THE MECHANISM OF THE TETRAZOLIUM REACTION IN CORN EMBRYOS[1]

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(WITH TWO FIGURES)

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The reduction of tetrazolium salts to insoluble, colored formazans by living tissues was pointed out by KUHN and JERCHEL in 1941 (**18**). The reaction has since been widely used as an indicator of viability or metabolic activity with a variety of tissues and tissue extracts from animals (**6, 19, 28, 31**), microörganisms (**5, 11, 13, 22**), and higher plants (**9, 12, 27, 32**), especially seeds (**8, 10, 20, 25**). The 2,3,5-triphenyltetrazolium salts (hereafter called tetrazolium) have been used most commonly although several other derivatives have been introduced recently (**1, 2, 28**).

The biological reduction of tetrazolium has generally been attributed to enzymatic action. This interpretation was first suggested by KUHN and JERCHEL (**18**) on the basis of the low oxidation-reduction potential (–0.080 volts) and the lack of reduction by dead tissues or by ascorbic acid, sulfhydryl compounds, or sugars at physiological pH values. MATSON *et al.* (**24**) first reported that a dehydrogenase preparation (the glucose-6-phosphate system) would catalyze reduction, and several others (**19, 22, 28, 31**) have used the reaction as a dehydrogenase indicator.

Because of the increasing use of the tetrazolium test in the measurement of seed viability a more thorough study of the mechanism of the reaction in seed tissues was undertaken in 1949. This paper reports the determination of optimum conditions and the kinetics of tetrazolium reduction by malic dehydrogenase, the role of diaphorase, and the relative activities of several diphosphopyridine-nucleotide-linked dehydrogenases in corn embryos. Since this work was completed JENSEN *et al.* (**16**) have published further evidence that several pyridine-nucleotide-linked dehydrogenases would reduce tetrazolium, and BRODIE and GOTS (**7**) have shown that diaphorase may participate in the reaction.

Materials and methods

All enzyme preparations were made from seed of open pollinated WF9 × 38–11 hybrid corn. The seeds were soaked at 30° C for 18 to 20 hours with the embryo side down and half immersed in water. At this stage of germination there was visible radicle growth but seldom any emergence. Excised embryos were ground in cold 0.02 M phosphate buffer (pH 8) in a Potter-Elvehjem homogenizer to give a 10% suspension on a fresh weight basis. Cellular debris was removed by centrifuging cold for seven to eight

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minutes at about 1000 times gravity and the supernatant, which contained all the enzyme activity, was stored in an ice bath. There was no appreciable loss of activity for several hours and high levels of activity remained even after several days in the refrigerator.

Tetrazolium reduction was measured at 30° C in 15 ml. graduated centrifuge tubes containing 3 ml. of reaction mixture including buffer, substrate, diphosphopyridine-nucleotide (DPN), tetrazolium, diaphorase, and enzyme. Optimum conditions and concentrations are described in the following section. The reaction was stopped after 2 to 10 minutes by the addition of 6 ml. of dioxane which precipitated the enzyme and dissolved the formazan (2,3,5-triphenylformazan). The latter was then extracted by shaking with 1.5 ml. of xylene and centrifuging to give about 4.6 ml. of nonaqueous layer. The concentration of formazan was measured by the optical

TABLE I

FACTORS AFFECTING THE REDUCTION OF TETRAZOLIUM BY THE MALIC DEHYDROGENASE SYSTEM. R REPRESENTS RELATIVE RATES AS PER CENT. OF THAT UNDER OPTIMUM CONDITIONS (R = 100). OPTIMUM CONDITIONS WERE USED FOR OTHER COMPONENTS IN EACH CASE. AMOUNTS OF DIAPHORASE ARE GIVEN IN ML. OF THE STOCK PREPARATION IN REACTION MIXTURE.

