2-(*m*-Nitrophenyl)benzoxazole was obtained from the first elute fraction by removal of the solvent (1.84 g., m.p. 211.5~212°).

Summary

Nickel peroxide, readily obtainable by the treatment of an aqueous solution of nickel sulfate with sodium hypochlorite in an alkaline solution, was shown to be a useful oxidizing agent for a preparation of substituted 2-phenylbenzoxazoles from the corresponding Schiff's bases in benzene or ether.

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155. Masahiro Nakadate, Chikako Matsuyama, and Michiya Kimura : Fundamental Studies on Clinical Chemistry. VII.[*1] Janovsky Reaction of Polynitrodiphenyl Compounds.

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Since Janovsky[1] found that *m*-dinitrobenzene produced a color with acetone in the presence of alkali, the reactions of various nitrobenzene derivatives with active methylene compounds have been studied by many groups of workers. *m*-Dinitrobenzene,[2] 3,5-dinitrobenzoic acid,[3] 1,3,5-trinitrobenzene,[4] picric acid,[5] 2,2′,4,4′-tetranitrobiphenyl[6] and some other *m*-dinitrobenzene derivatives have been employed for the chemical analysis of different kinds of active methylene compound. These methods, however, still have some defects in the following respects : (1) instability of coloration,[2] (2) interferences by some non-active methylene compounds,[7] (3) larger blank values of reagents in the cases of some polynitrobenzene derivatives, and (4) similar absorption spectra for the different active methylene compounds.[8] Moreover, sensitive methods have been required for micro amount of samples such as those from biological origins.

The present paper describes the studies on the polynitrodiphenyl derivatives which have been expected to give sensitive, stable and bathochromic coloration on the basis of preceding studies in this laboratory. Fifteen polynitrodiphenyl compounds such as 4,4′-dinitro-, 2,4-dinitro, 2,2′,4,4′-tetranitro-derivatives of biphenyl, diphenylmethane, diphenyl ether, diphenyl sulfide, diphenylsulfone, and stilbene were examined in anticipation of bathochromic light absorption in their color reactions with active methylene

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[*1] Part VI : Steroids, **4**, 255 (1964).

[*2] Kita-12-Jo, Nishi-5-Chome, Sapporo (中舘正弘, 松山智郁子, 木村道也).

[1] J. V. Janovsky : Ber., **19**, 2158 (1886); **24**, 971 (1891).

[2] *a*) W. Zimmermann : Z. physiol. Chem., Hoppe-Seyler's **233**, 257 (1935). *b*) W. D. Raymond : Analyst, **64**, 113 (1937). *c*) E. E. Epsteine : Clin. Chim. Acta, **7**, 735 (1962).

[3] *a*) W. D. Langley, M. Evans : J. Biol. Chem., **115**, 333 (1936). *b*) E. L. Pratt : Anal. Chem., **24**, 1324 (1952).

[4] M. Kimura : Yakugaku Zasshi, **71**, 991 (1951); T. Momose. *et al.* : *Ibid.*, **83**, 143 (1963).

[5] O.Folin : Z. physiol. Chem., Hoppe-Seyler's **41**, 223 (1904); N. Koishi : Seikagaku, **28**, 23 (1956) : T. Momose, *et al.* : Rinsho Kensa, **5**, 451 (1961).

[6] R. Mauli, Ch. Tamm, T. Reichstein : Helv. Chim. Acta, **40**, 284 (1957).

[7] F. Rappaport, J. Fischl, N. Pinto : Clin. Chem., **6**, 16 (1960).

