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Chromatography of riboflavin decomposition products

Part VI. Demonstration of non-volatile contaminants of ^{14}C-acetate by thin-layer chromatography*

It is well known that commercial radiochemicals may contain considerable amounts of contaminants which are due, at least partly, to self-irradiation of solid samples.[2] This does not apply only to such reactive organic substances as methionine, but, *e.g.*, to phosphate or iodide as well. We should like to report an observation which may serve as a warning to those concerned with the conversion of acetate into other products, especially in the case of enzyme-catalyzed reactions.

We have been trying to characterize a substance which was called 69 CX in one

* For the preceding communication, see ref. 1.

of our papers[3] and which originates from riboflavin in an acetate-containing medium. These studies will form the subject of a later communication. In order to check whether the carbon atoms of acetate are incorporated in the photolytic product 69 CX, some of the photolytic experiments were performed in a medium containing ^{14}C-acetate and the excess of acetic acid was removed by evaporation after acidification with hydrochloric acid. Substance 69 CX appears to be stable under these conditions. Controls of these experiments included acetate without riboflavin with light, acetate plus riboflavin without light and acetate without light after the addition of the photolysed sample of riboflavin in HCl-containing medium in which the substance 69 CX does not appear. A very rich pattern of spots was found on the autoradiograms of the respective control chromatograms but, fortunately, no spot was observed at the position corresponding to 69 CX in the two-dimensional system employed (TREADWELL *et al.*[4]).

Samples

Sodium 2-^{14}C-acetate Isocommerz Berlin, DDR, designated as "radiochemically pure" (less than 1 % impurities, expedited 6th April 1966, obtained through Messrs ÚVVVR, Prague; spec. activity 23.8 mC/mmole). An amount corresponding to 620 μC was dissolved and diluted with non-labelled sodium acetate to give 0.1 ml of an 0.4 *M* solution of sodium acetate to which was added 0.1 ml of an 0.4 *M* solution of non-labelled acetic acid (pH about 4.6).

To this acetate buffer, the following additions (0.2 ml) were made in respective cases:

Sample 1. Water.

Sample 2. Solution of riboflavin (150 mg/100 ml). Riboflavin was dissolved in 0.02 *N* NaOH and the solution made to pH 4.6 by concentrated acetic acid.

Sample 3. Riboflavin solution (150 mg/100 ml) in HCl-containing medium at pH 4.6 which had been photolysed in absence of acetic acid.

Each of the samples 1–3 (0.4 ml) was acidified with a few drops of 1 *N* HCl, evaporated, the residue dissolved in 0.4 ml 1 *N* HCl and again evaporated to dryness by warm air from the hair-drier under subdued light in a fume-cupboard. The residues were then taken up in 0.4 ml 1 *N* HCl and 20 μl aliquots were applied on the chromatograms.

Sample 4. 250 μC of sodium 2-^{14}C-acetate (same product as above) was dissolved in 25 μl water to give a 0.42 *M* solution which was then acidified with 60 μl of concentrated acetic acid and evaporated in a small tightly closed desiccator which contained, in separate containers, concentrated H_2SO_4 and solid NaOH*. The residue was then dissolved in 60 μl of concentrated acetic acid and evaporated again in the desiccator over H_2SO_4 and NaOH. After removing the dish from the desiccator, the residue was exposed briefly to a current of warm air and redissolved in 125 μl of 50 % acetic acid. An aliquot (20 μl) was applied on a chromatogram.

Thin-layer chromatography

Kieselgel G (Merck) layers were spread by means of a rod and heated to 105° for 90 min. The layer was about 10 mg per cm^2 area. A mixture S 1, acetic acid–

* Radioactivity assay showed that both NaOH and H_2SO_4 participated in the absorption of acetic acid vapour.

2-butanone–methanol–benzene (5:5:20:70; v/v)[4], was used in the first and a mixture S 2, *n*-butanol–acetic acid–water (4:1:5; v/v), in the second dimension.

One-dimensional chromatograms of the same samples and of suitable reference samples of riboflavin photolysed in an acetate-containing medium were run on two sides of the two-dimensional quadrangle.

A tank of 9.5 l gas volume per 3 chromatograms (20 × 20 cm) was used. Two contralateral sides of the tank were lined up with Whatman 3 paper soaked with the solvent mixture one hour before the plate was inserted and development was started. The tank was wrapped in black paper.

Detection was carried out by viewing in U.V. light from an analytical lamp provided with Wood's filter (Philora, Luma or Osram), emission max. 366 nm, and by autoradiography. Dry chromatograms were placed in contact with an X-ray film (ORWO AF 4 for macro-autoradiography, VEB Filmfabrik Wolfen, DDR) and exposed for 3.5 days on an average.

