Experimental

Oxidation of Bis-(*p*-methoxyphenyl)-cyclohexene, I.—The cyclohexene, m.p. 86.5–88°, 2.98 g., was dissolved in 100 ml. of dioxane. The solvent had been purified by refluxing over sodium metal during forty-eight hours and distilling; it was stable toward dilute potassium permanganate. A saturated aqueous solution containing 6.32 g. of potassium permanganate was added in small portions. The solution was stirred and the *p*H measured with glass and calomel electrodes. As oxidation proceeded 1 *N* hydrochloric acid was added as necessary to maintain *p*H within the limits 7.0–8.5. An additional 50 ml. of dioxane was added at the half-way point to keep the organic material in solution. When the last permanganate had been added the purple color persisted for an hour during which the *p*H was constant. The solution was acidified, sufficient sodium metabisulfite added just to clear the solution; it was extracted six times with ether. The ethereal concentrate was dried by distilling benzene from it and treated with 60 ml. of absolute alcohol, 6.0 ml. of glacial acetic acid and 6 g. of Girard reagent "T." After two hours at reflux the solution was poured into 100 ml. of ice water and 9.54 g. of sodium bicarbonate and the mixture extracted with ether. Removal of ether left 1.45 g. of oily needles; partition of this material between saturated sodium bicarbonate and ether yielded 0.43 g. of an acid which was crystallized three times from benzene, m. p. 182.5–184.0°; m. p., with recrystallized anisic acid, 182.6–184.5° (micro).

The aqueous solution containing the carbonyl derivatives was made about one-half normal with hydrochloric acid and extracted with ether after standing eight hours. Back extraction of the ether layer with sodium bicarbonate solution, acidifying and extracting again with ether yielded 0.25 g. of crude acid which was recrystallized several times from benzene, with decolorization, to yield colorless plates, m. p. 141.2–142.0° (micro): m. m. p. 141.2–142.2° (micro) with purified synthetic γ-*p*-methoxybenzoylbutyric acid.[14]

p-Bromophenacyl γ-*p*-methoxybenzoylbutyrate was prepared with 9 mg. of the keto acid from oxidation. It was washed with dilute sodium hydroxide and recrystallized three times from alcohol-water, m. p. 106.3–107.5°. The same derivative prepared from the synthetic acid on a large scale melted at 108.1–108.9°; melting point of mixture, 106.8–108.0° (micro).

Anal. Calcd. for $C_{20}H_{19}O_5Br$: C, 57.29; H, 4.57. Found: C, 57.26; H, 4.58.

Oxidation of Bis-(*p*-methoxyphenyl)-cyclohexene, II.—Ozone in oxygen was passed through a solution of 70 mg. of the cyclohexene, m. p. 83–87°, in 80 ml. of ethyl acetate for two minutes, when absorption was complete as judged by iodine liberation in a potassium iodide solution beyond the absorption vessel. The ethyl acetate solution was shaken with water, dried and evaporated. The white, crystalline residue, 65 mg., was recrystallized twice from ethyl acetate; m. p. 145.0–146.5°. A mixture with synthetic 1,4-bis-(*p*-methoxybenzoyl)-butane,[15] m. p. 146.8°, was not depressed.

The dioxime was prepared from hydroxylamine hydrochloride in alcoholic potassium hydroxide with refluxing for ninety minutes, and purified from ethyl acetate; m. p. 201.5–202.5°. A sample of the synthetic diketone gave a dioxime, m. p. 205.5–206°. A mixture of the two melted in an intermediate range but showed no depression. Literature values for this dioxime vary from 201.5°[15] to 208°.[16]

Summary

The structures of 1,2-bis-(*p*-methoxyphenyl)-cyclohexene and 2,3-bis-(*p*-methoxyphenyl)-cyclohexene have been clarified through oxidation to known compounds. Ultraviolet absorption spectra of several derivatives are in agreement with the structures given.

[14] Plant and Thomlinson, *J. Chem. Soc.*, 856 (1935).

[15] Fuson, Kuykendall and Wilhelm, THIS JOURNAL, **53**, 4187 (1931).

[16] Zanden, de Vries and Westerhof, *Rec. trav. chim.*, **62**, 283 (1943).