[8] A. G. Green : Clin. Chim. Acta, **7**, 674 (1962).

compounds under the Canbäck condition*[3] and 2,2′,4,4′-tetranitrobiphenyl (III) was also examined under the Zimmermann condition.*[3] Active methylene compounds such as, for instance, 17-ketosteroids, cardenolides and creatinine, which have different types of active methylene group in their molecules, can generally give color reactions of different intensities and stabilities with *m*-dinitrobenzene and its derivatives.[2b,2c,3b] Acetone and cyclohexanone were, therefore, selected as the most simple standards of active methylene compounds. Absorption curves and wave lengths of the maximum absorption of colors produced by these polynitro compounds with acetone and cyclohexanone under the presence of definite amount of alkali are shown in Fig. 1 and Table I, respectively. Rates of color development which indicate also the stability of colors produced are given in Fig. 2.

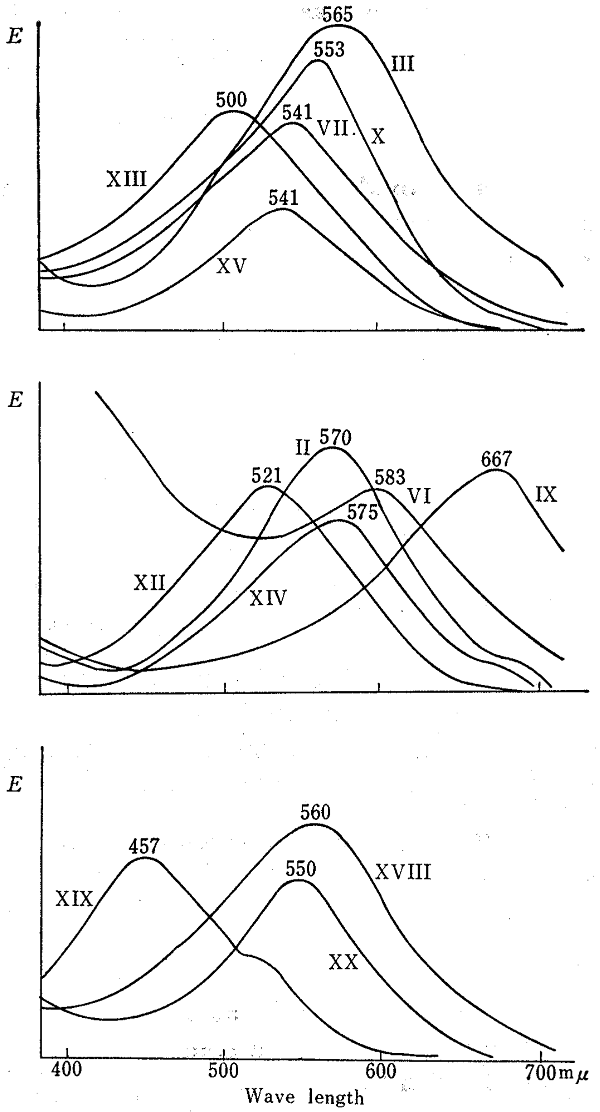

Fig. 1. Absorption Spectra of Color Reactions in Acetone

In the series of 4,4′-dinitro derivatives with an exception on bis(*p*-nitrophenyl)methane (IV), no Janovsky reactions were shown. As far as IV and bis(2,4-dinitrophenyl)methane (V) were concerned, their alkaline methylcellosolve solutions without any active methylene compounds gave rapidly an instable color having a maximum absorption at 588 mμ and a stable color having that at 702 mμ respectively.

In the ultraviolet absorption spectra of biphenyl compounds the *ortho*-substitution by alkyl groups affects some hypsochromic shift of their maximum absorptions with hypochromic changes.[9] 2-Nitrobiphenyl, on the contrary, shows a bathochromic shift as compared to biphenyl itself[10] and this is well explained by the increased resonance contribution of quinoid structures due to nitro group.[11] However, in the case of 2,4-dinitrobiphenyl (II) which is also supposed to be sterically hindered by *ortho*-nitro substituents, the maximum absorption exhibited a hypsochromic shift in comparison with that of 4-nitrobiphenyl ($\lambda_{max}=295$ mμ)[12] as expected. As to the visible regions, it was

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[*3] Norymbersky classified the Janovsky reaction according to the relative amount of reagents: Canbäck condition when the reaction is carried out with a large excess of the ketone and Zimmermann condition when it is carried out with a large excess of *m*-dinitrobenzene. C. S. Corker, J. K. Norymbersky, R. Thow: Biochem. J., **83**, 586 (1962).

