Journal of Chromatography, 132 (1977) 121–127
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CHROM. 9536

DETECTION REAGENT FOR ADENINE, GUANINE, URACIL, CYTOSINE AND THEIR ALKYLATED BASES, NUCLEOTIDES AND NUCLEOSIDES ON THIN-LAYER PLATES

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(First received May 4th, 1976; revised manuscript received July 5th, 1976)

SUMMARY

The detection of alkylated bases at the nanogram level is reported. The reaction is based on two steps, first, chlorination of the bases and, secondly, spraying the chlorinated product with *o*-tolidine solution to give a dark blue color. The detection of nucleotides and nucleosides is also possible. The optimum conditions and the possible mechanism of the reaction are discussed.

INTRODUCTION

The separation and detection of alkylated purine and pyrimidine bases, the products of a reaction between carcinogenic alkylating agents and DNA or RNA, are important aspects of chemical carcinogenesis. Research has been directed towards determining the methylated purine and pyrimidine base patterns of nucleic acids from animals exposed to carcinogenic alkylating agents. Paper and column chromatographic techniques were used for separating and estimating methylated base constituents[1–3] but these methods are tedious and time consuming. Thin-layer chromatography (TLC) has been employed for the separation of a number of methylated nucleic acid bases[4–6]. Recently, Issaq *et al.*[7] were able to separate a mixture of nineteen alkylated adenines and uracils by two-dimensional TLC, and to separate alkylated forms of nine uracils, ten adenines, eight guanines, and six cytosines by one-dimensional TLC.

Although alkylated bases have been separated by TLC, detection of these compounds at the nanogram level was not reported. These compounds may be detected at the 0.3–0.5 μg level by: (a) fluorescence quenching on fluorescent plates, (b) spray reagents, and (c) charring. Kochetkov *et al.*[8] reported interaction of 9-N-methyladenine and 1-N-methylcytosine in weakly acidic aqueous solutions. Later Leonard *et al.*[9] used the same reaction as a basis for a spray reagent to be used for detection of nucleotides and nucleosides on TLC plates. This method of detection[9] is dependent on the pH of the reagent and may vary from compound to compound. It

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also requires that the plate be placed in a developing tank with the reagent and heated for 15 min at 70°. The detection limit reported is 0.5 μg.

Since the extent of alkylation of any one base cannot be predicted, techniques for detecting the product of alkylation of the bases must be as sensitive as possible. This study presents a simple and sensitive method for detection of adenine, guanine, cytosine and uracil and their alkylated forms, and nucleotides and nucleosides by TLC at the nanogram level.

Among the reagents tested for the detection of the bases, chlorination followed by spraying with *o*-tolidine, after deaeration, gave the most satisfactory results. These reagents were previously used for the detection of barbiturates[10], amino and imino compounds[11–13] and for nitrogen-containing compounds which can be converted into chloramines[14–16].

EXPERIMENTAL

Apparatus

Standard glass tanks were used for plate development. A viewing cabinet with long-wavelength (366 nm) and short-wavelength (254 nm) UV lamps (Brinkmann, Westbury, N.Y., U.S.A.) was used to locate the spots on the plate.

Reagents

All solvents used were glass distilled (Burdick and Jackson, Muskegon, Mich., U.S.A.). The reagents were analytical grade. Sources of purine and pyrimidine bases are listed in Table I. Drummond micropipettes were used for spotting the samples on silica gel plates (EM Silica Gel 60 F-254; Brinkmann). The chlorine–tolidine reagent used was prepared according to Reindel and Hoppe[11]. 160 mg of tolidine were dissolved in 30 ml acetic acid, the solution was diluted to 500 ml with distilled water, and 1 g potassium iodide was added. Chlorine was generated by adding concentrated HCl to a $KMnO_4$ solution in a small beaker placed at the bottom of the developing tank.

Procedure

Solutions of adenine, uracil and cytosine bases were made in methanol, those of guanine bases were made in hot ethanol–water (1:1), and guanine solutions were made in ethanol–water–formic acid (50:50:5). Nucleotides and nucleosides were dissolved in water. After the guanine, adenine, uracil, cytosine, and their alkylated forms had been spotted on silica gel plates, the plate was developed in chloroform–methanol[7]. The nucleotides and nucleosides were spotted separately and developed in chloroform–methanol (90:10). After development, the plate was dried at 110° for 5 min until complete dryness was achieved. The plate was then set aside to cool to room temperature, placed in a developing tank, saturated with chlorine gas for 5 min or less. Excess chlorine was removed by allowing the plate to stand in the hood for 5 min or until the plate gave a negative test (no color reaction) when few microliters of reagent were spotted on the plate. When excess chlorine escaped, the plate was sprayed in the hood with the tolidine solution until the spots became visible.

