Sites of Electron Transfer to Tetrazolium Salts in the Succinoxidase System*

Marvin M. Nachlas, Stanley I. Margulies,† and Arnold M. Seligman

From the Departments of Surgery, Sinai Hospital of Baltimore, Inc., and The Johns Hopkins University School of Medicine, Baltimore, Maryland

(Received for publication, February 23, 1960)

With the increased use of tetrazolium salts in biological systems (1–20), and with the increased availability of tetrazolium salts with widely different oxidation-reduction potentials (3–5, 11, 14, 32), differences in the effect of anaerobiosis on the reaction rate (3, 6, 9, 10, 16, 19), in the inhibitory action of cyanide (6, 15, 18, 21–24), and in the inhibitory action of cyanide (6, 15, 18, 21–24), have become more evident. It has occurred to us that these differences could be due to the fact that the various tetrazolium salts accept electrons from different sites along the electron transport chain. Although recent publications have dealt with the sites of electron transfer to a variety of acceptors, little attention has been given to tetrazolium salts (25–27), other than to point out both similarities and dissimilarities with other oxidation-reduction dyes, such as methylene blue and 2,6-dichlorophenolindophenol (9, 28–31).

In order to test the above hypothesis, three pairs of tetrazolium salts were selected for study. These were two monotetrazolium salts, without and with nitro-substitution (2, 3, 5-triphenyl tetrazolium chloride and 2-p-nitrophenyl-3-p-iodophenyl-5-phenyltetrazolium chloride); two ditetrazolium salts without and with nitro-substitution (2, 2', 5, 5'-tetraphenyl-3, 3'- (3, 3'-dimethoxy-4, 4'-biphenylene) ditetrazolium chloride; and two ditetrazolium salts without methoxyl substitution, and without and with nitro-substitution (2, 2', 5, 5'-tetraphenyl-3, 3'- (p-hydroxyphenyl) ditetrazolium chloride; and 2, 2', 5, 5'-tetraphenyl-3, 3'- (p-iodophenyl) ditetrazolium chloride).

Not only do these nitro groups increase the oxidation-reduction potential (32), but they apparently make the tetrazolium salt a more active acceptor of electrons in dehydrogenase systems, whereas the methoxyl group increases the possibility of steric hindrance in reactions with components of dehydrogenase systems.

Although the final organization of electron transport in the succinoxidase system has not been determined, a simplified scheme which incorporates current opinion (33–39) is shown in Scheme 1, together with the special conditions or inhibitors that were used in this study. By notation of the effects of these inhibitors or conditions upon formazan production, inference was made concerning the site of electron transfer to various tetrazoles. It was expected that differences attributable to various degrees of disruption of the organized succinoxidase system might be evaluated by use of three types of enzyme preparations; purified soluble succinic dehydrogenase, homogenates, and tissue sections.

This report will show that the nitro-containing mono- and ditetrazolium salts accept electrons at an earlier stage of electron transport than those without this strong electron-negative group.

EXPERIMENTAL PROCEDURE

Preparation of Incubation Media—The reaction mixture used for both the biochemical and histochemical experiments consisted of the following reagents: sodium succinate (0.2 M), 1.0 ml; phosphate buffer (0.1 M), pH 7.7, 1.5 ml; gelatin (0.1%), 0.5 ml; tetrazolium salt (2 mg per ml), 1.0 ml; enzyme solution, 0.5 ml; and distilled water, 0.5 ml. The reagents may be mixed in any order and allowed to stand at room temperature for 10 minutes before use.

<table>
<thead>
<tr>
<th>Scheme 1</th>
</tr>
</thead>
<tbody>
<tr>
<td>Succinic Dehydrogenase → Cytochrome b → Factor (?) →</td>
</tr>
<tr>
<td>Cytochrome c → Cytochrome a → Cytochrome aa → O₂</td>
</tr>
<tr>
<td>Cyanide</td>
</tr>
</tbody>
</table>

* This investigation was supported by research grants (CY-2475 and H-3223) from the National Institutes of Health, Department of Health, Education, and Welfare, Bethesda, Maryland.

† National Institute of Health Student Scholar of Johns Hopkins University School of Medicine.