Malate		DPN		Diaphorase		Glutamate		Tetrazolium		pH		Oxalacetate	
M	R	Mg.	R	ml.	R	M	R	M	R	pH	R	M	R
0	0–3	0	0–3	0	3	0*	40	0.008	14	8	5	0	100
0.017	35	0.5	20	0.05	8	0**	25	0.02	100	8.9	25	0.001	20
0.033	60	1.0	55	0.20	60	0.25	100	0.04	75–100	9.5	100		
0.050	85	1.5	100	0.60	100	0.50	100			9.9	24		
0.067	100	2.0	100	1.20	100					10.4	9		

* Glycine buffer.
** Ammonia buffer.

density at 490 mμ in 1 cm. cuvettes in a Beckman spectrophotometer. During extraction and measurement the solution was protected from strong light.

Methylene blue reduction was measured by the time for complete decolorization at 30° C in Thunberg tubes using 3 ml. of a reaction mixture containing 0.033 M phosphate (pH 8), 1 mg. DPN, 1.7×10^{-5} M methylene blue, 0.6 ml. of diaphorase preparation, and varying concentrations of substrate and enzyme.

The reduction of tetrazolium by chemically reduced DPN was carried out in the following reaction mixture: 0.25 micromole of reduced DPN, prepared by HOGEBOOM's technique (**14**) and standardized spectrophotometrically, 12 micromoles of tetrazolium, and 0.6 ml. of diaphorase preparation, all in 5.7 ml. of carbonate-bicarbonate buffer (pH 9.7). After a 10 minute incubation at 30° C, the formazan was extracted by a dioxane-xylene mixture and measured spectrophotometrically.

The diaphorase preparation was made from pig heart by a modification of STRAUB'S method (**30**). The alkaline phosphate extraction was made in a Waring Blendor, and the supernatant, following 43° C incubation, was 0.8 saturated with ammonium sulphate in the cold. The precipitate, after being redissolved in water, was dialyzed free of ammonia; and the opalescent yellow solution was made slightly alkaline with Na_2HPO_4 and stored frozen. This preparation contained a significant amount of malic dehydrogenase activity which was destroyed by heating at 60° C for 10 minutes.

Other special materials used were the following: milk flavoprotein, prepared according to BALL (**3**); DPN (40%), 2,3,5-triphenyltetrazolium chloride, hexose-diphosphate, and hypoxanthine, Schwartz Laboratories; L-malic acid, L-glutamic acid, sodium α-glycerophosphate, Eastman; β-hydroxybutyrate, Fisher; oxalacetic acid, Organic Specialties.

Results

PROPERTIES OF THE MALIC DEHYDROGENASE-TETRAZOLIUM REDUCING SYSTEM

The enzyme preparation alone, dialyzed or undialyzed, caused very little reduction of tetrazolium. With the addition of any of several dehydrogenase substrates plus DPN, there was some increase in rate of reduction but maximum rates required the addition of a flavoprotein carrier, diaphorase. Since the malic system was especially active, it was chosen for detailed analysis of tetrazolium reduction by a DPN-linked dehydrogenase. Oxalacetate, product of the reaction, strongly inhibited the enzyme and had to be removed to maintain the rate. Cyanide, which has been used by others (**21**), increased the rate markedly at pH 8 but, at higher pH values, cyanide reduced tetrazolium and was unusable. Glutamic acid, acting presumably by transamination (**23, 26**), increased the rate several-fold and served both to bind oxalacetate and as a buffer. The inhibitive effect of added oxalacetate is shown in table I. The optimum pH of the system with glutamate was found to be about 9.5, and at pH 12 to 13 non-enzymatic reduction appeared. Other buffer salts, including borate, ammonia, and glycine, used with or without glutamate had variable effects on reaction rates but in no case gave a higher rate than gutamate alone. Phosphate generally increased the rates at pH 8 and occasionally at 9.5, but the results were not consistent and appeared to vary with the enzyme preparation. The effects of varying concentrations of malate, DPN, diaphorase, glutamate, and tetrazolium are shown in table I. The optimum levels indicated were used in all subsequent work. The low rate without malate showed that there was no significant glutamic dehydrogenase activity in either the diaphorase or the enzyme preparation at that level. Negligible rates without enzyme or with heated enzyme also showed the absence of malic dehydrogenase in the diaphorase preparation. Addition of nicotinamide (**21**) was not found to affect the rates or to reduce the DPN requirement.

