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Substituted triphenyl tetrazolium chlorides

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SUBSTITUTED TRIPHENYL TETRAZOLIUM CHLORIDES

BY

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INTRODUCTION

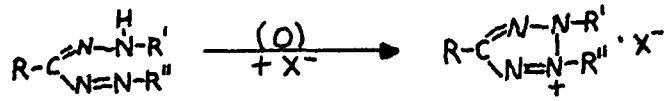
The work in this thesis is a continuation of a project started at the University of Richmond in 1952 by A. G. Richardson under the directorship of Dr. J. S. Pierce and Dr. George Z. Williams, Director of Clinical Pathology, National Institutes of Health, Bethesda, Maryland.

The purpose of the research was to prepare triphenyl tetrazolium salts, substituted, particularly in the ortho and para positions, with various electron accepting and electron donating groups. It was hoped that these substitutions would impart enough differences in the properties of the compounds so as to allow detection of certain biological reactions. The tetrazolium salts were sent to Dr. Williams for testing as soon as they were prepared. Time has not permitted the biological testing of the compounds prepared in this work to be completed. However, a number of the salts, as well as those prepared by Richardson have been tested in Dr. Williams' laboratories.

The substitutions which have been used were chosen from a number of groups which varied with respect to their electron donating and electron accepting power. The list as given in Fuson's "Advanced Organic Chemistry" was used as a guide. The groups listed in order of decreasing nucleophylic power are as follows: $-NH_2$, $-OH$, $-OCH_3$, $-CH_3$, $-H$, $-C_6H_5$, $-COCH_3$, $-NO_2$, and $-\overset{+}{N}(CH_3)_3$ (85). Piperonal, because of availability and electron donating power, was used to give a 3,4-methylenedioxy substitution on the phenyl group in position 5 of the tetrazolium molecule.

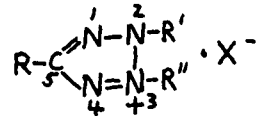
HISTORY

Tetrazolium salts are quartenary ammonium compounds which are formed from formazans by oxidative ring closure.

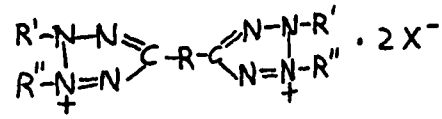


In contrast to the formazans, which are highly colored and insoluble in aqueous solvents, the tetrazolium salts are generally colorless or light yellow and have an appreciable solubility in water. As shown above, tetrazolium salts contain a tetrazole ring which has one quartenary nitrogen. Both mono- and bifunctional tetrazolium salts are known. Illustrative formulas are given below for the various types possible. As shown by type formula (I), there are three possible sites of substitution in a monotetrazolium salt. They are positions 2, 3, and 5. It is possible to substitute simple alkyl groups in position 5. However, because of the methods of syntheses available, positions 2 and 3 require aryl substituents . Because of

resonance of the double bonds, positions 2 and 3 are equivalent.



(I)



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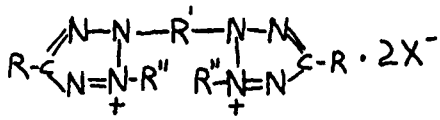

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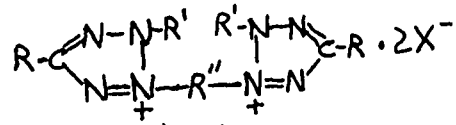
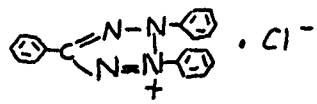
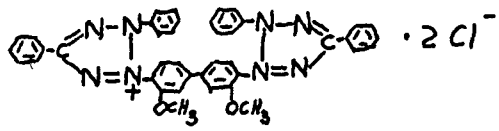


(IV)

The nomenclature of mono tetrazolium salts is relatively simple. The ring is numbered as shown in type formula (I). The individual salts are named by identifying the substituent groups and ending the name with the term "tetrazolium salt". For example, 2, 3, 5-triphenyl tetrazolium chloride has the following structure:

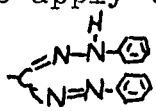


The naming of double tetrazolium salts presents somewhat more difficulty but is usually done by concluding the name with the term "tetrazolium salt" as is done for the mono salt. For example, 3,3'-(3,3'-dimethoxy-4,4'-diphenylene)-bis(2,5-diphenyl tetrazolium chloride) has the following structure:

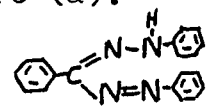


The nomenclature of the formazans, which are necessary for the formation of tetrazolium salts, is based on the hypothetical fundamental compound, $NH{=}N{-}CH{=}N{-}NH_2$, termed formazan. Thus  is 1, 3, 5- triphenyl formazan.

In 1892 von Pechmann (57) and Bamberger (3) independently discovered formazans. Jointly they proposed the name formazyl to apply to the basic structure (a).

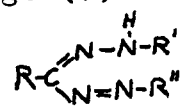


(a)

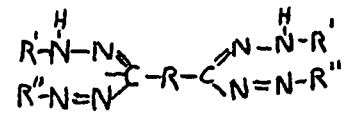


(b)

Thus 1, 3, 5-triphenyl formazan (b) was called formazyl benzene. Since that time, because of the difficulty in naming by this system, the nomenclature presented on page (5) has come into general use. The formulas of formazans which correspond to the types of tetrazolium salts given on page (4) are presented below:



(I)



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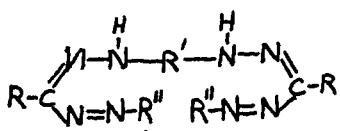
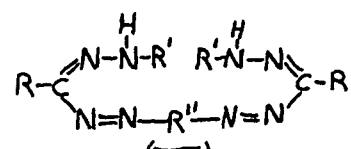


(III)

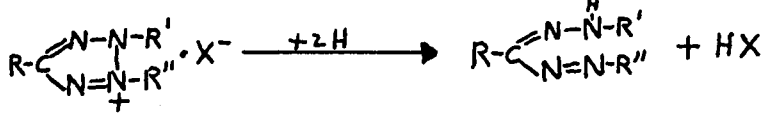


(IV)

The first tetrazolium salts were reported by von Pechmann and Runge in 1894 (56). Since that time many tetrazolium salts have been prepared. Their study received a great impetus in

1941 when Kuhn and Jerchel (39) reported the biological reduction of tetrazolium salts to formazans. While studying various bactericidal agents they observed that tetrazolium salts were being reduced to formazans by bacteria. This reaction was also observed in various other plant and animal tissues. In addition, it was noted that the formazans were being produced at action centers in the cells, especially the nuclei. This reduction to formazans was attributed to fermentative processes.

The reduction of tetrazolium salts to formazans by chemical means is probably their most characteristic reaction. This reduction appears to take place only at an alkaline pH. Various reducing agents have been employed, among them ammonium sulfide (80), sodium amalgan, sodium dithionite (30), ascorbic acid (42) and reductone (19). The reduction proceeds according to the following equation:



The work of a number of investigators has shown that the reduction of tetrazolium salts to formazans in biological systems is due to enzymatic activity. In particular the dehydrogenase system of enzymes, which utilize the pyridine-nucleotides, Coenzyme I and Coenzyme II, and the flavoproteins are felt to be responsible for the reduction. This point will be discussed in greater detail later.

With the discovery of biological oxidation, many possible uses for the tetrazolium salts were suggested. Most of the recent work with tetrazolium salts has been in their application to biological problems.

Lakon (43) substituted 2, 3, 5-triphenyl tetrazolium chloride (commonly called TTC) for sodium selenite in the "topographic" method for testing the germinating ability of seeds of oats, rye, wheat and barley. Viable seeds were shown to develop a red stain in the embryo, whereas non-viable seeds did not. TTC was also more effective than sodium selenite in indicating viability.

Many others have confirmed this work by Lakon. Shuel (71) tested the method using barley, oats and wheat, Matson and coworkers (47) in 1947 reported that the same staining could be observed in the fleshy part of apples, oranges and pears, in the gill areas of mushrooms, in carrot roots, potatoes, young leaves, bull spermotozoa and the blastoderm of hens' eggs. They also pointed out that sugars could not be responsible since they reduce at a pH of 11 or more and these reductions took place at a pH of 7 or less. When tissues were heated to 82° C. or higher, their ability to cause reduction of tetrazolium salts was lost.

Gunz (25) , working with fresh brewer's yeast, demonstrated that the ability of the yeast to reduce TTC could be removed by heating or by dialyzing the yeast solution. Both of these effects are characteristic of enzymes.

Mixner (50) confirmed Matson's work with bull spermatozoa by using bull semen. In addition, he showed that the reduction was a characteristic of the spermatozoa only. The plasma of semen had no ability to reduce TTC.