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11) B. Williamson, W. H. Rodebush: J. Am. Chem. Soc., **63**, 3018 (1941).

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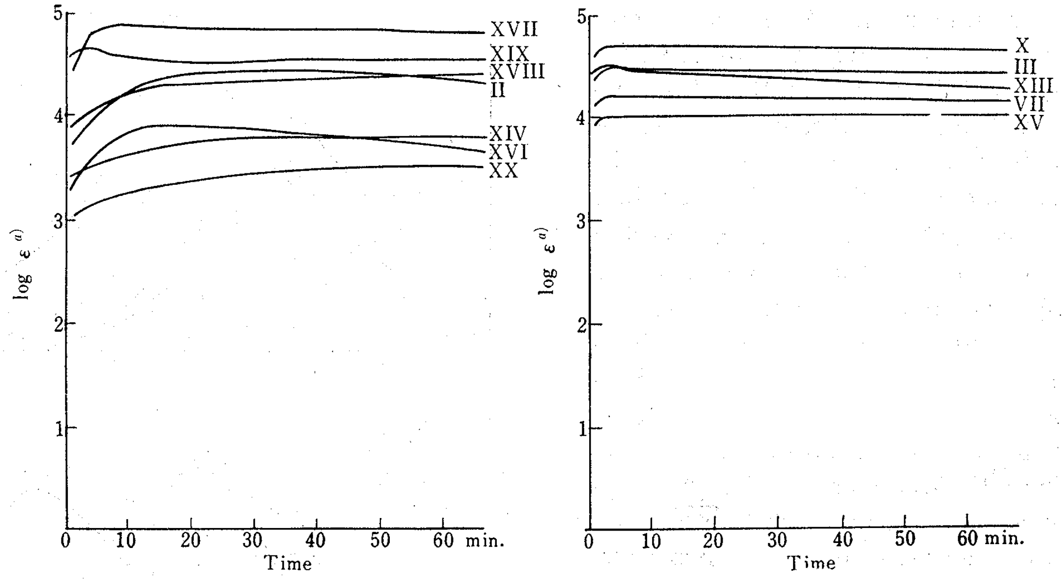

Fig. 2. Rates of Color Development in Acetone

a) Apparent extinction coefficient

observed that II exhibited more bathochromic shift of maximum absorption than that of the color produced by *m*-dinitrobenzene, but the apparent extinction coefficients smaller than expected. Most of the 2,4-dinitrodiphenyl compounds examined took generally more time for the development of stable colors.

