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A rapid test of the purity of tetrazolium salts (bis-) on thin-layer chromatograms

While tetrazolium salts find increasing application to biological problems, only a handful of communications[1–6] describe procedures by which the purity of these compounds may be tested. RIED AND GICK[1] chromatographed various derivatives of blue tetrazolium including the parent compound, while neotetrazolium and its chief contaminant have been separated by chromatography[4,6]. GLANTZ AND FRIED[3] used microelectrophoresis to separate a number of tetrazolium salts from their impurities; cathodic mobilities of the chief components were given at pH 8 and 9. Impurities in nitroblue tetrazolium have been detected by similar means[5]. The problem of purity is most acute in the case of bistetrazolium salts, which are not only of the greatest use in cytochemical studies, but which, by virtue of the mode of synthesis, are also more likely to contain monotetrazolium salts as contaminants. Determinations of the melting point are of no value in these cases, since the nature and extent of retention of solvents of crystallization affect the experimental values (*e.g.* refs. 7 and 8); in addition, the more complex salts melt with decomposition. A simple screening procedure is described, and data pertaining to bistetrazolium salts, their chief impurities, and the corresponding formazans are presented and discussed*.

Experimental

Saturated ethanolic solutions (3–10 μl) of the chlorides of various tetrazolium salts [blue tetrazolium, BT, 2,2′,5,5′-tetraphenyl-3,3′-(3,3′-dimethoxy-4,4′-biphenylene)-ditetrazolium chloride; blue tetrazolium (*p*-anisyl), BTA, 2,2′-diphenyl-5,5′-di-(4-methoxyphenyl)-3,3′-(3,3′-dimethoxy-4,4′-biphenylene)-ditetrazolium chloride; blue tetrazolium (piperonyl), BTP, 2,2′-diphenyl-5,5′-dipiperonyl-3,3′-(3,3′-dimethoxy-4,4′-biphenylene)-ditetrazolium chloride; blue tetrazolium (veratryl), BTV, 2,2′-diphenyl-5,5′-di-(3,4-dimethoxyphenyl)-3,3′-(3,3′-dimethoxy-4,4′-biphenylene)-ditetrazolium chloride; neotetrazolium, NT, 2,2′,5,5′-tetraphenyl-3,3′-(4,4′-biphenylene)-ditetrazolium chloride; nitroblue tetrazolium, NBT, 2,2′-di-(4-nitrophenyl)-5,5′-diphenyl-3,3′-(3,3′-dimethoxy-4,4′-biphenylene)-ditetrazolium chloride; *m*-nitroviolet tetrazolium, NVT, 2,2′-diphenyl-5,5′-di-(3-nitrophenyl)-3,3′-(4,4′-biphenylene)-ditetrazolium chloride; tetranitroblue tetrazolium, TNBT, 2,2′,5,5′-tetra-(4-nitrophenyl)-3,3′-(3,3′-dimethoxy-4,4′-biphenylene)-ditetrazolium chloride; violet tetrazolium (*o*-tolidine), VTT, 2,2′,5,5′-tetraphenyl-3,3′-(3,3′-dimethyl-4,4′-biphenylene)-ditetrazolium chloride], purchased from different sources between 1962 and 1968, were applied to commercially-prepared glass plates coated with Silica Gel F (DC-Fertigplatten Kieselgel F_{254}; E. Merck AG, Darmstadt, Germany), cut into suitable pieces (4 × 10 mm). The chromatograms were briefly subjected to a low pressure (50–75 μ Hg) before running with a mixture of 3-methylbutan-1-ol–formic acid–water (8:1:1, by vol.)[6] in sealed domestic jars. The dried plates were sprayed with an alkaline solution of sodium ascorbate to produce coloured formazans; R_F values were

* *Note added in proof.* Similar data from six monotetrazolium salts will shortly appear under *Society Proceedings* in the *Biochem. J.*

determined. The chromatograms were carefully washed, almost to the point of disintegration, in distilled water, and were allowed to dry overnight in the dark. Within 24 h silica gel bearing the spots was scraped off, extracted with pyridine (0.5–8 ml), and centrifuged. λ_{max} of each supernatant was determined in the visible region of the spectrum.

