A solution of 78 g. of anisic anhydride and 5 g. of nickel tetracarbonyl in 80 g. of benzene reacted under the same conditions to give 43 g. (88.6% conversion) of 4-methoxyphthalic anhydride, and 20 g. (68%) of anisole. The anhydride was identified by its melting point of 94–95° and by hydrolysis to 4-methoxyphthalic acid, m.p. 171–172°.[5]

Tracer Studies.—A sample of C^{14}-tagged benzoic anhydride with an activity of 2.08×10^6 counts/min./mmole was prepared from carboxyl tagged benzoic acid. A silver-lined shaker tube was charged with 15 g. (0.0664 mole) of the anhydride, 100 g. of benzene and 3 g. of $Ni(CO)_4$. The tube was sealed, pressured with CO to 100 atm. (total CO estimated as 1.26 moles including that bound as $Ni(CO)_4$) and heated at 300° for 2 hours. The product consisted of 4.3 g. of phthalic anhydride and 7.7 g. of recovered benzoic anhydride. The molar activity of the phthalic anhydride was 1.99×10^5 counts/min./mmole or 9.5% of the activity of the original benzoic anhydride. The recovered benzoic anhydride gave a value of 2.02×10^5 counts/min./mmole or 9.7% of the original value. Since a total of 1.39 eq. of carbonyl groups was charged, of which 0.133 was in the original tagged benzoic anhydride, complete equilibration with gaseous CO would reduce the molar activity of the benzoic anhydride to 9.57% of the original activity. In a second experiment, 0.044 mole of benzoic anhydride was treated with 3.09 moles of CO under similar conditions. The recovered benzoic anhydride showed a specific activity which was 2.26% of the original value. The value for complete equilibration should be 2.77%, so that agreement is within the limits of accuracy by which the equivalents of CO charged were measured.

Conversion of N,N-Dibenzoylaniline to N-Phenylphthalimide.—A solution of N,N-dibenzoylaniline (8 g.) in 200 g. of toluene containing 5 g. of $Ni(CO)_4$ was heated at 325° under 200 atm. CO pressure for 2 hours. The product contained 3.6 g. of N-phenylphthalimide (m.p. 203° after recrystallization from chloroform–methanol) in addition to recovered N,N-dibenzoylaniline. A mixed melting point with an authentic sample of N-phenylphthalimide, melting at 203°, was not depressed.

Anal. Calcd. for $C_{14}H_9O_2N$: C, 75.32; H, 4.06; N, 6.28. Found: C, 74.98; H, 4.13; N, 6.32.

WILMINGTON, DEL.

(5) 4-Methoxyphthalic anhydride is reported to melt at 93–96° and 4-methoxyphthalic acid at 168–170°; M. Freund and E. Göbel, *Ber.*, **30**, 1932 (1897).

[CONTRIBUTION FROM THE DAJAC LABORATORIES OF THE CHEMICAL DIVISION OF THE BORDEN COMPANY, AND THE DEPARTMENTS OF SURGERY, SINAI HOSPITAL OF BALTIMORE AND THE JOHNS HOPKINS UNIVERSITY SCHOOL OF MEDICINE]

Syntheses of Some *p*-Nitrophenyl Substituted Tetrazolium Salts as Electron Acceptors for the Demonstration of Dehydrogenases[1]

BY KWAN-CHUNG TSOU, CHAO-SHING CHENG, MARVIN M. NACHLAS AND ARNOLD M. SELIGMAN

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A number of *p*-nitrophenyl substituted tetrazolium salts have been synthesized for the histochemical demonstration of de, hydrogenase in tissues. One of these, 2,2′-di-*p*-nitrophenyl-5,5′-diphenyl-3,3′-(3,3′-dimethoxy-4,4′-biphenylene)-ditetrazolium chloride (XA, "nitro-BT") (Table IV) was found to be superior to any tetrazole so far developed for histochemistry and makes possible the demonstration of the dehydrogenases at the cytological level. It was also found that during the coupling of a tetrazotized biphenylenediamine with a phenylhydrazone, one of the diazonium groups could be replaced by hydrogen to form a phenyl group. Thus, in the formation of the above-mentioned ditetrazolium salt, a monotetrazolium salt, 2-*p*-nitrophenyl-5-phenyl-3-(3,3′-dimethoxy-4-biphenylyl)-tetrazolium chloride (IIIA) (Table III), was obtained concomitantly. The isolation of the mono- and diformazans is described and mechanisms for the formation of the monoformazans are discussed. The nature of the monotetrazole that is found as a contaminant in commercial preparations of ditetrazoles is thus ellucidated.

Tetrazolium salts form a unique class of oxidation–reduction indicators in the study of dehydrogenases.[2] The advantages of tetrazolium salts over the classical methylene blue reaction arise from the facts that (1) with the former compounds a color is produced on reduction (Fig. 1), while in the latter a decoloration occurs and (2) the formazans are not readily reoxidized in air, in contrast to methylene blue. The tetrazolium salts should have certain other properties in order to serve as an ideal electron acceptor for histochemical purposes; *i.e.*, (1) ease of reduction, (2) low lipid solubility (3) low light sensitivity, (4) amorphous or fine granular particle size, (5) insolubility in aqueous solution, (6) low solubility in common organic solvents and (7) aerobic as well as anaerobic reduction. In spite of the fact that a large number of tetrazolium salts have been synthesized in recent years,[3] one possessing all the desired features listed above has not yet been described.