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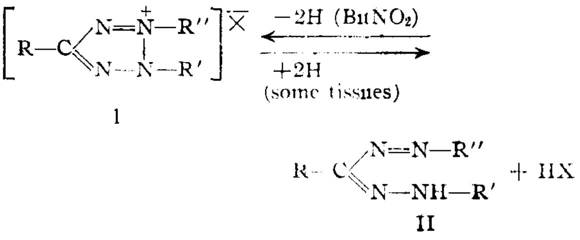
[CONTRIBUTION FROM THE CHEMICAL LABORATORY OF IOWA STATE COLLEGE]

Synthesis of Some Substituted Tetrazolium Chlorides[1,2]

BY SIDNEY W. FOX AND ELSIE HEMMINGSON ATKINSON

Although tetrazolium salts have been known to organic chemistry since 1894[3] and a variety of these compounds has since been synthesized, the biological properties of the tetrazolium salts have received attention only in the last decade.[4] The intrinsic characteristics responsible for the unique behavior of the tetrazolium series include the following:

1. They represent one of the few organic types colorless in the oxidized form, I, but colored in the reduced form (formazan, II).



2. The tetrazolium salt is frequently water-soluble, whereas the formazan is not, and the latter will, under proper circumstances, deposit as a colored material in the tissue which reduces it.
3. The oxidation-reduction potentials of many of the tetrazoles overlap values found in living systems.
4. The tetrazolium salts and formazans are sufficiently innocuous that their properties may be manifest directly in thriving cultures of micro-

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[2] From a thesis submitted by Elsie Hemmingson Atkinson to the Graduate School of Iowa State College in partial fulfillment of the requirements for the degree of Master of Science, 1949.

[3] von Pechmann and Runge, *Ber.*, **27**, 2920 (1894).

[4] Kuhn and Jerchel, *ibid.*, **74B**, 941 (1941).

organisms[5] and in studies in mammals.[6] The above attributes underlie the current investigative interest in the biological applications of tetrazolium salts, including their use as germinability indicators,[7] in studies of viability of sperm, and as localization agents in oncology.[8,6,9]

Many types of tetrazolium salts described in the literature result from variation in the R, R′ and R″ groups. Since in biological applications the 2,3,5-triphenyltetrazolium chloride has been most used, the point of departure in this study was the substitution of iodo and nitro groups in the phenyl rings. The corresponding formazans of interest in the present study are listed in Table I.

TABLE I

PROPERTIES OF FORMAZANS

Formazan	M. p., °C. Found	M. p., °C. Reported	N, % Calcd.	N, % Found
N,N′,C-Triphenyl	173–174	173.5[a]		
N-(*p*-Iodophenyl)-N′,C-diphenyl	187–188	185–186[b]		
N,N′-Di-(*p*-iodophenyl)-C-phenyl	169–170		10.01	9.88
N-(*p*-Iodophenyl)-N′-(*p*-nitrophenyl)-C-phenyl	185–186		14.88	14.73

[a] von Pechmann, *Ber.*, **27**, 1690 (1894). [b] Ref. 11.

Spectral absorption curves for the formazans in dioxane are presented in Fig. 1. It is clear that in the absence of interfering materials, wave lengths of about 500 mμ would be especially suitable for utilization of these properties in assays.

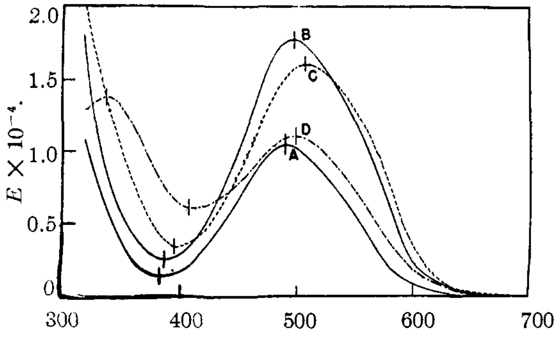

Fig. 1.—Absorption curves of formazans in dioxane solutions of 5×10^{-5} molarity: A is N,N′,C-triphenylformazan; B is N,C-diphenyl-N′-(*p*-iodophenyl)-formazan; C is N,N′-di-(*p*-iodophenyl)-C-phenylformazan; D is N-(*p*-iodophenyl)-N′-(*p*-nitrophenyl)-C-phenylformazan.

The tetrazolium salts and some of their properties are listed in Table II.

For the 2,5-diphenyl-3-(*p*-iodophenyl)-tetrazolium chloride, another melting point than that found here has been recorded.[10] The melting point observed in this Laboratory for the iodo derivative is within the 34° range which covers these four closely related chlorides. The value reported by Seligman, *et al.*, is 60° below that range. Since a comparably low value of 180° has been found for a hydrochloride hydrate of the parent triphenyltetrazolium in this Laboratory,[9] attempts to obtain an hydrochloride hydrate for the iodo compound, as a possible explanation of the discrepancy, were made. Such attempts were unsuccessful.[11]

The last column in Table II presents solubility values for solutions of tetrazolium chlorides which may have been in equilibrium but were not known to be. While such values are not as dependable as those obtained from concordant successive values from initially supersaturated and initially undersaturated solutions, they represent approximations of solubilities under conditions in which they are likely to be of practical interest.