Results

The results are shown in Table I. When treated with alkaline ascorbate, all samples gave rise to at least two formazan spots, the colours of which were characteristic and reproducible. The slower spots (range of R_F values, 0.33–0.57) predominated, in shades of purple and blue, and represented formazans derived from bistetrazolium salts. The faster spots (range of R_F values, 0.64–0.80) varied in colour from red to lilac. In all pairs for which sufficient data had been obtained, the wavelengths of maximal light absorption by solutions of bisformazans were found to be 55–80 nm higher than those shown by formazans derived from fast spots. The relative intensities of the fast and slow spots varied to some extent, depending on the batch; in the cases of BTA, BTP and BTV the fast spots were only faintly visible, and in recent samples of NBT and TNBT the relative intensities of the fast spots were low. Further subsidiary spots were sometimes detected; these were generally negligible, with the exception of the slow spots from NVT (R_F 0.18–0.24) and from VTT (R_F 0.24–0.34).

Discussion

The fast spot (R_F 0.73–0.80) obtained from NT has been identified as 2-(4-biphenylyl)-3,5-diphenyl tetrazolium chloride[4,6]; the origin of this impurity has been

TABLE I

R_F VALUES OF BISTETRAZOLIUM SALTS AND THEIR PRINCIPAL CONTAMINANTS, WITH COLOURS OF THE CORRESPONDING FORMAZANS AND λ_{max} VALUES OF SOLUTIONS IN PYRIDINE

Tetrazolium salt	*Tetrazolium salt; R_F range*	*Formazan*		*Tetrazolium salt; R_F range*	*Formazan*	
		Colour of spot	*λ_{max} (nm)*		*Colour of spot*	*λ_{max} (nm)*
	Slow major spot			*Fast minor spot*		
Blue tetrazolium	0.42–0.51	Blue	600	0.70–0.77	Reddish violet	545
Blue tetrazolium (*p*-anisyl)	0.46–0.50	Royal blue	620	0.71–0.73	Lilac (?)[a]	—[a]
Blue tetrazolium (piperonyl)	0.40–0.45	Royal blue	620	0.71–0.72	Lilac (?)[a]	—[a]
Blue tetrazolium (veratryl)	0.33–0.36	Royal blue	622	0.64–0.68	Lilac (?)[a]	—[a]
Neotetrazolium	0.50–0.57	Purple	565	0.73–0.80	Red	505
Nitroblue tetrazolium	0.48–0.55	Purplish-blue	590	0.72–0.80	Dirty red	525
m-Nitroviolet tetrazolium	0.33–0.40	Purple	560	0.66–0.72	Red	505
Tetranitroblue tetrazolium	0.35–0.42	Purplish-black	600	0.69–0.74	Dirty pinkish-red	520
Violet tetrazolium (*o*-tolidine)	0.46–0.53	Royal purple	580	0.73–0.77	Carmine	520
	Slow minor spot					
m-Nitroviolet tetrazolium	0.18–0.24	Carmine	515			
Violet tetrazolium (*o*-tolidine)	0.24–0.34	Carmine	522			

[a] Quantity present too small for accurate observation.

fully explained[6]. The other fast spots are very probably monotetrazolium salts containing 4-biphenylyl, 2-methoxy-4-(3-methoxyphenyl)-phenyl, or 2-methyl-4-(3-methylphenyl)-phenyl substituents, depending on whether benzidine, *o*-dianisidine[9], or *o*-tolidine were used in the synthesis of the formazans. The significance of these findings is important. Unless pure tetrazolium salts are employed, it follows that the composition of the mixture of reduction products which results from the demonstration of a tetrazolium reductase system with an impure salt will depend on the relative affinities of the system for the different electron acceptors available.

Ideally, in the preparation of bistetrazolium salts the bisformazan intermediates should be purified from monoformazan contaminants as extensively as possible prior to the oxidation step[2,9,10–12], although traces of impurity may persist even after prolonged extraction. Once oxidation has been carried out, various methods of purification may be applied; these include chromatographic separation on columns (*e.g.* refs. 4 and 13) or on thick-layer plates, column electrophoresis, and continuous-flow paper electrophoresis. A more convenient method involves precipitation from alcoholic solutions by a solvent of appropriately low polarity. Ethyl acetate is preferable to the commonly-used diethyl ether as a precipitant, because monotetrazolium salts tend to be more soluble in mixtures of alcohol and the ester than in alcoholic ether. Mixtures of the lower alcohols with ethyl acetate have been used in this way to purify BT[14] and NT[6]; repeated crystallization is unnecessary if the bulk of the impurities can be selectively extracted first[6].

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