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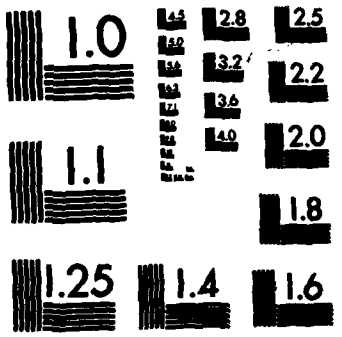
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CHEMICAL AND MOLECULAR BIOLOGICAL ASPECTS OF ALKYLHYDRAZINE-INDUCED CARCINOGENESIS IN HUMAN CELLS IN VITRO

Donald T. Witiak
College of Pharmacy

For the Period
December 1, 1981 - November 9, 1982

AIR FORCE OFFICE OF SCIENTIFIC RESEARCH
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1. ~~We have successfully developed~~ was developed a cation exchange HPLC technique which is rapid and provides a high resolution of pyrimidine oligonucleotides, methylated purines and a methylated pyrimidine ~~(Fig. 1).~~

2. Treatment of asynchronous cells at a low dose of 1,1-DMH, 1,2-DMH or treatment of synchronized cells at a transforming dose of 1,1-DMH did not increase the methylation of purine bases. However, a dose dependent increase in incorporation of radiolabel into DNA is observed. The ratio of O^6MeGua/N^7MeGua in 1,1-DMH treated cell varied between 0.5 - 0.8. In all experiments about 75-90% of the label was found in the apurinic acid fraction. This may be due to a) methylation of pyrimidines, b) ^{14}C entering the carbon pool and then being utilized in de novo synthesis of pyrimidines or c) formation of phosphotriesters.

3. The presence of detectable amount of phosphotriester or alkali labile alkylated bases in DNA due to methylation is tentatively ruled out since sedimentation analysis of methylated DNA in alkaline sucrose gradient did not show any single strand breaks even at concentrations as high as 5.0mM, ~~(Fig. 3,4).~~

4. A priliminary labelling experiment with ^{14}C-MAMA employing a transformation dose of MAMA showed distinctive labelling of 7-MeGUA and O^6MeGua, ~~(Fig. 5).~~ O6-MeGua,

5. Alkaline sucrose gradient analysis of DNA from MAMA treated cell show a dose dependent break in DNA (Fig. 6), investigations to determine whether the damage is due to formation of methylated bases or phosphotriesters is in progress.

5. An initial experiment to see the effect of 1,1-DMH and 1,2-DMH on unscheduled DNA synthesis (UDS) revealed that both compounds at a concentration of 0.026mM slightly increased the UDS but at a transforming dose (0.5mM), inhibition of DNA synthesis was more pronounced.

PROGRESS REPORT TO

AIR FORCE OFFICE OF SCIENTIFIC

RESEARCH DIRECTORATE OF LIFE SCIENCES

Bolling Air Force Base
Washington, D.C. 20332

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December 1, 1981 - November 9, 1982

Submitted by:

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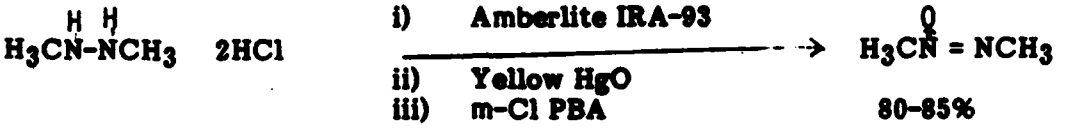
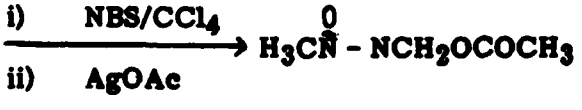
I. Synthetic Progress

Methylazoxymethanol acetate (MAMA) was prepared following essentially the method of Matsumoto et al[1] which consisted of oxidation of 1,2-dimethylhydrazine (1,2-DMH) in two steps to azoxymethane followed by further conversion by bromination and acetylation to MAMA. Modifications have been made in the isolation procedures enabling one to carry out the synthesis on a fairly small scale. (A typical batch in our laboratory employed 0.34 mmol of 1,2-DMH.2HCl).





