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ANALYSIS OF MUTAGENIC ACTIVITY IN CIGARETTE SMOKERS' URINE BY HIGH PERFORMANCE LIQUID CHROMATOGRAPHY

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Summary

Mutagenic activity in smokers' urine which had been concentrated by XAD-2 resin can be separated from approximately 90% of the non-mutagenic material by CH_2Cl_2 extraction. This extract appears to be stable for 3 months at −20°C. High performance liquid chromatography analysis of the CH_2Cl_2 extract showed multiple mutagenic non-polar fractions which were better activated by rat liver homogenates prepared from 3-methylcholanthrene treated rats than from rats treated with phenobarbital. Mutagenic activity in smokers' urine was extractable by acid, but not by base. The mutagens in smokers' urine appear to be a complex mixture of relatively non-polar chemicals.

There is reason to believe that environmental factors play an etiologic role in some human cancers (Peto, 1980). Most of the factors that have already been identified as human carcinogens are chemical in nature (IARC Working Group, 1980) and possess mutagenic activity (McCann et al., 1975a; Rinkus and Legator, 1979). The availability of short-term mutagenicity tests such as the Ames test (Ames et al., 1975) and the demonstration that most known carcinogens are mutagens (McCann et al., 1975a) make it possible to approach the problem of detecting and identifying some of the chemical causes of human cancer that are present in environmental samples and human body fluids and tissues.

Cigarette smokers' urine is mutagenic (Yamasaki and Ames, 1977). As the link between cigarette smoking and both lung and bladder cancer is fairly well established (USDHEW, 1979), we have begun to investigate the possibility of

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defining a relationship between cancer risk and the presence of mutagens in body fluids by studying the chemical nature and distribution of mutagenic activity in body fluid samples from cigarette smokers. This approach should be useful for developing procedures to detect and identify mutagenic activity in individuals receiving other environmental exposures to chemical agents which might place them at a high risk for developing cancer.

We have begun to characterize the chemical nature and biological activity of the mutagens in cigarette smokers' urine and report here on the results of high performance liquid chromatographic analysis of organic extracts of cigarette smokers' urine.

Materials ald methods

Materials

XAD-2 resin, obtained from Applied Sciences, was rehydrated by soaking for 15 min in methanol and backwashing extensively (3—6 h) with distilled water. 100 g of each batch of resin was assayed for the presence of acetone-elutable mutagenic activity. All batches had less than 50 revertants above background/100 g of XAD-2, or less than 1% of the activity obtained from smokers' urine using an equivalent amount of resin. Reagent grade solvents and chemicals were used throughout.

Urine collection

Urine samples were collected from smokers and non-smokers who were not taking any medication. Samples from Boston, MA were collected during working hours and immediately frozen at −20°C. Samples from Akron, OH were collected at the end of a work day, frozen and shipped in dry ice, then stored at −20°C. 24-h and other individual samples from the Republic of San Marino were shipped frozen with dry ice and stored at −20°C.

Sample preparation

Urine samples were thawed, neutralized to pH 7 with 5 N NaOH, and filtered to remove sediment (Whatman No. 1 followed by Whatman GF/A filters). Prior to adjustment, the pH of the urines ranged from 5.4 to 6.5 for smokers and from 5.6 to 6.2 for non-smokers. No relation between initial pH and mutagenic activity in smokers' urine was observed. The urine was passed over an XAD-2 column at a rate of 7 ml/min for a small (100 g) column or 60 ml/min for a larger (500—900 g) column. To improve the efficiency of extraction the amount of XAD-2 was increased from 1 to 10 g/100 ml urine (Yamasaki and Ames, 1977). The column was washed with distilled water (10 ml H_2O/100 ml urine) to remove trapped urine, and acetone (25 ml/100 ml urine) was used to elute material extracted by XAD-2 resin. The acetone eluate was dried in vacuo at 45°C, resuspended with water (20 ml H_2O/100 ml original urine) and neutralized to pH 7 with 0.2 N NaOH. The reconstituted fraction was then extracted 3 times with CH_2Cl_2 (7 ml CH_2Cl_2/100 ml original urine). CH_2Cl_2 extracts were dried with anhydrous Na_2SO_4, filtered through glass wool, evaporated in vacuo at 45°C, and stored at −20°C.

