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Tetrazolium salts

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TETRAZOLIUM SALTS

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THESIS

Submitted to the Graduate Faculty of the University of Richmond in Partial Fulfillment of the Requirements for the Degree of Master of Science.

by

Andrew Garnett Richardson

****

University of Richmond

June, 1956

Approved

[Signature]

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I. HISTORY

Tetrazolium salts, as the name indicates, are quartenary ammonium salts containing a tetrazole ring structure of one carbon and four nitrogen atoms, one of the latter being quartenary. Substitution is possible on positions 2, 3, and 5 of the ring as is shown in type formula (I). When these substituent groups are bifunctional ditetrazolium salts of types (II), (III) and (IV) result. Compounds bridged at both \( R' \) and \( R'' \) at the same time are theoretically possible, but no such cases have been reported, probably on account of the practical limitations of synthesis. The anion, which is indicated by \( X \), may be halide, sulfate, acetate, picrate, succinate, thiocyanate, or practically any atom or group capable of existing as a negative ion.

![Chemical Structures](image)

As might be expected from the ionic nature of these compounds they are more or less soluble in water and polar solvents,
although the solubility varies with complexity of the molecule. In non-polar solvents they are considerably less soluble. Their solutions are generally light yellow in color, but some are colorless.

In the presence of a hydrogen donor a tetrazolium salt accepts two atoms of hydrogen. The mechanism of this reaction has not been established, but the overall result is that the tetrazole ring is opened. One hydrogen atom unites with the nitrogen which has been left with two free electrons, and another hydrogen atom unites with the anion to form the corresponding acid. The ionic character of the compound is thus lost, and the resulting compound is known as a formazan.

\[
\text{R-C} + 2\text{H} \rightarrow \text{R-C} + \text{HX}
\]

The loss of ionic character is accompanied by striking differences in solubility between the two classes of compounds. The formazans are generally soluble in chloroform, benzene, toluene, ether, and similar solvents, less soluble in the lower alcohols, and practically insoluble in water, unless the molecules contain polar groups. The formazans are also intensely colored compounds, usually some shade of red, although blue formazans have also been reported (1), (2).
The reduction of tetrazolium salts to formazans can be carried out quite readily with a number of organic and inorganic reducing agents under relatively mild conditions. For example, the reduction takes place at room temperature with powdered zinc, sodium bisulfite, hydroxylamine, or the sodium salt of ascorbic acid. Further reduction beyond the formazan stage is possible, but more drastic conditions are required, such as the use of catalytically activated hydrogen, or sodium amalgam.

The step-wise reduction of tetrazolium salts was studied by Kuhn and Jerchel (3), and they gave the following mechanism for the reduction of triphenyltetrazolium chloride:

\[
\begin{align*}
\text{tetrazolium salt} & \quad \xrightarrow{2H} \quad \text{formazan} \\
\text{Na(Hg)} & \quad \xrightarrow{\text{amidazone}} \\
\text{hydrazone} & \quad \xrightarrow{\text{indeterminate products}}
\end{align*}
\]

\[
\begin{align*}
\text{(I-2)}
\end{align*}
\]
The present research was concerned only with the first stage, or the formazan stage, of the reduction. This reaction is unusual in that a colorless, or slightly colored compound is reduced to an intensely colored one. Such a condition is rarely encountered in organic chemistry. For example, most of the azo dyes are deeply colored in their oxidized form but much lighter colored in their reduced, or "leuco", form.

The first tetrazolium compounds were reported by H. von Pechmann and P. Runge (4) in 1894, but it was not until 1941 that any practical use for them was reported. At this time Kuhn and Jerchel (5) discovered the biological reduction of tetrazolium salts during the course of their studies on bactericidal agents. They found that these compounds were not particularly effective as germicides, but they caused the bacteria to turn red. These investigators postulated that the aqueous solution of the tetrazolium salt penetrated the cellular membranes of the bacteria and that reduction took place within the cells, due to the dehydrogenase systems present there. This hypothesis has been fairly well substantiated by subsequent investigations.

During World War II G. Lakon (6), a German biologist, used this property of tetrazolium salts to test the germinating ability of various grains. The seeds of the grain were covered with a 1% solution of triphenyltetrazolium chloride (commonly designated as TTC), and if the seeds were capable of germinating they turned red within a short time. If no change occurred they were considered to be incapable of growth. The favorable results of this test led subsequent investigators to apply it
to other biological systems.

Mattson and his associated (7) reported that TTC was reduced to triphenylformazan by apples, citron, oranges, mushrooms, young leaves, pollinated floral pistils, ox sperm and the blastoderms of eggs, in neutral solution. They suggested the use of tetrazolium salts as indicators for tissue viability in biological systems, but they also warned that such a test probably could not be used to prove the existence of life. In this connection the present study has shown that solutions of tetrazolium salts when allowed to stand in contact with dead animal or vegetable tissues usually turned red within a few hours due to conversion to the corresponding formazans. This could be due to the metabolic processes of the growing bacteria or to autolytic reactions in the dead tissues. Mattson's work also gave evidence that cellular enzyme systems were responsible for the tetrazolium reduction, since tissues which were heated to 82° lost their ability to reduce these compounds.

Later workers produced additional evidence for this mechanism. Roberts (8) reported that the reducing agent was heatlabile but was unaffected by cold, and Bielig's (9) demonstration of the pronounced dependence of the biological reduction on hydrogen ion concentration, as well as the variation of the reaction rate with temperature, pointed to the existence of enzyme systems which transfer hydrogen. Hölscher (10) found that such physiologically active substances as thiamine, riboflavin, folic acid, pantothenic acid, nicotinic acid, inositol and cytochrome C had no effect on the reduction.
Kuhn and Jerchel (5) had already shown that other cellular substances, such as glutathione, cysteine, ascorbic acid and glucose were not concerned, since they are not able to function as reducing agents below pH 9, whereas the tetrazolium reduction can occur at pH values as low as 6. Mattson (7) reported also that the dehydrogenase, coenzyme I, reduced TTC at pH 6.6, and the pyrimidine nucleotide dehydrogenase was effective at pH 7.0. These hydrogen-transfer systems are very wide-spread in living tissues.

Shelton and Sneider (11), however, did not agree that dehydrogenase activity is solely responsible for the biological reduction of tetrazolium compounds. They concluded from studies which they had made that the mediation of other enzyme systems may be required.

In general, biological reduction stops at the formazan stage, probably on account of the extremely low aqueous solubility of these substances. As Jerohel and Möhle (12) have noted, the presence of solubilizing groups, such as COOH or \( \text{SO}_3\text{H} \), permit further reduction of the formazan to colorless substances, so the corresponding tetrazolium salts are useless as indicators. Siegert et al (13) found that further reduction beyond the formazan stage occurs in the bacterial reduction of 5-methyl-2,3-diphenyltetrazolium chloride.

Since the work of Kuhn and Jerchel had indicated that the tetrazolium salts were not effective germicides Jerohel and Fischer (14) tested the toxicity of several of these compounds on fish (Barbus conchonius) by measuring the time of survival in solutions of the test compounds as compared
with the survival in pure water. The results showed that the compounds tested were not toxic below concentrations of 0.1%.

It is now generally conceded that the rate of biological reduction of tetrazolium salts depends on the enzymatic activity of the cells affected, regardless of whether or not dehydrogenases are solely involved. Wundt (15) demonstrated that the extent of tetrazolium reduction was proportional to the logarithm of the increase in growth of young cultures of streptococci. Since accelerated dehydrogenase activity is one of the principal characteristics of tumor cells, considerable work has been done in attempts to use the tetrazolium reduction to distinguish tumor tissues "in vivo". It is not difficult to identify isolated tumor cells, as for example in tissue removed by biopsy, but an agent which applied topically, or injected, could distinguish tumors "in vivo" by differential staining, would have immeasurable value in early diagnosis and as an adjunct to surgery. In this regard, Kieswalter (16) reported that enlarged tumors showed a nine-fold increase in the zymohexase and isomerase levels over normal tissues.

One of the earliest investigations of the tetrazolium reduction for tumor diagnosis was made by Straus and his associates (17). The report of this work was very favorable, and it stimulated further studies along these lines. Straus reported that his group was able to identify tumor tissues "in vivo" by the differential staining produced when suspected ulcerous lesions were painted with 1% TTC solution. A test on skin tumors by means of applied tampons soaked in 1% TTC solution was also made by Schuermann (18), and he
reported that some difficulty was encountered due to similarity of the formazan color with that of the blood, but that this difficulty could be eliminated after some practice. With tests on blood serum from 33 malignant tumors and 63 healthy controls Schuermann found distinctly more intense formazan color with all of the tumor sera within 20 minutes. Schümmelfeder (19) agreed with Straus and Schuermann that under certain conditions TTC could be used to differentiate malignant and nonmalignant tissues. All of these workers recognized that normal tissue cells also caused reduction of TTC but at a slower rate, thus permitting the tumor tissues to be identified by the more intense staining.

In contrast to these favorable results regarding the efficacy of the tetrazolium reduction in distinguishing neoplastic tissues Siegert, Brückel and Ried (13) found no definite results in quantitative measurement of the reaction differential. In this work formazan formation in blood serum was measured colorimetrically. The most distinct results were found with a serum dilution of 1 to 2700, determined after standing 24 hours. Even so, the number of errors and failures lay between 40% and 50%. The authors also reported that the errors due to bacterial contamination and granulating tissues in ulcerous lesions must always be considered, since bacteria also bring about the tetrazolium reduction. Seligman and associates (20) reported that diphenyl-p-iodophenyl tetrazolium chloride was not reduced any more extensively "in vivo" by Sarcoma 37 than by normal tissue. In another report these
investigators (1) gave the results of tests of 3,3'-(3,3'-(
         dimethoxy-4,4'-biphenylene)bis[2,5-diphenyl-tetrazolium chloride]
with Sarcoma 37, Baag lymphosarcoma, and Walker carcinoma, all
"in vivo". None of these tumors were able to show any differ-
ential reduction of the tetrazolium compound in comparison with
normal tissues. Franke (21) found that the bacterial contami-
nation was particularly confusing when the test objects were
ulcerous skin tumors, pleural puncture fluid, ascites, and
gastric juice. These were exactly the sort of cases in which
a definite tumor diagnosis would have been of particular impor-
tance.

Since these unfavorable reports were published the
interest in tetrazolium salts as a source of possible aid to
tumor diagnosis has decreased, at least in this country, in
contrast to the interest which was apparent in the scientific
literature following the first favorable reports in this respect.
However, research on tetrazolium compounds as a whole has not
abated. Their value in studies of cellular respiration and
for measuring dehydrogenase activity of tissue slices and
tissue homogenates, as exemplified by the work of Black and
Kleiner (22), has been definitely established. The enzymatic
activity is determined by extraction and colorimetric measure-
ment of the resulting formazan, thus giving the amount of
tetrazolium reduced per milligram of tissue.

Stimulated by the observations of Hölischer (10) that
reduction of TTC by tumor cells caused the cellular granules
to turn red from production of formazan while the cytoplasm
remained colorless, Seyfarth (23) used the tetrazolium reduction to investigate the controversial question whether the mitochondria are to be considered as cellular constituents or as individual bodies. His conclusion was that they were separate entities.

Wallhauser (24) made use of the dependence of TTC reduction upon the age of bacteria to carry out microbiological assays for antibiotics and germicidal agents. For example, he was able to determine the Aureomycin concentration in body fluids in 40 to 60 minutes by this method.

In other applications of the tetrazolium reduction Stüttgen (25) developed a method for detection of diabetes with TTC and gave the opinion that the reduction was due to the products of fatty acid degradation (acidosis). It may be recalled (page 6) that Kuhn and Jerchel had stated that glucose could not reduce tetrazolium compounds below pH 9. Schönberg (26) described a method for making bacterial counts of milk within a few minutes. Normally such counts require several hours incubation following inoculation of the culture media.

A rather unusual application of TTC was recently reported by Goldblith (27) in which the reduction was used to evaluate the effects of irradiation of canned foods by high energy cathode rays. Haussner (28) and Gierlach (29) had previously pointed out that TTC could be reduced by high-energy radiations.

Since this research problem was started in 1952 over 200 new tetrazolium compounds have been reported. This is
about four times the number of compounds which were known up to that time. About three-fourths of these new compounds have been the result of work of two research teams, one headed by Dr. Walter Ried of the University of Frankfurt and the other by Dr. A. W. Nineham of May and Baker. Ltd.

As can be judged from the foregoing review these fascinating compounds have tremendous possibilities for development in many different fields of science.
II. PURPOSE AND SCOPE OF THE RESEARCH

This work began as a result of a conference between Dr. J. Stanton Pierce, professor of chemistry at the University of Richmond and Dr. George Z. Williams, Chief of the Department of Clinical Pathology of the National Institute of Health. Dr. Williams was interested in obtaining new tetrazolium compounds for his investigations of tumor cells and cellular mechanisms.