TABLE I

LIST OF METHYLATED BASES, NUCLEOTIDES AND NUCLEOSIDES AND THE COLORS PRODUCED AFTER CHLORINATION AND SPRAYING WITH *o*-TOLIDINE

Compound	*Color*	*Solvent*	*Supplier**
Adenine	blue	methanol	Sigma
1-Methyladenine	blue	methanol	Sigma
N-6-Methyladenine	blue	methanol	Sigma
9-Methyladenine	blue	methanol	Cyclo Chem.
N-6-Dimethyladenine	yellow	methanol	Cyclo Chem.
9-Ethyladenine	blue	methanol	Cyclo Chem.
N-6-Dimethyl-9-ethyladenine	yellow	methanol	Cyclo Chem.
3-Methyladenine	yellow	methanol	Chem. Procurement Lab.
7-Methyladenine	blue	methanol	Chem. Procurement Lab.
6-Methoxypurine	blue	methanol	Aldrich
Uracil	blue	methanol	Sigma
3-Methyluracil	yellow	methanol	Sigma
5-Methyluracil	blue	methanol	Sigma
6-Methyluracil	blue	methanol	Sigma
1-Methyluracil	blue	methanol	Cyclo Chem.
1-Ethyluracil	yellow	methanol	Cyclo Chem.
1,3-Dimethyluracil	blue	methanol	Cyclo Chem.
1,5-Dimethyluracil	blue	methanol	Cyclo Chem.
1-Ethyl-5-methyluracil	yellow	methanol	Cyclo Chem.
5,6-Dimethyluracil	yellow	methanol	Chem. Fabrik
Cytosine	yellow	methanol	Sigma
1-Methylcytosine	yellow	methanol	Sigma
3-Methylcytosine	yellow	methanol	Cyclo Chem.
1,6-Dimethylcytosine	yellow	methanol	Cyclo Chem.
9-Ethylcytosine	blue	methanol	Cyclo Chem.
5-Methylcytosine	yellow	methanol	Chem. Fabrik
Guanine	blue	ethanol–water–formic acid (50:50:5)	Schwartz/Mann
6-O-Methylguanine	blue	ethanol–water (1:1), heating	Project 9 (Frederick Cancer Research Center)
7-Methylguanine	blue	ethanol–water (1:1), heating	Sigma
9-Ethylguanine	blue	ethanol–water (1:1), heating	Cyclo Chem.
N-2-Methylguanine	blue	ethanol–water (1:1), heating	Cyclo Chem.
1-Methylguanine	blue	ethanol–water (1:1), heating	Chem. Fabrik
3-Methylguanine	blue	ethanol–water (1:1), heating	Chem. Fabrik
9-Methylguanine	blue	ethanol–water (1:1), heating	Chem. Fabrik
Adenosine	blue	water	Sigma
2′-Deoxyadenosine	blue	water	Sigma
5′-Monophosphoric acid	blue	water	Sigma
Uridine	blue	water	Sigma
Cytidine	blue	water	Sigma
Guanosine	blue	water	Sigma
2′-Deoxyguanosine	blue	water	Schwartz/Mann
5′-Monophosphoric acid, sodium salt	blue	water	Sigma

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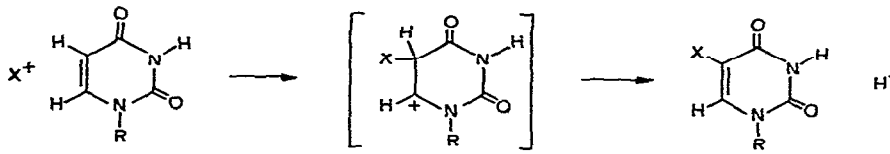
RESULTS AND DISCUSSION

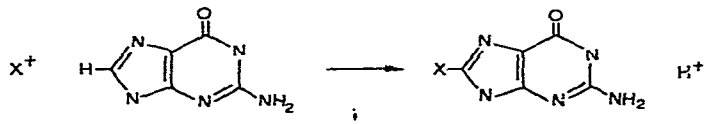
The reaction used for the detection of the purines and pyrimidines and their alkylated forms, nucleotides and nucleosides, is based on two steps: (a) halogenation and (b) reaction of the halogenated product with *o*-tolidine.

Halogenation

The mechanism of halogenation of purine and pyrimidine bases on TLC plates is unknown. However, halogenation in a non-aqueous medium is known[17] and is summarized below for the pyrimidines (i) and purines (ii)



R denotes hydrogen atom or carbohydrate
X denotes halogen

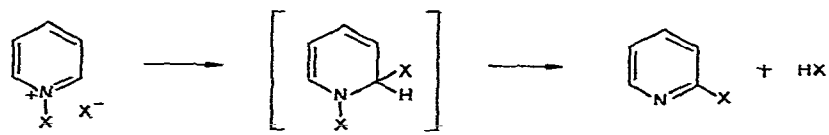


It is clear that the halogenation takes place through primary attack (substitution) by halogen on the C-5 atom of pyrimidine and the C-8 atom of purine. In the present study the halogen reactivity is $Cl_2 > Br_2 > I_2$, which is in agreement with the literature trend[17].