High performance liquid chromatography (HPLC)

CH_2Cl_2 extracts of urine concentrates were resuspended in methanol and centrifuged to remove methanol insoluble material which contained about 0.5% of the sample dry weight and no detectable mutagenic activity. The supernatant was chromatographed by reverse phase HPLC on a Whatman Partisil Magnum-9 column and a Perkin-Elmer Series 2 programmed for an elution gradient from 35% to 100% CH_3OH : H_2O changing at 3% per min with a flow rate of 5 ml/min. The column effluent was scanned continuously at 280 nm. Fractions were collected at 1-min intervals, dried in vacuo at 45°C and resuspended in methanol for mutagenicity testing.

Stability of urine extracts

In order to investigate the nature of the mutagens in smokers' urine, it was often necessary to retest stored material. To determine the validity of such results, the stability of the mutagenic activity in frozen material was determined. A CH_2Cl_2 extract of XAD-2 concentrate was divided into aliquots and dried in vacuo at 45°C. One aliquot was immediately tested for mutagenic activity and the others were stored at −20°C. Frozen aliquots which were thawed and tested for mutagenic activity were stable over a period of 2 months. A set of fractions from HPLC separation were also tested for stability. A portion of each fraction was tested before freezing. The remaining material was dried and stored at −80°C. The next day, the fractions were thawed, resuspended in methanol, and a portion removed and retested for activity (Table 1). No loss of activity had occurred. The remaining material was dried, stored for 4 additional days, and retested. 3 non-polar fractions lost 40—50% activity, but other fractions showed no loss of activity.

Mutagenicity testing

Samples were assayed for mutagenicity using the Ames/Salmonella test system (Ames et al., 1975). This test detects the reversion of specific histidine auxotrophs. Strains TA1538, TA98 and TA1537 carry frameshift mutations while TA1535 and TA100 carry a base-pair substitution. TA98 is TA1538 carrying the plasmid pKM101 which enhances mutagenicity (McCann et al., 1975b); TA100 is TA1535 carrying pKM101. Postmitochondrial rat liver supernatant (S9) was prepared from 100—150 g male Sprague—Dawley rats pretreated with Aroclor 1254, 3 methylcholanthrene, or phenobarbital (Ames et al., 1975). The protein concentrations (Lowry et al., 1951) were 29, 37, or 31 mg protein/ml S9 for the Aroclor, 3 methylcholanthrene, and phenobarbital preparations, resp. Except for the results presented in Tables 3 and 4, all tests were performed using strain TA1538 and 20 µl per plate of S9 from Aroclor induced livers. Positive controls (spot tests) were: 2-acetylaminofluorene for TA1538 and TA98, *N*-methyl-*N'*-nitro-*N*-nitrosoguanidine for TA1535 and TA100, 4-nitroquinoline-*N*-oxide for TA100, and 9-aminoacridine for TA1537. Acetylaminofluorene produced an average of 300 His^+ revertants/µg/plate (range 244—353 for 8 determinations) when tested against TA1538 in a plate incorporation test. All strains were tested for crystal violet sensitivity; TA100 and TA98 were tested for ampicillin resistance. Solvent controls were always negative compared to background.

TABLE 1

STABILITY OF MUTAGENIC ACTIVITY IN CH_2Cl_2 EXTRACT AND HPLC FRACTIONS FROM SMOKER'S URINE

Sample	Induced his^+ revertants/plate Days frozen							
	0	1	2	5	6	8	10	90
CH_2Cl_2 [a]	366	209	264	318	378	366	257	408
Fraction No. [b]								
1	0							
2	6							
3	0							
4	2							
5	7							
6	8							
7	0							
8	5							
9	12							
10	7							
11	23							
12	24							
13	33	33		59				
14	54	72		65				
15	106	161		168				
16	114	171		124				
17	207	182		160				
18	209	221		207				
19	208	256		239				
20	296	356		227				
21	199	153		96				
22	153	110		64				
23	100	93		49				
24	33	42		29				
25	56	49		34				
26	22	12		0				
27	10	6		0				
28	10	22		0				

[a] Averages of duplicate plates.

[b] Data from single plates; equivalent of 1700 ml of urine analyzed by HPLC, one-fourth fraction tested on each day.