Under the optimum conditions described, however, the reaction rates were not constant. Typical rate curves are shown in figure 1. In most

cases (curve 1) the rate increased during the first four or five minutes and then remained nearly constant. In fact, the first part of the reaction gave an approximately logarithmic increase in formazan concentration with time. In some cases (curve 2), however, no constant rate was reached during the 10 minute reaction period.

Since it appeared that the rate of tetrazolium reduction increased as the formazan concentration built up, at least to a certain level, the effect of adding formazan at the start of the reaction was investigated. Formazan was prepared both by enzymatic action and by dithionite reduction. In the latter case the suspension was stabilized with gelatin and washed with

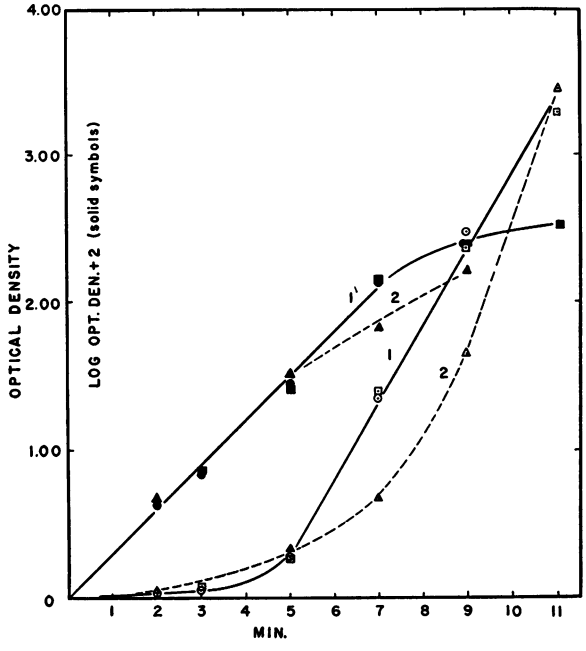

FIG. 1. The change in reaction rate with time. Optimum conditions were used. Curves 1 and 2 represent the types of rate curves observed. Outline symbols are for optical density, solid symbols for log of optical density.

oxygen to remove excess reducing material. Both type of formazan preparations were found to increase tetrazolium reduction under appropriate conditions. There was no evidence of formazan destruction in any of the reaction systems. Typical results are given in table II. Tetrazolium alone was not reduced by the formazan preparations nor was the complete enzymatic system affected by adding a dithionite gelatin blank (prepared without tetrazolium). Heating the enzyme, omitting malate, DPN, or diaphorase, or adding oxalacetate, all prevented extra tetrazolium reduction by added formazan, as well as blocking enzymatic reduction. Apparently the formazan effect involved the dehydrogenase itself. The effect was somewhat erratic, however, varying in magnitude and failing altogether in a few cases

(Exp. no. 6–27b). The reason for this variability was not clear, but it appeared to depend on the level of enzyme activity and the extent of the reaction. If the enzyme activity was such that the formazan concentration built up very rapidly in a normal system, *i.e.* without added formazan, an added amount at the start under similar conditions had a negligible effect on the enzymatic reduction. There was also evidence that the age of the homogenate may have affected the magnitude of the formazan effect. Whatever the basis of this effect may be, it seems to be involved in the production of the unusual rate curves observed.

TABLE II

FACTORS CONTROLLING THE FORMAZAN EFFECT. THE COMPLETE SYSTEM (CS) CONTAINED MALATE (M), DPN, DIAPHORASE (D), GLUTAMATE, TETRAZOLIUM (T), AND ENZYME AT OPTIMUM CONCENTRATIONS GIVEN IN TABLE I. FORMAZAN (F) CONCENTRATION WAS MEASURED BY OPTICAL DENSITY (OD). EXTRA $[F] = OD_{(CS + F)} - [OD_{(CS)} + OD_{(F)}]$. AMOUNT OF ADDED FORMAZAN IS INDICATED BY THE OD OF REACTION MIXTURE CONSISTING OF F ALONE.

