THIN-LAYER CHROMATOGRAPHY: A NON-SPECIFIC METHOD FOR DEMONSTRATING CARCINOGEN–DNA INTERACTIONS

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INTRODUCTION

The 8-methyl ether of xanthurenic acid (XAE), a urinary metabolite of the amino acid tryptophan, is excreted in amounts of nearly 1 mg per day by humans[1]. XAE has carcinogenic activity for the urinary bladder of the mouse[2,3] and may play a causal role in the production of spontaneous human bladder tumors. On the other hand, 2,6-quinolinediol[2], L-kynurenine and anthranilic acid have not been demonstrated to have carcinogenic activity for the bladder of the mouse. The mechanism of action of XAE as a carcinogen is unknown.

Recently, MALKIN AND ZAHALSKY[4] presented evidence suggesting that thin-layer chromatography (TLC) could be employed to study the interaction of water-soluble carcinogens with deoxyribonucleic acid (DNA) as a possible mechanism of carcinogenesis. The proposed method[4] was employed to investigate the possibility that an interaction of XAE with DNA was involved in the carcinogenic process. The utility of TLC has been studied and found to be inadequate to probe the interaction of water-soluble carcinogens with DNA when employed in the manner suggested by MALKIN AND ZAHALSKY[4]. The data supporting this conclusion are presented in this communication.

EXPERIMENTAL

Materials

XAE[1], 4-nitroquinoline-N-oxide (NQO)[5], L-kynurenine sulfate[6] and 2,6-quinolinediol[7] were synthesized by known methods[1,5–7]. Anthranilic acid and Eastman Chromagrams (type K301R) were purchased from Eastman Organic Chemicals (Distillation Products Industries, Rochester, N.Y., U.S.A.), calf thymus DNA from Sigma Chemical Co. (St. Louis, Mo., U.S.A.), and actinomycin D (AMD) was provided by Merck, Sharp and Dohme (West Point, Pa., U.S.A.).

Methods

Following incubation with DNA, an interaction between the carcinogen or test chemical and DNA was inferred on the basis of whether or not the carcinogen attained its normal R_F value or remained at the origin with the bulky DNA[4]. Eastman Chromagrams were developed with distilled water, a mixture of 0.015 M NaCl plus 0.015 M

sodium citrate (0.1 times SSC buffer), or methanol[4] in an ascending system. The water-soluble carcinogen NQO, reported by MALKIN AND ZAHALSKY[4] as interacting with calf thymus DNA in this test system, was utilized as a positive control. AMD, an antibiotic known to bind to DNA[8], was also studied. Anthranilic acid, L-kynurenine and 2,6-quinolinediol were employed as non-carcinogenic, negative controls for comparison with XAE.

RESULTS

The method described[4] was employed with incubation of XAE and DNA in a ratio of 1 μg of XAE to 10 μg of DNA. After spotting and developing the chromatogram, the XAE did not move from the origin, its normal R_F value in the 0.1 times SSC buffer solvent system being 0.58. This same phenomenon was observed using NQO and DNA in the same ratios (Fig. 1). No ultraviolet absorbing or fluorescing material was seen in the developing solvent. The use of heat to dry the spotted chromatograms prior to development did not alter the observations. When chromatograms were spotted in the dark to prevent extraneous ultraviolet light from decomposing the carcinogens[9] or test chemicals, the results were unchanged. The incubation of the DNA test compound mixture prior to chromatography, as described by MALKIN AND ZAHALSKY[4], was not necessary to demonstrate this apparent interaction. This method was non-specific for carcinogens alone, as similar results were obtained with 2,6-quinolinediol, L-kynurenine and anthranilic acid.

It was observed that during the development of the chromatograms the solvent initially moved around the spots of the DNA test compound causing slight elution of the test compound from the sides of the spot (Fig. 1). This phenomenon could easily be seen with all test compounds, except NQO, because their bright blue fluorescence made them easily distinguishable from DNA. The ultraviolet absorption of NQO was very similar to that of DNA, and the elution of NQO, as contrasted with

