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# Synthesis of *p*-Nitrophenyl Substituted Tetrazolium Salts Containing Iodine and Other Groups[1]

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The syntheses of several tetrazolium salts are described. These include (1) an improved method for preparing the ditetrazolium salt, Nitro-BT, (2) three iodo-5-phenyl-substituted analogs of Nitro-BT for possible use in electron microscopy and (3) five analogs of 2-*p*-nitrophenyl substituted monotetrazolium salts which contain 3-phenyl substituents of varying degrees of electronegativity for a study of their ability to act as electron acceptors with various dehydrogenases. Preliminary experiments with electron microscopy are given and the biochemical results with four dehydrogenases are summarized.

## Introduction

The histochemical demonstration of the succinic dehydrogenase system[2] was first proposed with a ditetrazolium salt (BT) and recently improved by the introduction of a monoformazan capable of forming stable chelates.[3] Further improvement was accomplished by the preparation of a nitro derivative of BT (2,2′-di-*p*-nitrophenyl-5,5′-diphenyl-3,3′-(3,3′-dimethoxy-*n*,*n*′-biphenylene)-ditetrazolium chloride (IX)), also known as Nitro-BT.[4] Enzymatic reduction of Nitro-BT proceeds readily to yield a diformazan (I) with particularly favorable properties for histochemistry, such as great insolubility in both aqueous and lipid media and substantivity for protein.[5] Histochemical methods which reveal localization of the dehydrogenases in the intracellular organelles of mammalian tissue are now possible, not only for the succinic dehydrogenase system[5] but for DPN diaphorase[6] and TPN diaphorase systems[7] as well. Because of its obvious importance, it was considered worthwhile to attempt to improve the yield of Nitro-BT and in addition to prepare some iodine-containing derivatives[8] in order to explore the possibility of histochemical study of the dehydrogenases at the resolution possible with the electron microscope.

The yield of the diformazan (I) in the original preparation[8] was only 18% on coupling tetrazotized dianisidine with the *p*-nitrophenylhydrazone of benzaldehyde in the presence of caustic potash at 0°. However, if the coupling reaction is conducted at −20 to −25°, the yield of I is raised to 55%. The mixture of monoformazan (V) and diformazan (I) formed in the condensation was best separated by extracting the mixture in a Soxhlet apparatus with benzene or dioxane. The final traces of monoformazan could not be efficiently removed by extraction alone, but only after repeatedly grinding the residue to a powder with mortar and pestle at intervals during the extraction. The period of extraction required 10–15 days to rid the material of all but the very last traces of monoformazan. At the end of this time, the extract was extremely pale in color. The diformazan (I) was oxidized in 5-g. lots and Nitro-BT (IX) was purified by crystallization from methanol in stout yellow needles which assayed correctly with one molecule of methanol of crystallization.[4]

Although it is not known whether iodine will be lost from iodinated analogs of the formazans in the hot electron beam of the electron microscope, three analogs were prepared to test this point and to see if the favorable properties of the nitrodiformazan would be retained after introduction of iodine in the three possible positions of the $C_5$-phenyl group.

The three nitrobenzaldehydes[9] were converted to the iodobenzaldehydes and condensed with *p*-nitrophenylhydrazine to give the three corresponding iodobenzaldehyde *p*-nitrophenylhydrazones. The hydrazones were coupled with tetrazotized di-*o*-anisidine and the diformazans (II, III, IV) separated from the monoformazans (VI, VII, VIII) by the modified procedure used for Nitro-BT. The formation of the monoformazans must have taken place according to the mechanism suggested by Tsou, *et al.*,[4] for V, and were separated by extraction with boiling benzene. The formazans were oxidized to the corresponding tetrazolium salts (X, XI, XII) and (XIV, XV, XVI) with isoamyl nitrite and crystallized from methanol. Melting points, yields and analytical data are given in Tables I–IV.

Of the three diiodo diformazan derivatives, only the *meta*-iodo (III) was sufficiently substantive to withstand the procedures of fixation, dehydration and embedding in methacrylate necessary for preparing the thin sections required for electron microscopy. Furthermore, in order to avoid oxidation of the formazan during polymerization of the methacrylate, α,α-azodimethylisobutyronitrile was substituted for the peroxide catalyst at the suggestion of Dr. K. C. Tsou. Electron photomicrographs of tissue sections stained for the succinic dehydrogenase system[5] revealed deposits of this diiodo diformazan in mitochondria similar in size and distribution to that obtained with Nitro-BT,[10] except that the deposits in favorable regions were

(1) This investigation was supported by research grants from the National Cancer Institute, National Institutes of Health, Department of Health, Education and Welfare, Bethesda, Maryland.