Results

CH_2Cl_2 extraction of XAD-2 concentrate

Yamasaki and Ames (1977) showed that the mutagenic activity in smokers' urine could be concentrated by XAD-2 resin. We have found that the mutagenic activity can be further purified by extraction of the XAD-2 concentrate with CH_2Cl_2. Using this procedure, 96—98% of the dry weight of non-smokers' urine remained in the aqueous phase (407, 487, 708, 730, and 739 μg dry weight/ml original urine in the acetone eluate as compared with 12, 18, 17, 13, and 15 μg dry weight/ml original urine in the CH_2Cl_2 extract, resp.) However, virtually all of the mutagenic activity is extracted by the CH_2Cl_2. Recoveries of

95 ± 19% of the mutagenic activity were observed in the CH_2Cl_2 extract as compared with the acetone eluate. For example, in one experiment the CH_2Cl_2 extract contained 82% of the mutagenic activity of the acetone eluate (136 out of 165 induced His^+ revertants per concentrate of 30 ml of urine), but only 11% of the dry weight (14 mg out of 130 mg per concentrate of 100 ml urine). Thus, CH_2Cl_2 extraction is a useful and efficient purification step prior to chromatographic or other chemical analysis.

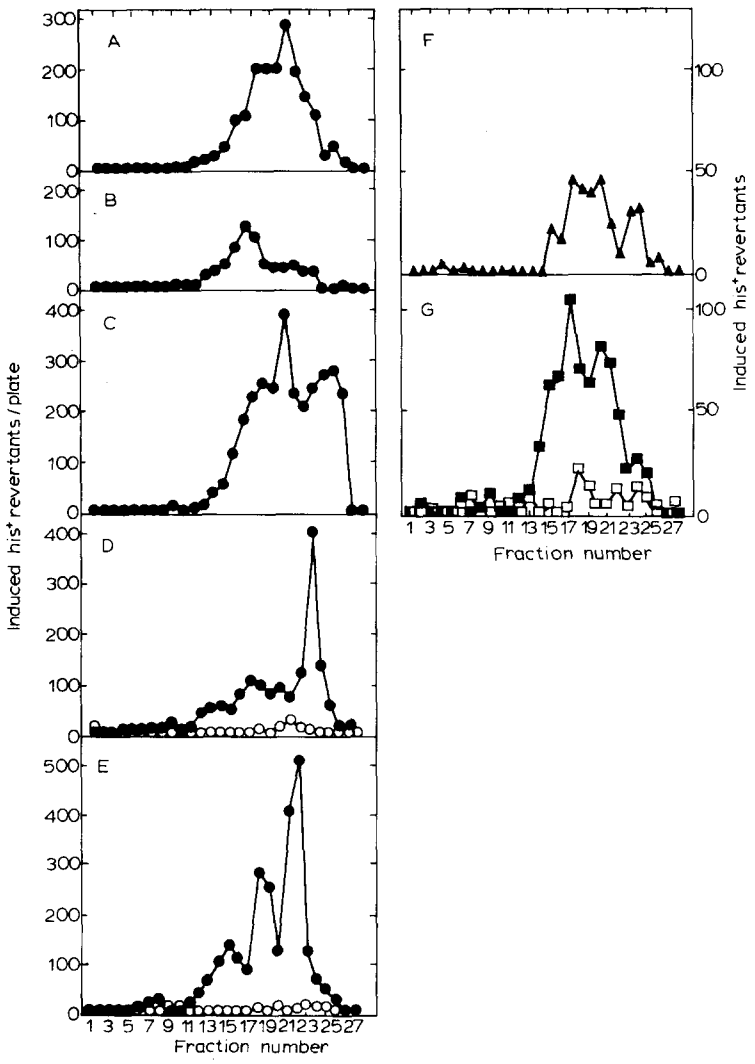

Fig. 1. HPLC fractionation of smokers' and non-smokers' urine. CH_2Cl_2 extracts of XAD-2 concentrates were dried, resuspended in methanol, and fractionated by reverse phase HPLC using a 35—100% CH_3OH : H_2O gradient changing at 3%/min with a flow rate of 5 ml/min. Fractions, collected at 1-min intervals, were tested for mutagenicity. Volumes from each fraction, equivalent to 250 ml of fractionated urine, were tested. B: a 5-l urine preparation; all other preparations are 1—2 l. A—E: a smoker (●) and pooled urines from non-smokers (○) from Boston, MA. F: pooled urines from smokers from Akron, OH (▲). G: urine from a smoker (■) and non-smoking spouse (□) from the Republic of San Marino (data from single plates).

