn frozen with as much as 60 per cent. moisture.

Summary

Freezing injury of seed corn could be estimated with fair accuracy from the development of embryo staining in a 0.05 per cent. solution of 2,3,5-triphenyltetrazolium chloride, provided the germination was moderately high and the corn had been stored for some time after freezing. The tetrazolium readings were commonly higher than the germination tests in sand, and did not give satisfactory estimates of percentages of abnormal seedlings.

When tetrazolium was used on freshly frozen, immature corn of 30 to 60 per cent. moisture content, staining of dead kernels tended to be more intense rather than spotted or absent as expected from tests of old seeds or those killed in hot water. This intensity we ascribe to increased permeability of the injured cells to the dye, which is then reduced to the colored form by substances present in the tissues before freezing. The dead kernels slowly lose the ability to reduce tetrazolium, and after several days or weeks can be tested by Lakon's method.

With experience, however, it is possible to estimate the injured seeds immediately after freezing, using tint, texture, structural appearance and other characteristics of the injured tissue instead of the presence or absence of a pink stain. The accuracy of such early estimates is not less than that obtained later. All tetrazolium tests tend to give a high estimate of germinability, particularly of badly injured seed, and the method

is not recommended for critical testing. It provides a means, however, for estimating the severity of frost damage to corn within two hours instead of one or two weeks.

IOWA STATE COLLEGE
AMES, IOWA

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