The properties of the tetrazolium compounds described in Section I make them uniquely suited to act as reduction indicators in biological systems. The fact that these compounds change from comparatively colorless to highly colored ones upon reduction is a decided advantage. The fact that solutions of the compounds are able to penetrate cellular membranes so that precipitation of the colored formazans takes place inside of the cells is also an advantage. When the process occurs in an appreciable number of cells it becomes readily visible without optical aids. The high degree of aqueous-insolubility of the formazans, in general, prevents further reduction beyond this stage, so that the dye is fixed in the tissues for long periods, yet the toxicity of both tetrazolium salts and formazans is sufficiently low to permit their use "in vivo" in effective concentrations. The dependence of the extent of tetrazolium reduction upon the dehydrogenase activity, or upon the growth rate of a cell, constitutes a property which makes them most valuable for studying cellular processes.
At the present time, the prevailing opinion is that the use of the tetrazolium reduction in tumor diagnosis is unreliable. However it is still within the realm of possibility that an effective compound of this type may be found. In spite of the relatively large number of tetrazolium compounds which have been synthesized very few of these have subjected to biological study. The compound which has been most extensively studied is TTC, and some investigators have drawn conclusions regarding the tetrazolium salts as a whole on the basis of work with this particular compound. However, the present work has shown that the properties of these compounds can be modified considerably by the nature of the substituent groups on the tetrazole ring.

This work dealt chiefly with tetrazolium salts having three simple aryl substituents, or in other words, triphenyl-tetrazolium salts bearing small substituent groups on one or more of the aromatic rings. It was found that the nature of these small groups had a marked effect on such properties as solubility, water/chloroform distribution coefficient and sensitivity of the compounds to reduction. In the previous studies very little attention has been paid to these properties, and very little effort has been made to correlate them with the chemical structure.

Perhaps the most important physical property of the tetrazolium salts, from the standpoint of their biological utility, is the reduction potential. Obviously, in order for a particular compound to be reduced by some substrate within
a cell it is necessary that the former should have the greater tendency to accept electrons. It is also apparent from work previously done with tetrazolium compounds that their reduction potentials overlap those of many substances present in living tissues. The investigations of these potentials, however, has been so meager that it is difficult to make any correlation with chemical structure at this time.

The reason for the lack of data on reduction potentials is the great difficulty encountered in determining this property of these particular compounds. The tetrazolium reduction proceeds readily with any one of a number of reducing agents, but the reverse oxidation requires special conditions, even though the system is thermodynamically reversible. Evidently the activation energy necessary for the reaction is comparatively high.

Jerchel (5), co-discoverer of the biological reduction of tetrazolium salts, made an attempt to determine the reduction potentials of some of these compounds by using the ingenious methods of Conant (30) and Fieser (31), according to which solutions of the salts were placed in contact with equimolar quantities of a known redox indicator system. After a few minutes a new equilibrium was established, and the reduction potential of the unknown was calculated by extrapolation of the straight-line plot of indicator potential readings vs change of potential in one-half minute. The values obtained for seven tetrazolium salts varied between -0.079 and -0.229 volt (TTC, -0.083 volt) at pH 6.72. The time factor, however,
made these values arbitrary. Pratt and Dufrenoy (32) found a value of -0.17 to -0.26 volt for TTC at pH 7, likewise measured by potentiometric methods. Using the polarographic technic Duskočil (33) found -0.160 volt for TTC and -0.185 volt for 2,3-diphenyl-5-methyl-tetrazolium chloride at pH 7.

Ried and Wilk (34) made a more extensive study of the polarographic method and determined the half-wave potentials of four mono- and six ditetrazolium salts. The reduction potentials may be calculated from the half-wave potentials for systems which are thermodynamically reversible. This work substantiated the assertions of earlier workers that the tetrazolium reduction proceeds step-wise, since the polarograms of Ried and Wilk show three distinct steps. The half-wave potentials of these steps were designated as alpha, beta and gamma.

Polarograms of the corresponding phenylhydrazone s used in the synthesis of each compound were also made, so these workers were able to show a correlation between the potentials of the aldehyde components and those of the complete compounds, thus indicating that the differences in potential of the salts studied were chiefly due to the aldehyde components.

It is interesting to note in this regard that Wedekind's (35), (36) study of the effect of various substituent groups on the ring-closure of formazans to form tetrazolium salts showed that the R groups (page 1) had a greater effect than the R' and R" groups. The R groups represent the aldehyde components. This will be discussed in more detail in Section V.

Five of the ditetrazolium salts of which Ried and
Wilk determined the half-wave potentials had the type structure IV (page 1) in which $R'$ was phenyl and $R''$ was diphenylene with only the $R$ groups varying. This condition presented an opportunity to compare the effects of these $R$ groups on the potentials. According to the experimental data of Ried and Wilk the relative ease of reduction of these compounds was found to be in the order in which the $R$ substituents were as follows: 4-pyridyl, 3,4-dimethoxyphenyl, 2-thiophenyl, 2-benzothiophenylene, p-methoxyphenyl, 2-pyridyl and phenyl. The authors reported that this order could not be certain on account of the difficulty of determining the first appearance of formazan color on the dropping mercury electrode, which was the point at which the readings were taken. With the mono-tetrazolium salts no comparison could be make, since the substituents were different on each position, although the triphenyl derivative was the most difficult to reduce (i.e. highest negative potential).

The original plan of this work was to prepare a series of triphenyl tetrazolium salts having small substituent groups on one or more of the aromatic rings, varying these groups with respect to electrophilicity or nucleophilicity, in order to obtain compounds which would differ as much as possible in their reduction potentials. In selecting the substituent groups the list given in Fuson's "Advanced Organic Chemistry" (37) was used as a guide. Omitting the first member of this list, the negative oxide ion, which was not available from a practical standpoint, the remaining groups, numbered in order of decreasing nucleophilic power, are as follows:
1. -NH₂
2. -OH
3. -OCH₃
4. -CH₃
5. -H
6. -C₆H₅
7. -COCH₃
8. -NO₂
9. -N(CH₃)₃

Tetrazolium compounds having some of these substituents were already known, and some of the compounds prepared in this study were reported by other investigators before the work was completed, but in view of the enormous number of structural combinations of these groups which are theoretically possible most of the compounds listed in Section VI are still new. As the work progressed it was discovered that certain combinations of these groups produced compounds which were extremely sensitive to reduction. Therefore efforts were made to develop this property as much as possible, rather than to utilize all of the groups just listed, as was planned at first.

Compounds which are highly sensitive to reduction, and which yield colored products, can be very useful for determination of biological substances and for following the course of biochemical reactions. It is a decided advantage that these are well adapted to colorimetric methods since the concentration of such test substances is usually quite low. TTC has been
used for the assay of cortisone and other alpha ketol steroids in pharmaceutical preparations. Some of the new compounds of this study were much more effective than TTC in detecting minute quantities of ascorbic acid.

The reason for preparing compounds with large variations in reduction potential is the possibility that a compound may be found the potential of which lies in a very critical range, so that it would show great differences in reactivity with substrates characterized by appreciable differences in enzymatic activity. The difference in dehydrogenase activity of tumor cells and normal cells is the basis for the original use of TTC to distinguish tumors, but the failure of this compound and the others similarly tested may have been due to the fact that their reduction potentials were not sufficiently critical.

The compounds prepared in the present study were turned over to Dr. Williams for biological testing as fast as they were completed. However, these tests of Dr. Williams were so extensive, involving animal experimentation, ultraviolet television microscopy and spectrophotometry, that it was realized that the results would not be known within the time allotted to the present research problem. Therefore some method was sought for roughly evaluating the desired properties of these compounds in order to determine whether the investigation was headed in the right direction. It was found that reduction by the corn seed embryo was a satisfactory method. Details of this method of testing are described in Section VII.
III. MECHANISMS OF FORMAZAN SYNTHESIS.

The formazans, which are precursors of the tetrazolium salts by all known methods of synthesis, were discovered simultaneous in 1892 by von Pechmann (38) and Bamberger (39). The structures of these compounds corresponding to the types of tetrazolium compounds given on page 1 are as follows:

\[
\begin{align*}
(\text{I}) & \quad \begin{array}{c}
\text{R}-\text{C} \\
\text{N}=\text{N}-\text{R}' \\
\text{H}
\end{array} \\
\text{N}=\text{N}-\text{R}''
\end{align*}
\]

\[
\begin{align*}
(\text{II}) & \quad \begin{array}{c}
\text{R}'-\text{N}-\text{N} \\
\text{C}-\text{R}-\text{C} \\
\text{N}=\text{N}-\text{R}' \\
\text{H}
\end{array} \\
\text{R}''-\text{N}=\text{N} \\
\text{N}=\text{N}-\text{R}''
\end{align*}
\]

\[
\begin{align*}
(\text{III}) & \quad \begin{array}{c}
\text{R}-\text{C} \\
\text{N}=\text{N}-\text{R}' \\
\text{H}
\end{array} \\
\text{N}=\text{N}-\text{R}'' \\
\text{R}''-\text{N}=\text{N} \\
\text{C}-\text{R}
\end{align*}
\]

\[
\begin{align*}
(\text{IV}) & \quad \begin{array}{c}
\text{R}-\text{C} \\
\text{N}=\text{N}-\text{R}' \\
\text{R}'-\text{N}=\text{N} \\
\text{C}-\text{R}
\end{array} \\
\text{N}=\text{N}-\text{R}'' \\
\text{N}=\text{N}
\end{align*}
\]

von Pechmann gave the name "formazyl group" to the structure (a) which follows. By this nomenclature structure (b) would be formazylbenzene, and we find it so designated in the older German literature, but modern German and American literature term these compounds as formazans. Thus (b) would be named N,N',C-triphenylformazan or simply triphenylformazan. By the I. U. C. system of nomenclature it would be termed $\alpha$-phenylazo-$\alpha$-phenylhydrazonotoluene. However the more common designation of formazan will be used throughout this thesis.
A dozen or more methods for the preparation of formazans have been reported in the literature, but only those methods will be listed here which are generally applicable.

A. The reaction of arylhydrazones with diazonium salts.

\[
\text{R-C} = \text{N-N-R'} + \left[ \text{N=N-R}'' \right]^+ \xrightarrow{\text{OH}^-} \text{R-C} = \text{N-N-R'} \quad \text{(III-1)}
\]

This reaction is the one which was used for most of the compounds of this study. It is the reaction which has been most thoroughly studied, and generally it is the most practical, if the required aldehyde is available, or can be readily prepared.

Usually the aryl hydrazone is prepared from an aldehyde and an arylhydrazone. von Pechmann pointed out that formazans could be prepared directly from aldehydes, but in such case hydrazones are the intermediate products, and two equivalents of the aldehyde are required. Such formazans are symmetrical in that the N and N' substituents are identical.
Busch and his associates (40), (41), (42), (43), (44) made a rather extensive study of Reaction A, above, and they found other products in addition to the formazans, depending on the experimental conditions. Busch postulated that the diazonium cation must initially couple to the beta-nitrogen atom and then rearrange to the methine carbon if the final product is to be a formazan. In other words, a diazo hydrazone, or tetrazene, is first formed, but this is unstable and rearranges to the formazan.

\[
\begin{align*}
\text{R-C-H} + [\text{N=N-R}^\equiv]^+ \times^- & \rightarrow \text{H} \text{H} \\
\text{R-C=N-N-R}' & \\
\text{[N=N-R}^\equiv]^+ \times^- \\
\text{H} \\
\text{N-N-R}' & \\
\text{R-C} \\
\text{N=N-R}'' & \\
\text{R-C} \\
\text{N=N-R}'' & \\
\text{(III-2)}
\end{align*}
\]

\[
\begin{align*}
\text{H} \text{H} \\
\text{R-C=N-N-Ar} + [\text{N=N-Ar}^\equiv]^+ \times^- & \rightarrow \text{H} \text{H} \\
\text{R-C} \text{N-N-Ar} & \\
\text{N-N-Ar}' & \\
\text{R-C} \\
\text{N-N-Ar} & \\
\text{R-C} \\
\text{N=N-Ar}' & \\
\text{(III-3)}
\end{align*}
\]
In support of this mechanism Busch demonstrated that aldehyde hydrazones in which the beta-nitrogen was tertiary were not able to produce formazans.

Busch also showed that aldehyde alkylhydrazones formed stable tetrazenes, so that rearrangement to the formazan did not occur as with arylhydrazones. His explanation was that:

"---the diazo fastens so firmly to the nitrogen atom that rearrangement to the carbonyl carbon, (i. e. formazan formation) does not take place". On the basis of modern electronic theory this is equivalent to saying that the presence of the nucleophilic alkyl group makes the nitrogen atom more negative, and consequently the positive diazo group is held too firmly for rearrangement to occur. Following this line of reasoning, tetrazenes formed from aryl hydrazones would be less stable, since the phenyl group is slightly electrophilic, and this would tend to weaken the bond between the beta-nitrogen and the diazo group.

Scott and his associates (45) gave a somewhat different concept of this mechanism. They postulated three possible modes of attack by the diazonium cation on the hydrazone molecule, namely: (1) reaction with the beta-nitrogen atom resulting in tetrazene formation, (ii) coupling on either of the aromatic rings, (iii) reaction directly with the methine carbon to produce a formazan. The last possibility is in direct contrast to Busch's theory. Scott also stated that an aryl group is activated by bonding with the beta-nitrogen in the hydrazone; so for this reason he prepared a class of formazans in which
the R' groups were both aliphatic and electrophilic.