Fractionation of CH_2Cl_2 extracts of smokers' urine by HPLC

To begin characterizing the nature of the mutagenic substances extracted from urine by CH_2Cl_2, extracts were subjected to HPLC separation using a reverse phase column and a CH_3OH : H_2O gradient. The distribution of mutagenic activity from several preparations over a period of months is displayed in Fig. 1. Figs. 1A—E represent profiles from several different urine collections from one smoker. Under the conditions used, pyrene eluted with a bandwidth of less than 1 fraction. The mutagenic activity in smokers' urine eluted in approximately 15 fractions. Recoveries of mutagenic activity from these fractionations were 102%, 53%, 92%, 92%, 56%, 37%, and 72% for Figs. 1A—G, resp. Considerable variation was noted in both the total amount of mutagenic activity per liter of urine and in the proportion of activity in any particular fraction. However, the mutagenic activity always eluted with the same, relatively non-polar portion of the gradient. When one preparation was divided in half and each half was run through the same HPLC protocol, little variation was seen between two consecutive fractionations of the same material. Thus, the mutagenic material in the urine behaves like a series of compounds which can exist in varying amounts.

Urine from 9 smokers and 5 non-smokers was analyzed by HPLC; several people were tested multiple times. Representative results are shown in Fig. 1. The chromatographic profiles of mutagenic activity of CH_2Cl_2 extracts from 5 samples from 1 male smoker from Boston, MA (Figs. 1A—E) can be compared with CH_2Cl_2 extracts of pooled urine from 4 male smokers from Akron, OH (Fig. 1F) and from a male smoker from the Republic of San Marino (Fig. 1G). The samples from Akron and the Republic of San Marino resemble the variable profiles seen from the Boston smoker's urine.

Non-smokers' urine samples were prepared in parallel with some of the smokers' urines (Figs. 1D, E, and G). With the exception of the non-smoking spouse of a smoker (Fig. 1G), all of the urines from non-smokers had no detectable mutagenic activity in either the CH_2Cl_2 extract of the XAD-2 concentrate or among the HPLC fractions. The non-smoking spouse of a smoker had 108 induced His^+ revertants/100 ml of urine as compared to 534 His^+ revertants/100 ml of urine from the smoking spouse. HPLC analysis of this non-smoker's urine indicated mutagenic activity chromatographically similar to that of the smoking spouse.

Since the fractions with mutagenic activity are contiguous, the chromatographic purity of the fractions was tested. Fractions 15 and 19 from a large preparation (5 l of urine, Fig. 1B) were rerun using the same HPLC protocol (Fig. 2). Both the absorbance and mutagenicity peaks of fraction 15 eluted earlier than fraction 19. Moreover, for a given fraction, the absorbance and mutagenicity eluted together. When other fractions of this preparation were rerun, their behavior was also chromatographically distinct.

Acid and base extraction of urine concentrates

In an attempt to further characterize the multiple mutagens in smokers' urine, CH_2Cl_2 extracts of XAD-2 concentrates were extracted with 1 N NaOH or 1 N HCl. Very little mutagenic activity was extracted by the basic wash, while much of the mutagenic activity was extracted into the aqueous phase of the