Experimental

All melting points are corrected.

All N analyses were performed by micro-Dumas.

2,3,5-Triphenyltetrazolium Chloride and Di-(2,3,5-triphenyltetrazolium Chloride) Hydrochloride Hydrate.—The preparation of these compounds has already been described.[9] Not only does the parent triphenyltetrazolium chloride yield a di-(tetrazolium chloride) hydrochloride hydrate of lower melting point, but two melting points have been found for the chloride both in the literature[3,4] and in the present study. While most samples prepared here have had a melting point of 243° (dec.), a few samples tested immediately after removal from the oven at 105–110° had the value 263° (dec.). On standing in the air or in a desiccator such samples later gave 243° (dec.).

N-(*p*-Iodophenyl)-N′,C-diphenylformazan.—In the simplified process employed, 21.9 g. (0.1 mole) of *p*-iodoaniline and 20 ml. of water were warmed together until the *p*-iodoaniline melted. Thirty ml. of concentrated hydrochloric acid solution was added slowly with vigorous shaking. The mixture was cooled rapidly with stirring, so that the *p*-iodoaniline hydrochloride precipitated in fine crystals. The suspension was cooled to 0–5°, and was then diazotized with a cold solution of 7.0 g. of sodium nitrite in 15 ml. of water to a starch-iodide end-point.

The diazotized *p*-iodoaniline and a solution of 19.6 g. of benzaldehyde phenylhydrazone dissolved in 1 l. of ethanol were added simultaneously with vigorous stirring to a solution of 28 g. of potassium hydroxide in 100 ml. of ethanol. The temperature was maintained at 20–30°. A red color developed at once, and within a few minutes a red precipitate was observed. Stirring was continued for an hour, and the mixture was then allowed to stand for at least an additional two hours. The mixture was then filtered, washed with about 200 ml. of water, followed by 100 ml. of ethanol. The formazan was reprecipitated by dissolving in hot dioxane (about 20 ml. boiling dioxane/g. of formazan) and by adding about one-third the volume of water slowly with stirring. The product was dark red with a metallic green luster, and had a m. p. of 187–188°. The yield was 45–60%.

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(5) Kuhn and Jerchel, *Ber.*, **74B**, 949 (1941).

(6) Straus, Cheronis and Straus, *Science*, **108**, 113 (1948).

(7) Lakon, *Ber. deut. botan. Ges.*, **60**, 299 (1942).

(8) Fox, *Iowa Agr. Expt. Sta. Rept. on Agr. Res.*, 198 (1948).

(9) E. H. Atkinson, Melvin and Fox, *Science*, **111**, 385 (1950).

(10) Seligman, Gofstein and Rutenburg, *Cancer Res.*, **9**, 366 (1949).

(11) Through the kindness of Dr. Seligman, who supplied a sample of his product, and of Dr. R. E. Rundle who prepared Debye–Scherrer photographs, the possibility of polymorphism of the two preparations in question has been recently investigated. The material of m. p. 232–233° (dec.) produced a definitely crystalline pattern, whereas an amorphous pattern resulted from the other material.

TABLE II

PROPERTIES OF TETRAZOLIUM CHLORIDES

	M. p., °C. (dec.)		Nitrogen, %		Chlorine, %		Iodine, %		Non-equil. soly., g./100 g. water at 25°
Tetrazolium chloride	Found	Reported	Calcd.	Found	Calcd.	Found	Calcd.	Found	
2,3,5-Triphenyl-	243	243[a] 263[b]							25
Di-(2,3,5-triphenyl) hydrochloride hydrate	180 (melt) followed by 243[c]		15.48	15.70[c] 15.43[c]	14.69	14.70[c] 14.58			16
2,5-Diphenyl-3-(*p*-iodophenyl)	232–233	170[d]	12.16	12.08 12.04	7.70	7.71 7.63	27.55	27.35 27.34	0.7
2,3-Di-(*p*-iodophenyl)-5-phenyl	256		9.55	9.58	6.04	6.08 6.01	43.27	43.27 43.27	0.1
2-(*p*-Iodophenyl)-3-(*p*-nitrophenyl)-5-phenyl	229		13.85	13.74	7.01	6.96 7.05	Not detd.		0.5

[a] Ref. 13. [b] Ref. 4. [c] Ref. 9. [d] Ref. 10.

2,5-Diphenyl-3-(*p*-iodophenyl)-tetrazolium Chloride.—Ten grams of the formazan was dissolved in 250 ml. of chloroform, and 250 ml. of methanol was added. To this mixture 6 ml. of freshly distilled butyl nitrite was added. Ten ml. of concentrated hydrochloric acid solution was added slowly with stirring. Some heating and effervesence occurred, and the solution was cooled in some cases. After a few minutes the red color of the solution disappeared, and a clear dark brown solution resulted. This became somewhat lighter upon standing.