Scintillation counting

Layers from finished chromatograms were scraped off, placed in scintillation vials to which were added 2.5 ml ethyl alcohol and 7.5 ml SLT-31 (Spolana N.P., Neratovice, ČSSR), containing PBD [2-(4-biphenyl)-5-phenyl-1,3,4-oxadiazole] and POPOP [1,4-bis-(5-phenyloxazol-2-yl-)benzene] in toluene, and counted using the Nuclear Chicago equipment Mark I. Counts were converted to disintegrations by means of the external standardization system and an efficiency *vs.* channel ratio plot, previously checked for samples containing silica gel sediment.

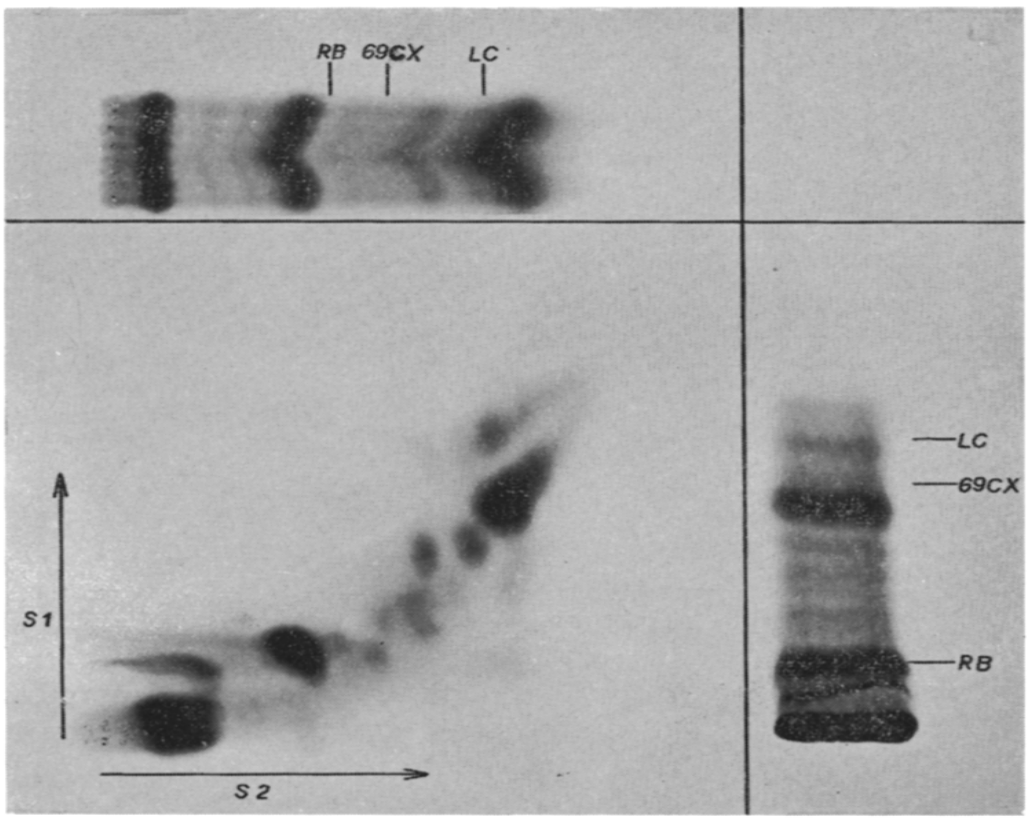

Fig. 1. Chromatogram of a sample obtained after acidification with HCl. For explanation see Fig. 2.