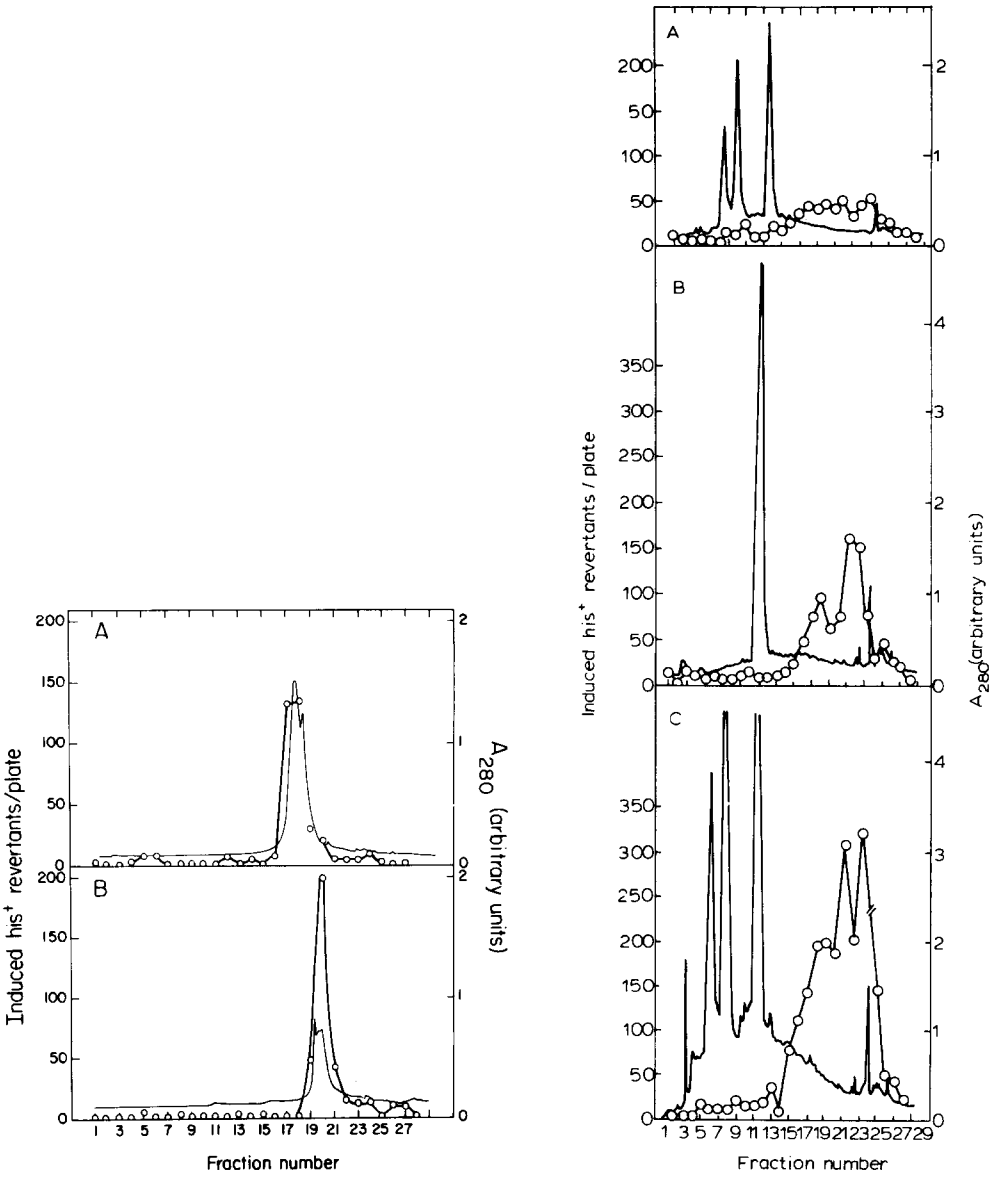

Fig. 2. Rechromatography of HPLC fractions. Fraction No. 15 (A) and 19 (B) from an initial separation shown in Fig. 1B were rechromatographed by the protocol described in Fig. 1. Distribution of mutagenicity (data from single plates, ○) and A_{280} (solid line) are shown.

Fig. 3. HPLC fractionation after acidic or basic treatment. A CH_2Cl_2 extract of XAD-2 concentrate equivalent to 2.2 l of smoker's urine was divided in thirds and partitioned between CH_2Cl_2 and either 1 N HCl or 1 N NaOH or distilled water. Those phases which contained mutagenic activity were chromatographed by HPLC as described in Fig. 1. Mutagenicity (○) and A_{280} (solid line) of the fractions are shown. A: aqueous phase of acidic extraction. B: CH_2Cl_2 phase of basic extraction. C: same phase analyzed in Fig. 1 (data from single plates).

acid wash (Table 2). Simple exposure of the CH_2Cl_2 extract to either 1 N NaOH or 1 N HCl was found to reduce mutagenic activity by amounts which could account for the failure to recover all the activity in the fractions. Both the acid

