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CHROMATOGRAPHIC METHODS FOR THE ISOLATION AND IDENTIFICATION OF THE PRODUCTS OF CHOLINE OXIDATION

D. SPEED AND M. RICHARDSON
Department of Botany, University of Durham (Great Britain)
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SUMMARY

The quaternary ammonium compounds associated with the oxidation of choline were separated by column chromatography on a buffered weakly cationic ion-exchange resin. These compounds were further characterized by thin-layer chromatography, paper electrophoresis, ion-exchange paper chromatography, and paper chromatography.

Analysis of the products of the oxidation of (^{14}C-methyl) choline by rat liver mitochondria revealed the presence of radioactively labelled betaine, betaine aldehyde and choline.

It has been known for some years that in animals choline is oxidized to betaine via the intermediate betaine aldehyde[1] and that the process involves the activity of two enzymes[2]. The choline oxidase enzyme responsible for the initial dehydrogenation of choline to betaine aldehyde has been partially purified from rat liver mitochondria[3–5]. The further oxidation of betaine aldehyde is brought about by a betaine aldehyde dehydrogenase. The question of the intra-cellular site of this enzyme in animals appears to be unsettled. Several workers[6–9] have reported betaine aldehyde dehydrogenase activity associated with mitochondria. On the other hand, other workers[10–13] have supported the idea that almost all of the betaine aldehyde dehydrogenase is in the cytoplasmic fraction and not in the mitochondria.

Although betaine is of widespread occurrence in higher plants[14] and fungi[15], little is known of the mode of biosynthesis of this compound in these organisms. There is some evidence from *in vivo* experiments that choline is readily oxidized to betaine in some plant tissues[16–20]. As yet all previous attempts to demonstrate the presence of the enzymes responsible for this oxidation have been unsuccessful[16,19].

Further experiments with plant systems in this laboratory were initially hampered by difficulties encountered during the separation of the products of the oxidation of choline. None of the previously reported methods for the separation of choline and betaine by column chromatography on ion-exchange resins[21–23] have dealt with the important intermediate betaine aldehyde or with the compound dimethylglycine, which is produced by the demethylation of betaine[19,20]. Furthermore

there are few available details of the behaviour of these compounds during electrophoresis and paper or thin-layer chromatography. Previous identifications of betaine aldehyde have been based upon simple chemical spot tests, melting point data and infra-red spectroscopy[12].

This report describes a simple buffered ion-exchange column procedure for the chromatographic separation of choline, betaine aldehyde, betaine and dimethylglycine. These compounds were isolated from the buffer effluent as their reineckate derivatives. These were then converted to the original compounds and further characterized by paper electrophoresis, thin-layer chromatography on Silica Gel G, paper chromatography and ion-exchange paper chromatography. The feasibility of these methods was then demonstrated by their application in the resolution of the radioactive products obtained when (^{14}C-methyl) choline chloride was oxidized by rat liver mitochondria.

MATERIALS AND METHODS

Quaternary ammonium compounds

Betaine aldehyde was synthesized by a slight modification of the procedure described by JELLINEK *et al.*[12] 2,2-Diethoxyethyltrimethylammonium iodide (Aldrich Chemical Co. Inc., Milwaukee, Wisc.) was dissolved in water, and the iodide converted to the chloride by treatment with freshly prepared silver chloride. After filtration, the aqueous solution was evaporated *in vacuo* at 50°. The residue, which was a pale yellow syrup, was then frozen in liquid nitrogen in a reaction vessel containing solid (frozen) concentrated hydrochloric acid in a second chamber. During this freezing process the entire reaction vessel was flushed with nitrogen for 30 min. The system was then placed under a vacuum and the contents heated to 55° before mixing. The hydrolysis was allowed to continue at this temperature for 2 h, after which time the remaining acid was removed in a vacuum desiccator containing sodium hydroxide. The product was recrystallised as described previously[12]. The other quaternary ammonium compounds used in this investigation were obtained from commercial sources.