TABLE I. Color Reactions and Ultraviolet Spectra of Polynitrodiphenyl Compounds

	with Acetone			with Cyclohexanone			Ultraviolet spectrum[b]		
	λ_{max} (mμ)	ε[a]	concn. (*M*)	λ_{max} (mμ)	ε[a]	concn. (*M*)	λ_{max} (mμ)	ε	concn. (*M*)
4,4′-Dinitrobiphenyl (I)[c]	—	—	—	—	—	—	308	19400	2×10^{-4}
2,4-Dinitrobiphenyl (II)	570	11700	3×10^{-4}	561	770	10^{-3}	286	10200	2×10^{-4}
2,2′,4,4′-Tetranitrobiphenyl (III)	565	18400	3×10^{-4}	550	20200	2×10^{-4}	240	34400	2×10^{-4}
Bis(*p*-nitrophenyl)methane (IV)	766	15300	5×10^{-4}	588	110	10^{-3}	275	19700	4×10^{-4}
Bis(2,4-dinitrophenyl)methane (V)	703	46800	10^{-4}	702	43500	10^{-3}	246	31000	2×10^{-4}
2,4-Dinitrophenyl phenyl ether (VI)	583	14700	2×10^{-4}	555	1100	10^{-3}	295	13300	4×10^{-4}
Bis(2,4-dinitrophenyl) ether (VII)	541	10100	10^{-3}	537	7960	5×10^{-4}	240	23000	3×10^{-4}
Bis(*p*-nitrophenyl) sulfide (VIII)[b]	—	—	—	—	—	—			
2,4-Dinitrophenyl phenyl sulfide (IX)	667	21400	4×10^{-4}	560	1220	10^{-3}	331	10900	5×10^{-4}
Bis(2,4-dinitrophenyl) sulfide (X)	553	52500	10^{-4}	540	13850	2×10^{-4}	242	23000	2×10^{-4}
Bis(*p*-nitrophenyl) sulfone (XI)[b]	—	—	—	—	—	—			
2,4-Dinitrophenyl phenyl sulfone (XII)	521	21600	3×10^{-4}	515	15630	3×10^{-4}	238	16800	5×10^{-4}
Bis(2,4-dinitrophenyl)sulfone (XIII)	500	17400	4×10^{-4}	525	16900	10^{-4}	243	32300	3×10^{-4}
2,4-Dinitrostilbene (XIV)	575	10000	10^{-3}	565	680	10^{-3}	245	1700	5×10^{-4}
2,2′,4,4′-Tetranitrostilbene (XV)	541	10700	10^{-3}	550	6980	10^{-3}	238	23000	3×10^{-4}
2,4-Dinitrotoluene (XVI)	576	8477	10^{-3}	545	450	10^{-3}	246	14100	5×10^{-4}
3,5-Dinitrotoluene (XVII)	570	83500	10^{-4}	560	770	10^{-3}	243	14000	4×10^{-4}
m-Dinitrobenzene (XVIII)	560	19250	2×10^{-4}	555	5360	5×10^{-4}	239	16800	5×10^{-4}
1,3,5-Trinitrobenzene (XIX)	457	26600	10^{-4}	528	14330	3×10^{-4}	229	24700	3×10^{-4}
3,5-Dinitrobenzoic acid (XX)	550	2590	10^{-3}	550	340	10^{-3}	238	20000	5×10^{-4}

a) Apparent extinction coefficient; b) No coloration under this procedure; c) in methylcellosolve.

2,2′,4,4′-Tetranitrobiphenyl (III) gave rapid and stable coloration with a larger apparent extinction coefficient. However, the expected bathochromic shift of maximum absorption was not shown. Ultraviolet spectra of the tetranitro compounds examined are shown in Table I, which proves some steric hindrances by the substituents located at 2,2′-positions as have been shown by alkyl radicals in the biphenyl and stilben derivatives[9] and gives a certain parallelism also in their reactions. Le Févre[13] reported that the twist-angle between two benzene nuclei was 90° in the case of 2,2′-dinitrobiphenyl. Williamson, *et al.*[11] showed that the extinction curve for this compound failed to differ greatly from the doubled extinction curve for nitrobenzene. Although the non-coplanarity of the rings in biphenyl does not much affect the transmission of substituents effect,[14] it seemed in the case of Ⅲ, as has been seen on bimesityl,[15] that the steric hindrance due to 2,2′-dinitro groups was much enough to show the similar absorption curve and doubly higher extinctions compared with those of *m*-dinitrobenzene respectively. In the color reactions of Ⅲ, on the other hand, the situation was slightly complicated. The wave lengths of their maximum absorptions did not alter greatly and their extinctions showed some unexpected results. On the contrary to the ultraviolet absorptions of polynitro compounds themselves, the extinction of maximum absorption in this color reaction for acetone did not differ from that given by *m*-dinitrobenzene. Nevertheless, an extremely increased intensity was observed in the case of cyclohexanone. Further investigation on these interesting results is in progress and will be reported at a later date.