Exp. no.	Reaction mixture	Time	[F]	Extra [F]
		min.		
4–13	F	20	1.31	....
	F + T	20	1.30	....
4–17	CS + F	10	2.96	0.89
	CS	10	0.76	....
	F	10	1.31	....
	CS − M	10	1.26	0
	CS − DPN	10	1.29	0
	CS − D	10	1.29	0
	CS + 0.001 M oxalacetate	10	1.40	0
4–19	CS + F	15	2.90	1.27
	CS	15	0.56	....
	F	15	1.07	....
6–27a	CS + F	15	1.10	0.43
	CS	15	0.24	....
	F	15	0.43	....
6–27b	CS + F	15	0.89	0
	CS	15	0.36	....
	F	15	0.58	....

Furthermore, reaction rates were not found to be directly proportional to enzyme concentration. Figure 2 shows the types of behavior observed ranging from curve 1 where increasing enzyme concentration gave a nearly linear increase in rate to curve 3 where a logarithmic increase resulted. The intermediate type, curve 2, was most commonly observed. The only conditions under which fair proportionality was achieved were with very low enzyme activities which gave formazan concentrations having optical densities below 0.2. These results again seem to indicate an accelerating effect of high formazan concentrations. With the present system no conditions have

been found which make tetrazolium reduction suitable for accurate dehydrogenase assay.

Tetrazolium reduction by chemically-reduced DPN

The role of diaphorase in DPN-linked tetrazolium reduction also can be demonstrated in the non-enzymatic reaction between dithionite-reduced DPN and tetrazolium. As shown in table III, no significant amount of formazan was produced unless diaphorase was added. The amount of reduced DPN was determined spectrophotometrically. Under the conditions used the reaction was apparently either slow or incomplete, but the catalytic action of diaphorase was clearly indicated.

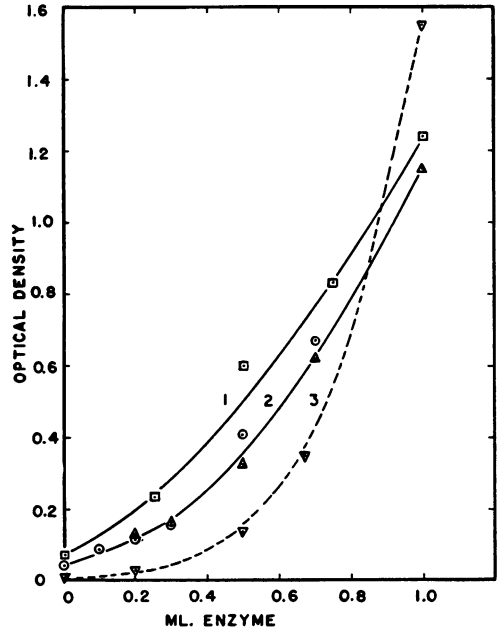

Fig. 2. Reaction rate as a function of enzyme concentration. Optimum conditions were used with a five minute reaction time.

The diphosphopyridine-nucleotide-linked dehydrogenases of the corn embryo

In addition to malate, four other substrates caused tetrazolium reduction in the standard DPN-diaphorase-homogenate system. Results are included in table IV. Alcohol dehydrogenase was the only one approaching the activity of the malate system. Both glutamic and β-hydroxybutyric dehydrogenases were very much less active. The reduction with hexosediphosphate was probably due to triosephosphate dehydrogenase after splitting of the hexose by aldolase. Some of the other substrates, especially glycerophosphate, gave slight reduction after much longer reaction times but no measurable amount of formazan during the standard 10 minute pe-

TABLE III

TETRAZOLIUM REDUCTION BY CHEMICALLY REDUCED DPN.

DPN · H_2	Diaphorase	Tetrazolium ml. 4%	Optical density 490 mμ, 3.6 ml.	Formazan
μM	ml.			μM
0.24	0.6	0.1	0.106	0.023
0.24	0	0.1	0.009	
0.24	0.6 (5 min. 100° C)	0.1	0.013	
0	0.6	0.1	0.006	

riod. The minimum reaction measurable under these conditions was equivalent to about 0.003 micromole of substrate oxidized.