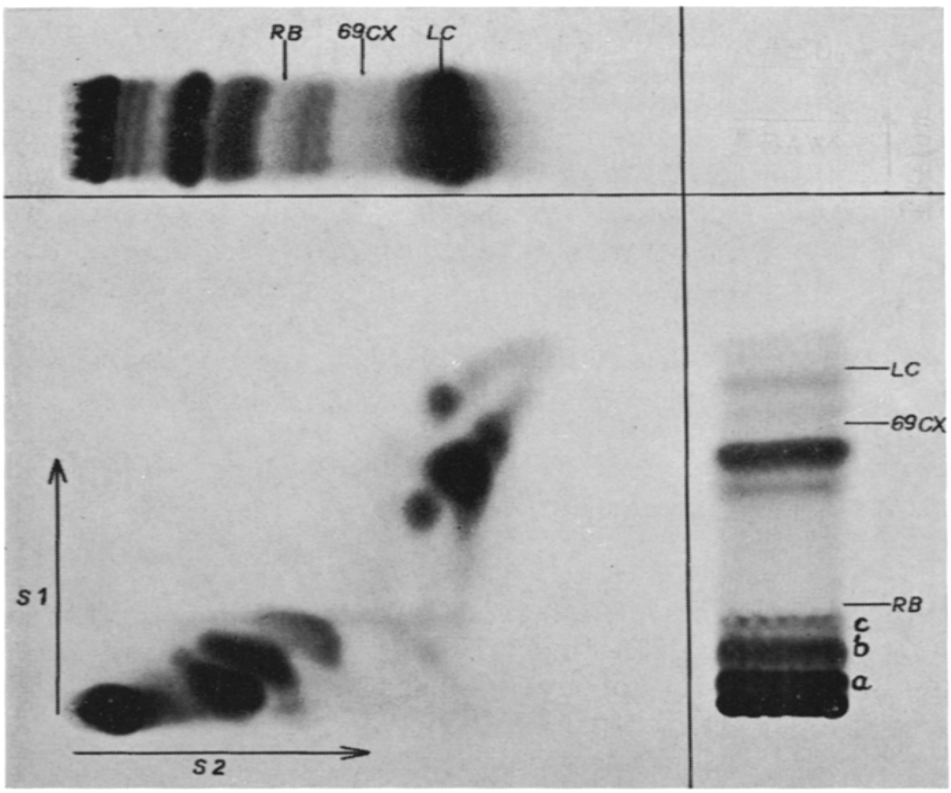

Fig. 2. Chromatogram of sample 4 obtained after acidification with an excess of unlabelled acetic acid. First run S 1, second run S 2. *RB*, *69 CX*, *LC*: Positions of riboflavin, 69 CX and lumichrome markers (spots not shown). *a*, *b*, *c*: Spots mentioned in foot-note on page 397.

Results

Samples 1–3. Autoradiograms of samples 1–3 were essentially alike without respect to the presence of riboflavin or its photolytic products. One of them is shown in Fig. 1.

The average radioactivity obtained from the whole individual one-dimensional chromatograms in S 1 (scintillation counting) was about 525,000 d.p.m. Since an aliquot corresponding approximately to 31 μC (*i.e.* 68,200,000 d.p.m.) of the original acetate sample was applied, this would represent on the average 0.77 % of the original sample.

Another preparation of radioactive sodium acetate (1-^{14}C, from the same producer, controlled by TLC and containing less than 1 % radiochemical impurities, expedited on 21st March 1967), was subjected to two-dimensional chromatography with following autoradiography and showed a similar pattern of spots, the major ones of which approximately corresponded, in shape and intensity, to those in Fig. 1. In this case scintillation counting was not carried out.

Sample 4. The preceding experiments open up a question whether the chromatogram only revealed radioactive non-volatile constituents already present in the commercial preparation or whether some of the components of the evaporated sample had not been formed due to the high concentration of HCl (azeotropic mixture) during evaporation and heating. In the last experiment to be reported an attempt was made to chase acetic acid and possibly other volatile acidic constituents in the presence of acetic acid instead of HCl and at room temperature.

The autoradiogram (Fig. 2) is not completely identical with that of samples 1–3 (Fig. 1). There are more of the fairly strong spots in Fig. 2, but most of the spots appear on both chromatograms in identical positions*.

When the layer material of the two-dimensional chromatogram and both one-dimensional runs of Fig. 2 was radio-assayed, the average radioactivity was slightly above 1 % of that of the respective aliquot of the original preparation of 2-^{14}C-acetate.

Sample 4 has thus shown the pattern of spots may differ to a certain extent, depending on whether radioactive acetic acid was volatilized by an addition of HCl or of an excess of unlabelled acetate, but that the amount of non-volatile radioactivity on the chromatogram has not diminished due to the absence of HCl during evaporation.

Conclusions

The calculations showed that the overall non-volatile radioactivity demonstrable by TLC did not exceed the limit of 1 % guaranteed by the producer, or at least did not exceed it to a large extent. Nevertheless, in incorporation experiments, substances which have been present in the acetate substrate might easily be attributed to incorporation products if appropriate controls were neglected. One of our experiments, in which the compound under investigation (69 CX) contained about 3000 d.p.m. in a chromatographic spot compared with nearly 3,000,000 d.p.m. of the whole group of chromatographically detectable acetate contaminants, is an example of a case where such controls are absolutely necessary.

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* An aqueous solution of sodium 1-^{14}C-acetate, dispatched on 21st March 1967, was chromatographed in system S 1 without previous acidification and evaporation. Though the whole autoradiogram was dark, as expected (exchange with acetic acid in the solvent system over the gas phase and incomplete removal of acetic acid from the chromatogram before autoradiography), several bands were conspicuous, some of the most prominent corresponding in position to bands a, b and c in Fig. 2.