Ion-exchange column chromatography

The weakly cationic resin Amberlite CG-50 (Chromatographic grade, Type 1, 100–200 mesh; Rohm & Haas, Co., Philadelphia, Pa.) was regenerated and buffered at pH 7.3 with phosphate citrate buffer as described previously for the Zeo-Karb 226 resin[20]. The quaternary ammonium compounds (2–3 mg) were dissolved in a minimal volume of phosphate–citrate buffer pH 5.3 and applied to a column (1.2 cm × 125 cm) of the resin. Elution of the compounds was effected with phosphate–citrate buffer pH 5.3 at room temperature. Fractions (10 ml) were collected at a flow rate of 60–80 ml/h.

Determination of quaternary ammonium compounds

Column effluent fractions were analyzed for quaternary ammonium compounds by measurement of the absorbance of their periodide derivatives at 365 mμ. Aliquot samples (0.5–1 ml) of the effluent fractions containing choline, betaine, or dimethylglycine were made up to 2 ml with 2 *N* HCl but samples containing betaine aldehyde

were adjusted to pH 7.3 as the formation of the periodide of this compound was greatly reduced under acid conditions. One millilitre of a reagent (10 g of resublimed iodine and 12.4 g of KI in 1 l of water) was then added to the solutions which were shaken and placed in an ice-bath for 20 min. Ten millilitres of 1,2-dichloroethane were then added and the two layers mixed by bubbling a fine stream of nitrogen through them for exactly 30 sec. The absorbance of the organic layer was determined in a Unicam S.P. 800 at a wavelength of 365 mμ within 10 min, and compared with standard curves obtained previously for each of the quaternary ammonium compounds. Table I shows the optical densities of the periodides of μmolar quantities of each of these compounds.

TABLE I

OPTICAL DENSITIES OF PERIODIDE DERIVATIVES OF THE QUATERNARY AMMONIUM COMPOUNDS ASSOCIATED WITH THE OXIDATION OF CHOLINE

Compound	$E^{\mu mole}_{1\,cm}$ *at 365 mμ*
Choline	2.134
Betaine aldehyde	1.476
Betaine	1.006
Dimethylglycine	0.610

Isolation of quaternary ammonium compounds from column effluents

The effluent fractions containing a quaternary ammonium compound were pooled and concentrated *in vacuo* at 50°. The concentrated solutions were then acidified with a little concentrated hydrochloric acid and the quaternary bases precipitated as their water-insoluble reineckate derivatives by the addition of excess saturated ammonium reineckate solution. The reineckates were collected by centrifugation and converted to the free bases by the method of BYERRUM *et al.*[19].

Thin-layer chromatography

The method used was essentially that employed for the separation of compounds related to carnitine by ENEROTH AND LINDSTEDT[24]. Plates of Silica Gel G (E. Merck AG, Darmstadt, Germany) with an approximate wet thickness of 0.4 mm were prepared using a Unoplan leveller (Shandon Scientific Co., London). The solvent systems investigated were: (I) methanol–acetone–HCl (90:10:10, v/v), (II) methanol–acetone–HCl (90:10:4, v/v), (III) methanol–0.88 ammonia (75:25, v/v), and (IV) methanol–dioxane–0.88 ammonia (30:45:25, v/v). For two-dimensional separations solvent system III was used for the first dimension and was followed by solvent system II in the second dimension. After drying in a stream of warm air for 2 h the plates were placed in a desiccator containing iodine vapour from a few crystals at the bottom. Spots of the quaternary ammonium compounds appeared after a few minutes as brown areas which were then outlined with a fine needle. The R_F values of the compounds investigated during this study are given in Table II.

TABLE II

THIN-LAYER CHROMATOGRAPHIC R_F VALUES ON SILICA GEL G OF THE QUATERNARY AMMONIUM COMPOUNDS ASSOCIATED WITH THE OXIDATION OF CHOLINE

Compound	*R_F values*			
	Solvent I	*Solvent II*	*Solvent III*	*Solvent IV*
Choline	0.37	0.31	0.01	0.01
Betaine aldehyde	0.48	0.42	0.06	0.03
Betaine	0.55	0.48	0.47	0.10
Dimethylglycine	0.59	0.51	0.60	0.18

Paper electrophoresis

The method used was a slight modification of that reported by BROCKHUYSEN *et al.*[25] for the separation of carnitine and several other quaternary ammonium compounds. Electrophoresis was carried out on strips (40 × 410 mm) of filter paper (Schleicher & Schüll No. 2043) impregnated with 0.3 *M* acetic acid–pyridine buffer pH 4.0, for 90 min at 340 V. The strips were dried in a current of circulating air for 24 h to remove all traces of the pyridine which would otherwise interfere with the location of the quaternary ammonium bases. The reagents used for the detection of the compounds were as described by CROMWELL AND RICHARDSON[20]. The relative mobilities of quaternary ammonium compounds in this system are given in Table III. No corrections were made for electro-endosmosis.

