Histochemical Journal, 1 (1968), 59-67

The purification and some properties of neotetrazolium chloride and its chief monotetrazolium salt contaminant

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Received 6 March 1968

Synopsis. A simple method which does not involve chromatography is described for the purification of neotetrazolium chloride (NT). Commercial samples of NT were shown to contain variable amounts of a major monotetrazolium salt contaminant, which was isolated and identified as 2-(4-biphenyl-)-3,5-diphenyltetrazolium chloride (BDTC). A qualitative method for checking the purity of NT and BDTC by thin-layer chromatography is described; with this technique the presence of traces of other tetrazolium salt contaminants was detected. The preparation of pure samples of formazan corresponding to NT and BDTC is described; molar extinction coefficients are given with sufficient other information to enable estimation of the amounts of NT and BDTC in mixtures of the two to be made.

Introduction

Neotetrazolium chloride (NT; 2,2′,5,5′-tetraphenyl-3,3′-(4,4′-biphenylene)-ditetrazolium chloride) is still the reagent of choice in the quantitative cytochemical demonstration of certain oxidative systems, despite such histochemical shortcomings as poor substantivity, inadequate localisation, and relative indifference as an indicator of tetrazole reductase activity. The advantages of NT are that, unlike nitro blue tetrazolium salt (nitroBT) and tetranitroBT, it is not enzymically reduced in incubating media (Kalina, 1966), and that the corresponding purple formazan is readily extracted from tissue sections. However, solutions of the formazan obtained cytochemically

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can show slightly differing spectra; for example, the wavelength of maximal light absorption can depend on the rate of formazan production. Examination of NT samples from different sources has revealed the presence of significant and variable amounts of a major contaminant, which on reduction yielded a red formazan. In the present study a simple method for the purification of NT is described, as well as the isolation and identification of the chief monotetrazolium salt contaminant. Some properties of these compounds are described.

Methods

All melting points (m.pt.) were uncorrected. All vacuum drying took place over activated charcoal and phosphorus pentoxide.

PURIFICATION OF NT

Commercial NT (10 g; 79% NT) was extracted with 200 ml chloroform in a Soxhlet thimble for 40–60 hr, the chloroform level always remaining well below the thimble top. NT separating from the cold extract was filtered, washed with a little chloroform, and returned to the thimble. This procedure removed the bulk of the monotetrazolium salts. The dried NT was extracted into 200 ml hot ethanol, treated with decolorizing charcoal, filtered hot and cooled before being added dropwise to 600–700 ml ethyl acetate with brisk stirring. The NT was filtered after 30–60 min, washed with ethanol–ethyl acetate (1 : 9, v/v) and dried at 15 mm and 110°C. A further purification by solution in ethanol and precipitation in ethyl acetate was sometimes necessary; second extractions with chloroform had no effect. The yield was 7.5 g of a strongly hygroscopic light yellow powder, m.pt. 297°C (dec.). The purity of samples is in the range 92–95% NT, on the basis of an assay procedure involving reduction and measurement of the resulting formazan (see later). Monotetrazolium salt contamination ought not to exceed 1%.

ISOLATION OF CHIEF TETRAZOLIUM SALT IMPURITY

The chloroform extract from NT (above) was evaporated and dried for 1–2 hr at 100–120°C. The residue was extracted with 15–20 times its weight of alcohol-free chloroform, treated with decolorizing charcoal, filtered, and washed with a little chloroform. The filtrate was added drop by drop to 5–10 volumes dry ether with magnetic stirring. The suspension was centrifuged; the resulting pellet was redissolved, decolorized and precipitated as before. The final residue was washed with a little dry ether before being centrifuged, decanted, and dried by cautious evacuation over the desiccants described. Once most of the ether had been removed, the product was dried at 15 mm and 100–105°C. The off-white amorphous solid turned yellow on exposure to light; m.pt. 175°C.

PREPARATION OF FORMAZAN FROM CHIEF TETRAZOLIUM SALT IMPURITY

The tetrazolium salt (150 mg) and ascorbic acid (200 mg) were dissolved in 100 ml 50% ethanol. Potassium hydroxide (200 mg) in 10 ml 50% ethanol was added drop by drop with stirring; 100 ml 0.5N HCl was added after 20–30 min, followed by 50 ml water. The precipitated formazan was filtered, washed extensively with water, and crystallised from 100 ml 90% aqueous ethanol. The formazan was dried at 15 mm and 105°C. The yield was 20 mg of dark red needles with a bronze reflex; m.pt. 176°C.