Roberts (62) has shown that the apical meristems, lateral meristems including cork cambium, vascular cambium, the inner and outer cortical regions, the intercalary systems of some monocots, zones in root tip tissue and zones in seed embryos all reduce TTC. This he attributes to enzymatic activity in these areas.

Bielig, Kausche and Hoardish (6) have used TTC to detect the loci of reduction in bacteria. By using various cocci and bacilli, they showed that the sites of activity were at the cell poles except in dividing cells in which cases there were some sites at the center of the cells. The optimal pH for reduction with Escherichia coli was 8.4 Heating to 70° C. deactivated the system.

Fred and Knight (24) used TTC to test the viability of cultures of Penicillum chrysogenium. Young cultures were more viable than older ones in that they reduced more TTC to the formazan. They also demonstrated that TTC was only reduced inside the cells of the fungus.

Koch-Wener, Barclay and Ebert (39) have used tetrazolium salts in testing the affect of various antibacterial agents on Mycobacterium tuberculosis. Isonizid and streptomycin are reported to remove the ability of the bacteria to reduce

tetrazolium salts provided the bacteria are not resistant. (Isonizid resistant bacilli still reduced tetrazolium salts.) Carper and Jones (4) reported that streptomycin had very little effect on the reduction of TTC by Mycobacterium tuberculosis whether resistant or sensitive. However, age and type of culture had a quantitatively detectable effect on the reduction. Older cultures were much less effective in forming formazan.

Somerson and Morton (73) used TTC and "neotetrazolium" salt 3,3'-(4,4'-diphenylene)-bis(2,5-diphenyl tetrazolium chloride) to demonstrate dehydrogenase in pleuropneumonia-like organisms. Some specificity of tetrazolium salt reduction was demonstrated in that an iodo-triphenyl tetrazolium and "blue tetrazolium" 3,3'-(3,3'-dimethoxy-4,4'-diphenylene)-bis(2,5-diphenyl tetrazolium chloride) were not satisfactory in detecting this activity.

Dianzani (16) has used neotetrazolium to demonstrate the location of enzymes in liver and kidney cells. He was able to localize different enzymes in the mitochondria by using different substrates.

Zweifach and coworkers (84) used TTC in studying the adrenal cortex. Because of the different metabolic activity of cells in different zones of the cortex, a differentiation of activity was achieved by differences in amount of reduction of TTC. Lagnado and Sourkes (44), by using a tetrazolium salt as a terminal hydrogen acceptor, were able to detect an

enzyme system in rat brain which affects the dehydrogenation of amines. Mui and Green (52) have used TTC as a terminal hydrogen acceptor in their work on the fatty acid oxidizing system in animal tissue. Others (55) have described the use of tetrazolium chlorides in the microdetection of corticosteroids.

In 1948 Straus, Cheronis and Straus (76) proposed the use of tetrazolium salts in detecting neoplasms. They reported there was a differential reduction of tetrazolium salts by excised, human carcinomatous tissue when compared with normal tissue. In a series of tests using TTC on various tissues, both normal and malignant, they showed that the malignant tissue always showed more formazan than did the normal after the same time interval. It was felt by these workers, as well as by others, that this phenomenon of tetrazolium reduction would offer a very valuable tool to the surgeon and diagnostician. The diagnosis of a tumor by microscopic study of a biopsy specimen as to its being benign or malignant is not usually too difficult for the experienced pathologist. This procedure, however, can at times be time consuming, dangerous and unreliable. The toxicity of tetrazolium salts as reported by Jerchel and Fischer (33) and by other workers (45),(66) is such that the doses required for in vivo tumor diagnosis would not be dangerous.

Schuermann (67) reported that TTC applied to ulcerated tissues in vivo was reduced relatively more rapidly by malignant than by benign tissue. In excised malignant tissue the red of formazan was produced before it appeared in normal tissue. Difficulty was reported because of the simularity of formazan color to that of blood but this difficulty can be eliminated with practice. Schuermann reported that in blood serum from 33 malignant tumors and 63 normal controls the formazan was produced more rapidly in all malignant cases over a 20 minute interval. Hsu and Hoch-Ligeti (29) reported that a number of malignant tumors reduced tetrazolium salts, however, epithelial carcinoma showed no differential staining from normal epithelial tissues. Schümmelfeder (64) also reported that under certain conditions TTC could be used to differentiate malignant and non-malignant tissues.

Others, in investigating the use of tetrazolium salts in tumor diagnosis, have not been so favorable in their reports. Pearson and Defendi (59) reported that a rat hepatoma which was induced by p-dimethylaminoazobenzene could not be distinguished from normal hepatic tissue by tetrazolium salts. Siegert, Brüchel and Ried (69) report they could find no definite results when quantitative measurements of reduction were made. Errors and failures of 40 and 50% were reported. Waldo, Zipf and Burton (77)

report that there appears to be a correlation between tetrazolium reducing activity in serum and the serum alkaline and acid phosphatase activity. Although acid phosphatase activity tends to be highest in prostatic carcinoma and alkaline phosphatase high in sclerosing osteogenic sarcoma, many other diseases can cause increased phosphotase activity. For example, liver cirrhosis, Cushing's syndrome, renal rickets and hypo- and hyperparathyroidism all are associated with high phophatase activity. Therefore, reduction of tetrazolium salts by blood serum can not be used as a means of tumor diagnosis.

Masouredin and associates (49) in an in vivo study using 2, 5 diphenyl - 3(p-radioiodophenyl) tetrazolium chloride found that transplantable mammary carcinoma and lymphomas contained less radioactivity than most normal tissue, indicating a serious defect in the use of tatrazolium salts for in vivo malignancy diagnosis. Black and coworkers (7) have reported that while tetrazolium salts may not be of use in the detection of malignancies in vivo, they have a definite value in staining frozen sections for microscopic examination, if the reduction is accelerated by light.

The original discovery by Kuhn and Jerchel (40) of the biological activity of tetrazolium salts, as previously mentioned, occurred when they were studying antibacterial agents. In this work they found that certain tetrazolium

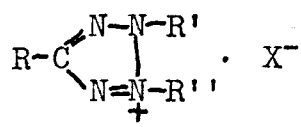
salts, ones in which the five positions of the tetrazole ring was substituted by an n-undecyl group, had a definite antibacterial action. Sewell and Hawking (70) have reported that triphenyl tetrazolium bromide and 2,5-diphenyl -3(p-tolyl) tetrazolium bromide are inactive against fillariasis. Other workers (46) have reported a slight activity against Influenza A and mouse pneumonitis virus in mice by tetrazolium salts substituted by amino groups.

A few non-biological uses of tetrazolium salts have been described. Weiner (82) has reported the use of TTC as an indicator for reducing substances in alkaline solution. Mattson and Jensen (48) have used TTC in the colorimetric determination of reducing sugars. Wollenfels (78) has used tetrazolium salts in paper chromotography to test quantitatively for reducing sugars. Steigman (74) has described the use of TTC in the detection of sugars in gelatins used in photography.

SUPPLEMENT TO HISTORICAL

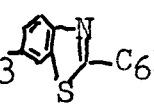
The literature has been reviewed with respect to all the tetrazolium salts which contain three phenyl groups or substituted phenyl groups on the tetrazole ring. A comprehensive review of tetrazolium salts by Nineham (54) contains tables of all the tetrazolium salts reported through 1953 and a large number which have been reported in 1955 and 1956. Also included in his tables are a number of tetrazolium salts which as yet have not appeared in the literature.

Table of Tetrazolium Salts



	R	R'	R''	X-	REF
1-	C_6H_5	C_6H_5	C_6H_5	Cl	56
2-	C_6H_5	C_6H_5	C_6H_5	Br	26
3-	$4\text{-}C_6H_5\text{-}C_6H_4$	C_6H_5	C_6H_5	Cl	17