It should be mentioned here that in the experimental work of this study the best yields of formazans were obtained in reactions in which the R' group was p-nitrophenyl. (See table of formazans, Section VI). The nitro group is strongly electrophilic, and in this position it inactivates the ring by electron impoverishment. So in this respect the results agree with Busch's theory. However, Scott stated that increasing the electrophilicity of the R' group diminished the possibility of tetrazene formation. If the electrophilicity of this group is increased, then the electron density of the nitrogen bound to it should be decreased, yet according to Busch the electrophilic diazo group must first attack this position in order to produce a formazan. This means that a center of high electron density would have to exist at this point. These divergent views are difficult to reconcile.

According to Hauser and Breslow (46) the diazonium cation may be represented by two resonant forms:

\[
\begin{align*}
\text{(a)} & \quad \text{N} \equiv \text{N}^- \text{Ar} \\
\text{(b)} & \quad \text{N=\text{N}^- \text{Ar}}
\end{align*}
\]

(III-4)

It may be assumed that as the diazonium ion approaches the electron donating molecule the cation assumes the form (b) in which the active nitrogen has only a sextet of electrons. The presence of an electrophilic group, like the nitro, in the
ortho or para position of the aromatic ring should cause resonant form (b) to contribute more to the structure of the ion and enhance its ability to couple, since the electron impoverishment of the ring accentuates the electron deficit of the active nitrogen.

Nineham (47) agreed with Scott that a p-nitro group in the phenylhydrazine moiety tends to reduce formazan formation. However, as was mentioned previously this is at variance with the results of the present study. Four of the formazans in the table of these compounds listed in Section VI were prepared by alternative reactions. In one case the nitro group was in the para position of the phenylhydrazine moiety. In the other case the nitro group was in the same position of the diazonium cation. The R groups were identical for each pair of reactions.

\[
\begin{align*}
R-C=N-N-\text{PhNO}_2^+ + [N=N-\text{Ph}]^+Cl^- & \rightarrow R-C=N-N-\text{PhNO}_2 \\
R-C=N-N-\text{Ph}^+ + [N=N-\text{PhNO}_2]^+Cl^- & \rightarrow R-C=N-N-\text{PhNO}_2
\end{align*}
\]
Also for each pair of reactions, identical products were obtained, but in each case the yield of formazan was substantially higher for reaction III-5 than for reaction III-6.

The reason for this anomalous behavior is not known, although it is possible that the more reactive ion might have a greater tendency to couple on either of the aromatic rings of the hydrazone than the unsubstituted ion.

The identicalism of the products of such pairs of reactions has been noted by Hunter and Roberts (48) as well as by Hausser and associates (28). The latter advanced the theory that imino hydrogen does not belong to either of the nitrogen atoms, but by forming a chelate bridge belongs to both of them at the same time. On this basis the two formazans in the above equations can be considered as mesomers and the position of the double bond would thus be immaterial. According to Hausser one could alternatively assume the existence of isomers in which the activation energy required for transition from one form to the other would be extremely small.

Hausser also postulated, in addition to the mesometric forms above, four theoretically possible forms of cis-trans isomers on the basis of the C-N and N-N double bonds:

\[
\begin{align*}
\text{cis-cis} & \quad \text{cis-trans} & \quad \text{trans-cis} & \quad \text{trans-trans} \\
\begin{array}{c}
\begin{array}{c}
\text{N} \\
\hline
\text{N}
\end{array}
\end{array}
\begin{array}{c}
\begin{array}{c}
\text{N} \\
\hline
\text{N}
\end{array}
\end{array}
\begin{array}{c}
\begin{array}{c}
\text{N} \\
\hline
\text{N}
\end{array}
\end{array}
\begin{array}{c}
\begin{array}{c}
\text{N} \\
\hline
\text{N}
\end{array}
\end{array}
\end{align*}
\]
Only two of these isomers are known at the present time, one red and one yellow. Since it has been observed ever since the early work on the formazans that these normally red substances undergo transformation to yellow compounds in certain solvents under the influence of light, these same investigators studied this phenomenon with the aid of absorption spectra measurements and showed that it was due to cis-trans isomerism. They postulated that the existence of these isomers does not preclude the aforementioned mesomers if one assumes a non-planar structure for the molecule, otherwise the hydrogen bridge would open or close with each cis-trans rearrangement. The yellow forms rearrange back to the red forms in the dark.

One should not get the impression that identical products always result from types of formazans similar to those shown in reactions (III-5) and (III-6). Exceptions were observed by Busch and Schmidt (44) and also by Hauser (28). In order to show this clearly the competitive reactions involved in attack of the diazonium cation on the hydrazone molecule may be represented as follows:
According to Busch and Schmidt the reaction must be conducted in alkaline medium to produce a formazan (b) whereas if the reaction mixture is acidic or neutral and the aromatic ring attached to the beta nitrogen atom is unsubstituted, then coupling tends to occur on this ring, preferably in the 4-position to produce a 4-phenylazophenylhydrazone. This agrees with Scott's theory that the beta-nitrogen activates the aromatic ring to which it is attached. The mechanism for the reaction as given by Busch and Schmidt is as follows:
These investigators reported that in the reaction between benzaldehyde phenylhydrazone and p-nitrobenzenediazonium chloride in benzene-absolute alcohol solution they also obtained the above product plus some benzaldehyde p-nitrophenylhydrazone.

Portions of the reactants underwent double decomposition and the resulting benzenediazonium chloride coupled with some of the unchanged benzaldehyde phenylhydrazone to produce the 4-phenyl-azo compound. It should be noted that the conditions were acidic in this case.

The alternative reaction which yields product (d), (III-7) causes considerable trouble if the aldehydic aromatic
ring has a para-substituted nucleophilic group (R). Such a group donates electrons to the ring and these tend to concentrate at the ortho positions, thus sensitizing these positions to attack by electrophilic moieties.

An unsubstituted aromatic ring, on the other hand, is inactivated by the methine unsaturation, according to Scott (45), so that alternative coupling does not tend to occur.

B. The reaction of diazonium salts with compounds containing an active methylene group.

This reaction is probably the second most useful one for preparing formazans. Its chief advantage is that in certain cases the group to be introduced on the formazyl carbon atom may be more accessible by way of a compound containing an active methylene group than by way of an aldehyde.

Although credit for development of the reaction to a general procedure for preparing formazans probably belongs to Ried and Hoffschmidt (49), credit for the basic principles can be shared by a number of investigators. Both von Pechmann (4) and Bamberger (39) had demonstrated that diazonium ions could replace hydrogen as well as other atoms or groups from an active methylene groups, in alkaline solution, to produce formazans. For example, Bamberger produced N,N'-diphenylformazan-C-carboxylic ester from aceto-acetic ester and benzene-diazonium chloride. The phenylhydrazone was formed as an intermediate product.
When acetoacetic acid was used instead of the ester the acetyl group was replaced to yield \( N,N' \)-diphenyl-C-phenylazoformazan.

Busch and Wolbring (50) used the analogous reaction of diazonium salts with malonic acid. However, they reported that sometimes the reaction did not go as expected, since in addition to the desired formazans phenylhydrazones and azo-oximes were produced also.

Another development of this reaction was contributed by B. Prager (51) who obtained a formazan from the reaction of oxalo-crotonic acid ethyl ester and benzenediazonium chloride in ammoniacal solution accompanied by oxamide cleavage. This reaction likewise proceeded by way of the intermediate phenylhydrazone.
In the same way Borsche and Manteuffel (52) prepared the next higher vinylog of this formazan from oxalosorbic acid ester. Note that the methylene group of malonic acid is activated by virtue of its position between the two carbonyls, whereas in ethyl oxalocrotonate the methylene group is separated from a carbonyl by a vinyl group, and in ethyl oxalosorbate it is separated from a carbonyl by two vinyl groups. Thus activity is transmitted along the carbon chain by the principle of vinylogy.

In 1946 Ragno and Bruno (53) reported the synthesis of formazans from phenylhydrazones of pyruvic acid.
The contribution of Ried and Hoffschmidt (49) was to demonstrate that formazans could be prepared generally from mono-substituted pyruvic esters. Since the latter can be prepared readily from practically any active methyl compound by condensation with ethyl oxalate, according to the procedure of Wisclicenus (54), this method has wide application. The synthesis of \( N,N'\text{di-}(p\text{-bromophenyl})-C-(2\text{-quinolyl})\text{formazan} \), as given by Ried and Hoffschmidt, is typical of the reaction:

\[
\begin{align*}
\text{CH}_2-C-C-0-C_2H_5 + \left[ \text{N=N-} \right] & \xrightarrow{2 \text{NH}_3} \text{C}_2H_5-OH \\
\text{H} & \xrightarrow{\text{H}_2N-C-C-NH_2} \\
\end{align*}
\]

The reaction was carried out in ammoniacal solution, and the authors stated that if sodium hydroxide was substituted for
ammonia then the reaction would stop at the intermediate aryl hydrazone. Only symmetrical formazans, in which \( R' = R'' \), were reported, but it appears quite possible that if it were desired one could use sodium hydroxide to obtain a hydrazone, isolate it, and then react it with a different diazonium salt. This would allow a wider choice of substituents for \( R' \) and \( R'' \). Investigation of this interesting possibility, however, was beyond the scope of the present research.

Ried and Hoffschmidt also reported a variation of this reaction by the conversion of heterocyclic acetonitriles to formazans:

\[
\begin{align*}
\text{[N=N-} \text{C}=\text{N-Br}^+ & \quad \text{NH}_3 \\
\text{H}_2\text{N-C}=\text{N-H} + \text{[N=N-} \text{C}=\text{N-Br}^+ & \quad \text{NH}_3 \\
\end{align*}
\]

(III-14)

The methylene group of the nitrile is activated by virtue of its bonding between the \(-N=CH-\) group of the heterocycle and the cyano group. The potential applications of reaction III-13 appear to be more favorable than those of reaction III-14. Note that in the former case the by-product formed is oxamide and in the latter case it is urea.
C. The reaction of arylhydrazines with various compounds.

Some of the compounds which have been reacted with phenylhydrazine to produce formazans are ethyl formate (38), ethyl nitrate (55), imino ethers (56), dichloroacetonilide (57) and benzotrichloride (58). However, these are special cases of this reaction and they are not generally applicable. The reactions of phenylhydrazines with acid hydrazides and with halogen hydrazides reported by von Pechmann (4), on the other hand, potentially have wide application, even though they have been little used.

\[
\begin{align*}
\text{R-C} & \quad + \quad \text{H}_2\text{N-N-C}_6\text{H}_5 \\
\text{N-N-C}_6\text{H}_5 & \quad \xrightarrow{\text{H}_2\text{O}} \\
\text{R-C} & \quad \text{H} \\
\text{N-N-C}_6\text{H}_5 & \quad \text{H} \\
\text{N-N-C}_6\text{H}_5 & \quad \text{H} \\
\end{align*}
\]

(III-15)
This method was not used in the present research, since the desired formazans were more conveniently accessible by way of methods A or B. One disadvantage of the method is that many acids do not form the required hydrazides. Stempel and Schaffel, (59) who prepared a number of hydrazides, reported that they were best prepared from the lower fatty acids. Weaker acids with phenylhydrazine, in general, yielded phenylhydrazides while stronger acids, such as chloroacetic, formed salts. Crotonic, salicylic, and halogen-substituted benzoic acids failed to react, although the unsubstituted benzoic acid was satisfactory. Halogen-substituted aliphatic acids merely split off halogen halide.

D. The reaction of aldehyde guanylhydrazones with diazonium salts.

This reaction was first studied by Wedekind (35), (36), but more completely by Scott and his associates (45)
who prepared a number of bisguanazyls. (See reaction III-18.)

\[
\begin{align*}
0 & \quad 0 \\
R-\text{CH} + H_2N-N-C-NH_2 & \rightarrow R-C=N-N-C-NH_2 \\
\left[ N=N-C_6H_5 \right]^+ & \\
N-N-C-NH_2 \\
R-C & \\
N=N-C_6H_5
\end{align*}
\]

(III-17)

\[
\begin{align*}
0 & \quad 0 \\
2R-\text{CH} + H_2N-N-C-N-NH_2 & \rightarrow (R-C=N-N)_2-C=NH \\
2 \left[ N=N-C_6H_5 \right]^+ & \\
\left( R-C \right) & \\
N=N-C_6H_5/2
\end{align*}
\]

(III-18)

In the first case, 1-aminoguanidine was reacted with an aldehyde to produce the corresponding guanylhydrazone, and this in turn was coupled with a diazonium salt to yield the formazan. In reaction III-18, diaminoguanidine and an aldehyde were used as the starting materials.
As mentioned before (page 22), Scott postulated direct attack of the diazonium cation on the methine carbon of the hydrazone for the mechanism involved in this reaction, which is in direct contrast to the concept of Busch. Despite the success of the reaction, however, Scott warned that aldehydic acylhydrazones do not in general yield formazans. The method would therefore be limited in application, and it is only mentioned here on account of Scott's views on the mechanism, which also applies to method A.

E. The reaction of aldehyde N,N'-disubstituted semicarbazones with diazonium salts.

\[
\begin{align*}
\text{R-C=N-N-C-H} & + [\text{N=N-R''}^+] \\
\rightarrow & \text{R-C-N-N-C-N} \\
\end{align*}
\]

(III-19)

This reaction, which was reported by Ried and Hillenbrand (60), has no practical value as far as the present research is concerned, but it is of interest here, because it is the only class of formazans so far reported which have not been successfully converted to tetrazolium salts.