† National Institute of Health Student Scholar of Johns Hopkins University School of Medicine.
and stored (without enzyme) at 4° for 1 week. The control tube contained fumarate (0.1 m) in place of succinate. When phenazine methosulfate was used, it was prepared freshly at a concentration of 8 mg per ml, and 0.5 ml was added to the media in place of distilled water.

**Histochemical Procedure**—Small blocks of liver (3 to 5 mm) from freshly exsanguinated rats were frozen rapidly by immersion in isopentane at -70° and then stored at -20°. Sections were cut in the cryostat (-20°) with a rotary microtome, fixed to cover slips by warming with a finger, and air-dried for 1 minute.

For the incubations with the nitro-substituted tetrazolium salts, 12 μ-thick sections were used, whereas for the others, sections were 50 μ thick. The thinnest sections were adequately stained after incubation at 37° for 15 minutes, whereas the unsubstituted tetrazolium salts and the thicker sections required 1 to 2 hours to stain adequately. After incubation, the sections were fixed in 10% formalin for 15 minutes and then were mounted on slides with glycerogel. Formazan deposition was estimated with magnification at 50 X on a scale from 2 - to 2 +. Xnus values indicate inhibition, plus values show activation, and 0 represents no change from the control sections.

**Biochemical Procedures**—Portions of the same rat livers which had been stored at -20° were homogenized in cold 0.1 m phosphate buffer (pH 7.7) to give a concentration of 200 mg of tissue (wet weight) per ml of buffer. Gross debris was removed by centrifugation for 30 seconds at 5000 r.p.m. Because of the different degrees of reactivity with the various tetrazolium salts, it was necessary to vary the concentration of the enzyme solutions as follows: INT, 3 to 4 mg per ml; nitro-BT, 10 to 15 mg per ml; nitro-NT, 30 to 40 mg per ml; NT, 50 to 60 mg per ml and TPT-BT, 200 mg per ml. When phenazine methosulfate was used, the concentrations of enzyme were halved. After 4.5 ml aliquots of the incubation media had been warmed to 37°, the homogenate solution was added. All reactions were run in duplicate. After 30 minutes of incubation with the nitro-substituted tetrazolium salts, it was possible to determine the color density directly in the photocell colorimeter (Klett-Summerson) with a 540 mμ filter. However, with the other tetrazolium salts the turbidity produced by the large amount of formazan into a solution in isopentane at -70° and then stored at -20°.

The incubation period was terminated by the addition to each tube of 0.5 ml of 40% trichloroacetic acid. This was not done in experiments with phenazine methosulfate. The tube was shaken with 5 ml of ethyl acetate, centrifuged briefly to clarify the solvent, decanted into a Klett tube, and the color density determined.

**Experimental Conditions and Reagents Tested**

**Anaerobiosis**—The enzymatic assays were performed under anaerobic conditions obtained by reducing the pressure to a few mm Hg with an oil pump and a dry air ice trap, during the incubation period. A convenient method was to place the reaction tubes in a vacuum desiccator. The tubes were partially sub-

1 The abbreviations used are: 2,3,5-triphenyl tetrazolium chloride, TPT; 2-p-nitrophenyl-3-p-iodephphenyl-5-phényltetrazolium chloride, INT; 2'2',5,5'-tetraphenyl-3,3'-(3,3'-dimethoxy-4,4'-biphenylene) ditetrazolium chloride, BT; 2',2'-di-p-nitrophenyl-5,5'-diphenyl-3,3'-(3,3'-dimethoxy-4,4'-biphenylene) ditetrazolium chloride, nitro-BT; 2',2',5,5'-tetraphenyl-3,3'-(p-biphenylene) ditetrazolium chloride, NT; 2',2'-diphenyl-5,5'-di-m-nitrophenyl-3,3'-(p-biphenylene) ditetrazolium chloride, nitro-NT. merged in 2.5 inches of water at 37° with a small quantity of boiling chips. The jar was sealed, evacuated, and the entire system placed in a 37° water bath. In some experiments, the system was also flushed several times with nitrogen.