	R	R'	R''	X⁻	Ref
4-	4-$O_2NC_6H_4$	C_6H_5	C_6H_5	Cl	2
5-	4-CNC_6H_4	C_6H_5	C_6H_5	Cl	2
6-	3-$HO_3SC_6H_4$	C_6H_5	C_6H_5	Betaine	20
7-	4-$H_2NC_6H_4$	C_6H_5	C_6H_5	Cl	2
8-	4-$CH_3OC_6H_4$	C_6H_5	C_6H_5	Cl	61
9-	3,4$(OCH_2O)C_6H_3$-	C_6H_5	C_6H_5	Cl	61
10-	4-CH_3CONH-C_6H_4-	C_6H_5-	C_6H_5-	Cl	2
11-	4-CH_3CONH-C_6H_4-	C_6H_5-	C_6H_5-	Br	47
12-	4(4'-H_2N-C_6H_4)SO_2 NH-C_6H_4-	C_6H_5-	C_6H_5-	OH	2
13-	4(4'-CH_3CONH C_6H_4)2 $SO_2NHC_6H_4$-	C_6H_5-	C_6H_5-	Cl	2
14-	4-$(CH_3)_3\overset{+}{N}C_6H_4$-	C_6H_5-	C_6H_5-	Cl	54
15-	4-$(CH_3)_3\overset{+}{N}C_6H_4$-	C_6H_5-	C_6H_5-	CH_3SO_4	54
16-	4-ClC_6H_4-	C_6H_5-	C_6H_5-	NO_3	18
17-	C_6H_5-	C_6H_5-	4-$CH_3C_6H_4$-	Cl	26
18-	C_6H_5-	C_6H_5-	4-$CH_3C_6H_4$-	Br	2
19-	C_6H_5-	C_6H_5-	4-iso-C_3H_7 C_6H_4-	Br	2
20-	C_6H_5-	C_6H_5-	4-n-$C_{12}H_{25}$ C_6H_4-	I	2
21-	C_6H_5-	C_6H_5-	4-$C_6H_5C_6H_4$-	Cl	2
22-	C_6H_5-	C_6H_5-	4-ClC_6H_4-	I	2
23-	C_6H_5-	C_6H_5-	4-IC_6H_4-	Cl	27
24-	C_6H_5-	C_6H_5-	4-$O_2NC_6H_4$-	Cl	2
25-	C_6H_5-	C_6H_5-	4-$O_2NC_6H_4$-	Br	41

	R	R'	R''	X⁻	Ref.
26-	C₆H₅-	C₆H₅-	4-HOC₆H₄-	Cl	30
27-	C₆H₅-	C₆H₅-	4-CH₃OC₆H₄-	I	30
28-	C₆H₅-	C₆H₅-	4-H₂NC₆H₄-	Cl	2
29-	C₆H₅-	C₆H₅-	4-CH₃CONHC₆H₄-	Cl	2
30-	C₆H₅-	C₆H₅-	4-CH₃CONHC₆H₄-	I	2
31-	C₆H₅-	C₆H₅-	2-Cl-4-H₂NC₆H₃-	Cl	2
32-	C₆H₅-	C₆H₅-	2-Cl-4-CH₃CONHC₆H₃-	Cl	2
33-	C₆H₅-	C₆H₅-	3-Cl-4-H₂NC₆H₃-	Cl	2
34-	C₆H₅-	C₆H₅-	4-CH₃OC₆H₄-	Cl	61
35-	C₆H₅-	C₆H₅-	2-O₂N-4-H₂NC₆H₃-	Cl	2
36-	C₆H₅-	C₆H₅-	3-HO-4-H₂NC₆H₃-	Cl	2
37-	C₆H₅-	C₆H₅-	4-(n-C₁₂H₂₅NH)-C₆H₄-	I	2
38-	C₆H₅-	C₆H₅-	NH₂(CH₂)₁₂NHC₆H₄-Cl	Cl	2
39-	C₆H₅-	C₆H₅-	4-(CH₃)₃$\overset{+}{N}$C₆H₄	Cl	54
40-	C₆H₅-	C₆H₅-		Picrate	37
41-	C₆H₅-	4-ClC₆H₄-	C₆H₅-	NO_3^-	32
42-	C₆H₅-	3-HOOCC₆H₄-	C₆H₅-	I	79
43-	C₆H₅-	4-C₆H₄-2-thiazolyl	C₆H₅-	Br	5
44-	C₆H₅-	4-COOHC₆H₅-	C₆H₅-	Br	21
45-	C₆H₅-	4-C₂H₅COO-C₆H₄-	C₆H₅-	Br	21

	R	R'	R''	X	Ref.
46-	C_6H_5-	3-Cl-C_6H_4-	C_6H_5-	NO_3	21
47-	C_6H_5-	4-Cl-C_6H_4-	C_6H_5-	NO_3	21
48-	C_6H_5-	3,4-diCl-C_6H_4-	C_6H_5-	NO_3	21
49-	C_6H_5-	3-$CH_3O$$C_6H_4$-	C_6H_5-	NO_3	21
50-	C_6H_5-	4-$NO_2C_6H_4$-	C_6H_5-	Cl	21
51-	C_6H_5-	2-$COOHC_6H_4$-	C_6H_5-	Cl	36
52-	C_6H_5-	3-$COOHC_6H_4$-	C_6H_5-	Cl	36
53-	C_6H_5-	4-$NH_2SO_2C_6H_4$-	C_6H_5-	Br	36
54-	4-BrC_6H_4-	2,4,6-$Br_3C_6H_2$-	C_6H_5-	Br	54
55-	4-$O_2NC_6H_4$-	C_6H_5-	4-$CH_3OC_6H_4$-	I	81
56-	3,4-$(CH_3O)_2C_6H_3$-	C_6H_5-	4-$CH_3OC_6H_4$-	I	2
57-	3,4-$(OCH_2O)C_6H_5$-	C_6H_5-	4-$CH_3OC_6H_4$-	Cl	61
58-	3,4$(CH_3O)_2C_6H_5$-	C_6H_5-	4-$CH_3OC_6H_4$-	Cl	61
59-	3,4-$(OCH_2O)C_6H_3$-	C_6H_5-	4-$CH_3OC_6H_4$-	Cl	61
60-	4-$CH_3OC_6H_4$-	C_6H_5-	4-$NO_2C_6H_4$-	Cl	61
61-	4-$CH_3CONHC_6H_4$-	C_6H_5-	4-$NO_2C_6H_4$-	Cl	61
62-	4-$H_2NC_6H_4$-	C_6H_5-	4-$O_2NC_6H_4$-	Cl	61
63-	4-OH-3,5-$(CH_3)C_6H_2$-	C_6H_5-	4-$CH_3OC_6H_4$-	Cl	61
64-	3-$CH_3OC_6H_4$-	C_6H_5-	3-$CF_3C_6H_4$-	Acetate	59
65-	4-$(CH_3)_3\overset{+}{N}C_6H_4$-	C_6H_5-	4-$(CH_3)_3\overset{+}{N}C_6H_4$-	CH_3SO_4	54
66-	4-ClC_6H_4-	C_6H_5-	4-$(CH_3)_3\overset{+}{N}C_6H_4$-	Cl	54
67-	4-$O_2NC_6H_4$-	C_6H_5-	4-$(CH_3)_3\overset{+}{N}C_6H_4$-	Cl	54

	R	R'	R''	X	Ref.
68-	4-HOC_6H_4-	C_6H_5-	4-$(CH_3)_3\overset{+}{N}C_6H_4$-	Cl	54
69-	4-HOC_6H_4-	C_6H_5-	4-$(CH_3)_3\overset{+}{N}C_6H_4$-	CH_3SO_4	54
70-	4-$H_2NC_6H_4$-	C_6H_5-	4-$(CH_3)_3\overset{+}{N}C_6H_4$-	CH_3SO_4	54
71-	4-$(CH_3)_3\overset{+}{N}C_6H_4$-	C_6H_5-	4-$NH_2C_6H_4$-	Cl	54
72-	4-$HOOCC_6H_4$-	C_6H_5-	4-$(CH_3)_3\overset{+}{N}C_6H_4$-	CH_3SO_4	54
73-	4-CNC_6H_4-	C_6H_5-	4-$(CH_3)_3\overset{+}{N}C_6H_4$-	Cl	54
74-	4-$(CH_3)_3\overset{+}{N}C_6H_4$-	C_6H_5-	2-HOC_6H_4-	Cl	54
75-	4-$(CH_3)_3\overset{+}{N}C_6H_4$-	C_6H_5-	2-$C_6H_5CH_2OC_6H_4$-	Cl	54
76-	4-$(CH_3)_3\overset{+}{N}C_6H_4$-	C_6H_5-	2-ClC_6H_4-	CH_3SO_4	54
77-	2-HOC_6H_4-	C_6H_5-	4-HOC_6H_4-	Cl	1
78-	4-BrC_6H_4-	C_6H_5-	4-BrC_6H_4-	Cl	2
79-	4-$O_2NC_6H_4$-	C_6H_5-	4-HOC_6H_4-	Cl	81
80-	4-$O_2NC_6H_4$-	C_6H_5-	4-$O_2NC_6H_4$-	Cl	61
81-	C_6H_5-	4-$C_6H_5C_6H_4$-	4-$C_6H_5C_6H_4$-	Cl	33
82-	C_6H_5-	4-I-C_6H_4-	4-I-C_6H_4-	Cl	23
83-	C_6H_5-	4-$O_2NC_6H_4$-	4-IC_6H_4-	Cl	23
84-	C_6H_5-	4-$H_2NC_6H_4$-	4-$(CH_3)_3\overset{+}{N}C_6H_4$-	CH_3SO_4	54
85-	C_6H_5-	3-ClC_6H_4-	3-ClC_6H_4-	NO_3	21
86-	C_6H_5-	4-$O_2NC_6H_4$-	4-$O_2NC_6H_4$-	Cl	21
87-	4-$C_6H_5C_6H_4$-	4-$C_6H_5C_6H_4$-	4-$C_6H_5C_6H_4$-	Cl	33
88-	4-$H_2NC_6H_4$-	4-$H_2NC_6H_4$-	4-$(CH_3)_3\overset{+}{N}C_6H_4$-	CH_3SO_4	54
89-	4-$CH_3OC_6H_4$-	4-$CH_3OC_6H_4$-	4-$CH_3OC_6H_4$-	Cl	21
90-	3,4-$(OCH_2O)C_6H_3$-	4-$NO_2C_6H_4$-	4-$CH_3OC_6H_4$-	Cl	61
91-	4-$CH_3OC_6H_4$-	4-$NO_2C_6H_4$-	4-$CH_3OC_6H_5$	Cl	61