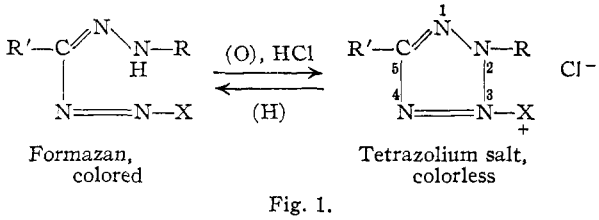


Fig. 1.

Among the commonly used biological tetrazolium salts[4] are: "TTC" (2,3,5-triphenyltetrazolium chloride), "BT" (2,2′,5,5′-tetraphenyl-3,3′-(3,3′-dimethoxy-4,4′-biphenylene)-ditetrazolium chloride), "NT" (2,2′,5,5′-tetraphenyl-3,3′-(*p*-biphenylene)-ditetrazolium chloride), and "INT" (3-*p*-iodophenyl-2-*p*-nitrophenyl-5-phenyltetrazolium chloride). The last reagent was found to reduce easily but was lacking in many of the other features essential for histochemical use; in fact, its crystalline nature resulted in the destruction of cellular detail in the tissue section. Since the introduction of the nitro group in "INT" markedly increased its ability to serve as an electron acceptor, and since it was known that the less soluble diformazans yielded blue rather than red pigments, it

(1) This investigation was supported by research grants (C-2530 and C-2478) from the National Cancer Institute, National Institutes of Health, Department of Health, Education and Welfare.

(2) H. A. Hölscher, *Z. Krebborsch*, **56**, 587 (1900); A. M. Seligman and A. M. Rutenberg, *Science*, **113**, 317 (1951); A. M. Rutenburg, R. Gofstein and A. M. Seligman, *Cancer Research*, **10**, 113 (1950).

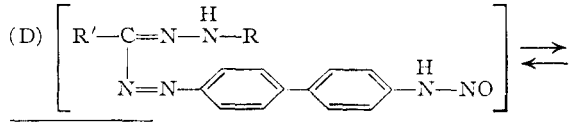
(3) A. W. Nineham, *Chem. Revs.*, **55**, 355 (1955).

(4) N. D. Cheronis and H. Stein, *J. Chem. Ed.*, **33**, 120 (1956).

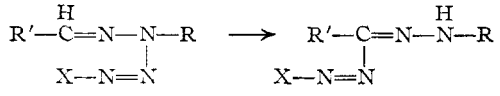
was anticipated that the introduction of a *p*-nitro group into a ditetrazolium salt such as "BT" would yield a more sensitive indicator of dehydrogenase activity and retain the favorable solubility and pigment qualities of the diformazans.

Benzaldehyde *p*-nitrophenylhydrazone, *p*-nitrobenzaldehyde phenylhydrazone or *p*-nitrobenzaldehyde *p*-nitrophenylhydrazone was coupled at 0°[5] with tetrazotized benzidine hydrochloride or tetrazotized *o*-dianisidine hydrochloride in the presence of alkali to form the formazan. Difficulty in purification soon led us to the conclusion that the crude formazan was in fact a mixture of formazans.[6]

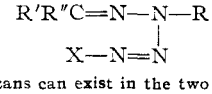
Careful fractionation resulted in the isolation of a monoformazan in addition to the expected diformazan. Rather unexpectedly this monoformazan is a product resulting from the coupling of the tetrazotized diamine to one mole of the hydrazone and the simultaneous reduction of one of the two diazonium groups. Even though it is well known that diazonium salts can be reduced to hydrocarbons by a number of reducing agents[7] such as hypophosphorous acid, reduction in this particular instance was difficult to explain, especially when even alcohols were rigorously excluded. Negative evidence that the reducing agent was not the formazan moiety was furnished by an attempted reaction of formazan and tetrazotized amine to form the tetrazolium salt. A plausible mechanism involves prototropy of an intermediate (D) to (E)[8] during the diazotization and followed by a novel rearrangement of the hydrocarbon and nitrous oxide[9] as



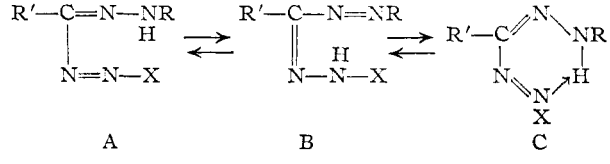
(5) The coupling mechanism of a diazonium salt to benzaldehyde phenylhydrazone seems to be *via* nucleophilic substitution on the methine carbon atom and not that of diazoamino rearrangement, *i.e.*



since ketone phenylhydrazone does not give the anticipated product



(6) Since the formazans can exist in the two tautomeric structures (A) and (B)



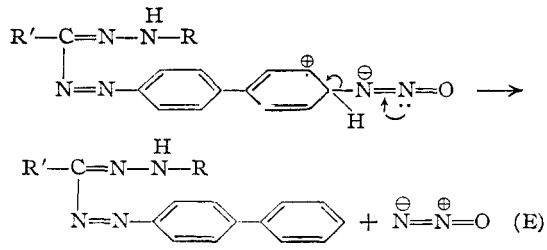
A B C

a considerable effort was devoted to the isolation of the two forms with no avail. Infrared evidence (3.18 μ NH-hydrogen bonding), however, suggests a chelating structure (C).

(7) R. C. Fuson, "Advanced Organic Chemistry," John Wiley and Sons, New York, N. Y., 1950, p. 560.

(8) It is noted that the resonance stability of ionic form (E) is greatly enhanced when one end of the molecule is a formazan since the positive charge can be distributed among the four nitrogens of the formazan molecule.