**Cytochrome Oxidase Inhibition**—This was accomplished by the use of KCN (1.0 x 10^{-3} m) dissolved in 0.1 m phosphate buffer (pH 7.7). The sections and the homogenates were preincubated with KCN for 30 minutes at room temperature before being added to the various reaction mixtures. The concentration of KCN was 5 x 10^{-3} m during the preincubation period, and 10^{-2} m during the enzyme assay. The histochemical and biochemical preparations were handled similarly. Control enzyme preparations were exposed to buffer alone during the preincubation period.

**Cytochrome c Removal**—The effect of removing cytochrome c was studied only with histochemical preparations. Sections fixed to cover slips were placed in 0.85% sodium chloride solution for 30 minutes (thin sections) or for 60 minutes (thick sections). That this procedure removed most of the cytochrome c from the tissues was verified by incubation of extra sections in the reaction mixture described for the histochemical demonstration of cytochrome oxidase (40). No significant staining was noted unless cytochrome c (1 mg per ml) was added. Occasionally, with the thicker sections all of the cytochrome c was not removed in 1 hour, so that a faintly positive reaction for cytochrome oxidase was noted. When this happened, the sections were allowed to soak for an additional 30 minutes in fresh 0.85% sodium chloride solution.

**Antimycin A**—This inhibitor was dissolved in absolute ethanol at a concentration of 1 mg per ml. The stock solution was stored at -18° and when used was diluted in water (1:100). For the histochemical studies, the sections were preincubated in antimycin A (1.2 μg per ml) for 10 minutes, and then transferred to the reaction mixture to which had been added 0.5 ml of antimycin A (12 μg per ml) instead of the 0.5 ml of distilled water. In the biochemical experiments, 5 μg of antimycin A were added to each 100 mg of tissue (wet weight) in the homogenate. After 10 minutes of contact with the inhibitor, the enzyme solution was added to the incubation mixture containing the tetrazole. Thus, during the incubation periods, the ratio of antimycin A to homogenate was unchanged for all tetrazoles. The enzyme preparations used as controls were exposed to an ethanol-buffer mixture of like concentration and for the same preincubation period.

**Purified Soluble Succinic Dehydrogenase**—To study the reactions of the various tetrazolium salts with the primary dehydrogenase, a 3% solution of succinic dehydrogenase was prepared from beef heart mitochondria acetone powder, according to the directions of Singer et al. (24).

**RESULTS**

**Influence of Anaerobiosis and Cyanide**—Anaerobiosis has been noted to enhance formazan production by the succinoxidase system in several histochemical studies (6, 7, 10, 13, 17) and in a few biochemical reports (3, 5, 9). This phenomenon was less evident in reports with nitro-substituted tetrazoles (16, 18, 19, 41, 42), and was confirmed in the present study with three nitro-substituted tetrazolium salts as compared to three unsub-

2 Prepared by Mr. Francis Stolzenbach, McCollum Pratt Institute, The Johns Hopkins University.
substituted tetrazoles. The results are shown in Table I. No significant amount of activation was obtained by anaerobiosis with the enzyme present in homogenates, although some variation was noted (occasional activation) with the unsubstituted reagents (TPT, BT, and NT). Thus, it appears that oxygen competes most successfully with the unsubstituted tetrazoles for the liberated hydrogens, when the succinoxidase system is relatively intact (in tissue sections), via cytochrome oxidase.

This hypothesis was supported by the experiments, with cyanide to block cytochrome oxidase (Table I). With both homogenates and tissue sections, complete inhibition (TPT and BT) or almost complete inhibition (NT) of tetrazolium reduction was observed. No inhibitory effect was noted for the nitro-substituted electron acceptors. Therefore, the data support the view that TPT and BT are reduced by accepting electrons at the terminal portion of the succinoxidase chain. The same statement appears to hold in large part for NT, except that a small but definite reduction occurs, even in the presence of cyanide, in the biochemical system and an even greater amount of formazan is produced in the cyanide-blocked histochemical system. The finding of some electron transfer to NT in the succinoxidase chain before cytochrome oxidase has been described by Oda and Okazaki (26). Although there are occasional activating effects with cyanide on the nitro-substituted tetrazolium salts which we cannot explain (Table I), nevertheless it is apparent that these acceptors receive electrons before cytochrome oxidase.