Table Ⅱ. Color Reactions of Various Active Methylene Compounds with 2,2′,4,4′-Tetranitrobiphenyl in the Presence of Alkali

	λ_{max} (mμ)	$\varepsilon^{a)}$	concn. (M)
Digitoxine	610	15900	2.6×10^{-4}
Dehydroepiandrosterone	535	1800	1.05×10^{-3}
Creatinine	555	15500	4.4×10^{-4}
Acetone	500	1480	2×10^{-3}
Cyclohexanone	540	19100	2×10^{-4}

a) Apparent extinction coefficient.

On the basis of these observations described above, 2,2′,4,4′-tetranitrobiphenyl derivatives were examined in their utilities for the colorimetry of active methylene compounds, and it was observed that most of them except Ⅲ gave hypochromic and instable color reactions under the Zimmermann condition. Therefore, Ⅲ was consequently selected for carrying out the color reactions under the colorimetric conditions for active methylene compounds such as digitoxin, dehydroepiandrosterone, creatinine, acetone, and cyclohexanone with the results shown in Table Ⅱ. On the contrary to the Canbäck condition the colors produced were less stable, and a different sensitivity was observed for each active methylene compound. It seemed most reasonable to conclude that creatinine and digitoxin were sensitive for Ⅲ and the possibility to introduce a novel method of colorimetry was suggested for them. The following paper will describe in this respect.

Experimental

Preparations——Since all the compounds synthesized by the authors were prepared according to previously established procedures, only references to the procedures used are made, with a statement of any departures therefrom.

4,4′-Dinitrobiphenyl (I) was prepared by the method of Gull, *et al.*[16] Colorless needles, from benzene, m.p. 237°; 2,4-dinitrobiphenyl (Ⅱ) was prepared according to Gull, *et al.*[16] Yellow needles, from

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[13] R. J. W. LeFévre, H. Vine: J. Chem. Soc., **1938**, 967.
[14] R. Baker, R. W. Bott, C. Eaborn, P. M. Greasley: *Ibid.*, **1964**, 627.
[15] L. W. Pickett, G. F. Watter, H. France: J. Am. Chem. Soc., **58**, 2296 (1936); M. T. O'Shaughnessy, W. H. Rodebush: *Ibid.*, **62**, 2906 (1940).
[16] H. C. Gull, E. E. Turner: J. Chem. Soc., **1929**, 491.