Ion-exchange resin paper chromatography

Chromatography of the quaternary ammonium compounds was carried out on Amberlite SA-2 ion-exchange paper (British Drug Houses Ltd.) using 1 *N* HCl as the developing solvent. It was found necessary to pre-wash these papers with 1 *N* HCl and distilled water to remove a substance which gave rise to anomalous blackening of X-ray films. Chromatography was also carried out on Amberlite WA-2 ion-exchange paper (British Drug Houses Ltd.) impregnated with 0.2 *M* phosphate–citrate pH 7.3 and the papers were developed with the same buffer at pH 5.3. The R_F values of these compounds in these two systems are given in Table III.

TABLE III

ELECTROPHORETIC MOBILITIES AND ION-EXCHANGE PAPER CHROMATOGRAPHIC R_F VALUES OF THE QUATERNARY AMMONIUM COMPOUNDS ASSOCIATED WITH THE OXIDATION OF CHOLINE

Compound	*Electrophoretic mobility (distance (in cm) moved in 90 min at 340 V and pH 4.00)*	*R_F values*	
		Amberlite SA-2 *Solvent: 1 N HCl*	*Amberlite WA-2* *Solvent: 0.2 M phosphate–citrate, pH 5.3*
Choline	12.2	0.59	0.42
Betaine aldehyde	11.5	0.51	0.39
Betaine	5.0	0.92	0.82
Dimethylglycine	5.5	0.86	0.85

Paper chromatography

The following solvent systems (all single phase) were used, and Whatman No. 3 MM paper employed:

(1) *n*-Butanol–acetic acid–water (4:1:2),
(2) Pyridine–*sec.*-butanol–water (6:3:1),
(3) *n*-Butanol–ethanol–acetic acid–water (9:1:1:2),
(4) 95 % ethanol–0.88 ammonia (95:5).

The reagents used for the detection of the spots of the quaternary ammonium compounds were as described previously[20].

Oxidation of (14C-methyl) choline and betaine aldehyde by rat liver mitochondria

Mitochondria were isolated from rat liver by differential centrifugation in isotonic sucrose (0.25 *M*) as described by SCHNEIDER AND HOGEBOOM[26]. The mitochondrial pellet was resuspended in a reaction medium containing 0.255 *M* sucrose, 0.0225 *M* KCl, 0.001 *M* potassium phosphate buffer pH 7.2 and $2 \cdot 10^{-5}$ *M* DNP[27].

The activity of the enzymes choline dehydrogenase and betaine aldehyde dehydrogenase in these preparations was followed polarographically using a simple platinum/silver oxygen electrode (Rank Bros., Bottisham, Cambridge, Great Britain). The reaction media were allowed to come to equilibria at 29° before the substrates [5 mmole(14C-methyl) choline chloride, specific activity 2 μC/mmole, 4 mmoles betaine aldehyde) were introduced into the reaction chamber via a movable well with a small opening.

At the conclusion of each incubation (10–40 min) the contents of the reaction vessel were removed and deproteinised by heating at 100° for 10 min. After filtration the pH of the filtrate was adjusted to 5.3 by the addition of 0.1 *M* citric acid. The radioactive products of the oxidation of (14C-methyl) choline in this filtrate were then resolved by column chromatography on CG-50 as described previously.

The radioactivity in each fraction of effluent buffer solution was determined by the liquid scintillation method described by RICHARDSON[28] using a Beckman liquid scintillation spectrometer series 200 B (Beckman Instruments Inc., Fullerton, Calif., U.S.A.).