Effect of Cytochrome c Removal—With TPT and BT, previous removal of cytochrome c resulted in the absence of formazan deposition in the sections in most instances. Occasional slight staining with TPT was believed to be due to incomplete removal of cytochrome c. When cytochrome c was returned to the reaction mixture (the control preparation), reduction of TPT and BT was restored. With NT, the sections were stained in the absence of cytochrome c about 56% as heavily as the control section, confirming the previous findings that some electron transfer with this tetrazole occurs before cytochrome c. With INT and nitro-BT, the sections devoid of cytochrome c stained as well as the controls, whereas with nitro-NT they were stained about one-half as strongly. This last tetrazole appears to differ from the other nitro-substituted reagents in that part of the electron transfer occurs before cytochrome c and an equal amount before cytochrome oxidase, as shown by the results with cyanide. This locates the site at cytochrome c, a finding similar to that obtained with potassium ferricyanide (43).

Effect of Antimycin A—This reagent, originally isolated by Strong et al. (44), has been used to block the respiratory chain at extremely low concentrations. Its site of action is believed to be on a factor between cytochromes b and c (25, 28, 37, 38, 45, 46). The influence of antimycin A on reduction of the various tetrazolium salts by the succinoxidase system is shown in Table II. In both biochemical and histochemical preparations, electron transfer to the unsubstituted tetrazoles is blocked completely (TPT and BT) or almost completely (NT). With INT and nitro-BT, the failure of antimycin A to prevent formazan production indicates that electrons are being transferred to these

### Table I

**Influence of anaerobiosis and cyanide on tetrazolium reduction by succinoxidase system**

<table>
<thead>
<tr>
<th>Tetrazolium salts</th>
<th>Anaerobiosis</th>
<th>Cyanide</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Homogenates</td>
<td>Sections</td>
</tr>
<tr>
<td>TPT</td>
<td>100</td>
<td>++</td>
</tr>
<tr>
<td>BT</td>
<td>100</td>
<td>++</td>
</tr>
<tr>
<td>NT</td>
<td>100</td>
<td>+</td>
</tr>
<tr>
<td>INT</td>
<td>100</td>
<td>0</td>
</tr>
<tr>
<td>Nitro-BT</td>
<td>100</td>
<td>0</td>
</tr>
<tr>
<td>Nitro-NT</td>
<td>100</td>
<td>0</td>
</tr>
</tbody>
</table>

* Each column in the table summarizes three or more sets of experiments, with liver tissue from a different rat in each experiment. The results of the experiments with homogenates are recorded as percentages of the control activities which are considered equal to 100%. Formazan deposition in the tissue sections was graded from 2- to 2+, the estimations being made at 50X magnification. Negative values indicate inhibition, positive values, activation, and 0 means that the test reaction was similar to the control.

† The results with this tetrazolium salt in the presence of cyanide were not always consistent. Occasionally, slight activation was noted, whereas on other occasions some inhibition was observed. An average of four experiments suggested no significant effect.

tetrazoles before the antimycin A-sensitive factor, e.g., either from the primary dehydrogenase or from cytochrome b. In contrast to these two nitro-substituted reagents, nitro-NT reduction was significantly impaired by antimycin A. Thus, both NT and nitro-NT appear to receive a small but significant percentage

### Table II

**Effect of antimycin A on reduction of tetrazolium salt by succinoxidase system**

<table>
<thead>
<tr>
<th>Tetrazolium salts</th>
<th>Antimycin A alone</th>
<th>Antimycin A + phenazine methosulfate</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Homogenates</td>
<td>Sections</td>
</tr>
<tr>
<td>TPT</td>
<td>0</td>
<td>--</td>
</tr>
<tr>
<td>BT</td>
<td>0</td>
<td>--</td>
</tr>
<tr>
<td>NT</td>
<td>99</td>
<td>0</td>
</tr>
<tr>
<td>INT</td>
<td>98</td>
<td>0</td>
</tr>
<tr>
<td>Nitro-BT</td>
<td>30</td>
<td>--</td>
</tr>
</tbody>
</table>