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(3) A. G. E. Pearse, *J. Histochem. & Cytochem.*, **5**, 515 (1957).

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(5) M. M. Nachlas, K. C. Tsou, J. DeSouza, C. S. Cheng and A. M. Seligman, *J. Histochem. & Cytochem.*, **5**, 420 (1957).

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(7) M. M. Nachlas, D. G. Walker and A. M. Seligman, *ibid.*, **4**, 467 (1958).

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TABLE I

MONOFORMAZANS, $R-C(=N-NH-C_6H_4-NO_2\text{-}p)-N=N-R'$

| Compd. | R | R′ | Empirical formula | Crystalline form | M.p., °C. | Yield, % | Analyses, % Carbon Calcd. | Carbon Found | Hydrogen Calcd. | Hydrogen Found | Nitrogen Calcd. | Nitrogen Found |
|---|---|---|---|---|---|---|---|---|---|---|---|---|
| VI | *o*-I-$C_6H_4$ | *o*-OMe-*p*-[*m*-OMe$C_6H_4$]$C_6H_3$ | $C_{27}H_{22}N_5O_4I$ | Brownish, dark amorphous powder | 205 dec., shrinks at 200 | 55 | 53.37 | 53.68 | 3.62 | 3.72 | 11.53 | 11.48 |
| VII | *m*-I-$C_6H_4$ | *o*-OMe-*p*-[*m*-OMe$C_6H_4$]$C_6H_3$ | $C_{27}H_{22}N_5O_4I$ | Brownish, dark prisms | 163 dec., shrinks earlier | 65 | ... | ... | .. | .. | 11.53 | 11.90 |
| VIII | *p*-I-$C_6H_4$ | *o*-OMe-*p*-[*m*-OMe$C_6H_4$]$C_6H_3$ | $C_{27}H_{22}N_5O_4I$ | Dark amorphous powder | 127 dec. | 45 | 53.37 | 53.74 | 3.62 | 3.70 | 11.53 | 11.38 |
| XXIV | $C_6H_5$ | *p*-OMe$C_6H_4$ | $C_{20}H_{17}N_5O_3$ | Red needles | 197 dec., shrinks at 193 | 56 | ... | ... | .. | .. | 18.66 | 18.50 |
| XXV | $C_6H_5$ | *p*-Cl-$C_6H_4$ | $C_{19}H_{14}N_5O_2Cl$ | Pink prisms with shining violet luster | 215 dec. | 29 | ... | ... | .. | .. | 18.45 | 18.43 |
| XXVI | $C_6H_5$ | *p*-Br-$C_6H_4$ | $C_{19}H_{14}N_5O_2Br$ | Pink rods with shining violet luster | 204–205 dec. | 47 | ... | ... | .. | .. | 16.51 | 16.53 |
| XXVII | $C_6H_5$ | *p*-COOH-$C_6H_4$ | $C_{20}H_{15}N_5O_4$ | Violet needles | 217 dec. | 28 | ... | ... | .. | .. | 18.00 | 17.44 |
| XXVIII | $C_6H_5$ | *p*-CN-$C_6H_4$ | $C_{20}H_{14}N_6O_2$ | Dark needles | 219–220 dec. | 32 | ... | ... | .. | .. | 22.70 | 22.54 |
| XXIX | $CH_3$ | *p*-I-$C_6H_4$ | $C_{14}H_{12}N_5O_2I$ | Red broken plates | 216 dec. | 44 | ... | ... | .. | . | 17.11 | 17.28 |