Fractions of the eluate containing a radioactive peak were combined and the quaternary ammonium compounds re-isolated and further purified for chromatography by the ammonium reineckate method.

Radioautographs of all chromatograms of 14C-labelled compounds were made with Ilford Industrial G X-ray films.

RESULTS AND DISCUSSION

Column chromatography

Betaine, dimethylglycine, choline and betaine aldehyde were well resolved in that order by a buffered column of CG-50 (1.2 cm × 125 cm) using 600 ml of phosphate–citrate buffer pH 5.3 (Fig. 1). The recoveries of these compounds on this column were always quantitative.

Thin-layer chromatography

Although no one single solvent system yielded complete resolution of the compounds under investigation (Table II), a satisfactory separation was achieved by

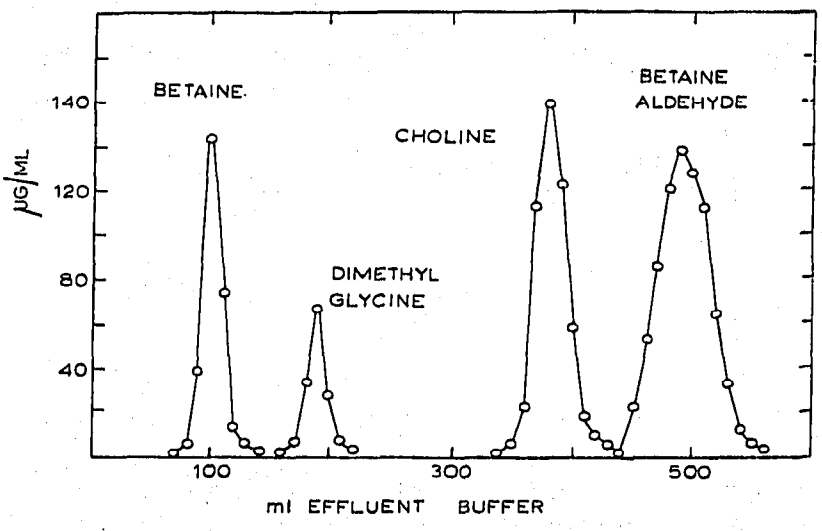

Fig. 1. Separation of betaine, dimethylglycine, choline and betaine aldehyde on a column (1.2 cm × 125 cm) of Amberlite CG-50 buffered at pH 7.3 and eluted with phosphate–citrate buffer pH 5.3.

a two-dimensional development using solvent system III in the first dimension and solvent system II in the second dimension. Best results were obtained when a thin section of the Silica Gel G was removed from the plate immediately below the solvent front left after the first development. Fig. 2 shows a typical autoradiograph of a two-dimensional TLC separation of the radioactive products of (^{14}C-methyl) choline oxidation by rat liver mitochondria.

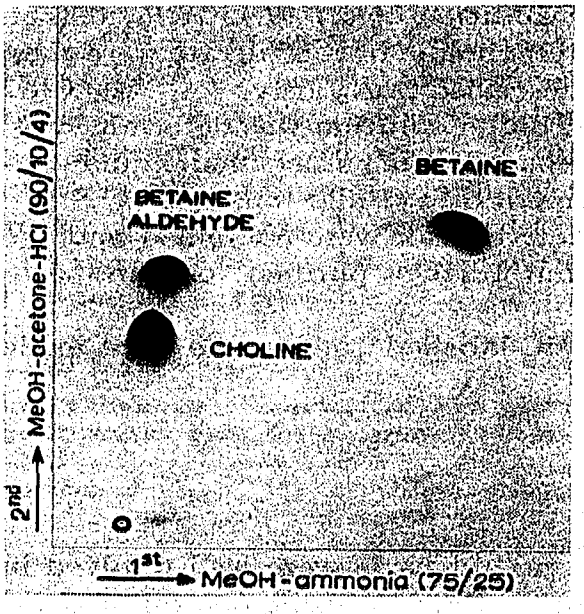

Fig. 2. Autoradiograph of the radioactive products of the oxidation of (^{14}C-methyl) choline by rat liver mitochondria separated by two-dimensional TLC on Silica Gel G.