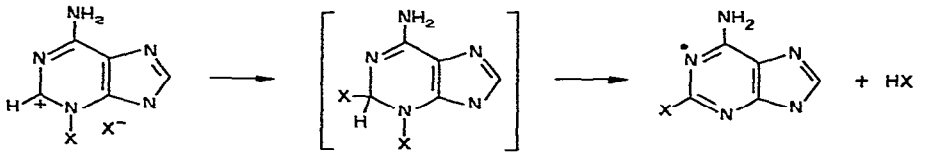
Kooyman[18] suggested the following speculative scheme for vapor phase halogenation of aromatic compounds

$$ArH + X_2 \rightleftharpoons (ArH^+ X_2^-)$$

This is summarized as follows for pyridine



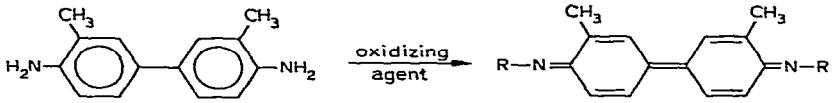
Halogenation of adenine may follow a similar path to that of pyridine



Halogenation can also take place at the N-1 atom to give the same product as that at N-3, or it can take place at N-7 or N-9 to give halogenation at the C-8 atom.

o-Tolidine reaction

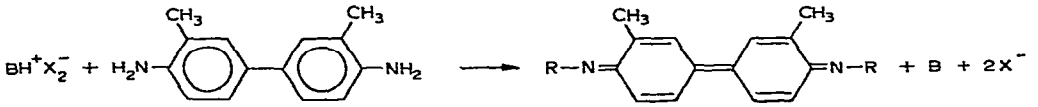
Reindel and Hoppe[11] reported the following reaction for the oxidation of *o*-tolidine



where R denotes Cl or H [(I) R = Cl; (II) R = H] the reaction goes to compound I. Further reaction of tolidine with the oxidizing agent possibly results in compound II, where R = H[11].

The reaction of chlorine with the base (B) may follow any of the following reactions: (a) chlorine association with the base, (b) charge-transfer type complex such that chlorine maintains its oxidation character[19], and (c) covalent bond but chlorine is bonded to the nitrogen where free chlorine can be generated again. In all the above cases, chlorine maintains its oxidation character and oxidizes *o*-tolidine. The reaction may be summarized as follows

$$B + X_2 \rightarrow BH^+X_2^-$$



We are fully aware that halogenation of purines and pyrimidines on TLC plates may not follow the above-mentioned mechanisms. The results of investigations of these mechanisms on TLC plates will be reported later. However, it is clear that chlorination of the bases takes place on silica gel plates because blue spots are produced when the plate is sprayed with the *o*-tolidine solution. *o*-Tolidine is known to react with chlorinated compounds to give a blue color[11,20].

To attain a maximum detection limit, the plate should be completely dry of developing solvent. This is achieved by drying the plate at 110° for 5–10 min, depending on solvent used. If the plate is not completely dry, the adsorbed solvent will react with the spray reagent after chlorination and give a dark-colored plate. It is

equally important that excess chlorine be allowed to escape from the surface of the plate since chlorine reacts with the raegent, giving a bluish color which will overshadow the spots. To determine if excess chlorine has escaped, a few microliters of reagent are spotted on the plate: a colorless spot indicates that no excess chlorine is present. A dilute spray reagent should be used to differentiate between the different purines and pyrimidines on the basis of color. A strong spray reagent gives a dark blue color for all the bases. It was observed that the color produced by the reaction of the reagent with the base may be a function of (a) amount of chlorine that reacted with the base, (b) concentration of the spot (amount per area), (c) concentration of the spray reagent, and (d) completion of the reaction.

Under optimum conditions, *i.e.*, saturated chlorine atmosphere in the tank to give complete reaction with the base, a dark blue spot indicates complete reaction of the chlorinated spot with the reagent.

When the spot is sprayed lightly, after chlorination with a diluted reagent (1:4) of that used in Experimental, the colors shown in Table I are observed. These colors require a concentration of 200 ng or more. It is clear from the above that the chlorine–tolidine reagent may be used not only to detect the bases but to identify them if a sufficient amount of compound is present. The selectivity and specificity of the reaction is under study and will be reported in a later communication.

It was also observed that the chlorine–tolidine reagent will react with the bases only when they are spotted on silica gel plates.

The chlorine–tolidine reagent was successfully used for the detection of 7-methylguanine in an extract of rat urine. The reagent was also used for the detection of adenosine, 2′-deoxyadenosine, 5-monophosphoric acid, uridine, cytidine, guanosine, 2′-deoxyguanosine, and 5′-monophosphoric acid sodium salt.

Bromine gives good results when substituted for chlorine in the above reagent. However, better sensitivity is achieved using chlorine.

Inorganic salts, such as $ZnCl_2$, $MgCl_2$, and $SnCl_2$, were used in place of KI to give approximately the same sensitivity except that a light blue color was obtained. The use of KI was preferred because the dark blue color obtained contrasts more sharply with the white background.

ACKNOWLEDGEMENTS

The authors would like to thank Dr. B. Shaikh for supplying a spiked extract of rat urine, and Dr. D. R. Crist and Dr. V. Horak, Department of Chemistry, Georgetown University, for helpful discussion. The research was sponsored by the National Cancer Institute under Contract No. N01-CO-25423 with Litton Bionetics, Inc.

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