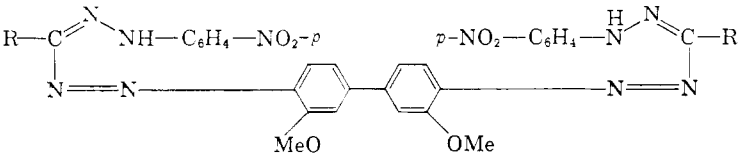
TABLE II

MONOTETRAZOLIUM CHLORIDES, $R-C(=N-N(-C_6H_4-NO_2\text{-}p)-)=N-N^+(-R')-Cl^-$

| Compd. | R | R | Empirical formula | Crystalline form | M.p., °C. | Yield, % | Analyses, % Carbon Calcd. | Carbon Found | Hydrogen Calcd. | Hydrogen Found | Nitrogen Calcd. | Nitrogen Found |
|---|---|---|---|---|---|---|---|---|---|---|---|---|
| XIV | *o*-I-$C_6H_4$ | *o*-OMe-*p*-[*m*-OMe$C_6H_4$]$C_6H_3$ | $C_{27}H_{21}N_5O_4ICl$ | Yellow prisms | 162 dec. | 66 | ... | ... | .. | .. | 10.84 | 10.40 |
| XV | *m*-I-$C_6H_4$ | *o*-OMe-*p*-[*m*-OMe$C_6H_4$]$C_6H_3$ | $C_{27}H_{21}N_5O_4ICl\cdot H_2O$ | Yellow amorphous powder | 143–144 dec. | 76 | 49.12 | 49.13 | 3.48 | 3.37 | 10.61 | 10.43 |
| XVI | *p*-I-$C_6H_4$ | *o*-OMe-*p*-[*m*-OMe$C_6H_4$]$C_6H_3$ | $C_{27}H_{21}N_5O_4ICl$ | Yellow amorphous powder | 177–178 dec. | 63 | ... | ... | .. | .. | 10.84 | 11.10 |
| XVII | $C_6H_5$ | *p*-OMe-$C_6H_4$ | $C_{20}H_{16}N_5O_2Cl\cdot H_2O$ (dried at 100°, 0.5 mm.) | Pale, yellow micro crystals | 187 dec. | 69 | 56.14 | 56.30 | 4.21 | 4.00 | 16.37 | 16.28 |
| XVIII | $C_6H_5$ | *p*-Cl-$C_6H_4$ | $C_{19}H_{13}N_5O_2Cl\cdot Cl$ (after drying at 100°, 0.5 mm ) | Pale, yellow needles | 253 dec. | 87 | 55.07 | 54.80 | 3.14 | 3.07 | 16.90 | 16.80 |
| | | | $C_{19}H_{13}N_5O_2Cl\cdot Cl\cdot 1.5\ H_2O$ (before drying) | Pale, yellow needles | | | 51.70 | 51.76 | 3.62 | 3.42 | 15.87 | 16.14 |
| XIX | $C_6H_5$ | *p*-Br-$C_6H_4$ | $C_{19}H_{13}N_5O_2Br\cdot Cl\cdot H_2O$ | Pale, yellow needles | 232–234 dec. | 83 | 47.85 | 47.74 | 3.15 | 3.17 | 14.69 | 14.62 |
| XX | $C_6H_5$ | *p*-COOH-$C_6H_4$ | $C_{20}H_{14}N_5O_4Cl\cdot 1.5H_2O$ | Pale, yellow prisms | 230 dec. | 78 | 53.27 | 53.23 | 3.77 | 3.53 | 15.53 | 15.32 |
| XXI | $C_6H_5$ | *p*-CN-$C_6H_4$ | $C_{20}H_{13}N_6O_2Cl\cdot H_2O$ | Colorless amorphous powder | 227 dec. | 67 | 56.82 | 56.97 | 3.55 | 3.30 | 19.89 | 19.70 |
| XXII | $C_6H_5$ | *p*-$NO_2$-$C_6H_4$ | $C_{19}H_{13}N_6O_4Cl\cdot H_2O$ | Pale, yellow prisms | 227–229 dec. after darkening at 215 | 83 | 51.52 | 51.64 | 3.39 | 3.26 | 18.99 | 18.97 |
| XXIII | $CH_3$ | *p*-I-$C_6H_4$ | $C_{14}H_{11}N_5O_2ICl\cdot H_2O$ (before drying) | Yellow amorphous powder | 175–185 | 66 | 36.40 | 36.66 | 2.81 | 2.95 | 15.17 | 15.20 |
| | | | $C_{14}H_{11}N_5O_2ICl$ (after drying) | Yellow amorphous powder | | | 37.87 | 37.50 | 2.48 | 2.68 | 15.79 | 15.70 |

TABLE III

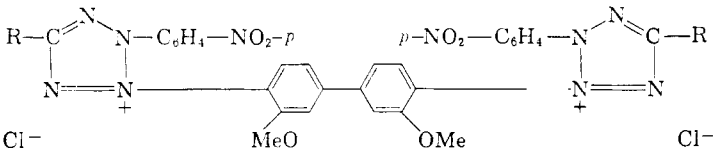
DIFORMAZANS,



| Compd. | R | Empirical formula | Crystalline form | M.p., °C. | Yield, % | Analyses, % Carbon Calcd. | Carbon Found | Hydrogen Calcd. | Hydrogen Found | Nitrogen Calcd. | Nitrogen Found |
|---|---|---|---|---|---|---|---|---|---|---|---|
| II | *o*-I-$C_6H_4$ | $C_{40}H_{30}N_{10}O_6I_2$ | Dark prisms | 263 dec. | 5 | ... | ... | .. | .. | 14.00 | 13.60 |
| III | *m*-I-$C_6H_4$ | $C_{40}H_{30}N_{10}O_6I_2$ | Dark prisms | ..... | 18 | ... | ... | .. | .. | 14.00 | 13.65 |
| IV | *p*-I-$C_6H_4$ | $C_{40}H_{30}N_{10}O_6I_2$ | Dark powder | 278–279 dec. | 32 | 48.00 | 47.60 | 3.00 | 2.99 | 14.00 | 13.70 |