The above substrates also were tested for methylene blue reduction using the same conditions with the Thunberg technique. Though rates by the two methods are not strictly comparable, the data in table IV do show that the embryo homogenate had a similar relative activity toward the various substrates in each case. Methylene blue reduction, however, was observed with several substrates which did not yield a measurable amount of formazan. Since complete methylene blue reduction was equivalent to 0.05 micromole of substrate oxidized and only one fifth as much enzyme was used in these cases, it was apparent that methylene blue was a more effec-

TABLE IV

COMPARISON OF TETRAZOLIUM AND METHYLENE BLUE REDUCTION BY VARIOUS SUBSTRATES IN THE CORN EMBRYO HOMOGENATE-DPN-DIAPHORASE SYSTEM, L-MALATE AT pH 9.5, ALL OTHERS AT pH 8.0.

Substrate	Methylene blue		Tetrazolium	
	Enzyme conc. (mg. fresh wt. per 3 ml.)	Time for complete reduction	Enzyme conc. (mg. fresh wt. per 3 ml.)	Optical density (10 min.)
		sec.		
L-Malate (0.05 M)	0.25	150	2.5	1.00
Alcohol (0.5 M)	0.25	240	2.5	0.100
β-Hydroxybutyrate (0.04 M)	10	100	50	0.083
Hexose-diphosphate (0.02 M)	10	180	50	0.072
L-Glutamate (0.05 M)	10	195	50	0.037
α-Glycerophosphate (0.03 M)	10	390	50	0
Citrate (0.1 M)	10	390	50	0
Succinate (0.1 M)	10	340	50	0
Glycine (0.05 M)	10	300	50	0
Hypoxanthine (0.01 M)	10	640	50	0
D-glucose (0.1 M)	10	600	50	0
Formate (0.1 M)	10	>600	50	0
D,L-Lactate (0.1 M)	10	>600	50	0
Hypoxanthine	0.1*	100	0.6*	1.375

*Ml. of milk flavoprotein.

tive hydrogen acceptor than tetrazolium. The results with malate and alcohol lead to the same conclusion.

Since reactions with citrate, succinate, glycine, and hypoxanthine required the addition of DPN, they were probably not catalyzed by the isocitric dehydrogenase-aconitase system or by succinic, amino acid or xanthine oxidases. These substrates must have been further metabolized to some DPN-linked dehydrogenase substrates (except, perhaps, in the case of citrate since there is evidence with some tissues (**15**) of conversion of DPN to TPN (triphosphopyridine nucleotide) which would allow isocitric dehydrogenase action and there is a recent report of a DPN-linked isocitric dehydrogenase (**17**)). Finally, no evidence for formic or lactic dehydrogenase activity was found by either method.

In addition to the DPN-linked dehydrogenases of corn embryo a milk flavoprotein containing xanthine oxidase was found to reduce tetrazolium (table IV). This observation and the diaphorase requirement of the DPN-linked systems indicate that flavoproteins are a characteristic part of the tetrazolium reducing mechanism.

Discussion

This work has shown that DPN-linked dehydrogenases coupled through diaphorase actively reduce tetrazolium. Substrates showed a wide range of activity from malate to glutamate and, with the exception of lactate, were the same as those giving a positive test with the corn embryo preparation of JENSEN *et al.* (**16**). The latter workers found reduction without added diaphorase using a saturated ammonium sulphate precipitate from an aqueous extract of acetone-dried embryos. However, no quantitative measurements were made and there is no indication that their conditions were optimum for tetrazolium reduction.

Optimum conditions for the malic dehydrogenase of corn embryo homogenates with tetrazolium as hydrogen acceptor were found generally similar to those reported for other tissues and acceptors. Optimum malate concentration agreed with that of BERGER and AVERY (**4**) with Avena coleoptile and thionine and with that of POTTER (**26**) with rat liver and the cytochrome system. The optimum DPN concentration was about half that found by Potter. The optimum pH was similar to that with Avena and with the dissociated kidney and liver preparations of HUENNEKENS and GREEN (**15**), who used 2,6-dichlorophenolindophenol, except that the optimum range was much narrower. The corn embryo-malate system, like the dissociated cyclophorase preparation, showed a much smaller response to oxalacetate-binding agents at pH 9.5 than at 8 but even at the higher pH, glutamate increased rates considerably.