* Designations here are similar to those described in Table I, and represent the averages of three or more determinations. In the experiments with homogenates, antimycin A was present at a concentration of 5 µg per 100 mg of tissue (wet weight). The same amount of antimycin A was used for the tissue sections, this representing much more inhibitor per milligram of tissue than was used with the homogenates. The inhibitor was preincubated with the enzyme for 10 minutes at room temperature for all the biochemical reactions. However, for the histochemical experiments, only the sections studied with the nitro-substituted tetrazolium salts were preincubated with antimycin A. Phenazine methosulfate was present in the reaction mixture at a concentration of 2.3 X 10^-4 M.*
of the total electrons transferred at some site before the antimony A-sensitive factor; TPT and BT are completely antimony A-sensitive; INT and nitro-BT receive electrons before the antimony A-sensitive site. The introduction of phenazine methosulphate into the reaction media results in the complete disappearance of the antimony A effects upon all the tetrazolium salts studied. These findings were expected in the light of Singer's work (23, 24, 29), which showed that phenazine methosulphate received electrons from the primary dehydrogenase, and that antimony A had no effect on the assay of succinic dehydrogenase in the presence of phenazine methosulphate when oxygen uptake was measured.

Experiments with Soluble Succinic Dehydrogenase—The important issue which remained unsettled was whether INT and nitro-BT could receive electrons directly from the primary dehydrogenase. Theoretically, two alternatives were available, either to block the reaction between cytochrome b and these tetrazoles, or to test them with an enzyme preparation devoid of cytochrome b. Since the former approach can not be used, tests were carried out with a 3% solution of soluble succinic dehydrogenase obtained from acetone powder of beef heart mitochondria (24). Neither INT nor nitro-BT were reduced in the reaction mixture when soluble succinic dehydrogenase was introduced. However, in the presence of phenazine methosulphate, all six tetrazoles were reduced, although at different rates. For example, the amounts of formazan produced per milligram of acetone powder were approximately 16.3, 26.7, 98.7, 602, 220, and 268 μg per hour, for TPT, BT, NT, INT, nitro-BT, and nitro-NT, respectively. The data support the conclusions that INT and nitro-BT receive electrons from the succinic dehydrogenase-cytochrome b complex, and that whereas the various tetrazoles studied are reduced at different sites along the electron transport chain, all can be reduced by the primary dehydrogenase through its interaction with phenazine methosulphate.

Experiments with Other Tetrazolium Salts—The more reactive tetrazolium salts have two features in common; namely, the presence of nitro groups and a higher oxidation-reduction potential. For example, $E_1$ at pH 7.0 and 22° was reported as -50 mv for nitro-BT' and -90 mv for INT, whereas for BT', NT', and TPT, the $E_1$ was -160 mv, -170 mv, and -460 mv, respectively (32). It is worth emphasizing that these measurements were made potentiographically with irreversible systems and therefore are not ideal. The question as to whether the nitro groups exerted their influence only by making the oxidation-reduction potential of the system more favorable, or by some other means, was tested by experiments with 3-(4,5-dimethyl thiasolyl-2)-2,5-diphenyl tetrazolium bromide (MTT) and 2,5-diphenyl-3-p-nitrophenyl tetrazolium chloride (DNT). The former reagent is reduced fairly rapidly by the succinoxidase system in tissue sections (17) and has an oxidation-reduction potential of -120 mv. On the other hand, the latter agent, which is similar to INT except for the absence of the p-iodo group, is reduced relatively slowly by homogenates. When these tetrazolium salts were tested with antimycin A-treated enzyme, reduction was noted to be one-half of that seen in the controls for both acceptors. That cytochrome oxidase was not involved in electron transfer to these reagents was determined by the failure of cyanide to produce a decrease in the production of either of the formazans, as compared with the enzymatic activity in the absence of cyanide. Thus, about one-half of the electron transfer to these tetrazoles occurs before the antimycin A-sensitive factor, and the remaining electrons are transferred at some point between this factor and cytochrome oxidase. Whether the latter site is similar for these two tetrazolium salts is not known. The results noted with MTT and DNT, as well as those with the six other tetrazoles studied, suggest that reaction rate does not correlate strictly with the site of transfer of electrons in the succinoxidase system.