TABLE IV

DITETRAZOLIUM CHLORIDES,



| Compd. | R | Empirical formula | Crystalline form | M.p., °C. | Yield, % | Analyses, % Carbon Calcd. | Carbon Found | Hydrogen Calcd. | Hydrogen Found | Nitrogen Calcd. | Nitrogen Found |
|---|---|---|---|---|---|---|---|---|---|---|---|
| X | *o*-I-$C_6H_4$ | $C_{40}H_{28}N_{10}O_6I_2Cl_2$ | Yellow prisms | 197 dec. | 78 | 44.89 | 45.20 | 2.62 | 2.70 | 13.09 | 12.70 |
| XI | *m*-I-$C_6H_4$ | $C_{40}H_{28}N_{10}O_6I_2Cl_2$ | Yellow powder | 156–158 dec. | 75 | 44.89 | 45.23 | 2.62 | 3.00 | 13.09 | 12.80 |
| XII | *p*-I-$C_6H_4$ | $C_{40}H_{28}H_{10}O_6I_2Cl_2$ | Yellow powder | 210 dec. | 56 | 44.89 | 44.60 | 2.62 | 2.90 | 13.09 | 13.04 |
| | | $C_{40}H_{28}N_{10}O_6I_2Cl_2 \cdot H_2O$ | ..... | ..... | .. | 44.15 | 44.44 | 2.76 | 3.01 | 12.87 | 13.04 |

smaller and rounder. The deposits were small (200–500 Å.) and appeared to be related to the underlying membranes of the mitochondria, both cristae and external membranes. The results appear to be better than those already obtained with long crystals of telurium.[11] A preliminary statement of the results with electron microscopy is given in the experimental part of this report.

It has been noted that Nitro-BT is a better electron acceptor than BT in the succinic dehydrogenase system[5] and that 2-*p*-nitrophenyl-3-*p*-iodophenyltetrazolium chloride (INT)[12] is a better electron acceptor than 2,3,5-triphenyltetrazolium chloride (TPT) in various dehydrogenase systems. However, we observed that 2-*p*-nitrophenyl-3,5-diphenyltetrazolium chloride[13] was a surprisingly poor electron acceptor using homogenates of rat tissue. This suggested to us that the electronic influence of the 3-*p*-substituent played an important role even in 2-*p*-nitrophenyltetrazolium salts. Further interest was provided by the observation that agents which worked equally well histochemically with fresh frozen tissue sections did not necessarily work equally well with all dehydrogenase systems when tissue homogenates were used. For example, INT and Nitro-BT are equally good electron acceptors in the lactic dehydrogenase system but INT is superior to Nitro-BT in homogenates containing the succinic dehydrogenase system.

In order to study the various electronic and inductive effects on the readiness with which electrons are accepted from various dehydrogenase systems, 2-*p*-nitrophenyltetrazolium salts containing *p*-substituents in the 3-phenyl nucleus in order of their increasing $\sigma$-constants were synthesized as follows: OMe, H,[13] Cl, Br, I,[12] COOH, CN, $NO_2$.[14] In order to test certain steric features, 2-*p*-nitrophenyl-3-*p*-iodophenyl-5-methyltetrazolium chloride (XXIII) was also synthesized.

## Experimental

**2,2′-Di-*p*-nitrophenyl-5,5′-diphenyl-3,3′-(3,3′-dimethoxy-4,4′-biphenylene)-diformazan (I).**—Finely powdered *o*-dianisidine hydrochloride (50 g.) was suspended in water (240 ml.) and concentrated hydrochloric acid (60 ml.) was added. The solution was cooled to 0° and was tetrazotized with a solution of sodium nitrite (24 g.) in 60 ml. of water. The resulting mixture was added to a well-stirred solution of *p*-nitrophenylhydrazone of benzaldehyde (77 g.) in tetrahydrofuran (700 ml.), followed by the addition of potassium hydroxide (40 g.), in 75 ml. of water. The temperature during mixing was maintained at −20 to −25°. After about 1 hr. it was allowed to come to room temperature, water (320 ml.) and methanol (700 ml.) were added. The suspension was filtered. The precipitate was washed with methanol until the washings were light brown. Then it was washed with boiling water (2400 ml.) and finally again with methanol. The crude product containing both mono (V) and diformazan (I) weighed 136 g.