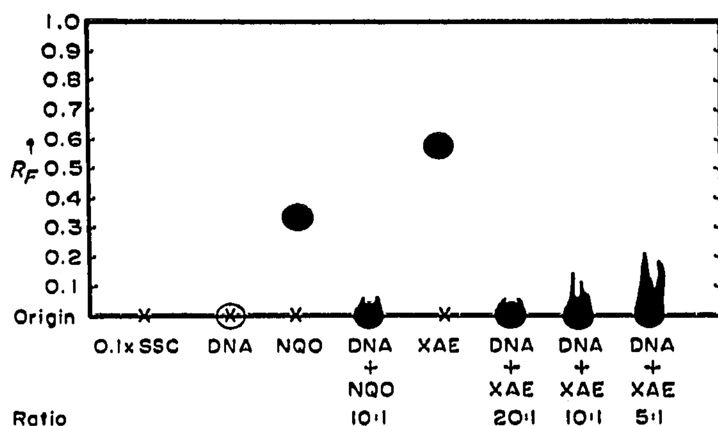

Fig. 1. Chromatogram showing "interaction" of NQO and XAE with native calf thymus DNA. Calf thymus DNA (100 μg/ml) and XAE or NQO (100 μg/ml) dissolved in 0.1 times SSC buffer at pH 7.2 were incubated with shaking at 37° in the dark for 10 min. Then 30 μl of the reaction mixture were spotted on Eastman Chromagrams and developed in tanks for 90 min with 0.1 times SSC buffer as solvent. Chromatograms were dried and examined with U.V. light (2537 Å). Note elution of carcinogens around edges of DNA spot and increased elution with decreasing DNA concentration.

that observed with the other test compounds, was not readily apparent. When mixtures of various ratios of DNA to XAE or NQO (20:1 through 5:1) were spotted, the amount of elution of XAE or NQO from the spot was inversely proportional to the amount of DNA in the spot (Fig. 1). The greater extent of elution of XAE from the spots with lower concentrations of DNA was probably due to decreased retardation of solvent flow through the DNA. It appeared that the DNA present acted to occlude the solvent from the spot and therefore did not permit normal solvent flow up the chromatogram. When XAE was spotted just above a spot of DNA with no physical contact between the two spots, the XAE did not attain its normal R_F value due simply to the retarded solvent flow imposed by the presence of the DNA (Fig. 2).

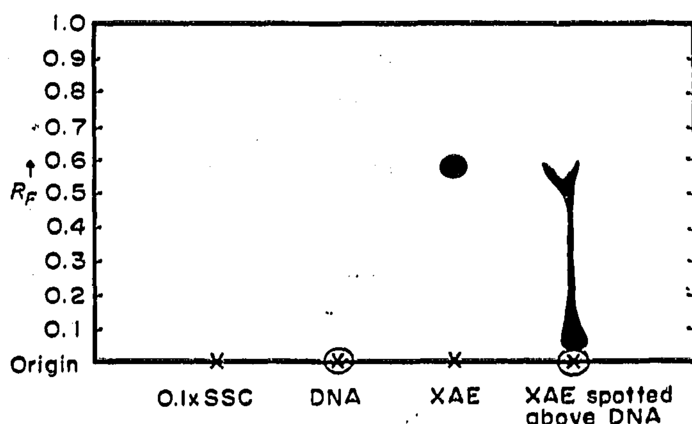

Fig. 2. Chromatogram demonstrating retardation of normal solvent flow by spot of DNA. XAE (0.75 μg) and DNA (7.5 μg) dissolved in 0.1 times SSC buffer were applied independently with no physical contact between the spots. The solvent employed for development was 0.1 times SSC buffer.

AMD and DNA were mixed in 0.1 times SSC buffer and incubated with suitable controls in the dark for 10 min. A portion of each reaction mixture was applied to the chromatogram in spots of varying diameters and the chromatogram was developed in methanol (Fig. 3). In contrast to the data reported by MALKIN AND ZAHALSKY[4], in the present experiment it was observed that all of the AMD migrated away from the DNA when the spot diameter was 6 mm or 10 mm (Fig. 3). Only when the spot diameter was 4 mm was any residual AMD observed at the origin.