	R	R'	R''	X	Ref.
92-	4-$CH_3OC_6H_4$-	4-$NO_2C_6H_4$-	4-$CH_3OC_6H_5$	Cl	61
93-	4-$CH_3OC_6H_4$-	4-$NO_2C_6H_4$-	4-$NO_2C_6H_4$-	Cl	61
94-	4-$O_2NC_6H_4$-	4-$NO_2C_6H_4$-	4-$O_2NC_6H_4$-	Cl	61
95-	$(CH_3O)_2C_6H_3$-	4-$O_2NC_6H_4$-	4-$O_2NC_6H_4$-	Cl	61
96-	$(CH_3O)_2C_6H_5$	4-$O_2NC_6H_4$-	4-$CH_3OC_6H_4$-	Cl	61

ENZYMES AND TETRAZOLIUM SALTS

Enzymes are well known in biochemistry as substances which play an indispensable role in the living organism. Various methods of study have been used in an effort to better understand enzymes and how they accomplish their function. Tetrazolium salts have been used in this work since the discovery of their biological reduction (40).

A classification of enzymes as given by Nielands and Stumpf in Outlines of Enzyme Chemistry (53) is as follows:

A - Enzymes catalyzing hydrolysis:

A typical hydrolase is a phosphatase which catalyzes the hydrolysis of organic phosphate to form an alcohol and phosphoric acid.

$$ROPO_3H_2 + H_2O \rightleftharpoons ROH + H_3PO_4$$

B - Enzymes catalyzing transference:

A typical transferase is creatinetransphosphorylase which catalyzes the transfer of phosphate from adenosine triphosphate to creatine.

$$ATP + \text{creatine} \rightleftharpoons ADP + \text{creatine-phosphate}$$

C - Enzymes catalyzing addition:

An enzyme involved in addition is aconitase which affects the change from citrate to isocitrate in the Kreb cycle.

Citrate $\rightleftharpoons$ cis-aconitate $\rightleftharpoons$ Isocitrate

D - Enzymes catalyzing isomerization:

An example is mutarotase, an enzyme which affects the isomerization of α - Glucose to β - Glucose.

α - Glucose $\rightleftharpoons$ β - Glucose

E - Enzymes catalyzing carboxylation:

An example of this type enzyme is pyruvate carboxylase, involved in the decarboxylation of pyruvate to form acetaldehyde.

Pyruvate $\rightleftharpoons$ acetaldehyde

F - Enzymes catalyzing respiration:

These enzymes are the ones involved in the redox reactions taking place in the living organism. Since practically all the known energy-yielding reactions in living organisms are redox in nature, these reactions occupy a very important place in biochemistry and enzymology. Cantarow and Schepartz (13) in their textbook of biochemistry classify the oxidative (respiratory) enzymes in four divisions.

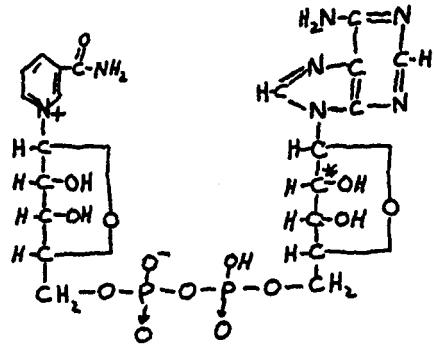
(1) The oxidases which act as electron carriers directly from the substrate to oxygen.

(2) The aerobic dehydrogenases which can transfer electrons to certain reducible dyestuffs, such as methylene blue, as well as oxygen.

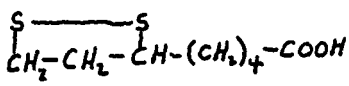
(3) Anaerobic dehydrogenases, whose reduced electron carriers are not directly oxidizable by oxygen, but require certain accessory carrier systems to complete the chain. These can also use dyestuffs as electron acceptors.

(4) The hydroperoxidases which include the peroxidases and catalases, and affect the reduction of hydrogen peroxide.

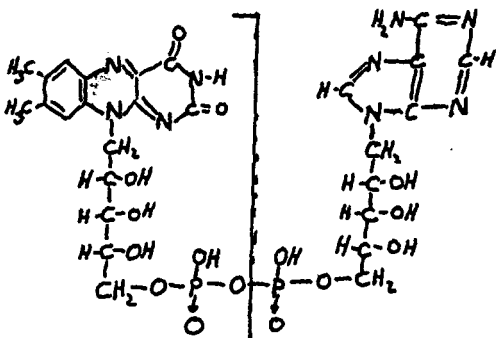
The oxidative enzymes all contain one of several different coenzymes or prosthetic groups. Coenzymes and prosthetic groups may be defined as organic, dialyzable, thermostable compounds which certain enzymes require for their function. If the compound is rather firmly attached to the protein it is called a prosthetic group. If the attachment is not very firm it is called a coenzyme. These groups are the actual carriers of electrons or hydrogen atoms in biological oxidations. Their structures are given below:



Diphosphopyridine Nucleotide (DPN). Triphosphopyridine has H_3PO_4 attached*. (TPN)

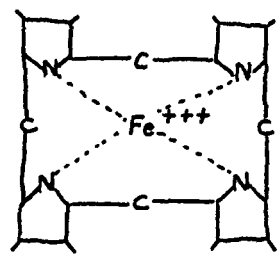


Lipoic acid



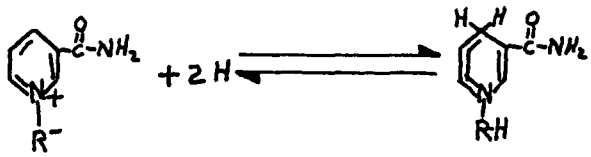
Flavin Mononucleotide (FMN)

Flavin Adeninedinucleotide (FAD)

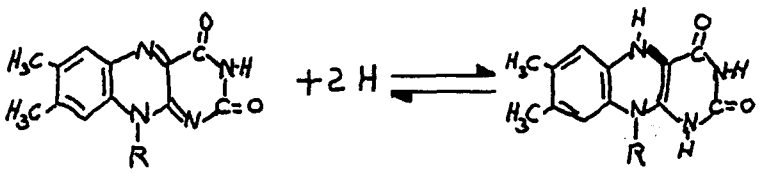


Iron phorphyrin
(skeleton structure)

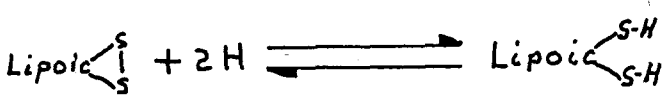
DPN and TPN are important cofactors for many anaerobic dehydrogenases (abbreviated deHase). FAD and FMN act as coenzymes or prosthetic groups for some of the deHases and also for some of the intermediate carriers which operate in conjunction with the anaerobic deHases. Lipoic acid functions in oxidative decarboxylations of certain α- keto acids in conjunction with thiamine pyrophosphate. The iron porphyrin skeleton occurrs in the cytochromes which function as intermediate electron carriers, in cytochrome oxidase and in catalase and peroxidase. The mechanisms of action of these groups are represented below:



Action of DPN and TPN:



Action of FMN and FAD:



Action of Lipoic Acid:

Representative of iron in Ferriprotoporphyrins:

In biological oxidations, as previously mentioned, electrons are transferred from the substrate molecules to pyridine nucleotides (DPN and TPN) or to flavoproteins (FMN and FAD plus an enzyme protein). Many flavoproteins are capable of reacting with molecular oxygen. However, this mechanism of completing electron transfer is not of much significance. Instead, reduced flavins are oxidized by the cytochromes. Biological oxidation can then be schematically represented in the way shown below (51) with electrons being transferred from the substrate along an oxidative chain to the terminal acceptor oxygen.

Substrate → pyridinenucleotide → flavoprotein → cytochromes → O_2

Substrate

Biological oxidations proceed in a stepwise fashion utilizing a series of enzymes.