The black precipitate was extracted in a Soxhlet apparatus with either dioxane or benzene, in three separate portions. After three days the residue in the thimble was dried, ground to a powder with mortar and pestle and the extraction was continued. This operation was repeated five times after every two days of extraction. The extract became practically colorless. The total residue from the three lots weighed 70 g. (55%), m.p. 255° dec. This compound was not completely free of the last trace of monoformazan but was considered good enough for further oxidation.

**2,2′-Di-*p*-nitrophenyl-5,5′-diphenyl-3,3′-(3,3′-dimethoxy-4,4′-biphenylene)-ditetrazolium Chloride (Nitro-BT) (IX).**—Diformazan (I) (5 g.) was suspended in a mixture of tetrahydrofuran (80 ml.) and dioxane (80 ml.). Isoamyl nitrite (6 ml.) was added. A stream of dry hydrogen chloride was passed through the suspension at 0° until it was saturated with hydrogen chloride. Isoamyl nitrite (2 ml.) was added intermittently during the 2 hr. It was then stirred with a magnetic stirrer for 4 hr. at room temperature, treated with norite and filtered. Solvents were removed under reduced pressure. The residue was dissolved in al-

(11) R. J. Barnett and G. E. Palade, *J. Histochem. & Cytochem.*, **6**, (1958).

(12) S. W. Fox and E. H. Atkinson, THIS JOURNAL, **72**, 3629 (1950).

(13) J. N. Ashley, B. M. Davis, A. W. Nineham and R. Slack, *J. Chem. Soc.*, 388 (1953).

(14) D. Jerchel and H. Fischer, *Ann.*, **590**, 216 (1954).

cohol and was precipitated by adding the alcoholic solution to dry ether with good stirring (yield 4.5 g.). The yellow substance was crystallized from methanol in stout yellow needles, m.p. 184° dec.

*Anal.* Calcd. for $C_{40}H_{30}N_{10}O_6Cl_2 \cdot CH_3OH$: C, 57.95; H, 4.00; N, 16.49. Found: C, 57.90; H, 3.77; N, 16.51.

The best Nitro-BT obtained by this procedure when incubated with a tissue section resulted in a reddish cast to the section after mounting in glycerogel. The red component, however, was readily removed by washing the section in alcohol–xylol for 1 hr. and mounting in cedar oil. The red component was considered therefore, to be due to the monoformazan which had failed to be extracted from the diformazan during the process of separation because of inclusion within the particles of diformazan. To purify further, the crystallized Nitro-BT was reduced back to diformazan by using dextrose ammonium hydroxide. The separated dark product was then extracted with alcohol and xylol in a Soxhlet apparatus. At this time the red material could be removed completely within a few hours. The diformazan thus obtained melted at 270–272° dec. This sample of diformazan was reoxidized to Nitro-BT and the product thus obtained was still found to give a red color with tissue sections, although to a lesser degree.

It was also noted that tissue sections being extracted with alcohol and xylol, showed pink stained cell nuclei if the extraction was less than an hour. This suggested that the pink component had a weakly positive charge, and so it was suspected that isoamyl nitrite had produced some change during the process of oxidation, which might be responsible for the red color.

Other oxidizing agents such as sodium hypochlorite, chromic anhydride and lead tetraacetate were also used for oxidizing purified diformazan, but the respective salts thus obtained behaved in the same way as the Nitro-BT obtained with isoamyl nitrite.

Attempts to remove the fraction that was responsible for the red color, from the alcoholic solution of Nitro-BT by using activated charcoal or the ion-exchange resin (Amberlite IRC-50), were unsuccessful. Successive extractions of the aqueous solution of Nitro-BT with chloroform removed some red color, but still the Nitro-BT thus obtained was not histochemically pure.

The laboratory procedure which could be used for testing the purity of the sample of Nitro-BT is as follows:

Dissolve a milligram of Nitro-BT in water, to the solution add a pinch of gelatin and then a crystal of sodium sulfide. The dark blue material thus obtained was shaken in a test-tube with ethyl acetate. The ethyl acetate layer appears pink if the Nitro-BT sample contains the red material. The diformazan remains in the aqueous gelatin solution because of its marked substantivity for protein.