MeOH, m.p. 108~109°; 2,2′,4,4′-tetranitrobiphenyl (Ⅲ) was prepared by the method of Gull, *et al.*16) Pale yellow needles, from AcOH m.p. 165°, which were not depressed on admixture and had identical IR absorption spectrum with a sample prepared from 1-bromo-2,4-dinitrobenzene by the method of Ullmann, *et al.*17) Bis(*p*-nitrophenyl)methane (Ⅳ) was prepared by nitration of diphenylmethane according to Staedel.18) Pale yellow needles, from AcOH, m.p. 182°; bis(2,4-dinitrophenyl)methane (Ⅴ) was prepared according to Matsumura.19) Yellow plates, from AcOH, m.p. 171°; 2,4-dinitrophenyl phenyl ether (Ⅵ) was prepared by the method of Raiford.20) Yellow needles, from EtOH, m.p. 70°; bis(2,4-dinitrophenyl) ether (Ⅶ) was prepared according to Raiford.20) Colorless plates, from EtOH, m.p. 195°; bis(*p*-nitrophenyl) sulfide (Ⅷ) was prepared by the method of Bost, *et al.*21) Yellow powder, from EtOH, m.p. 174~175°; 2,4-dinitrophenyl phenyl sulfide (Ⅸ) was prepared according to Bost, *et al.*21) Yellow prisms, from EtOH, m.p. 121~122°; bis(2,4-dinitrophenyl) sulfide (Ⅹ) was prepared by the method of Twiss.22) Yellow needles, from AcOH, m.p. 193°; bis(*p*-nitrophenyl)sulfone (Ⅺ) was prepared by the oxidation of Ⅷ according to Ishidate, *et al.*23) Yellow needles, from EtOH, m.p. 282°; 2,4-dinitrophenyl phenyl sulfone (Ⅻ) was prepared by the oxidation of Ⅸ with potassium permanganate.23) Pale yellow needles, from EtOH, m.p. 157°; bis(2,4-dinitrophenyl)sulfone (XⅢ) was prepared by the oxidation of Ⅹ. Colorless prisms, from AcOH, m.p. 238°; 2,4-dinitrostilben (XIV) was prepared by the method of Tiel, *et al.*24) Yellow plates, from AcOH, m.p. 140°; 2,2′,4,4′-tetranitrostilben (XV) was prepared by the method of Green, *et al.*25) Yellow powder, from AcOH, m.p. 266°; 2,4-dinitrotoluene (XVI) was prepared by the nitration of toluene. Pale yellow needles, from EtOH, m.p. 70°; 3,5-dinitrotoluene (XVⅡ) was prepared by the nitration of aceto-*p*-toluidine according to Beilstein, *et al.*26) followed by deamination according to Weeler27) after hydrolysis. Yellow needles, from aq. EtOH, m.p. 93°; *m*-dinitrobenzene (XVⅢ) was obtained commercially and recrystallized from MeOH to give pale yellow needles, m.p. 89°; 1,3,5-trinitrobenzene (XIX) was obtained commercially and recrystallized from methanolic hydrochloric acid to give colorless needles, m.p. 121°; 3,5-dinitrobenzoic acid (XX) was prepared under the direction of "Organic Synthesis." 28) Pale yellow needles, m.p. 204°, from EtOH.

Solvents——Several organic solvents such as EtOH, acetone, pyridine, dioxane, dimethylformamide (DMF), and methylcellosolve were examined for the color reactions and it was observed as follows: most of polynitrodiphenyl compounds were slightly soluble in EtOH; DMF and pyridine gave high blank values of reagents; and low intensity and instability of colorations were shown in dioxane. Acetone and methylcellosolve were finally proved to be suitable solvents for this experiment. The former acted as an active methylene compound simultaneously and the latter was used for the color reactions with cyclohexanone.

Apparatus——The instruments used were a Shimadzu QB-50 Spectrophotometer for the estimation of absorbances and a Beckman DK Ⅱ Spectrophotometer for the measurement of absorption spectra.

Absorption Spectra——KOH ($0.25N$; 0.5 ml.) was added to a methylcellosolve solution (1 ml.)*4 of polynitrodiphenyl compound with cyclohexanone (3 ml.) and the mixture was then diluted to 10 ml. with EtOH. When acetone was used as a solvent and an active methylene compound simultaneously, KOH ($0.25N$; 0.5 ml.) was added to a solution (1 ml.) of nitro compound followed by dilution to 10 ml. with acetone. The diluted mixture was allowed to stand for 10 to 15 min. after shaking thoroughly, and then the light absorption was measured (Table I).

Absorption spectra (Table Ⅱ) of the color reactions of Ⅲ with various active methylene compounds were observed as follows: Solutions (1 ml.)*5 of active methylene compounds were added to a methylcellosolve solution ($10^{-2}M$; 1 ml.) of Ⅲ with KOH ($0.25N$; 0.5 ml.) and the mixtures were diluted to 10 ml. with EtOH by shaking thoroughly. Light absorptions were measured after 10 to 15 min.

The apparent extinction coefficients were given from the absorbances observed by dividing with the final concentrations of active methylene compounds.

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*4 The concentrations are shown in Table I.

*5 The concentrations are shown in Table Ⅱ.

17) F. Ullmann, J. Bielecki: Ber., **34**, 2177 (1901).

18) W. Staedel: Ann., **283**, 153 (1894).