(22% overall)

1,2-DMH generated in situ from its dihydrochloride with a weakly basic ion exchanger was stirred with yellow HgO. The azomethane thus liberated was trapped in CH_2Cl_2 containing m-chloroperoxybenzoic acid. In view of the small amounts involved it was highly inconvenient to isolate the product, azoxymethane, free of solid organic acids by distillation. Isolation was facilitated by passing the CH_2Cl_2 solution over basic alumina which rendered the product totally free of acids and in high yields (80-85% as estimated by NMR analysis of product + CH_2Cl_2; lit[2] yields 36%).

Further conversion of azoxymethane to MAMA was accomplished in 2 steps by bromination followed by acetylation, according to a published procedure[2] without isolation of the intermediate bromoazoxymethane because of its reported instability. MAMA was purified by chromatography over a silica gel column or preparative TLC. Overall yields of MAMA from 1,2-DMH.2HCl were about 22%. Several attempts at improving yields in the last two steps employing different conditions as well as reagents failed to afford better results.

Synthesis of high sp. activity ^{14}C-labelled 1,2-DMH.2HCl has been described in the previous report. Attempts to prepare labelled MAMA using this material have not given satisfactory yields in preliminary runs.

II. Biological Progress

A. Introduction

As stated earlier, studies by Milo et al[3] have revealed that in human fibroblast cells in culture, hydrazine, 1,1-dimethylhydrazine (1,1-DMH) and methylazoxymethanol acetate (MAMA) induced neoplastic transformation. Monomethyl hydrazine elicited only cellular toxicity. One approach to the understanding of differences between toxicity and carcinogenicity in terms of alkylation of DNA is to (a) analyse alkylated bases, and (b) study DNA damage and repair in cells treated with closely related analogues, but with different oncogenic potentials.

With this in mind we have undertaken the analyses of DNA in human fibroblast cells exposed to ^{14}C-labelled 1,1-DMH, 1,2-DMH and MAMA. The alkyl hydrazines used in our studies are of high specific activity (110 mCi/mmole) and were synthesized by methodology developed in our laboratories.

B. Results

1. Development of an HPLC technique for separation of apurinic acid and methylated purines and pyrimidines.

We have recently developed a cation exchange HPLC technique which is rapid and provides a high resolution of pyrimidine oligonucleotides, methylated purines and pyrimidines obtained by acid hydrolysis or neutral-thermal hydrolysis of alkylated DNA. Fig. 1 shows a typical HPLC profile of 10 purine and pyrimidine standards. This method is far superior to separation on Sephadex G-10 in that a) resolution is considerably improved and b) elution time is short.

2. Methylation of DNA in cultured human fibroblast cells.

Experiments were carried out using higher concentrations (0.166 mM) of 1,1- and 1,2-DMH. Additionally the effect of transforming and non-transforming doses (0.5 mM and 0.026 mM respectively) of 1,1-DMH were investigated in synchronized cells. A 6 fold increase in concentration of 1,1- and 1,2-DMH resulted in a 4 fold increase in ^{14}C incorporation into asynchronous cells. Utilization of a 19 fold increase in 1,1-DMH concentration as well as synchronized cells only increased the incorporation of label by 3 fold (Table 1).

Results of mild acid hydrolysis of alkylated DNA revealed that high alkylhydrazine concentrations did a) not lead to a detectable increase in methylation of adenine (position 3) or guanine (position O-6 and N-7), b) increase incorporation of ^{14}C-label into unmodified adenine and guanine. When the concentration of 1,1-and 1,2-DMH were increased 6-fold in asynchronous cells, there was a 3-6 fold increase (Table 2) in ^{14}C incorporation into these unmodified bases. Such treatment resulted in a concomitant decrease of 5-19 fold in methylated purines. Treatment of synchronized cells with a transforming dose of 1,1-DMH resulted in 90% incorporation of radiolabel into the apurinic acids (Table 3). A typical HPLC profile of hydrolysed alkylated DNA isolated from 1,1-DMH treated cells is shown in Figure 2. Interestingly, there are major peaks of radioactivity which do not co-elute with known major purine standards. Identification of these substances represent a significant goal of our work.