TABLE 2

ACID OR BASE EXTRACTABILITY OF MUTAGENIC ACTIVITY IN CIGARETTE SMOKERS' URINE

Fraction	Volume tested (ml equivalent urine)	Mutagenicity toward TA1538	
		per plate	per liter urine
CH_2Cl_2 extract			
Expt. 1 [a]	18	164 [b]	9111
Expt. 2	78	416 [c]	5333
Base washed CH_2Cl_2			
Expt. 1	23	81 [b]	3522
Expt. 2	78	236 [c]	3026
Basic extract of CH_2Cl_2			
Expt. 1	243	99 [c]	407
Expt. 2	78	28 [c]	359
Acidic washed CH_2Cl_2			
Expt. 1	243	108 [c]	444
Expt. 2	78	51 [c]	654
Acidic extract of CH_2Cl_2			
Expt. 1	24	71 [b]	2958
Expt. 2	78	183 [c]	2346

[a] Urine used in Fig. 3.
[b] Single plate determination.
[c] Average of duplicate plates.

extractable and base washed material were each fractionated by HPLC and compared with the normal, neutral CH_2Cl_2 extract described above. The distribution of the mutagenic activity in the HPLC fractions of the acid extractable and base non-extractable material was similar to that of the CH_2Cl_2 extract (Fig. 3). Recoveries from HPLC of mutagenic activity from these fractionations were 63%, 84%, and 30% for Figs. 3A—C, resp.

Activation of urine extracts by rat liver homogenates

Detection of the mutagenic activity in smokers' urine concentrates in strain TA1538 requires the addition of rat liver homogenates (S9, cf. Yamasaki and Ames, 1977). Aroclor 1254 has been found to be a general inducer of the rat liver enzymes; 3-methylcholanthrene (3MC) and phenobarbital (PB) each induce overlapping subsets of these enzymes (Guengerich, 1979). To test the possibility that certain mutagens in smokers' urine might have distinct metabolic activation requirements or that different S9 preparations might affect the pattern of mutagenic activity against the various tester strains, S9 from rats injected with Aroclor, 3MC, or PB was tested with each fraction in the 5 Salmonella strains.

Using TA1538, dose—response curves were determined for the 3 types of S9 varying either the amount of urine concentrate or the S9 concentration (Fig. 4). Aroclor and 3MC S9 were equally active at all concentrations of urine extract and were optimal at similar concentrations. The PB S9, on the other

TABLE 3

ACTIVATION OF HPLC FRACTIONS BY DIFFERENT RAT LIVER S9 PREPARATIONS

Sample	His^+ revertants/plate					
	Expt. 1			Expt. 2		
	A	3MC	PB	A	3MC	PB
Unfractionated [a] aliquot	281	ND [b]	ND	242	292	106
Fraction No. [c]						
1	7	0	0	6	3	0
2	5	0	0	0	9	0
3	3	0	6	0	0	0
4	0	1	1	7	4	0
5	0	0	0	1	6	2
6	3	4	0	8	4	4
7	9	4	3	4	7	8
8	24	16	7	10	2	1
9	35	23	7	18	1	0
10	19	20	15	14	3	2
11	31	23	11	18	2	1
12	40	33	9	26	67	10
13	65	81	19	54	47	12
14	113	127	27	92	82	21
15	149	130	59	132	112	8
16	160	205	55	131	85	26
17	229	330	101	194	264	66
18	208	357	60	164	236	76
19	269	383	94	154	224	107
20	153	261	158	235	278	146
21	119	212	95	216	310	134
22	91	176	95	100	141	86
23	141	120	7	116	126	37
24	78	127	18	57	83	36
25	83	85	0	62	91	51
26	33	60	17	29	29	10
27	12	42	2	19	6	13
28	11	35	5	14	10	2

[a] Averages of duplicate plates; 1/50 of CH_2Cl_2 extract per plate.
[b] ND, not done.
[c] Data from single plates; 1/4 fraction tested per plates.

hand, was 4—5-fold less active than other S9 preparations.