After four hours the solvent was distilled off under reduced pressure. One hundred ml. of dioxane was added, and the flask was swirled to loosen the product. The dioxane was removed by distillation under reduced pressure. Crystallization occurred during this stage. Fifty ml. of dioxane was added and the product was filtered. Recrystallization could be effected by dissolving the solid in the minimum amount of alcohol and reprecipitating with added ether, or by solution in hot water, decolorization with charcoal, and precipitation by the addition of concentrated hydrochloric acid solution. The yield of tetrazolium compound was 55–60%.

N,N'-Di-(*p*-iodophenyl)-C-phenylformazan.—Eight grams of benzaldehyde *p*-iodophenylhydrazone[12] was converted in a similar fashion to the diiodo compound. The yields of several such preparations were 5.8–7.0 g. (42–51%). The formazan was recrystallized from a dioxane–water mixture.

2,3-Di-(*p*-iodophenyl)-5-phenyltetrazolium Chloride.—By methodology similar to the above, 10 g. of the N,N'-di-(*p*-iodophenyl)-C-phenylformazan was oxidized to the tetrazolium salt. In the first preparation the residue was dissolved in hot water, decolorized with charcoal, and precipitated by the addition of concentrated hydrochloric acid. It was found, however, that the material was not readily soluble in water. The yield of tetrazolium salt was 20%. In a second oxidation of formazan, the residue obtained after removal of the solvent was dissolved in 150 ml. of ethanol, decolorized with charcoal and filtered. To the filtrate were added 500 ml. of water and about 50 ml. of hydrochloric acid. Five g. (47%) of the tetrazolium salt was thus obtained. Further recrystallizations were carried out on this material from water solutions to which hydrochloric acid was added. Recoveries ranging from low values to 90% were obtained.

N-(*p*-Iodophenyl)-N'-(*p*-nitrophenyl)-C-phenylformazan.—To 40 g. of potassium hydroxide dissolved in 250 ml. of ethyl alcohol were added simultaneously with stirring a solution of 24 g. of benzaldehyde *p*-nitrophenylhydrazone[13] dissolved in a mixture of 1 l. of ethanol and 100 ml. of dioxane, and a solution of diazotized *p*-iodoaniline. The latter was prepared by diazotizing 22 g. of *p*-iodoaniline in 100 ml. of concentrated hydrochloric acid with a solution of 6.9 g. of sodium nitrite in 15 ml. of water to a starch–iodide end-point. The mixture was allowed to stand overnight and was then filtered. The product was reprecipitated by dissolving it in 300 ml. of boiling dioxane, and then adding 100 ml. of water slowly with stirring. The yield was 17 g. (36%). The product melted at 185–186°. In another preparation the yield was increased to 58% by the recovery of additional formazan by the addition of water to the filtrate.

A magenta color developed during coupling in the presence of potassium hydroxide. In a later preparation of this formazan, dioxane was used to dissolve the *p*-nitrophenylhydrazone (40 ml. per g.), and sodium acetate was used in place of potassium hydroxide. These modifications were based upon the methods of Kuhn and Jerchel.[4] The magenta coloration did not develop, and the yield was over 50%.

2-(*p*-Iodophenyl)-3-(*p*-nitrophenyl)-5-phenyltetrazolium Chloride.—The method of von Pechmann and Runge,[3] with butyl nitrite, was followed for the oxidation procedure. Fifteen grams of the formazan gave a yield of 3.2 g. (20%).

Absorption Curves.—These were plotted from data obtained in the region of 320–1000 mμ on a Beckman spectrophotometer (Model DU, quartz). The formazans were made up in dioxane solutions of 5×10^{-5} molarity.

Acknowledgment.—The analytical services of Mr. Armand McMillan were of help, both in identification of the formazans and in unsuccessful attempts to identify tetrazolium salts by several types of reductive Kjeldahl analysis, most of which returned approximately three nitrogens of the four in the ring. Mr. Samuel Melvin conducted the first syntheses of the 2,5-diphenyl-3-(*p*-iodophenyl)-tetrazolium chloride.

Summary

The synthesis and physical properties of several triphenyltetrazolium chlorides, with substituents in the phenyl rings, and of the intermediate formazans, have been described. Spectral curves of the formazans have been presented.

AMES, IOWA RECEIVED NOVEMBER 11, 1949

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(12) Fichter and Philipp, *J. prakt. Chem.*, **74**, 312 (1906).

(13) Biltz and Sieden, *Ann.*, **324**, 321 (1902).