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THIN-LAYER CHROMATOGRAPHY OF TETRAZOLIUM SALTS AND THEIR FORMAZANS*

J. H. TYRER, M. J. EADIE AND W. D. HOOPER
Medical Professorial Unit of Queensland University, Royal Brisbane Hospital, Brisbane (Australia)

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SUMMARY

A satisfactory system for thin-layer chromatography of tetrazolium salts and their formazan reduction products has been devised. The methods developed permit the detection of contaminating compounds in commercial samples of tetrazolium salts. When working with formazans it is necessary to control the conditions of tetrazolium reduction or chromatography may lead to complicated and possibly confusing results.

INTRODUCTION

Tetrazolium salts are water-soluble, relatively colourless compounds which can be reduced to insoluble highly coloured formazans. This property is made use of in biology, and particularly in histochemistry, to permit the demonstration and accurate histological localization of sites of tissue oxidations[1].

Despite their wide use in qualitative histochemistry, tetrazolium salts have been relatively little used in quantitative work. The purity of some commercially available preparations of these salts is suspect[2–4], so that there is doubt about the nature of the reduction products that would have to be measured. Because of these uncertainties we have attempted to develop chromatographic systems to assess the purity of tetrazolium salts, by investigating both the salts themselves and their reduction products.

EXPERIMENTAL

Tetrazolium salts

The formulae of the ten tetrazolium salts studied are set out in Table I. Single commercial samples of nine of the salts were obtained (not all from the same manufacturer), together with several samples of the widely used nitroblue tetrazolium from two different suppliers. A sample of nitroblue tetrazolium was synthesized, following the method described by KARMARKAR *et al.*[5], condensing tetrazotized *o*-dianisidine

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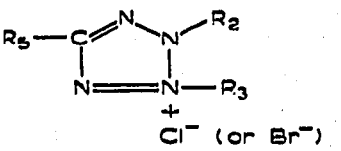
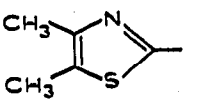
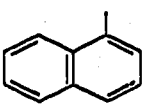
TABLE I

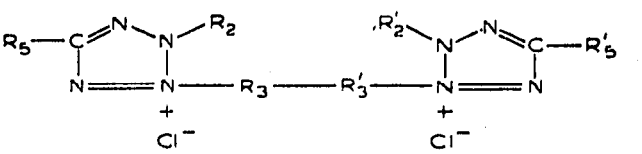
FORMULAE OF TETRAZOLIUM SALTS STUDIED

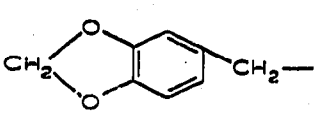
(a) Monotetrazoliums



	R_2	R_3	R_5
Triphenyl-tetrazolium (TTC)	C_6H_5	C_6H_5	C_6H_5
Iodonitro-tetrazolium (INT)	p-I–C_6H_4	p-NO_2–C_6H_4	C_6H_5
Monothiazolyl-tetrazolium (MTT)	C_6H_5		C_6H_5
Tetrazolium violet (TV)	C_6H_5		C_6H_5

(b) Ditetrazoliums



	R_2, R'_2	R_3, R'_3	R_5, R'_5
Neo tetrazolium (NT)	C_6H_5	C_6H_4	C_6H_5
Blue tetrazolium (BT)	C_6H_5	$C_6H_3(OCH_3)$	C_6H_5
Nitroblue tetrazolium (Nitro-BT)	p-NO_2–C_6H_4	$C_6H_3(OCH_3)$	C_6H_5
Tetranitroblue tetrazolium (TNBT)	p-NO_2–C_6H_4	$C_6H_3(OCH_3)$	p-NO_2–C_6H_4
Piperonyl tetrazolium blue (Pip-TB)	C_6H_5	$C_6H_3(OCH_3)$	
p-Anisyl tetrazolium blue (p-ATB)	C_6H_5	$C_6H_3(OCH_3)$	p-CH_3O–C_6H_4

with the p-nitrophenylhydrazone of benzaldehyde and oxidising the resulting formazan with isoamyl nitrite.

Thin-layer chromatography of the tetrazolium salts was carried out on 20 cm × 20 cm glass plates coated with an 0.25 mm layer of Merck Silica Gel G and dried at 100° for 2 h. The tetrazolium salts were dissolved in ethanol or methanol and were spotted on to the plates. Ascending chromatography was carried out on the plates at 37°, using as a developer a freshly made mixture of n-butanol–water–glacial acetic acid (78:17:5). After running, the plates were dried and the tetrazolium salts were demonstrated by their reduction to coloured formazans, effected either by spraying

the plates with alkaline ascorbate solution, or by exposing them to ammonium sulphide vapour.