Paper electrophoresis

Although the method of paper electrophoresis described facilitated the separation of the more amphoteric compound betaine from choline and betaine aldehyde, there was no appreciable resolution of the two latter compounds (Table III).

Ion-exchange paper chromatography

The separation of the quaternary ammonium compounds during chromatography on both types of ion-exchange paper (Table III) was similar to that obtained by electrophoresis. The more strongly charged compounds choline and betaine aldehyde were retarded to a greater extent than betaine or dimethylglycine. There was, however, no satisfactory resolution of choline from betaine aldehyde, or of betaine from dimethylglycine.

Paper chromatography

Table IV shows the R_F values of choline, betaine aldehyde, betaine and dimethylglycine obtained by paper chromatography in a number of different solvent systems. It is evident that no one solvent system completely separates these compounds.

TABLE IV

PAPER CHROMATOGRAPHY R_F VALUES OF QUATERNARY AMMONIUM COMPOUNDS

Key to solvent systems: 1 = *n*-Butanol–acetic acid–water (4:1:2).
2 = Pyridine–*sec.*-butanol–water (6:3:1).
3 = *n*-Butanol–ethanol–acetic acid–water (9:1:1:2).
4 = 95% ethanol–0.88 ammonia (95:5).

Compound	*R_F values for system No.*			
	1	*2*	*3*	*4*
Choline	0.65 (0.48) *	0.32	0.23	0.63
Betaine aldehyde	0.75	0.36	0.56	0.61
Betaine	0.53	0.31	0.29	0.47
Dimethylglycine	0.52	0.31	0.28	0.41

* The number in parentheses refers to a faint anomalous spot.

A fairly satisfactory separation was achieved, however, by a two-dimensional development using solvent system III in the first dimension and solvent system IV in the second dimension.

In general the somewhat poor separation of betaine and dimethylglycine by paper chromatography, electrophoresis and chromatography on ion-exchange papers was not a serious drawback as these two compounds were normally initially separated by ion-exchange column chromatography.

Oxidation of (^{14}C-methyl) choline by rat liver mitochondria

Two-dimensional TLC of the radioactive products of the oxidation of (^{14}C-methyl) choline by rat liver mitochondria revealed the presence of three radioactive compounds (Fig. 2). The R_F values of these compounds corresponded with those of authentic choline, betaine and betaine aldehyde.

These radioactive compounds were then initially purified by column chromatography on Amberlite CG-50. Fig. 3 shows the elution profile of the products of a typical experiment in which 10 μC (5 mmoles) of (^{14}C-methyl) choline were incubated with mitochondria for 40 min. Three peaks of radioactivity were obtained. The elution volumes of these peaks corresponded with those of authentic betaine (peak 1), choline (peak 2) and betaine aldehyde (peak 3).

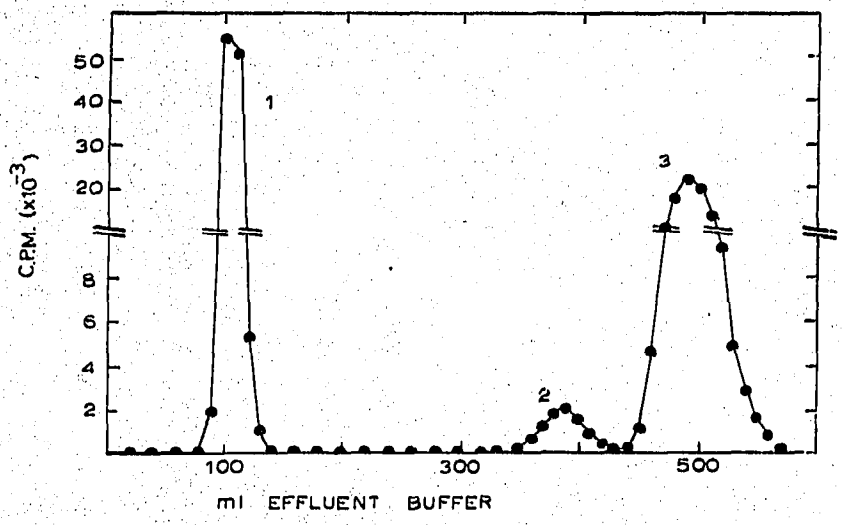

Fig. 3. Chromatographic separation of the radioactive products of the oxidation of (^{14}C-methyl) choline by rat liver mitochondria on a column (1.2 cm × 125 cm) of Amberlite CG-50 buffered at pH 7.3 and eluted with phosphate–citrate buffer pH 5.3. 1 = betaine; 2 = choline; 3 = betaine aldehyde.