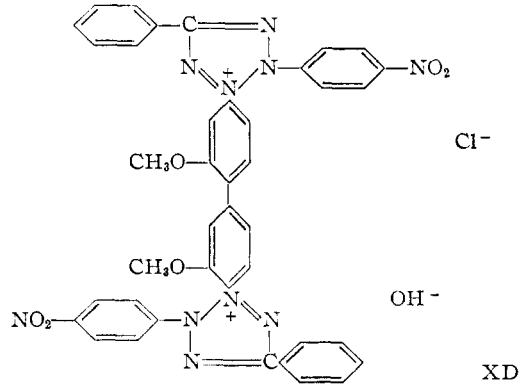
(9) N_2O is a resonance hybrid of $N{=}\overset{\ominus}{N}{=}\overset{\oplus}{O}$ and $\overset{\oplus}{N}{=}N{=}\overset{\ominus}{O}$, Syrkin and Dyatkina, "Structures of Molecules," Interscience Publishers, Inc., New York, N. Y., 1950, p. 259.



(E)

Further evidence will be necessary to establish this mechanism, but the importance of the presence of monotetrazoles as contaminants in commercial preparations of ditetrazoles recently has been shown by Burtner, Bahn and Longley.[10] A better yield of the diformazan may be achieved by premixing the tetrazotized diamine solution and the hydrazone solution. The physical constants and analytical data of the mono- and diformazans are listed in Tables I and II, respectively. Methoxy derivatives V and VI were obtained when alcohols were used in the reaction.

The tetrazolium salts (Tables III and IV) were prepared by the oxidation of the purified formazans as usual. Isoamyl nitrite was found to be the preferred reagent over that of lead tetraacetate or mercuric oxide. Many of them retained water or solvent of crystallization very tenaciously. Therefore analytical samples were only obtained after extensive drying *in vacuo*. In the case of 2,2′-di-*p*-nitrophenyl-5,5′-diphenyl-3,3′-(3,3′-dimethoxy-4,4′-biphenylene)-ditetrazolium chloride (XA, "nitro-BT") a half salt (XD) was obtained when recrystallized from water, and heated at 110° under vacuum to constant weight.



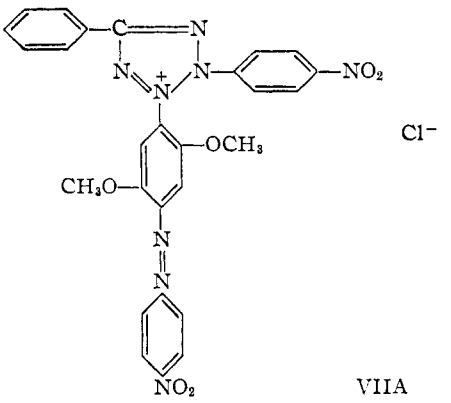
XD

2-*p*-Nitrophenyl-5-phenyl-3-(2,5-dimethoxy-4-*p*-nitrophenylazo)-phenyltetrazolium chloride (VIIA) was also prepared.

Extensive histochemical study so far has been made only with the ditetrazole (XA, "nitro-BT"). "Nitro-BT" was found to be reduced readily, even under aerobic conditions, by the succinic dehydrogenase system in frozen sections of tissues. In contrast to BT, very thin sections could be used aerobically and the dehydrogenase of organs where it was too weak to be demonstrated with BT were readily demonstrated with "nitro-BT." The dark blue pigment in these tissue sections was finely particulate, stable for over six months, and no crystallization was observed. An unexpected find-

(10) H. J. Burtner, R. C. Bahn and J. B. Longley, Abstracts of Histochemical Society Meeting, April, 1956.

ing was its marked insolubility in alcohol and xylene in tissue section.[11] These properties enabled de-



VIIA

hydrating and mounting the stained sections in oil for better optics. "Nitro-BT" is the first tetrazole to make possible the intracellular demonstration of organelles in frozen sections. Details of the use of this reagent in the cytochemical demonstration of the dehydrogenases will be presented elsewhere.

Experimental[12,13]

Unless specifically stated the formazans are prepared by the following general procedure. A tetrazotized solution of the diamine was mixed with a solution of the hydrazone at 0°. While the mixture was stirred vigorously, a base was added to initiate the reaction. After the addition, stirring was continued at room temperature for half an hour. The crude formazan was then isolated by the usual means as illustrated below and purified by careful fractionation into the mono- and diformazan.

The formazans are then oxidized to the tetrazolium salts by treating a suspension of the formazan with an excess of isoamyl nitrite and hydrogen chloride gas in an ice-bath. The mixture was then stirred at room temperature or in an ice-bath for a few minutes to several hours, depending on the ease of oxidation. Two to three drops of isoamyl nitrite were added at 10 to 15-minute intervals wherever necessary to speed up the reaction. The tetrazolium salts were then isolated in the usual manner as described in the example. Many of the salts showed a great tendency to occlude water or other solvent; therefore, pure analytical samples were possible only after numerous crystallizations and extensive drying.

The physical constants and analytical data of the formazans are listed in Tables I and II; the tetrazolium salts in III and IV.

Spectrophotometric Determination.—The absorption maxima of a few formazans in the wave length region 350–650 mμ were measured, chloroform being used as the solvent insofar as they were sufficiently soluble. These values were listed in Table V. A Bausch and Lomb Spectronic 20 Colorimeter was used for the measurements.