PREPARATION OF 1,3-DIPHENYL-5-(4-BIPHENYL-) FORMAZAN (DBF)

Well-powdered 4-biphenylamine hydrochloride* (2.055 g; 10 mmoles) was suspended in 2 ml conc. HCl (22 mmoles) and 4 ml water. The suspension was stirred at 0°C, diazotised with 0.76 g sodium nitrite (11 mmoles) in 1.5 ml water, and stirred for a further 20–30 min before it was added dropwise over 40 min to a stirred solution of 1.96 g benzaldehyde phenylhydrazone (10 mmoles) in 16 ml pyridine at 0°C. The reaction mixture was stirred at 0°C for a further 2.5 hr before being slowly added to 50 ml 30% aqueous ethanol, and washed in with 20 ml 50% ethanol. The dark precipitate was filtered, and washed successively with 50 ml portions of 30%, 40% and 55% ethanol, and water. The precipitate was boiled in 150 ml 67% ethanol and filtered; the process was repeated, and the product was dried at 15 mm. The yield was 2.42 g (6.4 mmoles; 64%) of a dark purple feathery powder. Analytically pure samples were obtained as dark red needles with a bronze reflex in 70–80% yield by crystallising 0.5 g from 500 ml 90% ethanol and drying at 110°C; m.pt. 180°C.

PREPARATION OF 2-(4-BIPHENYL-)-3,5-DIPHENYL TETRAZOLIUM CHLORIDE (BDTC)

DBF (1.88 g; 5 mmoles) was suspended in a mixture of 20 ml ethyl acetate and 2.8 ml freshly-prepared amyl nitrite (21 mmoles). Hydrogen chloride was passed over the magnetically-stirred purple suspension until a clear brown solution was obtained. The tetrazolium salt was precipitated by dropwise addition to 180 ml dry ether with magnetic stirring. The precipitate was centrifuged and washed twice with 70 ml dry ether before being dried by cautious evacuation over the desiccants described. The yield was 1.96 g of an off-white amorphous material containing about 90% monotetrazolium salt (4.3 mmoles; 86%). Analytically pure samples of the hemihydrate were prepared in 80–85% yield by dissolving this material in alcohol-free chloroform,

* Carcinogenic amine. See 'Precautions for laboratory workers who handle carcinogenic aromatic amines'; Chester Beatty Research Institute (1966).

treating with decolorizing charcoal, precipitating and washing with dry ether before finally drying at 15 mm and 105–110°C; m.pt. 179°C. The substance turned yellow on exposure to light.

PREPARATION OF PURE BIS-FORMAZAN CORRESPONDING TO NEOTETRAZOLIUM CHLORIDE

Purified NT (300 mg; 0.45 mmole) was dissolved in 25 ml water and filtered. 12 ml of a filtered aqueous solution containing 160 mg NaOH (4 mmoles) and 250 mg ascorbic acid (1.4 mmoles) were added dropwise with stirring. The precipitate was centrifuged, washed three times with water, once with ethanol, twice with water, and once more with ethanol (20 ml in each case) before being resuspended in water and filtered. The precipitate was dried at 15 mm and 100°C. The yield was 90 mg of a purplish-black powder (0.15 mmole; 33%); m.pt. 248°C (dec.).

MOLAR EXTINCTION COEFFICIENTS

Quantities of formazans (about 1 mg) were weighed to the nearest μg, dissolved in 10 ml pyridine and diluted 1 : 10. Extinctions were measured in standard 10 mm cuvettes on Hilger & Watts H.700 spectrophotometers with glass dispersion prisms.

PURITY TESTS

Qualitative

Tetrazolium salt samples were separated on silica gel G (Merck A.G., Darmstadt, Germany), spread on 76 × 25 mm microscope slides. The solvent mixture used was pentanol–formic acid–water (8 : 1 : 1, v/v/v.). Dried plates were sprayed with an alkaline ascorbate solution to produce coloured formazans.