The incomplete reduction of tetrazolium by dithionite-reduced DPN which was observed may be explained by the recent report of BRODIE and GOTS (**7**) that the reaction is complete only under anaerobic conditions and that the optimum pH is lower than that used here.

The marked diaphorase stimulation of the corn embryo-tetrazolium system was also true of the Avena coleoptile-thionine system. Malic dehydrogenase in homogenates (**26**) or cyclophorase (**15**) preparations from mammalian tissues, however, do not require added diaphorase, though purified preparations may require it (**21**). The difference between the plant and animal preparations may be merely in the amount or stability of the diaphorase or in the degree of association with the dehydrogenase. However, the nature of the hydrogen acceptor may also be involved. The corn embryo-malic system showed a much smaller response to added diaphorase with an indophenol dye as acceptor (unpublished results), similar to that of Huennekens and Green, than with tetrazolium or methylene blue.

The malic dehydrogenase-tetrazolium reaction with the corn preparations was peculiar in the unusual changes in rate with time and enzyme concentration. The product of the reaction, triphenylformazan, in some way increased dehydrogenase reaction. Whether this action depends on the insolubility of the formazan or the low potential of the system, both of which distinguish it from other common hydrogen acceptors, or whether some other unknown property of the system is involved has not been determined. The same type of corn preparations with an indophenol acceptor, however, has shown no such behavior (unpublished results).

Another peculiarity in the tetrazolium method described is the high concentration of acceptor (0.02 M) required for maximum rates. Most of the other acceptors have been used at a level at 10^{-4} or 10^{-5} M. This fact and the lower rate of reduction of tetrazolium compared with methylene blue seem to indicate that the former is a less efficient acceptor. However, the tetrazolium method is aerobic and, although the formazan is not autoxidizable, it is possible that oxidases may compete as hydrogen acceptors. In fact, some evidence was found that cyanide, which poisons metal-containing oxidases as well as binding oxalacetate, would allow somewhat higher rates than glutamate at pH 8 though the point has not been adequately studied. With intact oat embryo tissue unpublished data have shown that tetrazolium reduction decreased with increase in oxygen atmospheres from 5 to 100%. The effect of oxygen tension on reduction rates in homogenates has not yet been fully investigated. Significant oxidase interference would be most likely with the higher homogenate concentrations and may explain the failure to observe tetrazolium reduction with some substrates active in the Thunberg technique. The failure of formazan production in the triosphosphate-DPN system of BRODIE and GOTS (**7**) also indicates that competing hydrogen acceptors may function under aerobic conditions.

It should be emphasized again that reduction resulting from the addition of a given substrate does not prove that dehydrogenase action is on that substance alone, especially if the activity is low. The behavior of succinate in the present work and that of JENSEN *et al.* (**16**) illustrates the caution required in basing an assay procedure (**19**) on what may be a multiple enzyme system where there is no certainty as to which is the limiting step.

For further elucidation of the mechanism of tetrazolium reduction it will be desirable to extend the present results with purified enzyme systems.

At present a positive tetrazolium reaction in seed viability tests may be due to the action of pyridine-nucleotide-linked dehydrogenases or possibly to aerobic dehydrogenases such as xanthine oxidase. A weak or negative reaction may indicate (a) deterioration or lack of the dehydrogenases or diaphorase, (b) insufficient supply of substrates, or (c) competitive action of aerobic hydrogen acceptors.

Summary

Reduction of triphenyltetrazolium chloride by corn embryo tissues is catalyzed by diphosphopyridine-nucleotide-linked dehydrogenases, particularly the malic and alcohol systems, and is mediated by diaphorase. Optimum conditions and kinetics of the malic system were investigated, and a comparison of tetrazolium and methylene blue reduction with various substrates was made. The aerobic dehydrogenase, milk xanthine oxidase, also catalyzes the reaction.

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