2-*p*-Nitrophenyl-5-phenyl-3-*p*-biphenyl formazan (I) and 2,2′ - Di - *p* - nitrophenyl - 5,5′ - diphenyl - 3,3′ - (*p* - biphenylene)-diformazan (VIII).—A suspension of 2.57 g. (0.01 mole) of benzidine hydrochloride in 15 cc. of water and 2.5 cc. of concentrated hydrochloric acid was tetrazotized with 1.52 g. (0.022 mole) of sodium nitrite dissolved in 4 cc. of water. The completeness of the tetrazotization was tested by a potassium iodide–starch paper. More sodium nitrite solution was used if the reaction was found to be incomplete.

The tetrazotized solution was then added to a solution of 4.82 g. (0.02 mole) of benzaldehyde *p*-nitrophenylhydrazone dissolved in 40 cc. of tetrahydrofuran. During the addition a few gas bubbles were observed in the solution. To this mixture was now added a solution of 2.24 g. (0.04 mole) of potassium hydroxide dissolved in 8 cc. of water. The dark formazan, a black precipitate, formed almost instantaneously. The resulting mixture was then stirred at room temperature for thirty minutes and the precipitation was completed by the addition of 40 cc. of isopropyl alcohol. The crude product was collected on a Buchner funnel and washed with about 70 cc. more of isopropyl alcohol until the washings turned from dark brown to wine red. It was again washed with 100 cc. of boiling water, dried and weighed 4.20 g.

Fractional Separation of the Mono- and Diformazan I and VIII.—The crude product isolated as above was now extracted once with 90 cc. of boiling tetrahydrofuran and the extract was evaporated down to about 10 cc. and poured into 50 cc. of methanol. A purplish black precipitate was obtained which was washed with methanol until the washings were free from a yellow tinge. It was found to decompose at about 160°, weighed 0.91 g. (2.16%) and analyzed to be the monoformazan I. The analytical sample was prepared by overnight extraction with 70 cc. of acetone in a Soxhlet extractor. The boiled extract was decolorized with carbon black and the filtrate concentrated to about 10 cc. and poured into 50 cc. of methanol. Recovery was 0.67 g.; m.p. 153° dec. The analytical data are listed in Table I.

The insoluble black powder of the crude product from the tetrahydrofuran weighed 0.74 g. (10.7%), m.p. 234° dec. This was analyzed to be the diformazan VIII. An analytical sample was prepared by recrystallization from dimethylformamide and washed with acetone. The melting point remained unchanged. The analytical data are listed in Table II.

2-*p*-Nitrophenyl-5-phenyl-3-(4′-ethoxy-4-biphenylyl)-formazan (V).—When the above experiment for preparing the formazan by adding the tetrazotized solution to the hydrazone solution dissolved in a mixture of dimethyl sulfoxide and ethanol containing potassium hydroxide or pyridine was repeated a solid product was obtained, which after two precipitations from its solution in chloroform by isopropyl alcohol weighed 2.43 g. (52.2%) and melted at 173° dec. An analytical sample was made by dissolving it in chloroform and precipitating it by five times its volume of nitromethane, m.p. 189° dec. Analysis showed it to be an ethoxy compound V (Table I) one of the two diazonium chloride groups of the tetrazonium salt was replaced by an ethoxy group. No diformazan was isolated. The same ethoxy compound was obtained when pyridine was used in place of potassium hydroxide.

2,2′-Di-*p*-nitrophenyl-5,5′-diphenyl-3,3′-(*p*-biphenylene)-ditetrazolium Chloride (VIIIA).—To 0.34 g. (0.005 mole) of diformazan VIII suspended in a mixture of 15 cc. of dioxane and 15 cc. of tetrahydrofuran was added ten drops of isoamyl nitrite. The mixture was cooled in an ice-bath and saturated with hydrogen chloride gas. It was then stirred at room temperature for 20 minutes until the purple solution turned greenish and some yellow precipitate appeared. Two drops of isoamyl nitrite were added at ten-minute intervals. The use of a magnetic stirrer was recommended as the stirring bar also pulverizes the formazan thus facilitating the solution.

The yellow precipitate was collected by filtration after cooling the reaction mixture in ice for thirty minutes. It weighed 0.32 g. and decomposed at about 185°. After reprecipitation from a solution in a minimum amount of methanol by five times its volume of dioxane a light yellow solid was obtained. It weighed 0.28 g. (70.0%), m.p. 222–224°, dec.

Recrystallization from a minimum amount of hot methanol gave an analytically pure product as light yellow solid, m.p. 222–223° dec. Analysis showed that the compound contained 2.5 moles of water of crystallization. The presence of water was verified by a drop of tetraisopropyl titanate in an anhydrous dimethylformamide solution of the compound. The test solution turned cloudy and a precipitate followed.[14]

2,5-Di-*p*-nitrophenyl-3-*p*-biphenylylformazan (II) and 2,2′,5,5′ - Tetra - *p* - nitrophenyl - 3,3′ - (*p* - biphenylene)-

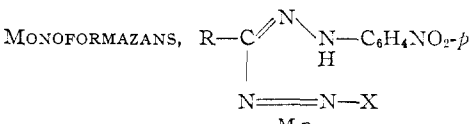
(11) This was in sharp contrast to the ready lipid solubility of the diformazan, a *m*-nitroditetrazole 2,2′-di-(*p*-nitrophenyl)-*m*-(5,5′-di-3-nitrophenyl), available commercially from Synthetic Labs., 5558 Ardmore Ave., Chicago, Ill.

(12) All melting points are corrected.

(13) Analyses were performed by Dr. C. K. Fitz, Needham Heights, Mass.; Micro Chemical Laboratory, Massachusetts Institute of Technology, Cambridge, Mass.; and Micro Tech Laboratories, Skokie, Ill.