The reaction is also interesting, because it introduces a non-aryl group on the imino nitrogen atom of the formazan. In this respect it resembles reaction III-17.
IV. OXIDATIVE RING-CLOSURE OF FORMAZANS

The oxidation, or more strictly speaking, the dehydrogenation, of a formazan produces the corresponding tetrazolium salt.

\[
\begin{align*}
\text{R-C} & \quad \frac{\text{H}}{\text{N-N-R'}} \quad \overset{-2\text{H}}{\text{HX}} \quad \text{N-N-R'} \\
\text{R-C} & \quad \frac{\text{N=N-R''}}{\text{X}^-} \quad \text{N-N-R'}
\end{align*}
\]

(IV-1)

It is evident that this reaction is the reverse of the one shown on page 2.

H. von Pechmann originally prepared tetrazolium bases by oxidation of formazans with yellow mercuric oxide in alcoholic solution. Treatment of the base with isoamyl nitrite and hydrogen chloride gas then produced the chloride salt. Kuhn and Jerohel (5) recommended the use of lead tetraacetate as the oxidizing agent. Fichter and Schiess (61) used nitric acid, and Benson and his associates (62) used t-butylhypochlorite. Ashley (63) reported that aqueous sodium hypochlorite converted triphenylformazan smoothly into triphenyltetrazolium chlorate, some inorganic hypochlorite decomposing at the temperature of the reaction to give the chlorate ion. This salt was then converted to the chloride by reduction with ferrous iron, the tetrazolium nucleus being unaffected.
Kuhn and Münzing (64) found that N-bromo-succinimide, N-chlorosuccinimide, N-bromophthalimide, N-chlorophthalimide, and N-bromoacetamide were good dehydrogenating agents for formazans and gave the following mechanism:

\[
\text{R-C} \quad \text{N-N-R}^n \quad + \quad \text{R-C} \quad \text{N=N-R}^n
\]

Dr. Walter Ried* of the University of Frankfurt very kindly sent details of a method which he originated for the oxidation of formazans. This procedure consisted in treating the formazan suspended in alcohol with hydrogen peroxide and hydrochloric acid in the presence of vanadium pentoxide catalyst. The method was very useful in the present work, because the reaction was easily controlled and there was less tendency to tar formation than with the lead tetraacetate method. The latter procedure, however, was preferred with formazans which were more difficult to oxidize, as for example the tris-

* Private communication.
(p-nitrophenyl) derivative.

According to Wedekind and Stauve (36), certain aromatic substituent groups at R' and R'' tend to retard the oxidative ring closure. They reported that with N-(m-carboxyphenyl) -N'-o,(m,p)-nitrophenyl-C-phenyl formazan the amounts of unoxidized material recovered when the nitro group was in the ortho, meta, and para positions were 43%, 18% and 40% respectively. The oxidant used was gaseous nitrous acid in alcoholic HCl.

With the compounds of the present series a single p-nitro group at R' caused no adverse effect on the ring-closure, as has been explained before.

Wedekind and Stauve showed that X in the system,

\[
\begin{array}{c}
N-N-\text{H} \\
\text{X}-\text{C} \\
N=N-\text{N} \\
\end{array}
\]

had a greater effect on the ring-closure than Y and Z in the following system:

\[
\begin{array}{c}
N-N-\text{H} \\
\text{Y}-\text{C} \quad \text{=} \\
N=N-\text{N} \\
\end{array}
\]

This is not as anomalous as it may seem at first. In the usual way of writing the formazan structure, the distance from
the carbon substituent to either nitrogen substituent appears to be greater than the distance between the two nitrogen substituents. However, in free rotation around the carbon atom, the converse is true part of the time, as follows:

![Chemical Structure]

The conclusions of Wedekind and Stauve are rather remarkable, because they were based upon experimental evidence alone and did not have the advantage of modern electronic concepts. These investigators measured the effect of eight formazyl-carbon substituents on the ring-closure from the yields of tetrazolium compounds with all other conditions remaining constant. They found that the tendency to ring-closure decreased for the substituents in the following order CN, C₆H₅, COOC₂H₅, C₂H₅CO, C₆H₅N=N, CH₃CO, CH₃, COOH, H. Since the only aromatic ring which was bound directly to the formazyl carbon has no other substituent group there was no possibility of comparison with compounds of the present series in this respect.

Both the lead tetraacetate method and the hydrogen peroxide method of oxidation suffer from the disadvantage that a water-soluble metallic salt must be separated from the water-soluble product. In the first case the salt is lead chloride and in the latter case vanadium chloride. For products which have a favorable chloroform/water distribution coefficient a
clean separation can be made by extraction of the aqueous solution with chloroform. However, this condition did not apply to very many of the products of this research, but in such cases it was found that extraction could be made with n-butanol. The separation was not as clear cut as it was with chloroform due to the appreciable solubility of n-butanol in water, but by repeated extractions a fairly good separation could be achieved.

The method of Kuhn and Münzing, using N-chlorosuccinimide, was found to be useful for the more complex tetrazolium salts. This is probably the method of choice for formazans having groups which are sensitive to hydrolysis, since no heating is required, and the product precipitates directly from the reaction solution. The authors used ethyl acetate as solvent. However any other solvent will suffice in which both the formazan and the reagent are soluble and which the tetrazolium salt is insoluble. These conditions limit its usefulness, and it was found that the procedure was not satisfactory if the product did not crystallize directly from the mixture. When the solution had to be worked up to obtain the product the yields were very poor. For most of the compounds of this study there was not a sufficient solubility differential between them and the reagent.

The oxidation of formazans to tetrazolium compounds by nitrous acid is the oldest procedure, since it was first used by von Pechmann (4). In his work the nitrous acid was used directly, and it was also produced in the reaction mixture
from isoamyl nitrite and hydrochloric acid.

Ried, Gick and Oertel (65) reported good results with nitrous acid, only they used isoamyl nitrite and glacial acetic acid as a source of the nitrous acid. This procedure of course produced tetrazolium acetates.
V. DISCUSSION OF EXPERIMENTAL WORK

As mentioned before, Reaction A, page 20, was used in preparing most of the compounds of this study. Nearly all of the aldehydes required for preparing the phenylhydrazones are known, and in most cases are commercially available. The only arylhydrazines which were used successfully were the unsubstituted phenylhydrazine and p-nitrophenylhydrazine. Attempts to use 2,4-dinitrophenylhydrazine failed at the formazan stage.

Two aldehydes prepared in this work were 4-hydroxy-3,5-dimethylbenzaldehyde, reported by Gattermann (66) and p-dimethylaminobenzaldehyde methobromide. The latter was prepared by heating p-dimethylaminobenzaldehyde with methyl bromide in a sealed tube.

Since the investigations of Busch, previously mentioned, indicated that the presence of a strong nucleophilic group activated a benzene ring to alternative azo-coupling, some protective device was sought which would make introduction of such a group possible.

The first group in the list given on page 17 in order of nucleophilic power is the primary amino group. Since the nucleophilicity of this group is considerably reduced by acetylation a formazan was prepared from p-acetamidobenzaldehyde p-nitrophenylhydrazone and benzenediazonium chloride. The corresponding tetrazolium chloride was also prepared, but a subsequent attempt to hydrolyze the acetamido group to the free amine resulted in decomposition of the product. Recently
Ashley and his associates (63) reported the synthesis of 2,3-diphenyl-5-(p-aminophenyl) tetrazolium chloride. In the present work a reattempt to prepare the corresponding 2-(p-nitrophenyl) compound, mentioned above, was successful when Ashley's conditions were used. The yield, however, was low.

In this research an indirect method was used to introduce the hydroxyl group, which is also a very strong electron donor. The only positions on the benzene ring of the aldehyde moiety of an aryl hydrazone which would theoretically be subject to attack by a diazonium cation are the positions ortho and para to the nucleophile. (See compound (d) of reaction III-7.) It was reasoned that if these sensitive positions were blocked, then the alternative coupling could be avoided. Therefore, 4-hydroxy-3,5-dimethylbenzaldehyde was selected as the starting material.

Although this aldehyde is known it is not commercially available, and considerable difficulty was encountered in preparing it. Three procedures were used, namely those of Gattermann (67), Duff (68), and Reimer-Tiemann (69). Only the last two procedures were successful, and in both of these the yields were not particularly good. These reactions are represented schematically as follows, starting with 2,6-dimethylphenol:
In regard to activation of the aldehyde ring by the hydroxyl group Ried (65) was able to prepare 2-(pyridyl-2)-3-(p-chlorophenyl)-5-(o-hydroxyphenyl)tetrazolium acetate in 28% yield, based on the hydrazone. In the present study, 2,3-di-(p-nitrophenyl)-5-(o-hydroxyphenyl)tetrazolium acetate was obtained in yield of 15%. It is difficult to understand why the hydroxy group does not activate the aldehyde ring to alternative
azo-coupling just as much in the ortho position as in the para. However, Hausser, Jerchel and Kuhn (70) and Ashley (63) also reported failures in the latter case.

Early in the present research an attempt was made to prepare the following tetrazolium compound:

\[
\text{N}_3\text{N}^+\text{C}_6\text{H}_4\text{N}^+(\text{CH}_3)_3\text{Br}^-
\]

It was felt that the presence of such strong electrophilic groups would impart interesting properties to the compound.

The amine required for the diazonium salt was prepared according to the following steps, starting with N,N-dimethylphenylenediamine:

\[
\text{N}^+(\text{CH}_3)_2 \xrightarrow{\text{Ag}_2\text{O}} \text{N}^-(\text{CH}_3)_2 \xrightarrow{\text{CH}_3\text{Br}} \text{N}^-(\text{CH}_3)_2 \xrightarrow{\text{H}_2\text{O}, \text{HBr}} \text{N}^-(\text{CH}_3)_2\text{Br}^-
\]

\[(V-4)\]
Diazotization of this amine and reaction with the p-dimethyl-aminobenzaldehyde-p-nitropherylhydrazone methobromide, in pyridine solution, however, failed to yield a formazan.

Recently, Nineham (47) likewise reported failure of this same hydrazone to react with benzenediazonium chloride, but he gave no details of his procedure. However, he postulated that the failure was due to the electrophilic nitro group in the para position of the phenylhydrazone moiety, and he stated that he had successfully carried out analogous reactions when the phenylhydrazone was unsubstituted.

It is the opinion of the writer that the failure of this reaction in the present work was due to the difficulty of isolating the formazan, since the dimethylammonium ion increases the solubility. Furthermore, as was previously mentioned, the presence of a nitro group in the para position of the phenylhydrazone moiety in compounds of the present series actually produced better yields than in those cases where the nitro group was in the para position of the diazonium cation.

According to the list on page 17, the methoxy group stands next to the hydroxy group in order of nucleophilic power, yet the former apparently has very little tendency to promote alternative azo-coupling. Therefore, in the present study this group was found to be the most useful nucleophile. Also the nitro group was found to be the most useful electrophile.

In most instances para-substituted groups were used on account of their availability as starting materials. None
of the other groups of the list on page 17 were used in this work since their nucleophilic or electrophilic power is relatively weak. The methylenedioxy group which was used in this work is not given on Fuson's list. It was used mainly because of the availability of piperonal as a starting material. No trouble was encountered in the use of this group, although the compounds in which it was used were somewhat more difficult to purify than the corresponding methoxy compounds.

As was previously stated, Reaction A (page 20) was the one used to prepare most of the formazans in this research. The initial procedure was to use methanolic potassium hydroxide buffered with sodium acetate as the reaction medium. This procedure was used by the earlier investigators, although some used other alcohols and alkalies. The mixture was cooled close to zero, and the aqueous diazonium salt and the arylhydrazone in methanol, or ethanol, were added with stirring.

The method has certain disadvantages. In the first place the arylhydrazones have only moderate solubility in the lower alcohols, and the solubility decreases markedly as the molecule becomes more complex. Fox and Atkinson (71) overcame this difficulty to some extent by using a mixture of alcohol and dioxane. The chief disadvantage of the method, however, is that alcoholic alkali tends to have a reducing action upon formazans. When pyridine was substituted as a solvent, as was proposed by Kuhn and Jerchel (5), the yields immediately improved.

In this work all of the diazonium salts were prepared in aqueous solution. However, other workers (63) have used
glacial acetic acid with hydrochloric acid when the diazonium salts had a low aqueous solubility. In such cases sufficient pyridine must be used to render the mixture alkaline.

Reaction B (page 29) was used to introduce the phenanthridyl and the cholesteryl groups on the carbon atom of the tetrazole ring. This substitution was not possible by way of an aldehyde, according to any known procedure. The phenanthridyl group was chosen first, because it was a fairly large group, and its introduction was possible by way of 9-methylphenanthridine and the corresponding substituted pyruvic ester.
9-Methylphenanthridine was prepared by the method of Morzan and Walls (72) and ethyl 9-phenanthridylpyruvate by the method of Wislicenus (54) as modified by Borsche and Manteuffel (52). One critical feature of this synthesis occurs in the preparation of the puruvic ester. Considerable care must be
exercised to exclude moisture. Good dehydrated ethanol can be made from magnesium methoxide as described in "Organic Syntheses" (73). The procedure causes some methanol to be present in the ethanol, but this does no harm.