DISCUSSION

Any procedure which interfered with the action of cytochrome oxidase prevented electron transfer to two of the three unsubstituted tetrazolium salts, TPT and BT. Likewise, NT received the majority of available electrons after cytochrome oxidase, with a small but definite amount of reduction occurring before cytochrome c. Nitro-NT, on the other hand, accepted electrons from the succinoxidase system at multiple sites, namely, a small proportion before the antimycin A-sensitive factor, some after this factor, and uniquely over half at the level of cytochrome c. INT and nitro-BT were reduced by the succinoxidase system in homogenates and sections even in the presence of antimycin A, but were not reduced by the preparation of soluble succinic dehydrogenase. The most readily acceptable explanation is that INT and nitro-BT receive electrons from cytochrome b, or from the succinic dehydrogenase-cytochrome b complex. It is probably valid to assume that succinic dehydrogenase is not denatured during the isolation and purification procedures, in such a way as to block reaction with the tetrazole but preserve reactivity with phenazine methosulphate. This assumption has been challenged by Wainio and Cooperstein (47). Ultimate proof will be provided only when cytochrome b is isolated, or when some specific inhibitor of cytochrome b is found.

No significant differences were noted between TPT and BT in both the ease of reduction by and the site of electron transfer from the succinoxidase system. It is noteworthy that the oxidation-reduction potential of BT is 300 mv more positive than that of TPT (32). On the other hand, with a 10-mv difference between BT and NT, the latter reagent is more rapidly reduced by the enzyme system. Possibly, steric factors due to the methoxy groups in BT are responsible for this result. INT with an oxidation-reduction potential equal to -90 mv (32) is reduced after cytochrome b ($E'_1 = 0.0 v$), whereas BT and NT which are, respectively, 70 and 80 mv more negative than INT, are not reduced significantly until the electrons have been passed to cytochrome oxidase ($E'_1 = +280 mv$) (25, 47, 48-53). Even though cytochrome oxidase in the reduced state transfers its electrons to BT and NT anaerobically, it also does so in the presence of oxygen. Although our evidence is indirect, Oda et al. (54) claimed to have shown this electron transfer to NT in histochemical preparations, using cytochrome c as substrate and p-phenylenediamine to keep this cytochrome in the reduced state. Without cytochrome oxidase, no tetrazolium reduction was obtained. Apparently cytochrome oxidase is an enzyme capable of transferring electrons to tetrazolium salts as well as to oxygen.

SUMMARY

The sites of electron transfer from the succinoxidase system to six commonly used tetrazolium salts have been demonstrated. The various points along the respiratory chain were tested by
the use of purified soluble succinic dehydrogenase, inhibition by antimycin A, removal of cytochrome c, inhibition with cyanide of cytochrome oxidase, and anaerobiosis. Homogenates were selected for study of the disrupted system, and tissue sections were found to receive all of their electrons \(2,3,5\text{-tri phenyl tetrazolium chloride, 2',2',5,5'-tetr phenyl-3,3'-(3,3'-di methoxy-4,4'-biphenylene) ditetrazolium chloride, and 2',2',5,5' tetraphenyl-3,3'-(p-biphenylene) ditetrazolium chloride reacted before the antimycin A sensitive factor, presumably after cytochrome c. The other tetrazole, 2',2'-diphenyl-5,5'-di-m-nitroph enyl-3,3'-(p-biphenylene) ditetrazolium chloride reacted to a slight extent both before and after this factor, but mainly at cytochrome c. Since the use of different tetrazolium salts for the assay of the succinodase system may yield findings which appear to be contradictory, the results will be easier to understand if the site of reaction in the electron transport chain is taken into consideration.

**Acknowledgments**—The technical assistance of Mrs. Hannah Wasserkrug, Miss Rita Rubinstein, and Mr. Jerome Goldberg is gratefully acknowledged.

**REFERENCES**

Sites of Electron Transfer to Tetrazolium Salts in the Succinoxidase System
Marvin M. Nachlas, Stanley I. Margulies and Arnold M. Seligman


Access the most updated version of this article at http://www.jbc.org/content/235/9/2739.citation

Alerts:
- When this article is cited
- When a correction for this article is posted

Click here to choose from all of JBC's e-mail alerts

This article cites 0 references, 0 of which can be accessed free at http://www.jbc.org/content/235/9/2739.citation.full.html#ref-list-1