(14) N. D. Cheronis, "Micro and Semi-Micro Methods," in Weissberger, ed. "Technique of Organic Chemistry," Vol. VI, Interscience Publishers, Inc., New York, N. Y., 1954, p. 422.

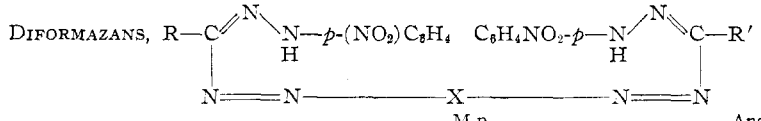
TABLE I

MONOFORMAZANS, 

	X	R	Empirical formula	M.p., °C., dec.	Yield, %	Carbon Calcd.	Carbon Found	Analyses, % Hydrogen Calcd.	Hydrogen Found	Nitrogen Calcd.	Nitrogen Found
I	*p*-Biphenylyl	C_6H_5	$C_{25}H_{19}N_5O_2$	153	21.6	71.25	71.3	4.54	4.4	16.62	16.3
II	*p*-Biphenylyl	*p*-$NO_2C_6H_5$	$C_{25}H_{18}N_6O_4$	171	49.3					18.02	18.8
III	3,3′-$(CH_3O)_2$-4-biphenylyl	C_6H_5	$C_{27}H_{23}N_5O_4$	202	51.6	67.35	67.44	4.81	4.78	14.55	14.63
IV	3,3′-$(CH_3O)_2$-4-biphenylyl	*p*-$NO_2C_6H_5$	$C_{27}H_{22}N_6O_6$	185	23.4	61.59	61.3	4.21	4.3	15.96	16.0
				239	3.8						
V	4′-(C_2H_5O)-4-biphenylyl	C_6H_5	$C_{27}H_{23}N_5O_3$	189	52.2	69.66	70.46	4.98	4.47	15.05	15.66
VI	3,3′4′-$(CH_3O)_3$-4-biphenylyl	C_6H_5	$C_{28}H_{25}N_5O_5$[a]	206	21.2	65.74	66.38	4.93	4.79		
VII	[2,5-$(CH_3O)_2$-4-*p*-NO_2-phenylazo]-phenyl	C_6H_5	$C_{27}H_{22}N_8O_6$	228	5.1	58.48	58.18	4.00	4.21	20.21	20.17

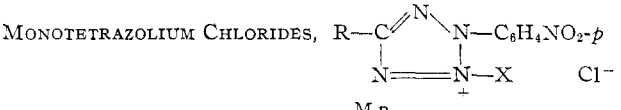
[a] Compound was obtained when methanol was used as a solvent in the preparation of the diformazan (X) (Table II). It was precipitated from a chloroform solution by methanol.

TABLE II

DIFORMAZANS, 

	X	R	Empirical formula	M.p., °C., dec.	Yield, %	Carbon Calcd.	Carbon Found	Analyses, % Hydrogen Calcd.	Hydrogen Found	Nitrogen Calcd.	Nitrogen Found
VIII	*p*-Biphenylene	C_6H_5	$C_{38}H_{28}N_{10}O_4$	234	10.7	66.27	65.5	4.10	4.3	20.34	20.2
IX	*p*-Biphenylene	*p*-$NO_2C_6H_5$	$C_{38}H_{26}N_{12}O_8$	269	49.3	58.61	58.5	3.37	3.5	21.59	21.3
X	3,3′-$(CH_3O)_2$-4,4′-biphenylene	C_6H_5	$C_{40}H_{32}N_{10}O_6$	252	18.2	64.16	63.9	4.31	4.6	18.71	18.6
XI	3,3′-$(CH_3O)_2$-4,4′-biphenylene	*p*-$NO_2C_6H_5$	$C_{40}H_{30}N_{12}O_{10}$	300	12.2	57.28	57.6	3.61	3.8	20.04	19.8

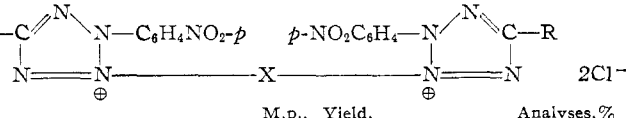
TABLE III

MONOTETRAZOLIUM CHLORIDES, 

	X	R	Empirical formula	M.p., °C., dec.	Yield, %	Carbon Calcd.	Carbon Found	Analyses, % Hydrogen Calcd.	Hydrogen Found	Nitrogen Calcd.	Nitrogen Found
IIIA	3,3′-$(CH_3O)_2$-4-biphenylyl	C_6H_5	$C_{27}H_{22}N_5O_4Cl\cdot 2H_2O$	134	29.0	58.75	58.71	4.75	4.53	12.69	12.56
IVA	3,3′-$(CH_3O)_3$-4-biphenylyl	*p*-$NO_2C_6H_5$	$C_{27}H_{21}N_6O_6Cl$[a]	136	94.0	57.81	57.6	3.77	4.0	14.98	14.8
VIA	3,3′,4′-$(CH_3O)_3$-4-biphenylyl	C_6H_5	$C_{29}H_{24}N_5O_5Cl$[b]	200	64.0	61.59	61.97	4.43	4.62		
VIIA	2,5-$(CH_3O)_2$-4′-(NO_2)-4-azophenyl	C_6H_5	$C_{27}H_{21}N_8O_6Cl$[c]	148	83.7	55.06	55.1	3.59	3.8	19.03	19.3

[a] Recrystallized from boiling water. [b] Precipitated from chloroform solution by benzene. [c] Precipitated from chloroform solution by petroleum ether.