Formazans

The formazans of nitroblue tetrazolium and tetranitroblue tetrazolium are insoluble in common solvents at room temperature. Therefore the most practicable way of obtaining formazans for thin-layer chromatography was to spot tetrazolium salts onto chromatography plates, and then to reduce the spots *in situ*. Ammonium sulphide was satisfactory for the reduction when dropped on to the tetrazolium spots on the plates. If the plates were then warmed the ammonium sulphide evaporated quickly and left no obvious residue. Alternatively, ammonium sulphide vapour could be used. As will be mentioned later, the conditions of tetrazolium reduction determined the nature of the reaction products, and hence the appearance of the developed chromatogram.

The formazans were produced by reducing tetrazolium salts on glass plates prepared as described above. Ascending chromatography was carried out at 37°, with a mixture of hexane–dichloromethane (2:3), as developer. The coloured formazans were easily seen during development, and were stable for at least some hours afterwards on the dried plates.

RESULTS

Tetrazolium salts

The *n*-butanol–water–acetic acid (78:17:5) system provided reasonably adequate separations of the various tetrazolium salts from one another, as well as from

TABLE II

R_F VALUES OF TETRAZOLIUM SALTS AND FORMAZANS

The colours mentioned beside the tetrazolium R_F values refer to the colours of the reduction products by which the positions of the tetrazolium salts were demonstrated.

Compound	*R_F values of tetrazolium salts ± S.D.*[a]			*R_F values of formazans ± S.D.*[a]		
TTC	0.60		± 0.03	0.88		± 0.02
INT	0.77		± 0.05	0.80		± 0.04
MTT	0.54		± 0.04	0.18		± 0.03
TV	0.79		± 0.03	0.89		± 0.01
NT	0.32	(violet, major component)	± 0.04	0.73	(violet, major component)	± 0.03
	0.24	(red)	± 0.04	0.34	(red)	± 0.05
	0.41	(violet)	± 0.03	0.82	(violet)	± 0.03
	0.83	(red)	± 0.04	0.87	(red)	± 0.03
BT	0.27		± 0.04	0.72		± 0.04
Nitro-BT	0.39		± 0.05	0		0
TNBT	0.24	(blue black)	± 0.04	0	(blue-black)	0
	0.78	(brown)	± 0.04	0.19	(brown)	± 0.04
Pip-TB	0.27	(blue, major component)	± 0.04	0.38	(blue, major component)	± 0.03
	0.33	(blue)	± 0.04	0.25	(blue)	± 0.03
p-ATB	0.29	(blue, major component)	± 0.04	0.31	(blue, major component)	± 0.05
	0.40	(blue)	± 0.04	0.34	(blue)	± 0.05
	0.49	(blue)	± 0.03	0.38	(blue)	± 0.05

[a] Average of ten chromatograms.

any impurities present. Other combinations of the components of this developer were less satisfactory. The R_F values that were obtained are set out in Table II. Most of the commercial samples of tetrazolium salts yielded more than one fraction after chromatography, and these fractions often reduced to differently coloured substances. It appeared that many of the tetrazolium salts were contaminated, possibly with other tetrazoliums.

The various commercial samples of nitroblue tetrazolium used in these studies contained different contaminants, but these could largely be removed by recrystallisation prior to chromatography; these contaminants were not found to any appreciable extent in the specimen of nitroblue tetrazolium that was synthesized personally according to the method of KARMARKAR *et al.*[5].

Formazans

The results of formazan chromatography depended to some extent on the conditions of tetrazolium reduction. The most simple situation occurred when the tetrazolium salts were reduced strongly with ammonium sulphide, and the plates were then dried for 5 min in a 100° oven before development. The R_F values of the formazans produced under these conditions are set out in Table II, reasonably well defined zones being obtained after development. (All of the tetrazolium salts studied have R_F values of 0 with the developer used for the formazan chromatography.) It can be seen that many tetrazolium salts yield more than one coloured product, and that the number of products corresponds with the number of components in each commercial sample of tetrazolium salt, as determined by chromatography of the unreduced salts (see above). This finding is further evidence for the contamination of some of the commercial products. The major formazan products of nitroblue tetrazolium and tetranitroblue tetrazolium gave R_F values of 0, but even with solvents at the polar end of the eluotropic series we found it impossible to get these formazans to run on silica gel or alumina.