Quantitative

Ethanolic solutions of NT were prepared at accurately known concentrations. Aliquots (100 μl; 0.5–2 mg/ml) were pipetted in duplicate into 10 ml volumetric flasks. Solutions of NT, its impurity, or mixtures of both, diluted if necessary in ethanol, were also examined. All organic solvents were completely removed *in vacuo*. The solid deposits were dissolved in 2 drops 50% aqueous pyridine, and mixed with a drop of saturated sodium sulphide solution. After 10–15 min the solution was made up to 10 ml with dry pyridine, and extinctions measured against a pyridine blank at 505 and 567 nm (*cf.* Jones, 1966). Absolute amounts of NT (m.wt. 667.6) and BDTC (m.wt. 410.9) were calculated from these data, the molar extinction coefficients, and extinction ratios (see below), assuming a quantitative reduction of tetrazolium salts to the corresponding formazans by sodium sulphide.

ELEMENTARY ANALYSES

Analyses were carried out on a Technicon C H & N Analyser at the School of Pharmacy, University of London.

Results

MOLAR EXTINCTION COEFFICIENTS (ε) AND EXTINCTION RATIOS

Greatest absorption of visible light by solutions of the formazan corresponding to NT occurred at 567 nm; ε_{567} was 40,900 $\pm$ 90, and $\varepsilon_{505}/\varepsilon_{567}$ was 0.775. Solutions of DBF showed greatest light absorption at 505 nm; ε_{505} was 19,250 $\pm$ 130, while $\varepsilon_{567}/\varepsilon_{505}$ was 0.677. The extinction of a pyridine solution of the formazan (10 μg/ml) corresponding to the chief tetrazolium salt impurity was 0.505 at 505 nm; the absorption maximum lay between 505 and 510 nm. If this material is assumed to be DBF, then the figure derived for the molar extinction coefficient is 19,000 $\pm$ 110. All determinations were carried out in quadruplicate.

IDENTIFICATION OF THE CHIEF TETRAZOLIUM SALT IMPURITY

When examined by thin-layer chromatography followed by reduction with alkaline ascorbate, commercial samples of NT always gave rise to two major components, a predominant purple spot (R_f 0.4–0.55) and a red spot (R_f 0.7–0.8). Samples of BDTC showed the fast-running red spot as the only significant component. When BDTC and impure NT were applied together, the resulting pattern could always be predicted from separate applications, although the overloading of mixtures tended to increase the R_f of NT and to decrease that of the monotetrazolium salt; there was never more than one major red spot. Solutions of the chloroform extract of impure NT in pure chloroform gave rise predominantly to the same red spot, a minor NT spot, and several very small spots of reddish hue which were just visible (approximate R_f values 0.2, 0.3, 0.5 and 0.65). An attempt to isolate one of these (R_f 0.65) from 250 mg dried chloroform extract on large thick-layer plates failed. No other solvent mixture was used, since numerous attempts to improve the separation of the red and purple spots were unsuccessful. The purified material from the chloroform extract always contained the fast-running red spot, with the purple spot present only in trace amounts.

Elementary analyses and mixed m.pts of the monoformazan and monotetrazolium salt obtained by synthesis and by isolation from commercial NT are shown in Table 1. Samples of the tetrazolium salt and formazan isolated from impure NT always melted at temperatures slightly lower than the synthesised materials; this was probably due to residual traces of NT, which were never entirely eliminated. It is concluded that the chief monotetrazolium salt contaminant of impure NT is BDTC.

Table 1. Elementary analyses and m.pts. of monoformazans and monotetrazolium salts

Compound	*Theory*				*Found*				*m.pt.* (°C)	*Mixed m.pt.* (°C)
	C	H	N	Cl	C	H	N	Cl		
1,3-Diphenyl-5-(4-biphenyl-) formazan (DBF; synthesised)	79.76	5.36	14.86		79.43	5.43	14.32		180	178
Ditto (from NT)					79.36	5.38	15.16		176	
2-(4-Biphenyl-)-3,5-diphenyl tetrazolium chloride[1] (BDTC; synthesised)	71.50	4.80	13.34	8.44	72.00	4.86	13.42	8.64	179	176
Ditto[1] (from NT)					70.99	4.66	13.18	8.84	175	

[1] Hemihydrate.

PURITY OF NT

Since tetrazolium salt impurities other than BDTC are present in NT to such a small extent, and since all give rise to reddish formazans similar in colour to those from BDTC, estimates of the amounts of NT and of other tetrazolium salt contaminants together in commercial samples can be obtained (Table 2). There appears to have been no recent improvement in purity.