TABLE IV

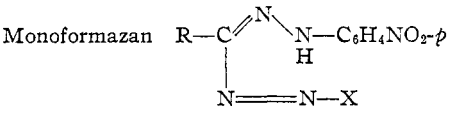
DITETRAZOLIUM CHLORIDES, 

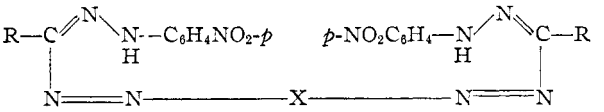
	X	R	Empirical formula	M.p., °C., dec.	Yield, %	Carbon Calcd.	Carbon Found	Analyses, % Hydrogen Calcd.	Hydrogen Found	Nitrogen Calcd.	Nitrogen Found
VIIIA	*p*-Biphenylene	C_6H_5	$C_{38}H_{26}N_{10}O_4Cl_2\cdot 2^1/_2H_2O$	22	70.0	56.86	56.84	3.90	4.11	17.46	17.46
IXA	*p*-Biphenylene	*p*-$NO_2C_6H_5$	$C_{38}H_{24}N_{12}O_8Cl_2\cdot C_3H_8O_2\cdot 2H_2O$	234	57.4	51.31	51.4	3.78	3.7	17.51	17.0
XA	3,3′-$(CH_3O)_2$-4,4′-biphenylene	C_6H_5	$C_{40}H_{30}N_{10}O_6Cl_2\cdot 3H_2O$	156	84.5	55.11	54.73	4.16	4.42	16.07	15.72[a]
XB			$C_{40}H_{30}N_{10}O_6Cl_2\cdot C_3H_7OH$	211	..	58.84	59.3	4.36	4.1		
XC			$C_{40}H_{30}N_{10}O_6Cl_2$	..	..	58.75	58.11	3.70	4.08		
XD			$C_{40}H_{30}N_{10}O_6Cl_2OH$	134	..	60.11	60.26	3.91	4.19	17.52	17.82
XIA	3,3′-$(CH_3O)_2$-4,4′-biphenylene	*p*-$NO_2C_6H_5$	$C_{40}H_{28}N_{12}O_{10}Cl_2$[b]	159	74.0	52.93	52.7	3.11	3.3	18.52	18.6

[a] Calcd.: Cl, 8.14. Found: Cl, 8.24. [b] Precipitated from methanol solution by isopropyl alcohol.

TABLE V

ABSORPTION SPECTRA OF FORMAZANS[a]

Monoformazan 

Diformazan 

	X	R	Absorption maxima, mμ			
			λ_{max}	ϵ_{max}	λ_{max}	ϵ_{max}
I	*p*-Biphenylyl	C_6H_5	375	19,800	500	18,600
II	*p*-Biphenylyl	*p*-$NO_2C_6H_5$	375	52,600	470	41,700
III	3,3′-$(CH_3O)_2$-4-biphenylyl	C_6H_5	375	8,500	520	12,200
IV	3,3′-$(CH_3O)_2$-4-biphenylyl	*p*-$NO_2C_6H_5$	390	30,500	510	30,400
VII	[2,5-$(CH_3O)_2$-4-*p*-NO_2-phenylazo]-phenyl	C_6H_5	370	9,600	460	8,900[b]
VIII	*p*-Biphenylene	C_6H_5	355	37,900	510	55,000

[a] 10^{-5} *M* solution in chloroform. [b] An additional maximum: λ_{max} 620 mμ, ϵ_{max} 20,100.

diformazan (IX).—A tetrazotized solution of benzidine hydrochloride 2.57 g. (0.01 mole) was prepared as described in the preparation of compounds I and VIII. As this solution was added to a solution of 5.72 g. (0.02 mole) of *p*-nitrobenzaldehyde *p*-nitrophenylhydrazone dissolved in 60 cc. of dimethylformamide the hydrazone soon partly precipitated out and the mixture became a deep red paste. When the potassium hydroxide was added the paste became thinner and the red particles turned black. After the reaction mixture was stirred for half an hour it was filtered and due to the pasty character of the mixture, filtration was very slow. The filtrate thus obtained was poured into 500 cc. of methanol to get a black precipitate which decomposed slowly at about 165°. The crude product weighed 1.17 g. (25.1%).

The residue from the above reaction mixture obtained after filtration was washed with 200 cc. of dimethylformamide then with 300 cc. of boiling water and again with dimethylformamide until the washings turned from deep green to light red. A black powder was obtained which melted at 267° dec. and weighed 3.84 g. (49.3%). The analytically pure sample was made by dissolving it in boiling dimethylformamide and precipitating it by adding two volumes of methanol. The precipitate was then washed with acetone until the washings were slightly purple, m.p. 269° dec. A diformazan IX was indicated by analysis.

2,2′,5,5′-Tetra-*p*-nitrophenyl-3,3′-(*p*-biphenylene)-ditetrazolium chloride (IXA).—The oxidation of diformazan IX (0.78 g., 0.001 mole) suspended in 15 cc. of methyl cellusolve by isoamyl nitrite took four hours in an ice-bath using a magnetic stirrer. A light brown precipitate was obtained after filtration, weighed 0.82 g., m.p. 232° dec. For analytical purposes it was dissolved in a minimum amount of dimethylformamide and reprecipitated by 20 times its volume of dioxane. The recovery was 0.55 g. (57.4%), it decomposed at 234°. Analysis showed that the compound contained both methyl cellosolve and water in the molecule IXA.

Oxidation by mercuric oxide at 100° with a suspension of VIII in dimethylformamide only led to decomposition but no product VIIIA. Lead tetraacetate could be used for oxidation but it was very difficult to remove all the lead salt from the product.