**Iodobenzaldehyde *p*-Nitrophenylhydrazone.**—To the hot alcoholic solution of *p*-nitrophenylhydrazine (0.7 g. in 7 ml.) was added a solution of *o*, *m* or *p*-iodobenzaldehyde[9] (1 g.) in alcohol (3 ml.). Glacial acetic acid (0.8 ml.) was added. Immediate separation of the corresponding hydrazone was observed. The mixture was cooled and filtered. Crystallization occurred from dioxane in yellow needles. *o*-Iodo derivative, m.p. 244°; *m*-iodo derivative, m.p. 212°; and *p*-iodo derivative, m.p. 201°.

*Anal.* Calcd. for *o*-iodobenzaldehyde *p*-nitrophenylhydrazone, $C_{13}H_{10}O_2IN_3$: N, 11.40. Found: N, 11.23.

The procedures below were used to prepare the *o*-, *m*- and *p*-iodo derivatives of the formazans and tetrazolium salts All the melting points were determined on a Fisher–Johns melting point apparatus (and the analysis were performed after rigorous drying over phosphorus pentoxide, at 100° under vacuum). Yields, melting points and analytical data are given in Table I–IV for the three isomers.

**2-*p*-Nitrophenyl-5-iodophenyl-3-(3,3′-dimethoxy-4-biphenyl)-formazan (VI, VII, VIII) and 2,2′-Di-*p*-Nitrophenyl-5,5′-di-iodophenyl-3,3′-(3,3′-dimethoxy-4,4′-biphenylene)-diformazan (II, III, IV).**—Finely powdered *o*-dianisidine hydrochloride (1.52 g.) was suspended in water (8 ml.) and concentrated hydrochloric acid (3.2 ml.). It was then tetrazotized at 0 to 5° with a solution of sodium nitrite (0.72 g.) in water (4 ml.). After about five minutes the solution was added to a vigorously stirred solution of iodobenzaldehyde *p*-nitrophenylhydrazone (3.66 g.) in tetrahydrofuran (80 ml.), immediately followed by a solution of potassium hydroxide (6.0 g.) in water (20 ml.). The temperature during mixing was maintained at −20 to −25°. After 2 hr. the mixture was allowed to come to room temperature. It was diluted with water, and the precipitate was filtered, washed with hot methanol (200 ml.) and water.

**Fractional Separation of the Mono (VI, VII, VIII) and the Diformazan (II, III, IV).**—The crude product obtained in the above experiment was extracted in a Soxhlet apparatus with tetrahydrofuran and then with alcohol for hours. The extract was concentrated and diluted with petroleum ether. The dark precipitate of monoformazan thus obtained was purified by repeated precipitations from benzene solution (see Table I).

The insoluble dark precipitate which remained in the thimble was crystallized from pyridine in irregular plates. The compound analyzed for the diformazan (see Table III).

**2-*p*-Nitrophenyl-5-iodophenyl-3-(3,3′-dimethoxy-4-phenyl)-tetrazolium Chloride (XIV, XV, XVI).**—Monoformazan (VI, VII, VIII) (0.2 g.) was dissolved in tetrahydrofuran (20 ml.) and ten drops of isoamyl nitrite were added. The solution was cooled in an ice-bath and saturated with dry hydrogen chloride. The solution was stirred for 2 hr. at room temperature, treated with Norite and filtered. The solvent was removed under vacuum and the oily precipitate was dissolved in absolute alcohol (5 ml.). The alcoholic solution was then poured into dry ether (100 ml.). The yellow precipitate thus obtained was purified by repeated precipitations in ether (see Table II).

**2,2′-Di-*p*-nitrophenyl-5,5′-di-iodophenyl-3,3′-(3,3′-dimethoxy-4,4′-biphenylene)-ditetrazolium Chloride (X, XI, XII).**—A suspension of diformazan (II, III, IV) (0.24 g.) in a mixture of tetrahydrofuran (15 ml.) and dioxane (15 ml.), containing isoamyl nitrite (0.5 ml.), was saturated with dry hydrogen chloride at 0°, stirred for 4 hr. at room temperature, treated with Norite and filtered. The filtrate was distilled over the steam-bath, under vacuum. The residual reddish oily matter solidified on contact with dry ether. It was purified by repeated precipitations with ether from an alcoholic solution, and it was then crystallized from methanol in yellow rods (see Table IV).