When the tetrazolium spots were less strongly reduced, and the plates were run as soon as the spots had dried (at room temperature) a rather more complicated picture than that described above occurred. Although the formazan fronts ran to the same R_F values as described above, "tails" extended from the front, back to the origin, and the origin contained material of colour similar to the formazan which had run. A similar procedure of reduction and development was carried out after each tetrazolium spot had been placed at one corner of a separate plate. After the first run the plates were allowed to dry well and then were re-run at right angles in the same developer as that used for the initial run. The formazan which had moved previously, its tail, and the formazan which had remained at the origin now all ran to the same R_F value, similar to that of the formazan which had run initially. This suggested that the mono- and ditetrazoliums studied could form a temporary coloured product during their reduction to definitive formazan. However, during the second run (which was carried out at right angles to the first), the formazan zone which had originally moved, and which had again run on the second development, left a small residue at the secondary origin. For the monotetrazolium salts this residue was similar in colour to the formazan which ran: the situation was hard to assess visually for the contaminated ditetrazoliums (and of course nitroblue tetrazolium and tetranitroblue tetrazolium formazan never left the primary origin): on re-running uncontaminated

blue tetrazolium, the residue which remained at the secondary origin was not blue, the colour of the definitive formazan, but was pink. If exposed to ammonium sulphide, this pink spot became blue, and then after drying ran with the R_F value of the definitive diformazan. It thus appeared that, when tetrazolium salts were being reduced, there was a reversible interconversion between the definitive formazan and other reduction products, though the equilibrium in the reaction favours the definitive formazan. In connection with blue tetrazolium one component of this reversible reaction appeared to be an intermediate reduction stage.

If tetrazolium spots on plates were weakly reduced, but then dried well before development, most or all of the formazan was of the definitive variety and ran as a zone in hexane–methylene dichloride (2:3) with the R_F value described above. However, the origins of the ditetrazolium salts, but not the monotetrazoliums, contained coloured material after the plates had been developed. If these plates, after drying, were re-run in methanol–water (8:1) the unreduced tetrazolium and the coloured material at the origin ran. The coloured material was red to blue in shade for each of the ditetrazoliums studied, did not run in a discrete zone, and was reasonably similar in R_F value for each salt studied. On exposure to ammonium sulphide the coloured zones assumed the colour of the corresponding definitive diformazans, and then ran with the R_F values of the appropriate diformazans when the plates were well dried and developed again in hexane–dichloromethane. It appeared that, if initial reduction was weak, ditetrazolium formed relatively stable intermediate reduction products, which could be separated chromatographically from the formazans which were also produced in the reduction.

DISCUSSION

There have been some previous chromatographic studies of individual tetrazolium salts. BURTNER *et al.*[2] carried out paper chromatography to demonstrate contamination of their specimen of neotetrazolium. TSOU AND SU[6] referred to chromatography of tetranitroblue tetrazolium but gave very little detail. OKUI *et al.*[7] carried out thin-layer chromatography on silica gel, using as developer water-saturated *n*-butanol, to demonstrate contamination of neotetrazolium. These workers also detected a coloured intermediate during reduction of pure neotetrazolium. GOSZTONYI *et al.*[8], working with paper chromatography and the Bush 'A' and 'B5' systems as developers, noted the two blue formazans derived from pure blue tetrazolium, and also the red intermediate, and claimed that other mono- and ditetrazoliums also yielded two formazans, which were probably isomers.

The present study has shown that thin-layer chromatography with suitable solvent systems can be usefully applied to a wider range of tetrazolium salts than those considered in the studies mentioned above. Also chromatography of the products of weak reduction of tetrazolium salts could show whether one was dealing with a mono- or a di-tetrazolium, though chromatography of formazans could lead to complex findings, because of the presence of possible reduction intermediates or isomers, unless tetrazolium salts were strongly reduced, and the resulting formazans adequately dried, prior to chromatographic development.

The present study has not determined the nature of the tetrazolium reduction products which occur in addition to the definitive formazans. Formazan isomerism

is known[9], and it has been suggested that ditetrazoliums can reduce first at one end of the molecule and then at the other, a possibility which could explain ditetrazolium intermediates. MAENDER AND RUSSELL[10] have shown the existence of a free-radical intermediate during reduction of the monotetrazolium triphenyl-tetrazolium chloride, though the possibility of such intermediates had been suspected earlier[11]. More recently DEGUCHI AND TAKAGI[12] have confirmed this observation, and NEUGEBAUER[13] has deduced the structure of the radical intermediate. It seems possible that free radical intermediates also occur in the reduction of ditetrazolium salts.

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