The lack of increase in DNA methylation with increasing hydrazine concentration may reflect isotope dilution or DNA repair. Sedimentation on alkaline sucrose gradients should be reflective of methylation and could serve to differentiate reasons for this apparent lack of increase. In fact, results of these experiments (Figures 3 and 4) showed no visible difference in the sedimentation profile of DNA from untreated and treated (various concentrations from 0.026-50mM of 1,1- or 1,2-DMH). An initial experiment on unscheduled DNA synthesis (UDS) in 1,1 and 1,2-DNA treated cells done in presence and absence of microsomal extract induced a slight enhancement of repair synthesis at 0.026mM concentration and an inhibition at 0.5 mM concentration.

A DNA labelling experiment using ^{14}C-MAMA at a transforming dose in synchronized cells showed a significant amount of radiolabel in methylated bases (Figure 5). Further quantitation is in progress.

Analysis of MAMA-treated cellular DNA, in contrast to 1,1 or 1,2-DMH-treated DNA, sedimented more slowly than the control DNA (Fig. 6). These data indicates that the DNA damage is due to either alklali labile alkylated bases, phosphotriesters or both. Investigations are underway to determine which mechanism results in single strand breaks.

3. Conclusion

Under conditions of varying concentration, treatment of asynchronized or synchronized cells with 1,1-or 1,2-DMH did not markedly affect methylation of purine bases. Using higher concentrations of these hydrazines resulted in a concentration-dependent increase in incorporation of label into DNA.

The ratio of O^6 to N^7-methylguanine ranged between 0.5-0.8 in 1,1-DMH and 0.4-0.5 in 1,2-DMH-treated cellular DNA.

In all experiments 75-90% of the radiolabel was found in the apurinic acid fraction. This was shown not to be attributable to phosphotriester formation since no single strand breaks could be detected by alkaline sucrose density gradient sedimentation of treated DNA.

Further investigations planned using MAMA (a potent carcinogen) should provide more detailed information about events leading to chemical transformation. Use of restriction enzyme analysis of DNA treated with carcinogenic and non-carcinogenic structurally related analogues should reveal molecular differences reflective of carcinogenicity vs. genotoxicity.

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2. Matsumoto, H., Nagahama, T. and Larson, H.O. (1965), Studies on methylazoxymethanol, the aglycone of cycasin: A synthesis of methylazoxymethyl acetate, Biochem. J., 95, 13C-14C.

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Table 1

Dose dependent incorporation of ^{14}C 1,2,-DMH and 1,1-DMH into DNA of human foreskin fibroblast cells.

	Asynchronized cells[a]		Synchronized cells[b]
Conc.	1,2-DMH	1,1-DMH	1,1-DMH
mM	DPM/mg DNA	DPM/mg DNA	DPM/mg DNA
0.026	35,060[c]	31,800	80,617
0.166	1,53,671	1,29,646	———
0.5	———	———	2,52,861

a Cells in 150 ømm culture dishes at 70-80/ confluency were treated for 24 hrs. with 1,1- or 1,2-DMH.

b Cells blocked in G_1 phase by arginine and glutamine deprivation were treated 10 hrs after release with 1,1 DMH for 12 hrs (0.026 mM) or 4 hrs (0.5 mM).

Table 2

Methylation of DNA from randomly proliferating human foreskin fibroblast cells treated with alkylhydrazines.

	1,1-DMH		1,2-DMH	
Fraction	0.026mM	0.166mM	0.026mM	0.166mM
Apurinic acid	87.56[a]	76.24	87.9	61.6
3.MeAde	3.18	0.64	2.9	0.5
7 MeGua	1.27	0.23	2.7	0.17
O^6 MeGua	1.09	0.09	1.33	0.07
Guanine	2.99	9.20	2.45	16.5
Adenine	3.8	11.6	2.60	21.1
O^6 Gua/N^7MeGua	0.86	0.4	0.49	0.42

[a]Percentage of adducts

Table 3

Methylation of DNA from synchronized human foreskin fibroblast cells treated with 1,1-DMH

Fraction	0.166mM	0.5mM
Apurinic acid	85.7[a]	0.1
3-MeAde	1.3	0.26
7-MeGua	0.5	0.24
O^6-MeGua	0.3	0.18
Guanine	7.6	2.3
Adenine	5.6	1.7
O^6MeGua/N^7MeGua	0.6	0.75