**2-*p*-Nitrophenyl-3-*p*-cyanophenyl-5-phenyl Monoformazan (XXVIII).**—Benzaldehyde *p*-nitrophenylhydrazone (2.4 g.) was dissolved in pyridine (25 ml.). The solution was cooled to 0°. To a solution of *p*-cyanoaniline (1.18 g.) in glacial acetic acid (1 ml.) and water (3 ml.), concentrated hydrochloric acid (3 ml.) was added, and it was diazotized with a solution of sodium nitrite (0.58 g.) in water at 0°. The latter solution was filtered and was slowly added to the solution of benzaldehyde *p*-nitrophenylhydrazone with continuous stirring. The color of the solution changed to red. It was stirred for 4 hr. at 0° and then it was left at room temperature overnight. The next day it was diluted with water, and the precipitate thus obtained was washed with hot water. It was dried and crystallized from benzene in clusters of dark needles. The crystals were filtered and washed with dry ether.

The corresponding 3-*p*-nitrophenylformazan[14] and 3-*p*-carboxyphenylformazan (XXVII) were prepared in the same manner. The other derivatives listed in Table I were also prepared in a similar manner except that instead of pyridine, a mixture of dioxane and tetrahydrofuran was used as a solvent. An aqueous solution (10 ml.) of sodium hydroxide (10%) was used as an alkaline condensing agent (see Table I).

**2-*p*-Nitrophenyl-3-*p*-cyanophenyl-5-phenylmonotetrazolium Chloride (XXI).**—The above formazan XXVIII (0.15 g.) was dissolved in tetrahydrofuran (20 ml.). Isoamyl nitrite (0.2 ml.) was added, and the solution was cooled to 0°. A stream of dry hydrogen chloride was passed through the solution until saturation was obtained. The bath was removed and the solution was stirred at room temperature for 2 hr. The color of the solution changed to yellow. The solvent was removed on a steam-bath, and the residue was dissolved in a few drops of alcohol. On addition of dry ether a flocculent precipitate separated. It was filtered and purified by repeated precipitations with ether or it was crystallized from alcohol.

The corresponding methoxy (XVII), chloro (XVIII), bromo (XIX), carboxy (XX), nitro (XXII)[14] and 2-*p*-nitrophenyl-3-*p*-iodophenyl-5-methyltetrazolium chloride were prepared in a similar manner (see Table II).

Most of the monotetrazolium chlorides contained 1 or 1.5 molecules of water of crystallization according to analysis. In some cases water of crystallization was removed after drying the sample at 140° under reduced pressure (0.5 mm.).

In other cases the water could not be completely removed, as indicated by analysis. On recrystallization or exposure to air for a few days, analysis showed reconstitution of water of crystallization.

**Histochemical Experiments with the Electron Microscope.**—The iodo-nitro tetrazoles (X, XI, XII) were compared with Nitro-BT as to their effectiveness as reagents in histochemical experiments for the demonstration of succinic dehydrogenase activity. Frozen sections and small pieces of rat heart muscle that were frozen and thawed or washed in 0.44 *M* sucrose to destroy endogenous dehydrogenase activity were used as the source of the enzyme system. The tissues were incubated in a medium containing a tetrazole, sodium succinate, phosphate buffer (*p*H 7.6), potassium cyanide, activators and enough sucrose to raise the over-all osmolar concentration to 0.44 *M*. Of the iodo-nitro tetrazoles tested, only *m*-iodo-nitro-BT (XI) proved to be an adequate histochemical reagent. However, the rate at which this tetrazole was reduced to its corresponding formazan in the presence of succinate and the active enzyme system was approximately eight to ten times slower than that of Nitro-BT. Examination of frozen sections of heart muscle incubated in the medium for the demonstration of succinic dehydrogenase activity with *m*-iodo Nitro-BT (XI) revealed that the mitochondria were stained by the formazan. In addition, the diformazan (III) of this ditetrazole, like Nitro-BT, was highly substantive and proved to be insoluble in ethyl alcohol and permount, allowing permanent sections to be made.

The substantivity and the insolubility of the formazan of Nitro-BT suggested its possible use as a histochemical reagent that could be adapted to electron microscopy.[8,10,15] It was decided, therefore, to test iodo-nitro-BT as a reagent and compare it with Nitro-BT for the demonstration of the sites of succinic dehydrogenase activity with the electron microscope. It was first determined that the diformazans of both Nitro-BT and *m*-iodo-nitro-BT were opaque to electrons. This was performed by reacting the ditetrazoles with glutathione and drying suspensions of the diformazan in ethanol on carbon-coated grids that were examined with the electron microscope. Alternatively the formazans were dehydrated with ethyl alcohol, embedded in N-butyl methacrylate, with ultraviolet light as catalyst; and thin sections (250–400 Å.) were also examined.