As mentioned in Section III, this method of synthesizing formazans was reported by Ried very recently, and no other work has appeared yet in regard to it, but it is interesting to note that all of the eight formazans which Ried prepared by this method had p-bromophenyl or p-chlorophenyl as N and N' substituents. In the present work a small amount of the above pyruvic ester was reacted with diazotized aniline under the same conditions, and a mixture of products were obtained which were not identified. Since Ried demonstrated that a phenylhydrazone is the intermediate product of such a reaction, the same conditions for coupling of the cation would prevail as described for reaction (III-13), so that the presence of inactivating groups on the aromatic rings should facilitate a more clear cut reaction.

Due to the importance of cholesterol in biochemical processes, Dr. Williams was interested in testing a tetrazolium compound which contained a cholesterol group. Since 6-methylcholesterol has been reported by Ushakov and Madaeva (74) and also by Goguadze (75) it appeared possible to substitute the cholesterol-6 group on the tetrazole carbon atom by means of Ried's method, provided the presence of the 5,6-double bond in cholesterol was sufficient to activate the adjacent methyl group.
The preparation of 6-methylcholesterol was first attempted by Goguadze's method. This procedure gave the following reaction sequence, with only that fragment of the cholesterol molecule shown which is concerned in the reaction:

![Chemical structure](image)

The 6-bromo derivative was prepared without difficulty, but the Grignard reaction then failed, and it is rather surprising that Goguadze supplied no experimental data for his work, particularly in view of the fact that there is considerable evidence to show that vinyl bromides are inactive toward Grignard reagents.

The reaction of Ushakov and Nadaeva, as modified by Fieser and Rigaudy (76), was then tried. In this procedure 6-methylcholesteryl acetate was attained by way of cholesterol-alpha oxide, which in turn was prepared by the method of Chrakavorty and Levin (77). The following scheme shows the complete synthesis of the tetrazolium compound stating with phthalic anhydride. Again only the reactive portion of the cholesterol molecule is shown in the intermediate steps.
\[
\begin{align*}
\text{O} & \quad \text{H}_2\text{O}_2 \quad \longrightarrow \quad \text{C} - \text{OH} \\
\text{cholesterol} \\
\text{HO-CH}_2\text{CH}_2\text{OH} & \quad \text{CH}_3\text{MgI} \\
\text{Ac}_2\text{O}, \text{H}_2\text{SO}_4 & \\
\text{AcO-CH}_2\text{CH}_2\text{CH}_2\text{CH}_3 & \quad \text{(EtCOO)}_2, \text{EtOK} \\
\text{HO-CH}_2\text{CH}_2\text{OH} & \quad \text{N=N-Br}^+\text{Cl}^- \\
\text{H}_2\text{HCl} & \quad \text{NH}_3 \\
\text{HOH}, \text{HCl} & \\
\text{CH}_3\text{CH}-\text{CH}_2\text{CH}_2\text{CH}_3 & \quad \text{Cl}^- \\
\text{HO-CH}_2\text{CH}_2\text{CH}_2\text{CH}_2\text{CH}_3 & \\
\text{N=N-Br}^+\text{Cl}^- & \quad \text{Cl}^- \\
\text{HO-CH}_2\text{CH}_2\text{CH}_2\text{CH}_2\text{CH}_3 & \\
\text{N=N-Br}^+\text{Cl}^- & \\
\text{CH}_3 & \\
\text{CH}_3 & \\
\text{Cl}^- & \quad \text{Cl}^- \\
\text{(V-7)}
\end{align*}
\]
The reaction of cholesterol with monoperphthalic acid also produces cholesterol-beta-oxide in addition to the alpha isomer. However, the former is more soluble in methanol, and so the two can be separated by fractional crystallization.

Ushakov and Madaeva reported that refluxing cholesterol-alpha-oxide with methyl magnesium iodide in benzene solution for 5 hours produced 6-methyl-3,5-cholestanediol, but that continuation of the reaction for 7 hours produced 6-methylcholesterol. However, Fieser and Rigaudy reported that when they tried both reaction periods the diol was the only product obtained.

In the present research the reaction time was prolonged to 10 hours producing a 48% yield of the diol, but no 6-methylcholesterol could be identified. The diol was therefore converted to the 6-methylcholesteryl acetate by dehydration with acetic anhydride and sulfuric acid according to the procedure of Fieser and Rigaudy. These investigators had next hydrolyzed the acetyl group. However, in the present research the hydrolysis was postponed until the final step in order to protect the hydroxyl group from oxidation.

The pyruvic ester was prepared similarly to 9-phenanthridyl pyruvate in about 50% yield, but when an attempt was made to convert this to the formazan by the method of Ried and Hoffschmidt (49) the desired product was not obtained.

Since a number of sterols are available commercially a small amount of 5-pregnene-3-ol-20-one was purchased.
This compound has a nuclear structure similar to cholesterol, and it was believed that the hydrogen atoms of carbon 21 should be activated by the adjacent carbonyl group and should react with ethyl oxalate in a manner analogous to that just described.

The reaction proceeded as predicted, and analysis of the product for carbon and hydrogen agreed reasonably well with the calculated values.

Unfortunately, however, the formazan reaction failed like that of the cholesterol derivative. There was not sufficient time to repeat these reactions, because in each case it would have meant starting the whole synthesis over. The reason for the failures is not known. It is possible that the desired products were formed but could not be isolated from the reaction mixtures, since the color changes appeared to be similar to those which took place during the other formazan reactions.

It has been mentioned before that the formazan synthesis generally yields a very impure product. This is easily understandable on account of the many side reactions which are
possible. In spite of this, however, the procedures described in the literature do not as a rule say much about purification except to state that the compounds were recrystallized from some solvent. The solvents which have been mentioned are benzene, chloroform, methanol, ethanol, ethyl acetate, acetone, dioxane, pyridine, xylene, petroleum ether and mixtures of these. Sometimes these were mixed with water when miscible.

None of these were found to be entirely satisfactory. Even after recrystallizing several times the product was seldom sufficiently pure for analysis, and the losses in yield were considerable. Also the formazans are not stable to prolonged heating in organic solvents and they tended to form tars. Treatment of crude formazan solutions with decolorizing carbon did not help and sometimes yielded a product which was less pure than before.

The impurities were generally other products of coupling, and some unreacted hydrazone was usually present. Removal of these by fractional crystallization was difficult, because their solubility characteristics are very similar to those of the formazans.

Since the formazans are known to form metallic complexes with copper, cobalt and nickel it was hoped that the compounds of this study could be purified this way. Hunter and Roberts (48), Jerchel and Fischer (14), and Wizinger and Biro (78) investigated these complexes in detail and proposed the following structure for them:
Nickel chelates of some of the formazans of the present series were prepared, but it was found that the formazans could not be regenerated without decomposition. Neither was it possible to prepare the tetrazolium salts directly from the complex.

The above authors used the metallic complexes to identify their formazans but this was found to be impractical in the present work.

In this work, chromatography was tried in attempt to purify the formazans, first by using a packed column of silicic acid and filter pulp in equal parts. Also a filter paper column was used. This was prepared from 200 two-inch filter paper disks clamped tightly together. A benzene solution of the formazan was allowed to drip slowly on the top of the column and soak through. When the column was saturated separation of the components was obtained by adding a mixture of benzene and chloroform. The zones were then separated and eluted with chloroform. This procedure was tedious and not entirely satisfactory.

M. Ragno and S. Bruno (79) showed that formazans in which $R'$ or $R''$ constituted an aromatic nitro group were capable
of forming salts with sodium ethoxide or potassium ethoxide and that these could be easily decomposed to regenerate the formazan. Many of the compounds of this study conformed to this type, but the method was considered too involved to be practical.

Fortunately it was discovered that most of the tetrazolium chlorides could be prepared from formazans which were only washed with hot water and dried. The final product required more purification but the overall yield was better than those obtained from pure formazans. However, it was necessary to obtain pure formazans for analysis. Since all of the compounds of this study were prepared several times alternative methods of purification were tried. In some cases the simplest way to obtain a pure formazan was to make the tetrazolium salt, purify it, and reduce it to the formazan.

The reduction procedure was fairly simple. The tetrazolium salt was dissolved in water and some sodium ascorbate added and the mixture stirred. After about five minutes it was extracted with chloroform, and the extracts washed with water and filtered through a plug of cotton. Evaporation of the solvent yielded almost pure formazan, although it was usually recrystallized once more. The reduction can also be performed with zinc dust, but some of the formazan will undergo further reduction.

The purification of tetrazolium salts presented additional problems. As other investigators (63) have pointed out, the crystalline forms are not characteristic and vary widely with the conditions of crystallization. Many of the
salts formed molecular compounds with the solvent, and pro-
longed vacuum drying was necessary to remove the solvated
molecules.

Most of the purification procedures for tetrazolium
salts reported in the literature recommend that the crude pro-
duct be dissolved in ethanol or methanol followed by precipi-
tation by addition of ether. This method did not produce sat-
isfactory results with the compounds of this study, because
the impurities were generally not ether-soluble and tended to
cooprecipitate. The products were also so soluble in alcohol
that considerable loss resulted. Another disadvantage of
using ether was that it caused darkening and partial lique-
faction of the tetrazolium crystals upon exposure to air.
So it was necessary to remove the ether by vacuum evaporation.

In the present investigation solvents such as
chloroform, acetone, benzene, or 2-butanone were satisfactory
for recrystallization in certain cases. However, chloroform
tended to darken those compounds which were most sensitive to
reduction when the wet crystals were exposed to air.

Recrystallization from hot water was possible with
a few of the compounds which had very low aqueous solubility.
This method of recrystallization caused some conversion to the
corresponding tetrazolium hydroxides which were somewhat more
water-soluble. However, when hydrochloric acid was added to
these solutions the tetrazolium chlorides precipitated.

In many instances a resinous, gummy substance gave
considerable trouble in purification of the tetrazolium salts.
This was apparently an oxidation by-product. It was soluble in organic solvents but insoluble in water and tended to pass through filters. Neither silica gel nor active carbon were satisfactory adsorbing agents for removing it. Besides, the latter usually contained some metallic oxides which contaminated the acid solution. Filtration through a glass column packed with glass wool gave the most satisfactory results.

Since most of the tetrazolium salts of this study were prepared by the hydrogen peroxide method (page 39), which makes use of vanadium pentoxide, the crude products contained some vanadium chloride which imparted a greenish color to the crystals. The procedure for the removal of this substance depended upon the solubility of the particular tetrazolium compound. Since vanadium chloride is water-soluble it was possible to wash it from the more difficult soluble tetrazolium salts. For those salts which had a favorable chloroform-water distribution coefficient, such as the unsubstituted 2,3,5-triphenyltetrazolium chloride, the product could be extracted from the aqueous mixture with chloroform. For compounds of moderate aqueous solubility separation could be effected by repeated extractions with n-butanol followed by vacuum distillation of the solvent.

Most of the tetrazolium compounds prepared in this work were white or cream-colored when freshly prepared, but they almost immediately darkened to various shades of yellow. This occurred even when considerable effort was made to vacuum dry the precipitates and to protect them from light.
Other investigators have called attention to the light-sensitivity of this class of compounds. Hausser, Jerchel and Kuhn (28) showed that irradiation of alcoholic or aqueous solutions of triphenyltetrazolium chloride with ultra-violet light brought about its conversion to 2,3-diphenylene-5-phenyltetrazolium chloride.

\[
\begin{align*}
\text{C} & \quad \begin{array}{c}
\text{N} \quad \text{N} \\
\text{N} \quad \text{N}
\end{array} \\
& \quad \begin{array}{c}
\text{Cl} \\
\text{Cl}
\end{array} \\
\quad \Downarrow \\
\text{C} & \quad \begin{array}{c}
\text{N} \quad \text{N} \\
\text{N} \quad \text{N}
\end{array} \\
& \quad \begin{array}{c}
\text{Cl} \\
\text{Cl}
\end{array}
\end{align*}
\]

This conversion to the diphenylene derivative, which has been investigated in detail only in the case of TTC, does not completely explain the coloration of the dry crystals of tetrazolium salts. In the present study it was noted that when the salts were well purified and dried there was no appreciable lowering of the melting point after appearance of the yellow color. The color usually appeared within an hour or two after filtration of the crystals from the mother-liquor, but it was apparently not progressive. It was more pronounced when the damp crystals were allowed to dry spontaneously in air.

Analysis of the tetrazolium salts also presented difficulties. Compounds containing an azo linkage do not generally yield ammonia nitrogen upon decomposition, so it was to be expected that an attempt to determine nitrogen by the
Kjeldahl method failed with the products of this study. Accurate chloride determination was difficult due to the fact that the presence of excess chloride ion was necessary for precipitation of the corresponding salts.

The only practical recourse appeared to be determination of nitrogen by the Dumas method which requires combustion of the organic molecule to elementary nitrogen. Since facilities for combustion determinations were not available for the present work the nitrogen analyses were made by a commercial laboratory. The results, however, did not agree very closely with the calculated values.

Jerchel and Fischer (85) reported that TTC formed a double salt with mercuric chloride. It was found that similar salts were produced when saturated mercuric chloride solution was added to aqueous solutions of the tetrazolium salts of the present study. In every case the double salts were considerably less soluble than the corresponding chlorides, and it was possible to recrystallize them from dilute alcohol.

The analytical data of Jerchel and Fischer indicated that their salt was a simple addition compound formed by one molecule each of TTC and HgCl₂. This was found to hold true for the tetrazolium chlorides of the present study.