19) K. Matsumura: J. Am. Chem. Soc., **51**, 817 (1929).

20) L.C. Raiford, J.C. Colbert: *Ibid.*, **48**, 2652 (1926).

21) R.W. Bost, J.O. Turner, R.D. Norton: *Ibid.*, **54**, 1985 (1932).

22) D.F. Twiss: J. Chem. Soc., **1914**, 1678.

23) M. Ishidate, T. Nambara: Yakugaku Zasshi, **79**, 653 (1959).

24) J. Tiel, R. Escales: Ber., **34**, 2843 (1901).

25) A.G. Green, J. Baddiley: J. Chem. Soc., **1908**, 1725.

26) F. Beilstein, A. Kuhlberg: Ann., **158**, 341 (1871).

27) H.L. Weeler: Am. Chem. J., **44**, 144 (1910).

28) Organic Synthesis, **22**, 48 (1942).

Rates of Color Development——At the wave lengths of maximum absorption, gradual changes of absorbances of these colored solutions described above were measured against the ethanolic solutions of polynitrodiphenyl compounds with KOH. Fig. 2 indicates the changes of the apparent extinction coefficients which were given from the absorbances described above by dividing with the final concentrations of nitro compounds.

This work was supported in part by a Grant-in-Aid for Fundamental Scientific Research from the Ministry of Education, for which the authors express their appreciation.

Summary

This work was undertaken to obtain a more satisfactory reagent for the assay of active methylene compounds than has been used. Biphenyl, diphenylmethane, diphenyl ether, diphenyl sulfide, diphenylsulfone and stilbene with nitro groups in 4,4′-, 2,4-, 2,2′,4,4′-positions were prepared and their color reactions with acetone and cyclohexanone in the presence of alkali were examined.

4,4′-Dinitro compounds gave negative Janovsky reaction. Bis(*p*-nitrophenyl)-(IV) and bis(2,4-dinitrophenyl)methane (V) gave blue color in methylcellosolve with addition of alkali without active methylene compound.

2,4-Dinitro compounds showed bathochromic shift as compared with usual reagents such as *m*-dinitrobenzene, 1,3,5-trinitrobenzene and picric acid. The intensities of colors by these reagents were lower.

2,2′,4,4′-Tetranitro compounds showed higher intensities and sufficient stabilities of the color produced, but no bathochromic shifts. These tetranitro compounds also gave the specific color reactions for active methylene compounds such as acetone, cyclohexanone, 17-ketosteroids, creatinine and cardiac glycosides.

2,2′,4,4′-Tetranitrobiphenyl (III) could be the reagent that has higher sensitivity, sufficient stability and specificity in color reaction of active methylene compounds with alkali.

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156. **Tamotsu Okumura, Yoshio Nozaki, and Daisuke Satoh**: Studies on Digitalis Glycosides. XIX.[*1] Microbiological Transformation of Digitoxigenin Derivatives by *Absidia orchidis*.[*2]

(*Shionogi Research Laboratory, Shionogi & Co., Ltd.*[*3])

A previous paper[1] of this series reported the 1β-, 5β-, and 7β-hydroxylations of digitoxigenin (I) by *Absidia orchidis*, a microorganism known to hydroxylate Reichstein's substance S (II) at 6β-, 11α-, and 11β-positions.[2] Subsequently it was found that the

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[*1] Part XVIII: This Bulletin, **11**, 576 (1963).

[*2] A part of this paper was presented at the 83rd Annual Meeting of the Pharmaceutical Society of Japan, November 2nd, 1963 at Tokyo College of Pharmacy.

[*3] Fukushima-ku, Osaka (奥村 保, 野崎義雄, 佐藤大助).

[1] Part XVII. H. Ishii, Y. Nozaki, T. Okumura, D. Satoh: This Bulletin, **11**, 156 (1963).

[2] O. Hanc, A. Capek, B. Kapac: Folia Microbiologica, **6**, 392 (1961).