Two steps in the procedure of fixation and embedding for electron microscopy required that the diformazan be subjected to oxidizing agents that could possibly solubilize these products; the fixative, osmium tetroxide; and the catalyst for polymerization of methacrylate, 2,4-dichlorobenzoyl peroxide. In addition, it was possible that the osmium tetroxide might react with the diformazan and provide additional electron opacity. However, when the diformazans were fixed in osmium tetroxide before ethanol dehydration and embedding in methacrylate, no increase or decrease in electron opacity was noted in comparison with unfixed diformazan. These experiments also indicated that brief fixation in osmium tetroxide did not oxidize the diformazans to the soluble ditetrazoles. However, when 2,4-dichlorobenzoyl peroxide was used as catalyst for polymerization, some but not all of the diformazan was oxidized. To avoid this loss of final product, $\alpha,\alpha$-azo-dimethyl-isobutyronitrile was tested and proved satisfactory as a catalyst for polymerization which did not affect the solubility of the diformazans.

In applying these reagents to histochemical tests combined with electron microscopy, small blocks of washed or frozen and thawed heart muscle of the rat were used. After incubation in the medium referred to above,[5] the blocks were fixed briefly in osmium tetroxide, embedded in *n*-butyl methacrylate, and the sections viewed with an RCA (EMU-2E) electron microscope. The product of histochemical reactions, the diformazans of Nitro-BT and *m*-iodo-nitro-BT were localized within or on the surface of mitochondria. The size of the deposits of diformazan depended on the duration of incubation, the size of the block of tissue and the portion of the block from which the sections were made. At sites of high activity in the periphery of the block, the dense deposits were roughly circular and varied in diameter from approximately 600 to 2000 Å. In more favorable regions closer to the center of the block, the deposits were small (200–500 Å.) and appeared to be related to the underlying membranes of the mitochondria, both cristae and external membranes. In optimal circumstances where the growth of the deposits of final product had not proceeded at the enzyme sites, the localization of the diformazan was restricted to the membranes of the mitochondria. This was especially true of the diformazan of *m*-iodo-nitro-BT (III) which was produced by reduction of the tetrazole at slower rate. This is in agreement with the cytochemical localization of the succinic dehydrogenase system.[3,5,16] It should be noted that some mitochondria of identical structure evinced no staining reaction regardless of position in the tissue block. In control experiments (omission of substrate or inhibition of enzyme system), no product was deposited. In these experiments, it could not be demonstrated with assurance that the diformazan of iodo-nitro-BT (III) was more electron dense than the formazan of Nitro-BT (I); the uncontrolled factors being the amount of diformazan deposited in a unit time, the thickness of the section, and the extent of decomposition of the diformazan in the electron beam.

**Colorimetric Experiments with Four Dehydrogenases.**—The series of 3-phenyl substituted 2-*p*-nitrophenyltetrazolium salts were compared in their ability to be reduced by the dehydrogenase systems in rat liver homogenate with the four enzymes, succinic, lactic, $\beta$-hydroxybutyric and isocitric dehydrogenase. Phenazine methosulfate was used as the intermediate electron carrier for the transfer of electrons from either the reduced enzyme or co-enzyme to the tetrazolium salt. The appropriate substrate was dissolved in the appropriate buffer together with gelatin and the phenazine methosulfate. The optimum *p*H of each enzyme (7.7 to 9.0) was used. The tetrazolium salt (2 mg./ml.) was added and finally the liver homogenate (5 mg./ml.) was introduced. After incubation at 37° for 15 minutes the color density of the colloidally suspended formazan was measured with a Klett colorimeter. Color density and formazan concentration were found to have a linear relationship.

With each enzyme, maximum reduction was obtained with 3-*p*-iodophenyl-2-*p*-nitrophenyltetrazolium chloride (INT). Surprisingly low activity (0.02 to 0.1) was observed with 2-*p*-nitrophenyl-3,5-diphenyltetrazolium chloride as compared to INT. When the 3-*p*-phenyl hydrogen atom was replaced by groups with increasing electronegativity, such as COOH, Cl and Br, progressively greater formazan production was noted with each enzyme. Substitution of cyano and nitro groups, which are more electronegative than iodo, did not result in greater formazan production than was observed with INT. The possibility that steric influences were detrimental to the electron transfer must be considered. Replacement of the 5-phenyl with a 5-methyl group resulted in significant decreases in formazan formation by all four dehydrogenase systems. This emphasized the importance of the 5-phenyl group in facilitating the opening of the tetrazolium ring, as was already demonstrated for the 2-*p*-nitrophenyl group.

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