Each of these reactions can be represented, at least theoretically, by an equilibrium constant which, in turn, can be used to derive oxidation-reduction potentials. Since an oxidation-reduction potential is a characteristic of a half reaction, such as :

$$DPN^+ + H^+ \quad 2e^- \rightleftharpoons DPNH,$$

an oxidation-reduction potential can be obtained for all the known steps in this series.

It must be noted here that in contrast to the physical chemist who defines as negative the potentials of systems that tend to oxidize the reduced members of other systems, the biochemist defines the potentials of reducing systems as negative. Thus, in biochemistry a system with hydrogen gas at one atmosphere equilibrated with a pH 7.0 buffer has a potential of - 0.42 volt.

Some representative oxidation reduction potentials which are frequently encountered in biochemical work are given below (51):

Reaction	E_opH7.0@25°C.(volts)
O_2-H_2O	0.815
O_2-H_2O_2	0.682
Ferricyanide-Ferrocyanide	0.36
Cyto-c(Fe^{+++})-Cyto-c(Fe^{++})	0.25
Methylene blue - Leucomethylene blue	0.011
Fumarate-Succinate	0.000
Oxoloacetate-Malate	-0.160
Riboflavin-Leucoriboflavin	-0.219
Acetaldehyde-Ethanol	-0.200
DPN-DPNH	-0.320
H^+ (10^{-7} M)-H_2(1Atms)	-0.420
Acetate-Acetaldehyde	-0.468

The oxidation-reduction potentials of some tetrazolium salts have been measured by Jerchel and Möhle (34) and by Ried and Wilk (59). Jerchel and Möhle obtained a redox potential of -0.08 volts for TTC on the hydrogen electrode scale. This value fits nicely into the table of potentials given above. It is noted that this potential is more positive than that of DPN-DPNH or that of riboflavin-leucoriboflavin (FMN or FAD). Therefore, one would expect TTC to accept electrons from either a pyridine mucleotide or a flavoprotein depending on which was present in the enzyme system under study. That TTC does accept electrons from DPNH has been shown by Jensen (37) and coworkers. Working with the deHases of glucose, alcohol, malic acid, β -hydroybutryic acid, lactic acid, 3-phospho-glyceraldehyde and α-glycerphosphate, they showed that when DPN was added to the pure enzymes plus substrate, TTC was reduced.

Other evidence has been presented which indicates that not in all cases can DPN-DPNH transfer electrons directly to a tetrazolium salt. Brodie and Gots (5) working with a flavin enzyme extracted from E. coli could reduce TTC and neotetrazolium in the presence of reduced DPN(DPNH) at a pH of 7.8. No other dehydrogenose activity was detected for the enzyme. It did not reduce the tetrazolium salts in the presence of TPNH. That the enzyme

mediated the transfer of hydrogen to the tetrazolium salts from DPNH was further shown by removal of activity by dialysis with restoration by addition of FAD.

Shelton and Schneider (68) have shown that although tetrazolium salts are reduced by many deHase systems, not all systems will reduce them. Crystaline lactic deHase and alcohol deHase failed to reduce tetrazolium salts in the presence of the substrate, whereas DPN-cytochrome-c reductase (a flavo protein) and xanthine oxidase were able to do so rapidly and progressively. They suggest the mediation of other enzymes in the reduction of tetrazolium salts by certain deHases.

Kan (38) has offered additional support to Shelton and Schneider's idea of the necessity of mediating enzymes. He has shown an obligatory component of the glycolytic-TTC reducing system to be present in mitochondria. This component appears to be a flavo-protein part of the cytochrome-c reducing system.

Sugimura and Ono (75), working with pigeon breast succinoxidase, have shown the necessity of a cofactor for reduction of TTC. They have obtained it from hot water extracts of tissues and in crude coenzyme A preparations from hog liver. The factor has been shown to contain ribose, phosphorus, sulfur and a base. However, it did not contain pantothenate, which is an integral part of coenzyme A.

Forbes and Sevag (22) have shown that amino acids, while unable to serve as substrates for the reduction of TTC by resting cells of Micrococcus pyogenes various aureus, are the most effective of the compounds tested in accelerating the reduction when added to the reaction system in combination with glucose. They believe the amino acids act catalytically to increase the availability of hydrogen for TTC reduction, perhaps by interaction with flavoprotein.

In their work, published in 1957, Sourkes and Lagando (72) measured the activity of solutions of enzymes using TTC and neo-tetrazolium as hydrogen acceptors. With tryptamine, benzylamine, choline and succinate, some reduction took place. However, the addition of a cofactor markedly accelerated the reaction. Adenosine monophosphate (AMP), purine, hypoxanthine and inosine could be used as cofactors. Adenine and uric acid were inactive. The participation of purines, nucleosides and nucleotides in enzymatic reductions is specific for tetrazolium salts among hydrogen acceptors tested. The ones tested were ferricyanide, methylene blue, 2,6-dichlorophenol, idophenol and oxygen.

The compounds prepared in this work are being employed by Drs. G. Z. Williams and A. C. Peacock (83) of the National Institutes of Health in a study of cell metabolism. These workers are endeavoring to see if any specific chemical reaction in a living cell can be detected by a particular

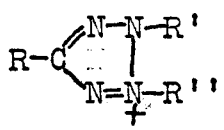
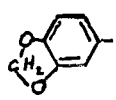
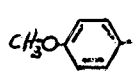
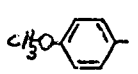
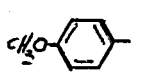
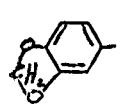
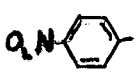
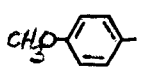
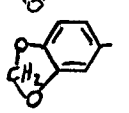
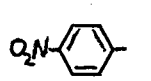
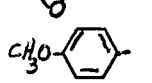
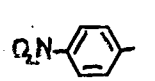
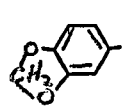
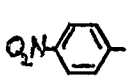
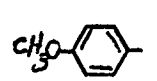
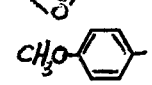
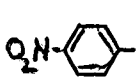
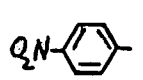
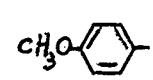
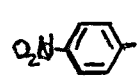
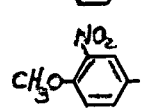
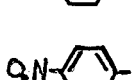
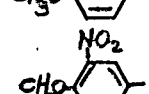
tetrazolium salt. To date, they have studied twenty-four salts, some of which were prepared by Richardson (61). Thirteen tetrazolium salts have been submitted to Dr. Williams recently and about ten more salts will be given to him at the close of the present project.

The compounds were studied using cysteine, the DPNH-Diaphorase system, and the succinic dehydrogenase and malate-DPN systems of washed mouse liver mitochondria. On the basis of the tests made to date, no definite picture has developed. However, quantitatively important differences have been noted among these tetrazolium salts. The data for these studies are presented in Table (I).

Several reports, as pointed out in the earlier discussion, have shown that tetrazolium salts may serve as hydrogen acceptors in the DPNH-Diaphorase reaction. The extent of participation of the various tetrazolium salts in the reaction was compared. In order to have some reference, the reduction of 2,6-dichlorophenol-indophenol by the system was compared to the reduction of the tetrazolium salts. In all cases 2,6-dichlorophenol-indophenol was more rapidly reduced.

As shown in Table (I), there was no reduction of tetrazolium salts which did not contain a nitrophenyl group at some position in the molecule. Among the nitrophenyl containing tetrazolium salts, there was a considerable variation in rate of reduction. However, no definite correlation to

TABLE I

Tetrazolium Chlorides  . Cl^-			Ratio Succinate/Malate	DPNH-DIAPHORASE	Cysteine
R	R'	R''			
			*	-	-
			-	-	-
			-	-	-
			-	-	-
			-	-	-
			3.05	+	+
			2.33	+	+
			2.05	+	+
			1.85	+	+
			1.25	++	++
			1.13	++	+
			0.95	±	+
			0.83	++	++
			0.53	++	++
			0.48	-	+
			0.26	++	++

Courtesy Dr. A.C. Peacock

structure can be made except to note that the tetrazolium salts with two nitrophenyl groups were for the most part more reactive.

Cysteine, which contains a sulfhydryl group, will under certain conditions readily lose hydrogen to a tetrazolium salt. At a pH of 7.4 those tetrazolium salts containing two nitrophenyl groups reacted at once. Those with one nitrophenyl group reacted more slowly, if at all. When studied in the presence of serum all of the mononitrophenyl tetrazolium salts reacted but more slowly than those tetrazolium salts with two nitrophenyl groups. The tetrazolium salts which contained no nitrophenyl groups did not react, even in the presence of serum.