The advantage of this double salt was that an accurate method was available in the present case for determination of mercury colorimetrically by means of the orange-colored complex which mercuric ion forms with diphenylthiocarbazone, A modification of the A. O. A. C. "dithizone" method (86) for mercury was used. Another advantage of this procedure was that
it was possible to carry out the analysis with very small samples.
VI. EXPERIMENTAL WORK

Aldehydes

p-Dimethylaminobenzaldehyde methobromide and 3,5-dimethyl-4-hydroxybenzaldehyde, which were known compounds but not commercially available, were prepared as follows:

4-Dimethylaminoacetanilide

Twenty seven and two tenths g. (0.2 mole) of N,N-dimethylphenylenediamine and 22.5 g. (0.22 mole) of acetic anhydride were heated together under reflux for two hours. Upon cooling the mixture set into a crystalline mass. This was dissolved in about 100 ml. of chloroform, treated with active carbon, filtered, and ligroin was added until a precipitate began to appear. The mixture was then heated to boiling and allowed to cool slowly. The resulting crystals were collected on a filter. Yield 23 g. (65%) m.p. 130-131°.

p-Acetamidophenyltrimethylammonium bromide

Seventeen and eight-tenths g. (0.1 mole) of 4-dimethylaminoacetanilide was mixed with an excess of methyl bromide (50 ml. of 25% methanolic solution) and heated in a sealed tube for 15 hours at 83°. The methanol was removed by evaporation to yield 16 g. of the crude quartenary salt. This was recrystallized from methanol-ether mixture. Yield 14 g. (51%) m. p. 265°.

p-Aminophenyltrimethylammonium bromide hydrobromide

Thirteen and seven-tenths g. (0.05 mole) of p-
acetamidophenyltrimethylammonium bromide was dissolved in 100 ml. of ethanol to which 10 ml. of hydrobromic acid (an excess) was added, and the mixture was evaporated to dryness on a steam bath. The residue was recrystallized from methanol. Yield 12.5 g. (80%) m.p. 235°.

p-Dimethylaminobenzaldehyde methobromide

Fifteen g. (0.1 mole) of p-dimethylaminobenzaldehyde, dissolved in 40 ml. of methanol, was mixed with 60 ml. of a 25% methanolic solution of methyl bromide (an excess) and heated in a sealed tube for 18 hours at 73°. The methanol was removed by evaporation, the residue taken up in boiling water, treated with active carbon, filtered, and allowed to cool slowly. The yield of crystalline product was 15 g. (61%) m.p. 73°-75°.

3,5-Dimethyl-4-hydroxybenzaldehyde

Gattermann method - Thirty six and six-tenths g. (0.3 mole) of 2,6-dimethylphenol was dissolved in 750 ml. of dry ether contained in a 5-liter three-necked flask fitted with a mercury-sealed stirrer in the center neck. A tube from a hydrogen chloride generator led in through one neck to discharge beneath the surface of the solution and an outlet tube led from the other neck to the top vent of the hood in which the apparatus was placed. The flask was set into an ice-salt bath and the stirrer started. Then 69 g. of zinc cyanide, 7.5 g. of potassium chloride, and 78 g. of anhydrous aluminum chloride dissolved in 300 ml of dry ether were added in the order named. Dry hydrogen chloride was then bubbled through the
mixture for four hours, after which the contents of the flask were poured into a 2-liter beaker about half full of cracked ice. When the ice had melted the aqueous and ethereal solutions were separated and the aqueous phase washed with two small portions of ether. The washings were added to the rest of the ether solution. The aqueous phase was heated on the steam bath for 1 hour, but when it gave no test for aldehyde it was heated for two hours longer and tested again. The ethereal solution was evaporated until a brown oil remained. When this also gave no test for aldehyde it was heated for one hour with dilute hydrochloric acid. No crystalline material could be isolated from this portion. Also neither phase formed a precipitate with 2,4-dinitrophenylhydrazine reagent.

Duff method - One hundred and fifty g. of glycerol and 35 g. of boric acid were heated in a beaker at 170° to expel all water. Then 25 g. of hexamethylenetetramine was added. The mixture was stirred and brought to 160°, at which point 24.4 g. (0.2 mole) of 2,6-dimethylphenol was added all at once, and the mixture was stirred vigorously. Stirring was continued for 15 minutes while maintaining the temperature at 150-155°. The thick, brown liquid was then left to cool. When the temperature had dropped to 100° the mixture was transferred to a flask set up for steam distillation, using a solution of 30 ml. of sulfuric acid in 100 ml. of water to rinse out the beaker. This was added to the mixture. A rapid current of steam was then passed through the mixture which was kept at the boiling point. The product was not distillable with steam,
but this procedure served to hydrolyze the intermediate imine and to remove the unchanged aldehyde. Five g. of the latter was recovered. After cooling, the mixture was extracted with four 50 ml. portions of ether and the solvent was removed by evaporation to yield 13 g. of crude product. This material was dissolved in ethanol and treated with sodium bisulfite solution and allowed to stand 24 hours. The precipitate was washed with ether, then dissolved in 200 ml. of dilute sulfuric acid and heated on a steam bath. After cooling the mixture was extracted with ether, the ether evaporated and the residue recrystallized from hot water. Yield 10 g. (33%) of very white needles m.p. 115-116°.

Reimer-Tiemann method - Thirty g. (0.25 mole) of 2,6-dimethylphenol in 100 ml. of ethanol was mixed with 200 ml. of 40% sodium hydroxide solution in a 500 ml. flask fitted with a stirrer, dropping funnel, and reflux condenser. Forty ml. of chloroform (about 0.5 mole) was then added from the dropping funnel at such a rate as to maintain the mixture at gentle reflux while stirring. After addition of the chloroform was complete the mixture was boiled for one hour longer. The condenser was then adjusted for distillation and the alcohol and excess chloroform distilled off. The mixture was cooled and adjusted to pH 3.5 with concentrated hydrochloric acid. Sufficient water was added to dissolve the salt and the mixture was extracted with a total of 200 ml. of ether in three portions. The crude product was dissolved in hot dilute alcohol, treated with active carbon, filtered and recrystallized.
Yield 11 g. (29%) m.p. 115-116°.

Aldehyde Phenylhydrazones

These products were prepared by dissolving the aldehyde in the required amount of methanol and adding, slowly with stirring, an equivalent quantity of the phenylhydrazine. Precipitation began immediately in almost every case, although p-nitrophenylhydrazine reacted slower than the unsubstituted compound. When the former was used, 1 ml. of glacial acetic acid per mole of hydrazine was also added. In the case of p-nitrobenzaldehyde-p-nitrophenylhydrazone the reaction mixture was heated on the steam bath until precipitation occurred.

p-Nitrobenzaldehyde-2,4-dinitrophenylhydrazone required special conditions and was prepared by the procedure of Iddles (80) as follows:

Fifteen g. (0.1 mole) of p-nitrobenzaldehyde was dissolved in 100 ml. of methanol and added in small portions with stirring to a solution of 19 g. (0.096 mole) of 2,4-dinitrophenylhydrazine in 200 ml. of 2N hydrochloric acid. About 50 ml. more of 2N hydrochloric acid was then added, the mixture was stirred mechanically for 30 minutes and then allowed to stand for sixteen hours. Finally the precipitate was collected by suction filtration and washed thoroughly with water. Yield 27 g. (82%) m.p. 320°.

The phenylhydrazones which were prepared in this work are summarized as follows:
### Arylhydrazones

RCH = NNHR'

<table>
<thead>
<tr>
<th>R</th>
<th>R'</th>
<th>m.p. (°C)</th>
<th>Reference</th>
</tr>
</thead>
<tbody>
<tr>
<td>C₆H₅</td>
<td>C₆H₅</td>
<td>157-158</td>
<td>(a)</td>
</tr>
<tr>
<td>p-CH₃O-C₆H₄</td>
<td>C₆H₅</td>
<td>120-121</td>
<td>(b)</td>
</tr>
<tr>
<td>3,4-OCH₂O-C₆H₃</td>
<td>C₆H₅</td>
<td>106</td>
<td>(c)</td>
</tr>
<tr>
<td>p-O₂N-C₆H₄</td>
<td>C₆H₅</td>
<td>153-154</td>
<td>(d)</td>
</tr>
<tr>
<td>C₆H₅</td>
<td>p-O₂N-C₆H₄</td>
<td>190</td>
<td>(e)</td>
</tr>
<tr>
<td>p-CH₃O-C₆H₄</td>
<td>p-O₂N-C₆H₄</td>
<td>165</td>
<td>(f)</td>
</tr>
<tr>
<td>3,4-OCH₂OC₆H₃</td>
<td>p-O₂N-C₆H₄</td>
<td>199-200</td>
<td>(g)</td>
</tr>
<tr>
<td>p-O₂N-C₆H₄</td>
<td>p-O₂N-C₆H₄</td>
<td>249</td>
<td>(h)</td>
</tr>
<tr>
<td>3,5(CH₃)₂-4-OH-C₆H₂</td>
<td>C₆H₅</td>
<td>140-142</td>
<td>(66)</td>
</tr>
<tr>
<td>3,4(CH₃O)₂-C₆H₃</td>
<td>p-O₂N-C₆H₄</td>
<td>206</td>
<td></td>
</tr>
<tr>
<td>p-O₂N-C₆H₄</td>
<td>2,4(O₂N)₂-C₆H₃</td>
<td>320</td>
<td></td>
</tr>
<tr>
<td>p-CH₃CONH-C₆H₄</td>
<td>p-O₂N-C₆H₄</td>
<td>269</td>
<td></td>
</tr>
<tr>
<td>3,5(CH₃)₂-4-HO-C₆H₂</td>
<td>p-O₂N-C₆H₄</td>
<td>248</td>
<td></td>
</tr>
<tr>
<td>p-(CH₃)₃⁺N-C₆H₄ Br⁻</td>
<td>p-O₂N-C₆H₄</td>
<td>173-175</td>
<td></td>
</tr>
</tbody>
</table>

(a) *Ber.* 2, 887.
(b) *Ann.* 248, 103.
(c) *Ann.* 248, 103.
(d) *Ber.* 20, 1343.
(e) *Ann.* 324, 321.
(f) *Ber.* 16, 63.
(g) *Dictionary of Organic Compounds*, p. 503.
(h) *Ber.* 32, 1813.
Formazans

Method A - One tenth mole of the arylhydrazone was dissolved in about 400 ml. of a 3 to 1 mixture of pyridine and methanol. This proportion was varied somewhat depending on the solubility of the hydrazone. The mixture was cooled to 0°C by surrounding the vessel with dry ice, and the diazonium salt, prepared from the appropriate amine, 20 ml. of hydrochloric acid, 20 ml. of water, and sufficient cracked ice to maintain the temperature at 0°C, was added in small portions with stirring. The stirring and cooling were maintained for two hours. Then an equal volume of water was added and after standing overnight the precipitated formazan was filtered with suction, washed with hot water and dried in air. Generally the product was used without further purification as explained in the preceding section.

Method B - One-tenth mole of the mono-substituted pyruvic ester was dissolved in a solution of 1000 ml. of water, 200 ml. of 29% ammonia and 350 ml. of methanol, with warming. The solution was filtered, cooled to 0°C by the external application of dry ice and diazotized with a solution of 0.2 mole of the appropriate amine, 50 ml. of concentrated hydrochloric acid, 50 ml. of water and 14 g. (0.2 mole) of sodium nitrite, at 0°C with rapid stirring. The stirring was continued for two hours after all the reactants had been added. The precipitated formazan was filtered with suction and washed thoroughly with water. The crude product was dried in air then dissolved in xylene and the oxamide removed by filtration. Vacuum distillation of the solvent at 50°C yielded the formazan which was used without further purification.
Formazans

\[
\begin{array}{ccc}
R & R' & R'' & \text{Yield} & \text{m.p.} & \text{Ref.} \\
C_6H_5 & C_6H_5 & C_6H_5 & 40 & 173-174 & (4) \\
p-CH_3O-C_6H_4 & C_6H_5 & C_6H_5 & 45 & 156-158 & (41) \\
3,4-OCH_2O-C_6H_3 & C_6H_5 & C_6H_5 & 38 & 155-156 & (81) \\
C_6H_5 & C_6H_5 & p-CH_3O-C_6H_4 & 44 & 131-132 & (84) \\
p-CH_3O-C_6H_4 & C_6H_5 & p-CH_3O-C_6H_4 & 40 & 148-150 \\
3,4-OCH_2O-C_6H_3 & C_6H_5 & p-CH_3O-C_6H_4 & 70 & 185-186 \\
3,4-OCH_2O-C_6H_3 & p-CH_3O-C_6H_4 & C_6H_5 & 70 & 180-181 \\
CH_3O-C_6H_4 & p-CH_3O-C_6H_4 & C_6H_5 & 75 & 186-187 \\
CH_3O-C_6H_4 & p-CH_3O-C_6H_4 & p-CH_3O-C_6H_4 & 85 & 182-183 \\
CH_3O-C_6H_4 & p-CH_3O-C_6H_4 & p-CH_3O-C_6H_4 & 80 & 117-118 \\
p-CH_3O-C_6H_4 & p-CH_3O-C_6H_4 & C_6H_5 & 78 & 145-147 \\
p-CH_3O-C_6H_4 & p-CH_3O-C_6H_4 & C_6H_5 & 65 & 146-148 \\
3,5-(CH_3)_2-4-HO-C_6H_2 & C_6H_5 & p-CH_3O-C_6H_4 & 30 & 95-96 \\
p-CH_3O-C_6H_4 & p-CH_3O-C_6H_4 & p-CH_3O-C_6H_4 & 40 & 257 \\
3,4-(CH_3O)_2-C_6H_3 & p-CH_3O-C_6H_4 & p-CH_3O-C_6H_4 & 38 & 170-172 \\
CH_3O-C_6H_4 & p-CH_3O-C_6H_4 & p-CH_3O-C_6H_4 & 44 & 118-120 \\
4-CH_3CONH-C_6H_4 & p-CH_3O-C_6H_4 & C_6H_5 & 25 & 135-136 \\
3,4(CH_3O)_2-C_6H_3 & p-CH_3O-C_6H_4 & p-CH_3O-C_6H_4 & 72 & 170 \\
C_6H_5 & p-CH_3O-C_6H_4 & C_6H_5 & 70 & 164-166 \\
CH_3O-C_6H_4 & p-CH_3O-C_6H_4 & p-CH_3O-C_6H_4 & 43 & 186-187 \\
p-CH_3O-C_6H_4 & p-CH_3O-C_6H_4 & p-CH_3O-C_6H_4 & 38 & 185-186 \\
3,4-OCH_2O-C_6H_3 & C_6H_5 & p-CH_3O-C_6H_4 & 38 & 185-186 \\