[a] Percentage of adducts

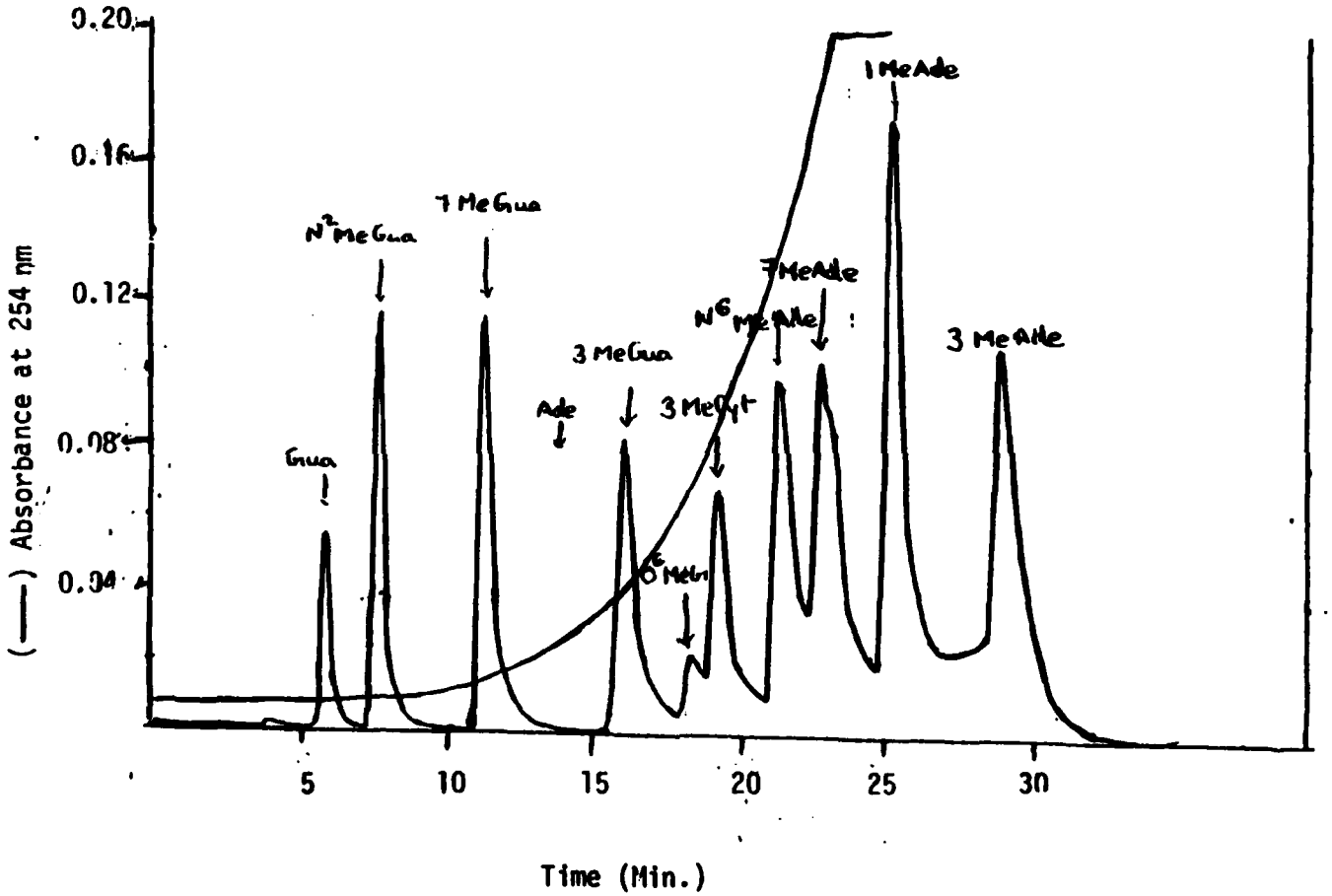

Figure 1. HPLC profile of methylated purine and pyrimidine standards separated on strong cation exchange column. (Partisil 10SCX 9M in series with Partisil 10SCX). The bases were eluted at 4ml/minwith a buffer which changed from 20mM ammonium formate in 6% methanol pH 4.0 to 200 mM ammonium formate in 8% methanol over 25 min. using a concave gradient exp. 5 in LDC system.