When mixtures of DNA and XAE or NQO of various ratios (20:1 through 1:1) were applied in 5 times 1 cm bands at the origin rather than spotted on the chromatograms, the solvent flow was impeded proportional to the amount of DNA in the mixture (Fig. 4). This method of application forced the solvent to flow directly through the DNA test compound mixture rather than around it, and the test compounds in all cases studied were completely eluted from the DNA band (Fig. 4). Prior incubation of the XAE or NQO with DNA[4] did not prevent the test compound from being eluted from the DNA band. A band of DNA of high concentration (200 μg/5 cm^2)at the origin completely blocked solvent flow up the chromatogram. With a series of analyses using this banding technique, a plot[10] of solvent height (cm) above the origin against mg DNA/5 cm^2 showed the retardation of solvent flow, as judged by solvent height above the origin after 90 min, to be a linear function of the DNA concentration (Fig. 5). When a mixture of DNA and AMD (10:1) in 0.1 times SSC buffer was applied in

bands on the chromatograms and developed with methanol[4], the AMD was eluted to its normal R_F value of 1.00 by this solvent system. No AMD could be visually or spectrophotometrically detected remaining at the origin with the DNA.

DISCUSSION

Though AMD was demonstrated to bind to DNA, employing another method[11] than the one utilized in this study, one would not expect the binding to DNA to be demonstrated with the present technique. REICH *et al.*[8,12–14] suggested that the mechanism of binding of AMD to DNA involved hydrogen bonding of the actinomycin

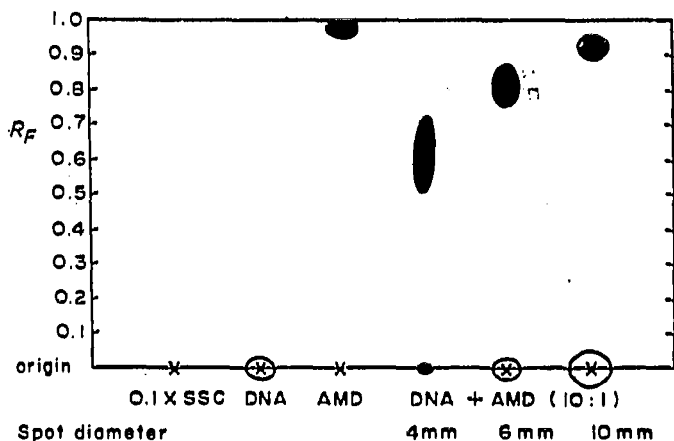

Fig. 3. Chromatogram illustrating effect of spot size on R_F value. AMD (100 μg/ml) and DNA (1 mg/ml) in 0.1 times SSC buffer were incubated with controls in the dark for 10 min. Following incubation, 30 μl of each reaction mixture were applied to the chromatogram in spots of varying diameters. The chromatogram was developed in methanol. After drying, the yellow AMD could be seen directly.

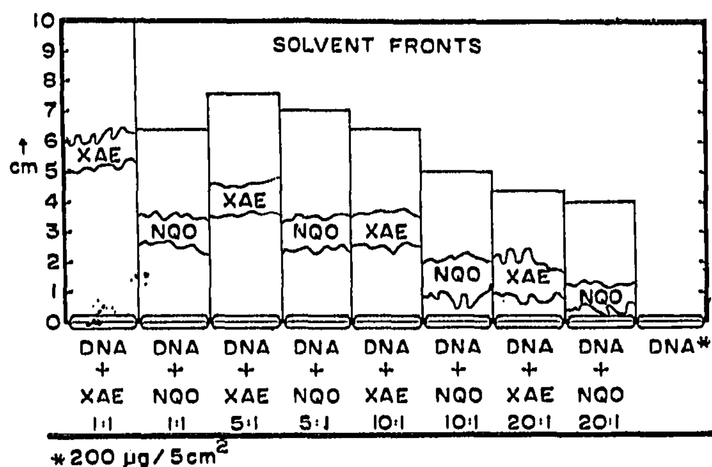

Fig. 4. Chromatogram showing retardation of solvent flow proportional to the amount of DNA in the band. Mixtures were incubated as described[4] and applied in 5 times 1 cm bands at the origin of one chromatogram which was divided vertically by scoring the chromatogram with a pencil. This method of application of the reaction mixtures forced the solvent (0.1 times SSC buffer) to pass directly through the DNA, eluting all of the XAE and NQO from the DNA. DNA of high concentration (200 μg/5 cm^2) was capable of completely blocking solvent flow at the origin.