2-*p*-Nitrophenyl-5-phenyl-3-(3,3′-dimethoxy-4-biphenylyl)-formazan (III) and 2,2′-Di-*p*-nitrophenyl-5,5′-diphenyl-3,3′-(3,3′-dimethoxy-4,4′-biphenylene)diformazan (X).—A suspension of 12.69 g. (0.04 mole) of finely powdered *o*-dianisidine hydrochloride in 60 cc. of water and 10 cc. of concentrated hydrochloric acid was tetrazotized with a solution of 6.07 g. (0.088 mole) of sodium nitrite dissolved in 15 cc. of water. The resulting mixture was added to a solution of 19.30 g. (0.08 mole) of benzaldehyde *p*-nitrophenylhydrazone dissolved in 180 cc. of tetrahydrofuran followed by a solution of 8.98 g. (0.08 mole) of potassium hydroxide.

After the reaction mixture was stirred for half an hour, 80 cc. of water and then 180 cc. of methanol were added and the mixture filtered to yield a black precipitate with gold reflex. The precipitate was washed with about 500 cc. of methanol until the washings turned from dark to light red. Then it was washed with 600 cc. of boiling water and again with methanol. It weighed 16.6 g. after drying.

The black precipitate was extracted with 340 cc. of boiling dioxane. The filtered extract was added to 250 cc. of water. A black precipitate separated (monoformazan III), m.p. 202–204° dec., weighed 9.90 g. (51.6%). An analytical sample was made by dissolving the compound in tetrahydrofuran and reprecipitating it with half its volume of water, m.p. 210° dec.

The insoluble portion from the dioxane extraction was proved to be diformazan X, weighed 5.45 g. (18.2%), m.p. 252–253° dec. An analytical sample was made by dissolving the compound in boiling dimethylformamide and running the filtered solution into five times its volume of acetone. A fine black precipitate was obtained, m.p. 257° dec.

Attempted Reaction of Formazan and Tetrazolium Salt.—A solution of monoformazan III in tetrahydrofuran was shaken for several minutes with an aqueous tetrazotized solution of *o*-dianisidine. After evaporating out the low boiling tetrahydrofuran the solution was neutralized with aqueous sodium hydroxide and decolorized with carbon black. It was expected that the filtrate would now contain some tetrazolium salt as a result of oxidation of III (the tetrazonium salt from *o*-dianisidine being reduced). To the filtrate was now added some chloroform and some crystals of sodium hydrosulfite with shaking. Any tetrazolium salt present would not be reduced back to the formazan and impart a purple color to the chloroform layer. But the chloroform layer remained colorless and hence the test was negative.

When in place of *o*-dianisidine, the stabilized salt Naphthanil Diazo Blue B[15] was used directly in the above reaction, only the monoformazan III was obtained in a 35.3% yield, and no diformazan X could be isolated.

2-*p*-Nitrophenyl-5-phenyl-3-(3,3′-dimethoxy-4-biphenylyl)-tetrazolium Chloride (IIIA) and 2,2′-Di-*p*-nitrophenyl-5,5′-diphenyl-3,3′-(3,3′-dimethoxy-4,4′-biphenylene)-ditetrazolium Chloride (XA).—The oxidation of 0.48 g. (0.001 mole) of monoformazan III took half an hour at room temperature. A dark brown solution was obtained. To this solution was added enough hot water until it became cloudy. It was then boiled with carbon black. On acidification with hydrochloric acid a gummy yellow solid was obtained. This was twice purified by boiling with water to give a light brown glossy solid, m.p. 134° dec., yield 0.16 g. (29.0%). Analysis indicated that it contained two molecules of water (IIIA), Table III.

The oxidation of 3.74 g. (0.005 mole) of diformazan X took two hours at room temperature. The resulting deep brown solution was treated with carbon black and evaporated to a heavy gum. This was dissolved in 20 cc. of hot ethanol and on adding 200 cc. of anhydrous ether a light straw colored precipitate was obtained. It weighed 2.77 g., m.p. 162° dec. After concentrating the filtrate from the main product, a second crop of 0.92 g. was obtained, m.p.

150°. The total yield was 3.69 g. (84.5%). Wet ether was not used because it precipitated a gummy product. An analytical sample was prepared by repeating the precipitation as just described, m.p. 156° dec. Analysis showed that it contained three molecules of water (XA) (Table IV).

The water of crystallization in compound XA could be replaced by isopropyl alcohol (XB) (Table IV) by boiling the hydrate in this solvent. The newly formed compound had a melting point of 211° dec. This new solvent in the molecule could be removed to form an anhydrous compound XC by drying the solvate XB at 100° under vacuum for five and one half hours. When the hydrate was dried at 100° to constant weight under vacuum, analysis indicated that one of the two chloride ions was converted to the hydroxide ion XD (Table IV).

Compound XA could also be prepared by oxidizing the diformazan X with lead tetraacetate, but the compound could then only be purified by repeated recrystallizations from boiling water to remove the last trace of lead salt. The yield as a result was very low.

When the crude product of formazan containing both the mono-(III) and diformazan (X) was subjected to oxidation a mixture of the two corresponding tetrazolium salts were obtained which could not be separated after repeated precipitations employing different organic solvents. The mixture had a melting point in the range of 225–260° dec. When it was repeatedly recrystallized from boiling water only a negligible amount of the ditetrazolium salt XA was isolated.