Individual fractions obtained by HPLC separation were tested for activation by the 3 types of S9 to see if any of the fractions had a pattern of activation which differed from that of the unfractionated material (Table 3). The pattern of S9 activation of each fraction was similar to that of the unfractionated extract, i.e., 3MC S9 was better than or equal to Aroclor S9 while PB S9 exhibited little to no activation. To further characterize the S9 dependence of the urine's mutagenic activity, S9 from Aroclor, 3MC, or PB pretreated rat livers were used to activate urine concentrates tested against 5 Ames tester strains. Only TA1538, TA98, and TA100 showed significant mutagenic activ-

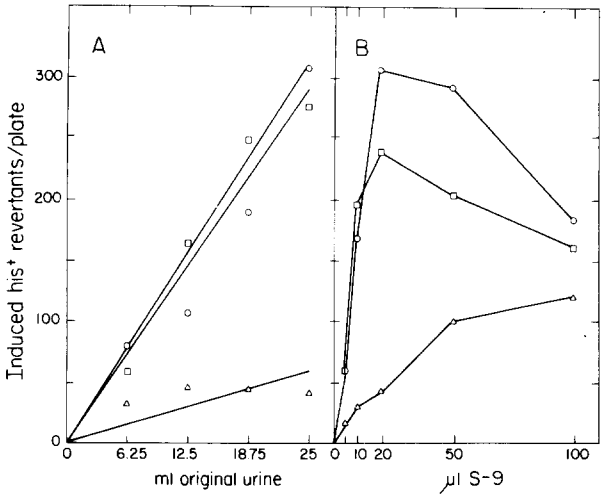

Fig. 4. Dose—response curves of S9 and smoker's urine concentrates. S9 was prepared from rat livers pretreated with Aroclor 1254 (□), 3-methylcholanthrene (○), or phenobarbital (△). 20 µl of S9 was used to activate varying amounts of CH_2Cl_2 extracts of smoker's urine (A). A concentrate equivalent to 25 ml of original urine was tested with varying amounts of S9 (B). Each point represents the average of 4 plates (2 Expts with duplicate plates).

TABLE 4

COMPARISON OF ABILITY OF DIFFERENT RAT LIVER S9 PREPARATIONS TO ACTIVATE URINE CONCENTRATES TO MUTAGENS FOR *Salmonella typhimurium*

Strain		His$^+$ revertants/plate [a]			
		No S9	A	3MC	PB
TA1538	Expt. 1	0	311	254	38
	Expt. 2	2	432	439	102
TA98	Expt. 1	0	428	427	48
	Expt. 2	16	445	536	134
TA1535	Expt. 2	0	11	5	8
TA100	Expt. 2	0	154	203	64
TA1537	Expt. 1	0	33	26	4
	Expt. 2	1	27	48	11

[a] Averages of duplicate plates; equivalent to 25 ml urine per plate.

ity (Table 4). Smaller increases in reversion incidence were observed with TA1537. No mutagenic activity was seen towards TA1535. For all strains the mutagenic activity detected in the presence of 3MC S9 was equal to or greater than that using Aroclor S9; mutagenic activity using PB S9 was significantly lower.

Discussion

The experiments presented in this paper represent a preliminary purification and characterization of the mutagenic activity extracted from smokers' urine

by XAD-2 resin. When XAD-2 concentrates were partitioned between water and CH_2Cl_2 at neutral pH, virtually all of the mutagenic activity was extracted into the organic phase while approximately 90% of the dry weight of the XAD-2 concentrates remained in the aqueous phase. When this CH_2Cl_2 extract was analyzed by HPLC, the mutagenic activity was observed to have a complex chemical composition. The elution profile for mutagenic activity revealed that many relatively non-polar substances present in cigarette smokers' urine possessed mutagenic activity and that their relative amounts varied considerably among individuals as well as among samples from one person.

To test the possibility that the chromatographically separated fractions might differ with respect to their metabolic activation requirement, the mutagenicity of each fraction was determined in the presence of different sources of S9. CH_2Cl_2 extracts were also examined in each of the 5 tester strains to see if mutagenic activity towards strains carrying frameshift or base-pair substitution mutations was a function of the source of S9. The outcome of these two efforts did not permit further classification of the material. Thus, at this level of resolution, the mutagenic activity in cigarette smokers' urine appears to be a complex mixture of chemicals with similar mutagenic specificity, metabolic activation requirements, and chromatographic behavior.

Since urine from only one "passive" smoker was available among our non-smoking population, the significance of the presence of similar mutagenic activity in a "passive" smoker's urine cannot be determined. However, the techniques described in this paper will be useful in investigating the possible presence of mutagens in urine due to "passive" smoking.

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