When malate and succinate were used as substrates with washed mitrochondria from mouse liver, the same type of reactivity, as with DPNH Diaphorase and cysteine, was noted with tetrazolium salts containing nitrophenyl groups. These tetrazolium salts without nitrophenyl present in the molecule were not reduced. One other important difference in the reactivity of tetrazolium salts with succinate and malate was noted. As shown in Figure (I), there is a considerable difference in the ability of malate and succinate to reduce different tetrazolium salts. 2-Phenyl-3-(p-nitrophenyl)-5-(p-methoxy phenyl) tetrazolium chloride is reduced much more strongly by succinate than by malate. 2,5-Di(p-nitrophenyl)-

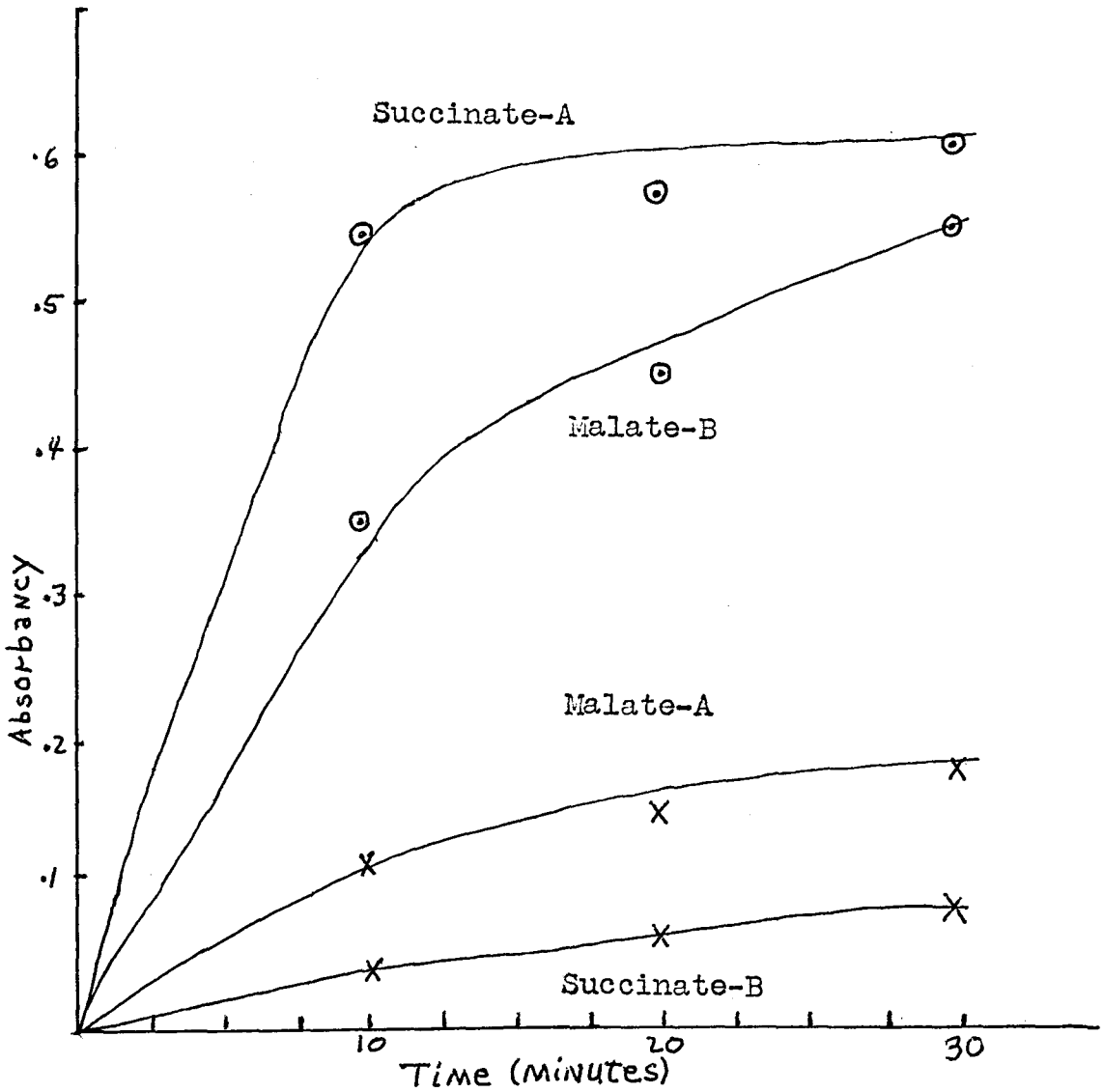


FIGURE I

2-Phenyl-3-(p-nitrophenyl)-5-(p-methoxyphenyl) tetrazolium chloride = A

2,5-Di-(p-nitrophenyl)-3-(o-methoxyphenyl) tetrazolium chloride = B

Courtesy Dr. A.C. Peacock

3-(-o-methoxyphenyl) tetrazolium chloride is reduced more strongly by malate than by succinate. These data suggested that a ratio could be formed which would indicate the relative activity of each tetrazolium salt with succinate and malate. This ratio was formed by dividing the amount of reduction with succinate by the amount of reduction with malate. If the ratio is greater than one, then more reduction occurred with succinate than with malate. If it is less than one, then more reduction occurred with malate. From the data of all the compounds studied, the tetrazolium salts were grouped into three classes: The malate preferring class with ratios of 0 to 0.5, the intermediate class with ratios of 0.75 to 1.5 and the succinate preferring class with ratios of 1.75 and higher. The succinate/malate ratios of the various tetrazolium salts are given in Table (I).

Four tetrazolium salts fall into the succinate preferring class. All four of these compounds have in common the possession of a single p-nitro group on one of the tetrazole ring nitrogens and either a p-methoxyphenyl or a 3,4-methylenedioxyphenyl group on the carbon of the tetrazole ring. If a second nitrophenyl group is added, succinate specificity is lost, although one tetrazolium salt, 2,3-di(p-nitrophenyl)-5-(p-methoxyphenyl) tetrazolium chloride, is borderline.

The three tetrazolium salts showing malate specificity all lack the p-methoxyphenyl or 3,4-methylenedioxyphenyl substitution in position 5 of the tetrazole ring. Two of the three possess two nitrophenyl groups. One of the three is at the upper limit of malate specificity.

As mentioned earlier, no definite picture has developed in regards to structure and specificity of reaction of tetrazolium salts. The presence of a nitro group in order for reduction to occur under the conditions of these experiments is established. However, when these compounds were tested with intact cells those which contained no nitro groups reacted quite well. Those containing nitro groups did not react well, if at all. No explanation for this phenomenom is known at present, although, differences in solubility or the ability to penetrate the cell membrane have been suggested as possibilities. That a 3,4-methylenedioxyphenyl group or a p-methoxyphenyl group must be present for succinate specificity is also indicated, but not definitely.

In hopes of clarifying the picture, more compounds containing electron donating groups and electron accepting groups have been prepared. A number of these tetrazolium salts are now being tested. Perhaps when these data are available, a more complete picture will be presented.

METHODS OF FORMAZAN AND TETRAZOLIUM SALT SYNTHESIS

As was mentioned in the historical discussion, the only known method of synthesis of tetrazolium salts is by the oxidation of formazans. Some have been prepared indirectly by alteration of a tetrazolium salt previously known, as for example, the preparation of a hydroxyphenyl derivative by hydrolysis of a methoxyphenyl derivative.

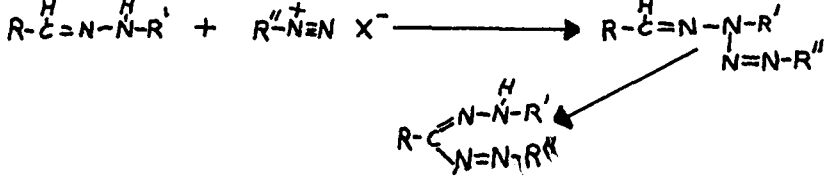
A thorough review of the synthesis of formazans and tetrazolium salts has been given by Nineham (54). Therefore, only a brief description of a few of the more important methods of formazan formation will be given. The methods employed in oxidation of formazans will be discussed briefly.

A - Methods of formazan formation:

I - Reaction of aldehyde-arylhydrozanes with a diazonium salt:

This method of synthesis is the one which has been used for the preparation of the majority of the formazans known. It is the method which was used for all the formazans employed in this work. The reaction is carried out in alkaline solution. Sodium hydroxide, sodium acetate, triethylamine and pyridine are some of the more frequently used bases.