2,5-Di-*p*-nitrophenyl-3-(3,3'-dimethoxy-4-biphenylyl)-formazan (IV) and 2,2',5,5'-Tetra-*p*-nitrophenyl-3,3'-(3,3'-dimethoxy-4,4'-biphenylene)-diformazan (XI).—*o*-Dianisidine (3.17 g., 0.01 mole) was used for the reaction. The hydrazone (5.72 g., 0.02 mole) was dissolved in 200 cc. of dimethyl sulfoxide. The crude product weighed 2.35 g. and the reaction was carried on in the usual manner.

Overnight extraction using the Soxhlet extractor with 200 cc. of tetrahydrofuran left 1.02 g. (12.2%) of a deep black powdered product that did not melt up to 300°. An analytical sample was prepared by leaching the powder with boiling dimethylformamide and then washing it with acetone. Upon analysis it was shown to be diformazan XI. The tetrahydrofuran extract on standing two days at room temperature deposited about 0.2 g. (3.8%) of purplish black crystals that melted at 239° dec. This was shown to be the monoformazan IV. The extract was next evaporated to dryness and the solid reprecipitated from its solution in dimethylformamide by methanol. A purplish black powder was obtained, weighed 1.23 g. (23.4%), m.p. 182°; after a second precipitation, m.p. 185° dec.

The compounds that had melted at 185° dec. and at 239° dec. were found to be polymorphous forms, because their solutions (10^{-5} *M*) in chloroform gave two identical and overlapping absorption curves in the wave length region 250–650 mμ. The lower melting compound on oxidation gave the corresponding tetrazolium salt IVA.

2-*p*-Nitrophenyl-5-phenyl-3-(2,5-dimethoxy-4-*p*-nitrophenylazo)-phenylformazan (VII).—Stabilized 2,5-dimethoxy-4-*p*-nitrophenylazobenzenediazonium chloride (20% pure) (17.49 g., 0.01 mole) was added to a solution of 2.41 g. (0.01 mole) of benzaldehyde *p*-nitrophenylhydrazone dissolved in 160 cc. of pyridine. The black precipitate was washed by boiling water and then by acetone until the washings were light brown, only 0.3 g. (5.1%) of the product was obtained. An analytical sample was prepared as fine black needles after overnight extraction using chloroform as a solvent with a Soxhlet extractor, m.p. 228° dec. Analysis showed that it was the formazan VII.

Acknowledgment.—Acknowledgment is due to Mr. James C. Stevens for technical assistance, and Dr. Eustace deSouza who collaborated in the histochemical studies.

LEOMINSTER, MASS.

[CONTRIBUTION FROM THE L. G. RYAN RESEARCH LABORATORIES OF MONSANTO CANADA LIMITED]

A New Molecular Rearrangement. II. Confirmation of Structures and Extension of the Rearrangement Reaction

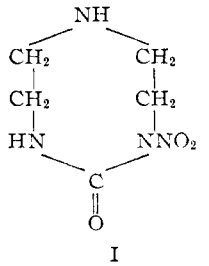
BY A. F. McKAY, W. G. HATTON AND R. O. BRAUN

RECEIVED JUNE 12, 1956

The hydrochloride obtained[1] in the treatment of 1-(β-hydroxyethyl)-2-nitriminoimidazolidine with thionyl chloride has been identified as 1-(β-aminoethyl)-3-nitro-2-imidazolidone hydrochloride. The nitrate salt of the latter compound has been synthesized by two different routes. On refluxing with propanol it is rearranged to 1-(β-nitraminoethyl)-2-imidazolidone. 1-(β-Nitraminoethyl)-2-imidazolidone was prepared from 1-nitro-2,3,5,6-tetrahydro-1-imidaz[1,2-a]imidazole nitrate by solution in aqueous alkali. The recently[1] described molecular rearrangement has been found to occur also in the reactions of amines or potassium cyanide with 1-(β-chloroethyl)-2-nitriminoimidazolidine in aqueous medium.

Recently[1] a new molecular rearrangement reaction was described. This rearrangement was observed with 1-(β-hydroxyethyl)-2-nitriminoimidazolidine on treatment with thionyl chloride. One of the isolated products was erroneously assigned structure I on the basis of a synthesis, which was found later to involve another molecular rearrangement.[2] It has been proven now that the rearranged product from 1-(β-hydroxyethyl)-2-nitriminoimidazolidine is the hydrochloride of 1-(β-aminoethyl)-3-nitro-2-imidazolidone. The latter compound on refluxing in ethanol rearranged to 1-(β-nitraminoethyl)-2-imidazolidone (V).

Since the mechanism[3] proposed for this rearrangement reaction involved the bicyclic intermediate II it was thought that 1-(β-nitraminoethyl)-2-imidazolidone (V) could be synthesized from the similar bicyclic compound 2,3,5,6-tetrahydro-1-imidaz[1,2-a]imidazole (III). Compound III on nitration in an absolute nitric acid–acetic anhydride medium gave 1-nitro-2,3,5,6-tetrahydro-1-imidaz-[1,2-a]imidazole nitrate (IV). This nitrate salt of the bicyclic compound IV was quite reactive and it



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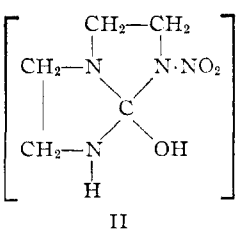

II

(1) A. F. McKay and J. R. Gilpin, THIS JOURNAL, **78**, 486 (1956).

(2) This synthesis will be reported later.

(3) It was pointed out previously[1] that this reaction could occur stepwise through the formation of a carbonium ion (SN1) or by the simultaneous progression of the several steps. This simultaneous making and breaking of the bonds would lead to rearrangement by an SN2 mechanism.