The quaternary ammonium compound contained in the pooled fractions of each peak was precipitated by means of ammonium reineckate. After recrystallisation from acetone the melting points of the reineckates thus obtained were found to be in good agreement with the published data for the authentic compounds, betaine reineckate, choline reineckate and betaine aldehyde reineckate[12].

After removal of the reineckate ions the original compounds were individually subjected to two-dimensional TLC followed by autoradiography. The results obtained confirmed that the radioactive quaternary ammonium compound in peak 1 was betaine, in peak 2 was choline and in peak 3 was betaine aldehyde (Fig. 4). Further confirmation of these identifications was obtained by electrophoresis, and by ion-exchange and paper chromatography.

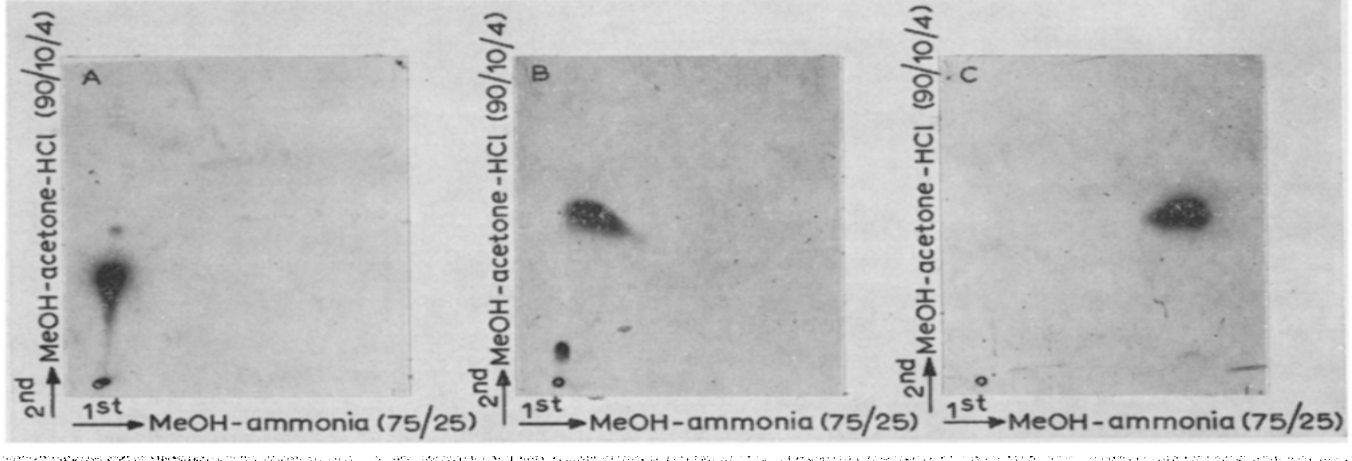

Fig. 4. Autoradiographs of two-dimensional thin-layer chromatographs of the individual radioactive products of the oxidation of (^{14}C-methyl) choline by rat liver mitochondria. A = radioactive quaternary ammonium compound (choline) in peak 2 after column chromatography on Amberlite CG-50 (see Fig. 3); B = peak 3 (betaine aldehyde); C = peak 1 (betaine).

The appearance of relatively large amounts of radioactive betaine in such reaction mixtures suggested that in addition to choline dehydrogenase in the mitochondria there was an enzyme capable of oxidizing betaine aldehyde. This suggestion was supported by the polarographic observation that a rapid uptake of oxygen occurred when betaine aldehyde (4 mmoles) was added to a suspension of rat liver mitochondria. The enzyme involved may be the NAD-dependent non-specific aldehyde dehydrogenase reported in rat liver mitochondria by GLENN AND VANKO[8], or a more specific betaine aldehyde dehydrogenase[7,9].

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