The mechanism of this reaction has been extensively studied by many workers. Busch and his co-workers (9-12) have shown that the mechanism may be represented as follows:



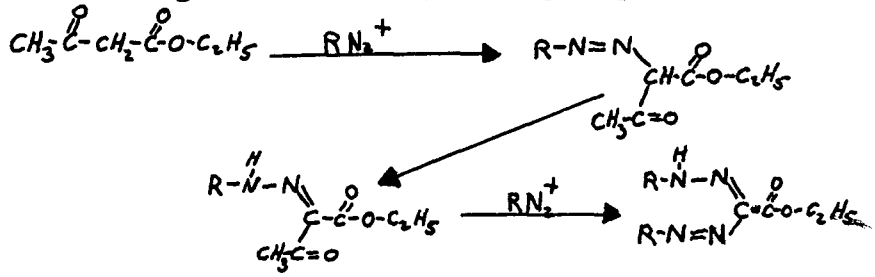
Scott and his co-workers (65) have questioned this as the only mechanism and have postulated three possible modes of attack by the diazonium cation on the hydrazone substrate.

(1) Reaction with the beta nitrogen atom with tetrazine formation.

(2) Coupling on either of the aromatic rings of the hydrazone.

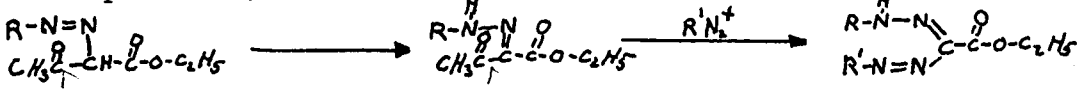
(3) Reaction directly with the methine carbon to produce a formazan.

II - By the reaction of diazonium salts with compounds containing active methylene groups (54) :



This method offers access to some formazans which are not available by the first method discussed. This reaction also requires strongly alkaline conditions since in nearly neutral solutions the Japp-Klingemann reaction for production of phenylhydrazones takes place.

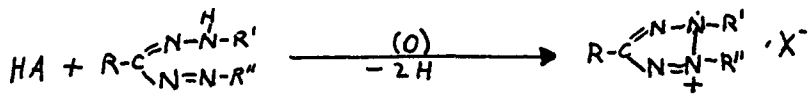
III - By the reaction of diazonium salts with aliphatic azo compounds (54) :



This reaction is a modification of reaction II in which the intermediate azo compound is isolated and then reacted with a different diazonium salt. It is thus possible to have two different N substituents which is not true in method II.

B - Oxidative ring closure of formazans:

The oxidative ring closure of formazans to form tetrazolium salts may be represented as follows:



The first reagent used in the oxidation of formazans was nitrous acid fumes (56) . Other oxidizing agents which are now used are mercuric oxide, isoamyl nitrite and lead tetra-acetate (40). Kuhn and Münzing (41) have described the use of halogenoimides. Tert-butyl hypochlorite has been used as an oxidizing agent for formazans by Benson and co-workers (4). It has been used in this laboratory

for the oxidation of tris-(p-nitrophenyl) formazan to the tetrazolium salt. Hydrogen peroxide in the presence of hydrochloric acid, with vanadium pentoxide as a catalyst, has been used quite successfully in the oxidation of formazans (60).

The effect of various substituent groups on the oxidative ring closure of formazans has been studied by Wedekind (79). He explained that the group R attached to the 3- carbon atom of the formazan chain, affected the ring closure by its effect on rotation of the chain. The more readily the group R permits rotation, the slower the oxidation. The following groups are arranged in order of increasing yield of tetrazolium salt: R=, -H, -COOH, $-CH_3$, $-COCH_3$, $-N{=}NC_6H_5$, $-COC_6H_5$, $-COOC_2H_5$, $-C_6H_5$and -CN. Wedekind also showed that the substitution of electrophylic substituents into an N-phenyl group, positions 1 and 5 of the formazan chain, reduced the yield of tetrazolium salt. This effect was greatest when the substitution was in the ortho position and least in the meta position. Nitro groups had the most marked effect.

EXPERIMENTAL

ARYL HYDRAZONES

All of the aryl hydrazones used in this study have been previously prepared by Richardson (61) and earlier workers. They were prepared, as is the custom, by the condensation of an aromatic aldehyde and aromatic hydrazone in ethanol solution. Acetic acid was used as a catalyst if necessary. A typical procedure is given below:

To a solution of 5.3 g. (0.05 mole) of benzaldehyde in 150 ml. of ethanol is added with stirring 5.4 g. (0.05 mole) of phenylhydrazine. The reaction is exothermic and proceeds rapidly to produce a white flocculant precipitate of benzaldehydephenylhydrazone.

When p-nitrophenylhydrazine is used, it is necessary to dissolve the hydrazine in hot ethanol. The aldehyde is then added to the solution of hydrazine. When p-nitrophenylhydrazine hydrochloride is used in place of the free

base, it is necessary to use sodium acetate in order to form p-nitrophenylhydrazine acetate. Otherwise the reaction will not proceed.

TABLE OF HYDRAZONES

R-CHN=NH-R'

	R	R'	M.P.(this work)	M.P.(Lit.)	Ref.
1-	C_6H_5-	C_6H_5-	157	157-158	(a)
2-	C_6H_5-	p-O_2N-C_6H_4-		110	(b)
3-	p-$CH_3OC_6H_4$-	C_6H_5-	119-121.5	120-120	(c)
4-	p-$CH_3OC_6H_4$-	p-$O_2NC_6H_4$-	160-162	165	(d)
5-	3,4-$OCH_2OC_6H_3$-	C_6H_5-	103-106	106	(e)
6-	3,4-$OCH_2OC_6H_3$-	p-$O_2NC_6H_4$-	200-203	199-200	(f)
7-	p-$O_2NC_6H_4$-	C_6H_5-		153-154	(g)
8-	p-$O_2NC_6H_4$-	p-$O_2NC_6H_4$-	248-251	249	(h)

References:

(a) Ber. 9, 887

(b) Ann. 324, 321

(c) Ann. 248, 103

(d) Ber. 16, 63

(e) Ann. 248, 103

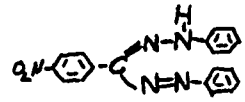
(f) Dictionary Organic Compounds, p. 503

(g) Ber. 20, 1343

(h) Ber. 32, 1813

FORMAZANS

The formazans used in this work were prepared according to method (I) as described on page 36.



The preparation of 1,5-diphenyl-3-(p-nitrophenyl) formazan is typical of the procedure followed.

To a solution of 4.65 g. (0.05 mole) of aniline in 40 ml. of 6 N. hydrochloric acid, at 0°, is added slowly a solution of 3.5 g. (0.05 mole) of sodium nitrite in 20 ml. of water until a positive test is obtained with potassium iodine-starch paper. The temperature is kept below 5° at all times. To the reaction mixture of p-nitrobenzaldehyde-phenylhydrazone in ethanol, as prepared above, is added 100 ml. of pyridine to yield a clear solution, which is then cooled to 10°. Benzenediazonium chloride is added in small portions with stirring over a period of 20 minutes. The reaction mixture is allowed to stand overnight at room temperature. Then 100 ml. of water is added and the reaction mixture is allowed to stand for several hours. 1,5-Diphenyl-3-(p-nitrophenyl) formazan is removed by filtration.

When p-nitroaniline, o-methoxy-p-nitroaniline and o-nitro-p-methoxy aniline are used to form diazonium salts, sulfuric acid is used in place of hydrochloric acid. The amine sulfate is soluble in hot 9 M. sulfuric acid solution. Upon cooling

to 0°, the amine sulfate precipitates, but as the diazotization proceeds, the diazonium sulfate goes into solution.

After filtration, the formazan is washed repeatedly with hot water to remove pyridine and pyridine salts. Early in the study attempts were made to recrystalize the formazans before they were oxidized to tetrazolium salts. Due to the tendency of most of the formazans to yield tars, little purification was achieved by the attempted recrystalizations. This observation is in line with the results reported by Richardson (61). Therefore, the washed formazans were oxidized to tetrazolium salts without attempting recrystalization.

TETRAZOLIUM SALTS

Three general methods have been used in this laboratory to oxidize formazans to tetrazolium salts.

Method A- Lead Tetraacetate Oxidation:

To the crude formazan dissolved in chloroform is added, in small portions, the calculated amount of lead tetraacetate. In the course of the addition the temperature rises, sometimes to 60°. However, no external cooling was used. The red to purple color of the formazan is replaced by a brown-yellow color. The solvent is evaporated on a hot plate until a very low volume is achieved. The last trace of solvent is removed on a boiling water bath. The resulting

residue is boiled with 200 to 500 ml. of water and 5 g. of charcoal for 15 to 20 minutes, and then filtered by gravity. A sufficient quantity of concentrated hydrochloric acid is added to precipitate the lead as lead chloride and to convert the tetrazolium acetate to the tetrazolium chloride. The lead chloride is removed by filtration and then washed with 50 to 100 ml. of alcohol, which is combined with the water extract. The product, if of low enough water solubility, can be precipitated by reducing the volume of water. Otherwise, extraction from the water solution by a suitable solvent is necessary. Chloroform can be used in many cases. The volume of the extracting solvent is reduced and the tetrazolium chloride precipitated by the addition of anhydrous ether. It is necessary to triturate some of the tetrazolium chlorides with several portions of ether in order to achieve crystalization. Recrystalization is carried out in most cases by use of mixed solvents such as ethanol and ether, chloroform and ether and methanol and ether.