All formazans in this table were prepared according to Method A.
Nickel Complexes of Formazans

One one-hundredths mole of the formazan dissolved in acetone was mixed with an equivalent quantity of nickel acetate dissolved in ethanol. The mixture was heated on a water bath for about fifteen minutes then allowed to stand until the next day. The precipitate was filtered, washed first with dilute alcohol and finally with dilute hydrochloric acid. The product was recrystallized from chloroform.

\[
\begin{align*}
&\text{R} \quad \text{R'} \quad \text{R''} \\
&p-\text{CH}_3\text{O-C}_6\text{H}_4 \quad p-\text{O}_2\text{N-C}_6\text{H}_4 \quad \text{C}_6\text{H}_5 \quad 285 \\
&3,4-\text{OCH}_2\text{O-C}_6\text{H}_3 \quad p-\text{O}_2\text{N-C}_6\text{H}_4 \quad \text{C}_6\text{H}_5 \quad >300 \\
&p-\text{CH}_3\text{O-C}_6\text{H}_4 \quad \text{C}_6\text{H}_5 \quad p-\text{O}_2\text{N-C}_6\text{H}_4 \quad >300 \\
&\text{C}_6\text{H}_5 \quad p-\text{O}_2\text{N-C}_6\text{H}_4 \quad p-\text{O}_2\text{N-C}_6\text{H}_4 \quad 260 \\
&p-\text{CH}_3\text{O-C}_6\text{H}_4 \quad \text{C}_6\text{H}_5 \quad p-\text{CH}_3\text{O-C}_6\text{H}_4 \quad 275
\end{align*}
\]

Tetrazolium Chlorides

Method A - Five, one-hundredths mole of the formazan was dissolved in 100 ml. of chloroform in a 400-ml. beaker and lead tetraacetate was added portionwise with stirring. The beaker was cooled externally to keep the reaction temperature below
20°. The characteristic color of the formazan was replaced by a yellowish brown color, usually within a few minutes. The chloroform was evaporated almost to dryness on a steam bath and the last few milliliters were evaporated in a current of air without heating. The residue was dissolved in 150 ml. of water and hydrochloric acid was added until no further precipitation occurred. The lead chloride was removed by filtration and the aqueous solution was either concentrated or extracted with a solvent to yield the crude product, depending on the solubility of the particular tetrazolium salt. (See table of physical properties)

Method B - Five one-hundredths mole of the formazan was suspended in 300 ml. of ethanol in a 600 ml. beaker. Then 0.1 g. of vanadium pentoxide was added followed by 15 ml. of hydrochloric acid and about 25 ml. of 30% hydrogen peroxide. The two liquids were added dropwise, simultaneously, over a period of 30 minutes. The reaction is exothermic, and too rapid addition of hydrogen peroxide causes a considerable portion of it to be wasted. Also, the formazans, in contrast to the tetrazolium compounds, are quite sensitive to acid hydrolysis, and if the reaction mixture is allowed to boil some of the formazan may decompose before it has an opportunity to react. The addition of hydrogen peroxide was continued until the characteristic color of the formazan was replaced by a yellowish brown and a clear solution resulted. The catalyst and any other insoluble particles were filtered out and the solvent was removed by vacuum distillation to yield the crude product.
Method C - One-tenth mole of the formazan was dissolved in a mixture of 400 ml. of glacial acetic acid and 40 ml. of isoamyl nitrite and heated on a steam bath for three hours, during which time the characteristic color of the formazan was replaced by yellowish brown. The mixture was then taken to dryness by vacuum evaporation.

The tetrazolium salts were recrystallized from the solvents indicated in the following table.
Table of Tetrazolium Salts

(See next page)
<table>
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<th>R</th>
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# Prepared by acid hydrolysis of the corresponding p-acetamido compound.
**TETRAZOLIUM SALTS**

\[
\begin{align*}
\text{R-C} & \quad \begin{aligned}
N-N-R' \\
N=N-R'' & \quad \text{X}^-
\end{aligned} \\
\text{N-N-R'} & \quad \text{HgCl}_2
\end{align*}
\]

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* Analysis by Clark Microchemical Laboratory, Urbana, Illinois. Recrystallization solvents: ME = methanol-ether; MD = methanol-dioxane; CPE = chloroform-petroleum ether; W = water; Acet. = acetone; DE = dioxane-ether; E = ether.
The syntheses of 2,3-di(p-bromophenyl)-5-(9-phenanthridy1) tetrazolium acetate and 2,3-di(p-bromophenyl)-5-(6-cholesterol) tetrazolium acetate are given separately, since they required special procedures.

2-Acetamidobiphenyl

Thirty-four g. (0.2 mole) of o-xenylamine was dissolved in 175 g. of pyridine which had been previously dried over solid potassium hydroxide, and 15 ml. (0.2 ml.) of acetyl chloride was added slowly with stirring. After standing for 20 minutes the mixture was poured into an excess of dilute hydrochloric acid solution containing pieces of ice. The product was filtered out and recrystallized from dilute ethanol. Yield 30 g. (73%) m.p. 120-121°.

9-Methylphenanthridine

Twenty-five g. (0.12 mole) of 2-acetamidobiphenyl was boiled under reflux with 30 ml. (0.3 mole) of phosphorous oxychloride. The reaction mixture was protected from moist air by a calcium chloride tube on the condenser. After one hour of heating the phosphorous oxychloride was distilled off and the residual gum was warmed with 400 ml. of 0.1 N hydrochloric acid. The mixture was cooled and the crystalline product filtered out. This was recrystallized from petroleum ether. However, this did not yield a very pure product, so it was vacuum distilled from a 200-ml. flask having a short neck and a side arm of 8 mm. bore. Yield 6.5 g. (36%) m.p. 84°.
Ethyl-9-phenanthridyl pyruvate

Four grams (0.1 gram atom) of potassium, cut into small pieces, was added to a solution of 25 ml. of anhydrous ether and 18 ml. (0.3 mole) of absolute ethanol contained in a 500-ml. round-bottom flask fitted with a reflux condenser. The rate of addition was sufficiently slow to prevent the reaction from becoming too violent. After the potassium was completely dissolved a solution of 7.5 g. (0.05 mole) of redistilled ethyl oxalate in 50 ml. of anhydrous ether was added in small portions, likewise through the top of the condenser, and after fifteen minutes, a solution of 5 g. (0.025 mole) of 9-methylphenanthridine in 50 ml. of anhydrous ether was added the same way. A drying tube was then attached to the condenser, and the mixture was allowed to stand for seven days. At the end of that time the precipitated yellow solid was filtered out by suction and immediately added to 100 ml. of dilute acetic acid. After two hours the product was collected by filtration and recrystallized from dilute ethanol. Yield 3.7 g. (50%) m.p. 183-184.

N,N'-Di-(p-bromophenyl)-C-(9-phenanthridyl) formazan

Three and seven-tenths g. (0.0125 mole) of ethyl-9-phenanthridyl pyruvate was dissolved in 200 ml. of a mixture of 25 ml. of ammonium hydroxide (29% NH) 40 ml. of methyl alcohol and 135 ml. of water. This was mixed slowly while stirring with the diazonium salt prepared from 4.3 g. (0.025 mole) of p-bromoaniline dissolved in 20 ml. of water and 6.3 ml. of concentrated hydrochloric acid and 1.75 g. (0.025
mole) of sodium nitrite in 20 ml. of water at 0°. The mixture was allowed to stand 24 hours and was then filtered. The precipitate was recrystallized from a mixture of pyridine and water. Yield 3 g. (53%).

2,3-Di-(p-bromophenyl)-5-(9-phenanthridyl) tetrazolium acetate

Three g. (0.0067 mole) of N,N'-di-(p-bromo-phenyl)-C-(9-phenanthridyl) formazan in 250 ml. of glacial acetic acid was mixed with 20 ml. of isoamyl nitrite and heated on a steam bath for three hours. At the end of that time the reddish color of the formazan had changed to yellowish brown. The solvent was removed by vacuum distillation leaving a brown oil. This was taken up in 20 ml. of methanol, treated with active carbon, filtered, and the filtrate concentrated by evaporation to 1 ml. Addition of ether precipitated the tetrazolium acetate. This was recrystallized from methanol-ether. Yield 0.1 g. (25%) Analysis by Clark Microchemical Laboratories, Urbana, Illinois. Calculated for C_{26}H_{19}N_{5}O_{2}Br_{2}: N, 11.3, Found 9.11.

Monoperphthalic Acid

In a 5-liter flask equipped with a mechanical stirrer and cooled with dry ice was placed 500 ml. of 15% sodium hydroxide solution. When the temperature had fallen to -10°, 210 ml. (approximately 2 moles) of 30% hydrogen peroxide which had been similarly cooled was added all at once. This caused the temperature to rise. When it had again fallen to -10°, 150 g. (1 mole) of phthalic anhydride which had been
finely ground in a mortar was added as quickly as possible with rapid stirring. As soon as the anhydride had dissolved 500 ml. (0.5 mole) of 20% sulfuric acid, which had been previously cooled to -10°, was added. It is important to add the acid as soon as possible after the anhydride, but not until the latter has dissolved. The solution was then filtered without suction through glass wool into a large separatory funnel and extracted once with 500 ml. of ether and then three times with 250 ml. portions. The combined extracts were shaken with three 150 ml. portions of 40% ammonium sulfate solution and dried for 24 hours in a refrigeration over 50 g. of anhydrous sodium sulfate. The peracid content of the solution was determined by removing a 10 ml. sample, adding 30 ml. of 20% potassium iodide solution and titrating with 0.1 N sodium thiosulfate solution. Yield 88.5 g. (48%) calculated from titration.

Cholesterol-α-oxide

To the ethereal solution of monoperphthatic acid (0.48 mole) obtained above, was added 93 g. (0.24 mole) of cholesterol dissolved in 400 ml. of ether. The mixture was heated under reflux for six hours and the solvent removed by vacuum distillation. The residue was then digested with 1 liter of chloroform previously dried over potassium carbonate. Filtration and vacuum distillation of the chloroform solution produced the crude cholesterol-α-oxide which was recrystallized twice from methanol to give 36 g. (37%) of the pure product, m. p. 141-143°.
6-β-Methylcholestane-3β,5α-diol

200 ml. of dry ether and 4.86 g. of magnesium turnings (0.2 mole) were placed in a 1-liter round bottom flask fitted with a reflux condenser and drying tube, and 28 g. of methyl iodide was added a little at a time through the top of the condenser. When the reaction had subsided and all of the magnesium had reacted 20 g. (0.05 mole) of cholesterol-α-oxide dissolved in 400 ml. of benzene was added all at once. The condenser was adjusted for distillation, and the ether was distilled off. When the temperature of the vapor had risen to 80° the condenser was readjusted for reflux, and the mixture was boiled for ten hours. The solvent was then removed by vacuum distillation, and the residue was recrystallized from methanol. Yield 10 g. (48%) m.p. 182-183°.

6-Methylcholesterol acetate

Ten g. (0.024 mole of the diol was refluxed with 200 g. of acetic anhydride for two hours. The solution was then cooled to 25°, treated with 2 ml. of concentrated H₂SO₄ and allowed to stand in a stoppered flask for three days. At the end of this time the mixture was poured onto 400 g. of cracked ice and brought to pH 6 with sodium hydroxide solution. The mixture was extracted with ether, the solvent evaporated, and the residue recrystallized from methanol. Yield 4 g. (40%) m.p. 115-116°.