Table 2. The purity of various samples of NT

Sample	*Source*	*Date of purchase*	*Percentage NT*	*Percentage monotetrazolium salt contamination, chiefly BDTC*
1	Firm A	1962	70	14
2	Firm B	?	69	15
3	,,	1966	74	14
4	,,	1967	74	16
5	,,	1967	79	13
6	Firm C	?	55	8
7	,,	1965	73	10
8	,,	1967	62	6
9[1]	—	—	92	<0.2

[1] Purified sample.

Purified NT samples produced only the one purple spot. In preliminary experiments to establish optimum conditions for purification, the presence of NT and BDTC in solutions and in mother liquors was qualitatively detected by thin-layer chromatography; where necessary, the actual amounts were estimated.

Discussion

The present results substantiate the work of Burtner, Bahn & Longley (1956, 1957), who used chromatography to separate two different coloured products from reduced NT, and who postulated that one of the compounds was derived from an unidentified monotetrazolium salt. The way in which such a substance could arise was originally explained by Wedekind (1898*a*). Normally, in the preparation of the formazan corresponding to NT, one molecule of tetrazotised benzidine condenses with two molecules of benzaldehyde phenylhydrazone (see also Seiler & Schmid, 1954). However, Wedekind (1898*a*,*b*) showed, by raising the reaction temperature, that one of the diazonium groups of tetrazotised benzidine decomposed in the presence of ethanol to form 4-biphenyl diazonium chloride, and he made use of this reaction to prepare DBF from benzidine and benzaldehyde phenylhydrazone. The extensive occurrence of this side reaction during the synthesis of the formazan corresponding to NT would result in the formation of significant quantities of monoformazan which, in the absence of adequate purification, would be subsequently oxidised to BDTC. Tsou, Cheng, Nachlas & Seligman (1956) demonstrated that similar monoformazans were obtained as by-products in the synthesis of other bisformazans, one diazonium group in a molecule of tetrazotised aryldiamine decomposing to give the parent hydrocarbon.

The isolation and identification of trace tetrazolium salt impurities in commercial NT was considered to be outside the scope of the present investigation. Such substances might include alkoxy monotetrazolium salts derived from alkoxy monoformazan by-products. Arylamine impurities in benzidine, such as semidine arising during the rearrangement of hydrazobenzene, could also yield formazans on diazotisation and coupling, which in turn would be oxidised to tetrazolium salts. The hazards of handling benzidine on a large scale may rightly deter manufacturers from purifying their product; the proportion of such tetrazolium salts may therefore be greater in future samples.

The concept of purity of tetrazolium salts requires clarification in a cytochemical context. Tetrazolium salts are notoriously difficult to obtain in an absolutely pure state, due to the occlusion of solvents from mother liquors (Ashley, Davis, Nineham & Slack, 1953; Tsou *et al.*, 1956). Melting points are not necessarily characteristic, as tetrazolium salts usually melt with

decomposition (Ashley *et al.*, 1953); the situation is worse with the more complex bistetrazolium salts. For example, the m.pt. of BDTC has been variously given as 175°C (Wedekind, 1898*a*), 242–3°C (Jerchel & Fischer, 1949), 238°C (Ashley *et al.*, 1953), and 179°C (present work). The m.pt. of NT has been described as 285°C (Wedekind, 1898*a*), 219°C (Seiler & Schmid, 1954), and 297°C (present work), all with decomposition. Fortunately the presence of contaminating solvents which do not interfere with cytochemical reactions is of little consequence. On the other hand, the presence under cytochemical conditions of even small amounts of impurities which behave similarly to the main material may be disastrous. The extent of contamination is unlikely to be accurately reflected in the elementary analysis. Screening of bistetrazolium salts by chromatography should therefore be regarded as an essential preliminary to any quantitative study.

In recent years various reports describing red reduction products from unpurified bistetrazolium salts have appeared. The formation of such compounds has been ascribed to half-reduction of the molecule. In the present investigation the finding that the nature of the reduction product is determined by that of the starting material is stressed (*cf.* Bush & Gale, 1958). No evidence for the existence of any intermediate stage in the reduction of NT has been adduced.

Acknowledgements

I thank Professor L. Young of St Thomas's Hospital and Professor C. Long of the Royal College of Surgeons for their interest and encouragement. I am grateful to Dr J. Perkins of King's College for the kind gift of an intermediate in the monoformazan synthesis. I am indebted to numerous friends and colleagues for helpful discussions, notably Dr A. W. Nineham of May and Baker Ltd.

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