Method B- Hydrogen Peroxide Oxidation:

The formazan is suspended in ethanol and 0.1 g. of vanadium pentoxide per. 0.05 mole of reactant is added. Then 15 ml. of hydrochloric acid and 25 ml. of 37% hydrogen peroxide per. 0.05 mole are added dropwise, simultaneously, over a period of 20 to 30 minutes. The temperature generally rises and external cooling is necessary to prevent loss of

hydrogen peroxide. The formazans are also quite sensitive to hot acid solutions so that a considerable amount of formazan may be lost by decomposition. In a few cases, however, it was found necessary to allow the temperature to rise to 40 to 50° in order for the reaction to proceed to completion. The solution becomes brownish-yellow as the end of the oxidation is approached. Any insoluble residue is removed by filtration and the solvent removed by vacuum evaporation. The crude product is worked up in a manner similar to that used for lead tetraacetate oxidations.

Method C- Isoamylnitrite Oxidations:

The formazan is dissolved in glacial acetic acid or a solution of chloroform and glacial acetic acid. Then 40 ml. of isoamylnitrite per. 0.1 mole of formazan is added. The resulting solution is heated on a steam bath for two to three hours, during which time the formazan color is replaced by a brown-yellow color. In some cases, the reaction is allowed to stand for several hours at room temperature before being heated. However, no difference in results can be detected. The solvent is removed by vacuum evaporation and the crude product removed from the flask with methanol. The methanol solution is poured into a large volume of water which is then boiled to remove the alcohol. Activated carbon is added and the solution boiled of 15-to 20 minutes, then

filtered by gravity. Sufficient hydrochloric acid is added to convert the tetrazolium acetate to the tetrazolium chloride. The product is then worked up in a manner similar to that given for method A.

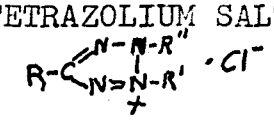
The data for the various tetrazolium salts prepared is summarized in the following table:

Table of

Tetrazolium Salts

(turn page)

TETRAZOLIUM SALTS



Chlorides

R	R'	R''	Method of ppn.
C_6H_5	C_6H_5	4-$CH_3COC_6H_4$	C
C_6H_5	C_6H_5	4- CLC_6H_4	C
C_6H_5	C_6H_5	2-O_2N-4-CH_3O-C_6H_3	C
C_6H_5	C_6H_5	2-CH_3O-4-O_2N-C_6H_3	C
C_6H_5	4-$O_2NC_6H_4$	4-$CH_3OC_6H_4$	C
C_6H_5	4-$O_2NC_6H_5$	4-$CH_3COC_6H_4$	C
C_6H_5	4-$O_2NC_6H_4$	2-$CH_3OC_6H_4$	C
C_6H_5	4-$O_2NC_6H_4$	2-O_2N-4-CH_3O-C_6H_3	C
C_6H_5	4-$O_2NC_6H_4$	4-$O_2NC_6H_4$	C
4-$CH_3OC_6H_4$	C_6H_5	4-$CH_3COC_6H_4$	C
4-$CH_3OC_6H_5$	C_6H_5	2-CH_3O-4-O_2N-C_6H_3	C
4-$CH_3OC_6H_4$	C_6H_5	2-O_2N-4-CH_3O-C_6H_3	C
$CH_3OC_6H_4$	4-$O_2NC_6H_4$	4-$CH_3COC_6H_4$	A
3,4-OCH_2O-C_6H_3	C_6H_5	4-$CH_3COC_6H_4$	C
3,4-OCH_2O-C_6H_3	4-$O_2NC_6H_4$	4-$CH_3COC_6H_4$	A
3,4-OCH_2O-C_6H_3	4-$O_2NC_6H_4$	4-$O_2NC_6H_4$	
4-$O_2NC_6H_4$	C_6H_5	C_6H_5	C
4-$O_2NC_6H_4$	C_6H_5	4-$CH_3OC_6H_4$	C
4-$O_2NC_6H_4$	4-$O_2NC_6H_4$	2-$CH_3OC_6H_4$	C

Recryst. Solvent	Yield %	m.p.	N calcd.	N Found	m.p.	Hg Calcd.	Hg Found
ME	42	210-211			123-125	30.94	
ME	20	263-264			249-251	31.31	
CE	17	163-164			184-185	29.44	29.27
CE	35	173-174			129-130	29.44	
ME	29	231-232			143-144	29.44	
ME	16.6	174-176			> 300	28.93	
W	91.6	203-205			187-188	29.44	
ME	15	150-152	18.48	17.96*			
ME	13	220-221			195-196	28.81	
ME	29	205-207			134-135	29.57	
W	47	137-138			224-225	28.20	
W	32	144-146			135-136	28.20	
ME	13.3	180-181			142-143	27.73	
ME	26	215-216			180-181	28.97	
ME	5.6	209-210					
W	32	249-250#			261-262	30.80	31.04
CE	24	186-187			141-142	29.44	
ME	68	159-161			178-180	27.62	

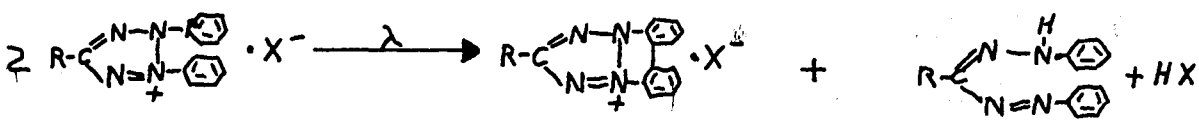
* Analysis by a commercial laboratory.
\# Ref. (2)
Recrystallization solvents: ME= methanol-ether; CE= Chloroform-ether; W= water.

Purification of tetrazolium salts presents many difficulties. Attempts have been made in this laboratory and by other workers to find satisfactory solvents for recrystalization. The solvents most frequently used are ethanol, methanol, acetone, ethyl acetate, chloroform and dioxane. Frequently, ether is used as a precipitating agent with one of the above solvents. Thus, the "recrystalizations" usually involve solution and precipitation without separation from those substances which have the same solubility properties as do the tetrazolium salts.

When ether is used as a precipitating agent, there is a great tendency for solvated tetrazolium salts to become gummy on exposure to air. This gum formation can be prevented in most cases by removal of ether and solvent in a vacuum desiecator soon after the precipitate is formed.

A few of the tetrazolium chlorides, as previously mentioned, are only slightly soluble in cold water and were recrystalized satisfactorily from dilute hydrochloric acid solution.

It is also necessary to avoid undue exposure of the tetrazolium salts to light. When exposed to light, tetrazolium salts are reduced to formazans and converted into "photo tetrazolium salts".



Even when exposed to light for a very short time, a tetrazolium salt will become yellow or pink. Therefore, all the compounds prepared were immediately placed in covered vacuum desiccators after crystalization.

Because of the difficulty in purification, preparation of samples of tetrazolium salts for analysis is very difficult and often results in a great loss of product. They cannot be analyzed by the Kjeldahl method, due to the tetrazole structure. The Dumas method can be used when pure tetrazolium salts are available.

Jerchel and Fischer (33) reported in 1949 the formation of a double salt with mercuric chloride and triphenyl tetrazolium chloride. Their data indicated a simple addition compound between mercuric chloride and TTC. Subsequent workers have reported the mercuric chloride double salts of other tetrazolium compounds. The formation of the mercuric chloride double salt leads to a marked drop in solubility from that of the tetrazolium chloride, it then being possible to recrystallize them from aqueous alcohol.

An unsuccessful attempt was made to titrate the mercury in the double salt with sodium cyanide. The end point was indefinite, partially due to formazan formation in the slightly alkaline solution.

Attempts were made to destroy the tetrazolium chloride and leave in solution ionic mercury free of interfering

substances. Heating the double salt with concentrated nitric acid and solid potassium permanganate failed to give complete decomposition of the tetrazolium salt.

Analyses were performed satisfactorily by solution of the tetrazolium chloride-mercuric chloride double salt in methanol or ethanol, accidification with hydrochloric acid and precipitation with hydrogen sulfide. The precipitated mercuric sulfide was filtered on a sintered glass crucible, washed and dried to constant weight.

The mercuric chloride double salts are prepared by the addition of a saturated solution of mercuric chloride to a solution of the tetrazolium chloride in hot water or ethanol. The double salt is removed by filtration and recrystalized from aqueous ethanol or aqueous methanol.

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