Ethyl cholesteryl-3-acetate-6-pyruvate

To a solution of 20 ml. of anhydrous ether and 4 ml. (0.1 mole) of absolute ethanol was added 0.8 g. (0.02 gram atom)
of potassium at such a rate that the solution was kept at reflux. After all the potassium had dissolved a solution of 1.5 g. (0.01 mole) of ethyl oxalate in 15 ml. of anhydrous ether was added dropwise, and after fifteen minutes a solution of 4 g. (0.01 mole) of 6-methylcholesteryl acetate in 15 ml. of ether was added. The flask was stoppered and allowed to stand for seven days. The precipitate was then filtered rapidly with suction and immediately mixed with 50 ml. of dilute acetic acid. After two hours the separated solid was collected on a filter, washed with water, and dried in air. Yield 2.0 g. (50%)

N,N'-Di-(p-bromophenyl)-C-(cholesteryl-3-acetate-6) formazan

Three and one-half g. (0.007 mole) of ethyl cholesteryl-3-acetate-6-pyruvate was dissolved in 125 ml. of aqueous methanol (85 ml. water, 15 ml. 29% ammonia and 25 ml. methanol) with warming. The filtered solution was cooled to 0° with dry ice and coupled with a diazonium salt solution prepared from 2.4 g. (0.014 mole) of p-bromoaniline, 15 ml. of water, and 3 ml. of hydrochloric acid treated with 1 g. of sodium nitrite in 5 ml. of water at 0°. The ammoniacal solution was stirred rapidly during addition of the diazonium solution and stirring was continued for two hours after the addition was completed. The product was then filtered with suction and washed well with water. Finally it was dried in air. Attempts were made to extract the formazan with xylene according to Ried's procedure (49) but without success. Other attempts to isolate a formazan were likewise unsuccessful.
Ethyl-5-pregnene-3-ole-20-one-21-pyruvate

This was prepared in exactly the same way as ethyl cholesteryl-3-acetate-6-pyruvate using 3 g. (0.01 mole) of 5-pregnene-3-ole-20-one (Bios Laboratories) as the active methyl compound. Yield 2.5 g. (60%) m.p. 207°. Analysis by Clark Microanalytical Laboratory, Urbana, Illinois. Calculated for C_{25}H_{36}O : C, 72.1%; H, 8.70%. Found C, 70.47%; H, 8.76%.

N,N'-Di-(p-bromophenyl)-C-(5-pregnene-3-ole-20-one-21) formazan

Two and one-half g. (0.006 mole) of ethyl-5-pregnene-3-ole-20-one-21-pyruvate was dissolved in 100 ml. of aqueous ammonical solution prepared from 12 ml. of ammonium hydroxide (29% ammonia), 20 ml. of methyl alcohol and 68 ml. of water. A diazonium solution was prepared from 2.1 g. (0.012 mole) of p-bromoaniline 15 ml. of water and 3 ml. of hydrochloric acid treated with 0.84 g. (0.012 mole) of sodium nitrite at 0°. The diazonium solution was added dropwise to the ammoniacal solution with rapid stirring and stirring was continued for two hours after the addition was completed. The crude product was filtered out with suction, washed with water and dried in air. No formazan could be separated or identified.
VII. COMPARATIVE PROPERTIES

As was mentioned in Section II, the sensitivity of the compounds of this study to biological reduction was tested on the corn seed embryo. Grains of dried seed corn were sliced in half longitudinally so that in each case the embryo was exposed. The hemispherical depressions of a spot plate were filled with different tetrazolium solutions (0.001 M). Then the seed sections were introduced, all at the same time, and the time of the first appearance of formazan color in the embryos was measured. Since the age of the corn and moisture content are factors in the reduction it was always necessary to use seed from the same ear of corn and preferably from the same zone of the ear in making comparison tests. The times, therefore, are relative rather than absolute, but it was possible to determine very quickly the comparative ease of reduction of a number of compounds by this procedure.

TTC was used as the standard of comparison, and the time required to reduce each compound was recorded as a ratio of the time required to reduce TTC. These ratios were found to be fairly reproducible with corn seed from different sources. More than a two hundred-fold difference in reduction time was shown by the compounds prepared in this study, and a few of the compounds were reduced 50 times more rapidly than TTC. The reduction time ratios listed in the table of physical properties represent averages of ten tests. All of the salts used in these tests were chlorides.
Since the compounds on which reduction potentials were determined by Jerchel and Mohle (12) and also those reported by Ried and Wilk (34) were different from those studied in this work, no correlation was possible. Jerchel and Mohle used only alkyl substituents on the tetrazole carbon (group R, page 1) for their compounds, and Ried and Wilk tested only two triphenyl mono-tetrazolium compounds, namely 2,3,5-tri-phenyltetrazolium acetate and 2-phenyl-3-(m-trifluoromethylphenyl)-5-(m-methoxyphenyl)-tetrazolium acetate.

It was found that the aldehyde component (group R, page 1) had considerable effect on the sensitivity of the compound to reduction as was indicated by Ried and Wilk, but the present work showed that the R' and R" groups also had marked effect. Note from the results shown in the table of physical properties that electron donor groups in R decreased the reduction time when either or both of R' and R" was a p-nitrophenyl group. The 2,3-di-(p-nitrophenyl)-5-(p-methoxyphenyl) salt was reduced eight times more rapidly than its isomer, 2,5-di-(p-nitrophenyl)-3-(p-methoxyphenyl)-tetrazolium chloride. In view of the foregoing, the comparatively rapid reduction of 2,3,5-tris-(p-nitrophenyl)-tetrazolium chloride was rather surprising.

In order to illustrate the relative rates of reduction of different tetrazolium compounds by the corn seed embryo a series of color photographs were taken at different time intervals during one of the tests just described. Due to the light-sensitivity of the tetrazolium salts the photographic flood lamps were turned on only during the actual film expo-
sures. The illumination accelerated the reduction time slightly in spite of this precaution, but apparently the relative reduction rates were not appreciably affected.
Appearance of Corn Seed During a Test

Time: 0.5 minute

Time: 4 minutes

Time: 14 minutes

Time: 32 minutes

Time: 67 minutes

Time: 177 minutes
Key to position of solutions on spot plate:

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<td>10.</td>
<td>2,3-di-(p-nitrophenyl)-5-(p-methoxyphenyl)</td>
<td>1.5</td>
</tr>
<tr>
<td>11.</td>
<td>2,3,5-tris-(p-nitrophenyl)</td>
<td>8.0</td>
</tr>
<tr>
<td>12.</td>
<td>2-(p-nitrophenyl)-3-(p-nitrophenyl)-5-(p-aminophenyl)</td>
<td>4.0</td>
</tr>
</tbody>
</table>
Physical properties

<table>
<thead>
<tr>
<th>Tetrazolium Chloride</th>
<th>Reduction time ratio</th>
<th>Solubility g./100g. of solvent at 25°C</th>
<th>$\text{H}_2\text{O}$ partition coefficient</th>
<th>$\text{CHCl}_3$ partition coefficient</th>
</tr>
</thead>
<tbody>
<tr>
<td>$R$</td>
<td>$R'$</td>
<td>$R''$</td>
<td>$\text{H}_2\text{O}$</td>
<td>$\text{CHCl}_3$</td>
</tr>
<tr>
<td>$p$-$\text{CH}_3\text{O}$-$\text{C}_6\text{H}_4$</td>
<td>$p$-$\text{O}_2\text{N}$-$\text{C}_6\text{H}_4$</td>
<td>$p$-$\text{CH}_3\text{O}$-$\text{C}_6\text{H}_4$</td>
<td>0.01</td>
<td>1.00</td>
</tr>
<tr>
<td>3,4-$\text{OCH}_2\text{O}$-$\text{C}_6\text{H}_4$</td>
<td>$p$-$\text{O}_2\text{N}$-$\text{C}_6\text{H}_4$</td>
<td>$p$-$\text{CH}_3\text{O}$-$\text{C}_6\text{H}_4$</td>
<td>0.015</td>
<td>0.99</td>
</tr>
<tr>
<td>$p$-$\text{CH}_3\text{O}$-$\text{C}_6\text{H}_4$</td>
<td>$p$-$\text{O}_2\text{N}$-$\text{C}_6\text{H}_4$</td>
<td>$p$-$\text{O}_2\text{N}$-$\text{C}_6\text{H}_4$</td>
<td>0.025</td>
<td>0.54</td>
</tr>
<tr>
<td>$p$-$\text{O}_2\text{N}$-$\text{C}_6\text{H}_4$</td>
<td>$p$-$\text{O}_2\text{N}$-$\text{C}_6\text{H}_4$</td>
<td>$p$-$\text{O}_2\text{N}$-$\text{C}_6\text{H}_4$</td>
<td>0.15</td>
<td>0.05</td>
</tr>
<tr>
<td>3,4-$\text{OCH}_3\text{O}$-$\text{C}_6\text{H}_4$</td>
<td>$p$-$\text{O}_2\text{N}$-$\text{C}_6\text{H}_4$</td>
<td>$p$-$\text{O}_2\text{N}$-$\text{C}_6\text{H}_4$</td>
<td>0.05</td>
<td>0.05</td>
</tr>
<tr>
<td>$p$-$\text{H}_2\text{N}$-$\text{C}_6\text{H}_4$</td>
<td>$p$-$\text{O}_2\text{N}$-$\text{C}_6\text{H}_4$</td>
<td>$\text{C}_6\text{H}_5$</td>
<td>0.06</td>
<td></td>
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<tr>
<td>$p$-$\text{CH}_3\text{O}$-$\text{C}_6\text{H}_4$</td>
<td>$p$-$\text{O}_2\text{N}$-$\text{C}_6\text{H}_4$</td>
<td>$\text{C}_6\text{H}_5$</td>
<td>0.10</td>
<td>0.88</td>
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<tr>
<td>$p$-$\text{O}_2\text{N}$-$\text{C}_6\text{H}_4$</td>
<td>$p$-$\text{O}_2\text{N}$-$\text{C}_6\text{H}_4$</td>
<td>$p$-$\text{CH}_3\text{O}$-$\text{C}_6\text{H}_4$</td>
<td>0.20</td>
<td>0.05</td>
</tr>
<tr>
<td>3,4-$\text{OCH}_2\text{O}$-$\text{C}_6\text{H}_4$</td>
<td>$\text{C}_6\text{H}_5$</td>
<td>$\text{C}_6\text{H}_5$</td>
<td>0.60</td>
<td>4.25</td>
</tr>
<tr>
<td>$\text{C}_6\text{H}_5$</td>
<td>$\text{C}_6\text{H}_5$</td>
<td>$\text{C}_6\text{H}_5$</td>
<td>1.00</td>
<td>0.25</td>
</tr>
<tr>
<td>$p$-$\text{CH}_3\text{O}$-$\text{C}_6\text{H}_4$</td>
<td>$\text{C}_6\text{H}_5$</td>
<td>$\text{C}_6\text{H}_5$</td>
<td>1.65</td>
<td>5.65</td>
</tr>
<tr>
<td>$p$-$\text{O}_2\text{N}$-$\text{C}_6\text{H}_4$</td>
<td>$p$-$\text{O}_2\text{N}$-$\text{C}_6\text{H}_4$</td>
<td>$\text{C}_6\text{H}_5$</td>
<td>2.80</td>
<td>0.72</td>
</tr>
</tbody>
</table>

The reduction time ratios listed here constitute averages of 10 tests, whereas the times listed on the preceding page refer only to the one test which was photographed.
Note that as more tetrazolium is converted to formazan, the embryonic cells gradually become deeper stained. However, the surrounding seed tissues, composed largely of starch cells, are not appreciably affected. Also the solutions retain the characteristic yellow color of the unreacted tetrazolium salts. The formazans which precipitate inside the embryonic cells are so insoluble in water that no outward diffusion of color is evident.

As mentioned before, considerable difference exists between the compounds of this study in respect to their relative solubility in water and chloroform. For this reason the distribution between these solvents was determined and is tabulated along with the reduction time. Although the three compounds most sensitive to reduction also have the highest water/chloroform distribution coefficient this parallel does not hold for the rest of the compounds.

According to Burger (87) the water/chloroform distribution coefficient of drugs in animal cells approximates the water/lipid distribution coefficient, and it is possible that the absorption of tetrazolium salts by plant or animal cells is affected by their relative solubility in the cellular constituents.

All of the tetrazolium salts of this study produced red formazans upon reduction except 2,3-di-(p-nitrophenyl)-5-(p-methoxyphenyl) tetrazolium chloride. This compound caused the corn seed embryo to turn a slate-blue which gradually darkened upon standing. (See photographs). It was noted that
the same color was produced when organic matter which was under­
going bacterial decomposition was treated with aqueous solutions
of this salt. Actually, the color of N,N'di-(p-nitrophenyl)-C-
(p-methoxyphenyl) formazan in dilute solutions appears violet
rather than blue, but it produces an indigo stain in animal
and vegetable cells.

It is believed that this compound should possess some
advantages over those which produce red formazans when used as
an indicator in animal tissues, due to confusion of the latter
with blood color. The fact that the disadvantages of red form­
azans led investigators to synthesize blue formazans has been
mentioned before. Ried and Gick (2), who prepared several
such compounds, however, expressed the opinion that blue color
could be obtained only from diformazans which contain the
p-(di-o-methoxy)-diphenylene moiety.
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For fifteen years I worked as an advertising agent. In 1939 I took a course in quantitative analysis at the Medical College of Virginia, and in the same year I went to work for William P. Poythress & Co. as a laboratory technician in chemical control. I have remained with this firm until the present time.

I attended the University of Richmond on a part-time basis for the 1949-1950 and the 1951-1952 scholastic years and on a full-time basis during the 1950-1951 school year. I received the Bachelor of Science degree in June 1952.