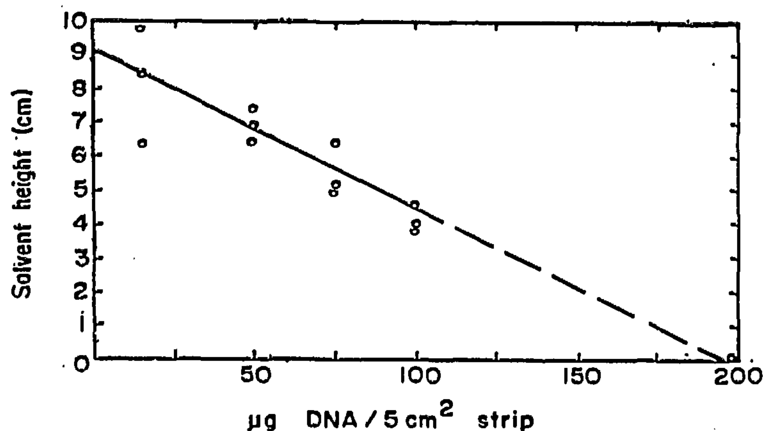

Fig. 5. Plot of solvent height (cm) above the origin against μg DNA/5 cm^2 of chromatogram at the origin. The DNA was applied to the chromatograms in 5 times 1 cm bands. The amount of retardation of solvent flow was a linear function of the amount of DNA applied to this area of the chromatogram. The slope of the regression equation best representing this linear relationship was computed by the method of least squares, and tested for statistical significance by the F test[10]. The slope was (—) 0.48 cm/μg DNA and was highly significant with a P value of less than 0.01.

chromophore to guanine residues in the DNA. The DNA–AMD complex could be dissociated readily by low concentrations of urea or extractions with organic solvents[8,14]. Thus it would be expected that the methanol employed as the solvent would disrupt the hydrogen bonds and cause the elution of AMD from the DNA band or spot. KAHAN *et al*[11] reported that 5.6 mμmoles of AMD bound to 100 mμmoles of calf thymus deoxynucleotides, approximately 50 μg of AMD per mg of DNA or a ratio of DNA/AMD of 20:1. In the present experiment (Fig. 3) and as described by other workers[4] a mixture of DNA and AMD in a ratio of 10:1 was employed. At this ratio it would be anticipated, if AMD did bind to DNA in this system, that only about one half of the AMD would remain at the origin with the DNA and that the remainder would migrate away from the origin when the reaction mixture was chromatographed as described. However, MALKIN AND ZAHALSKY[4] failed to record and apparently did not observe any migration of AMD, in contrast to that demonstrated in Fig. 3.

From the preceding observations we have concluded that the utilization of TLC by the method described by MALKIN AND ZAHALSKY[4] is an unreliable, inappropriate, and non-specific method of demonstrating carcinogen–DNA interactions because: (1) with aqueous solvents, the DNA occludes the solvent from the spot and does not permit normal separation of the test compounds from the DNA; (2) employing aqueous solvent systems, the presence of the DNA retards normal solvent flow up the chromatogram and does not allow the test compound to attain its normal R_F value; (3) systems using organic solvents would disrupt hydrogen bonds in compounds that used this type of bond to interact with DNA; and (4) the test compounds are often difficult to distinguish from the DNA spots on the chromatogram making interpretation very difficult.

Our observations do not preclude the interactions of XAE or NQO with calf thymus DNA, however, the use of TLC cannot be used as proof of interaction. It is possible that the method of banding the reaction mixtures on the chromatogram,

described in this communication, could serve as a qualitative demonstration of interaction but this method would be restricted to compounds that could be separated from DNA in aqueous buffer solvents, and that could be easily distinguished from DNA on the chromatograms.

ACKNOWLEDGEMENT

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SUMMARY

The utilization of thin-layer chromatography revealed an apparent interaction of native calf thymus DNA with two carcinogens: 4-nitroquinoline-N-oxide and the 8-methyl ether of xanthurenic acid. Similar results with 2,6-quinolinediol, L-kynurenine and anthranilic acid were observed. The observed "binding" of these compounds to DNA at the origin of the chromatograms was related to physical occlusion of the solvent by the DNA and retardation of normal solvent flow through the DNA spot. The test compounds were physically trapped within the DNA spot and could not be eluted by the solvent to give their normal R_F values. These inherent problems with thin-layer chromatography have precluded its use as positive proof of interaction of water-soluble carcinogens with DNA.

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