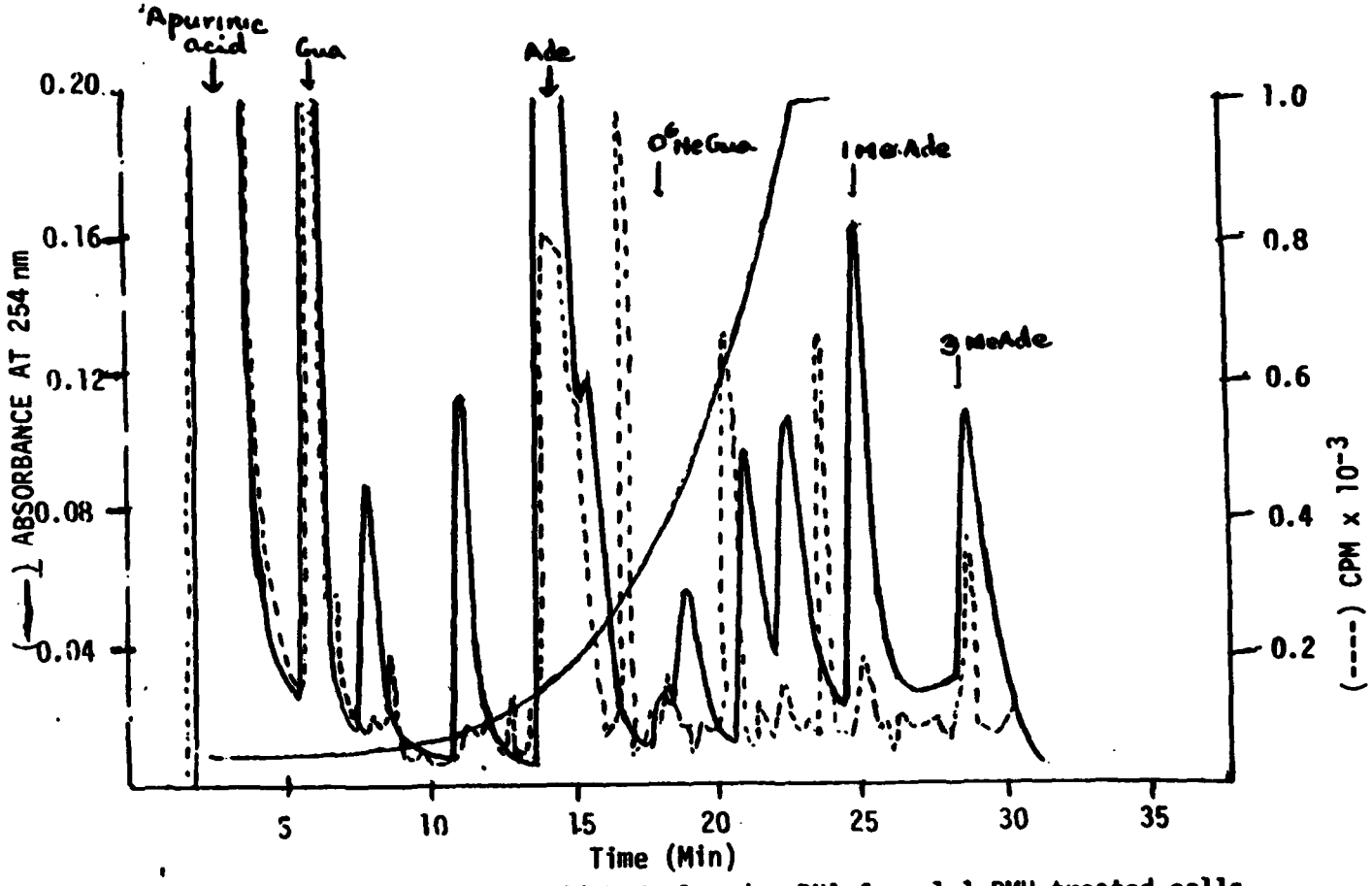

Figure 2. HPLC profile of acid hydrolysed DNA from 1,1-DMH treated cells. The sample was hydrolysed in 0.1N HCl at 70°C for 30 min and chromatographed with standards on a strong cation exchange column. (Partisil 10SCX 9M in series with Partisil 10SCX). Methylated bases were eluted at 4ml/min with a buffer which changed from 20mM ammonium formate in 6 % methanol pH 4.0 to 200mM ammonium formate in 8 % methanol pH 4.0 over 25 min using exp. 5 in LDC system. (----) represents radioactive profile.

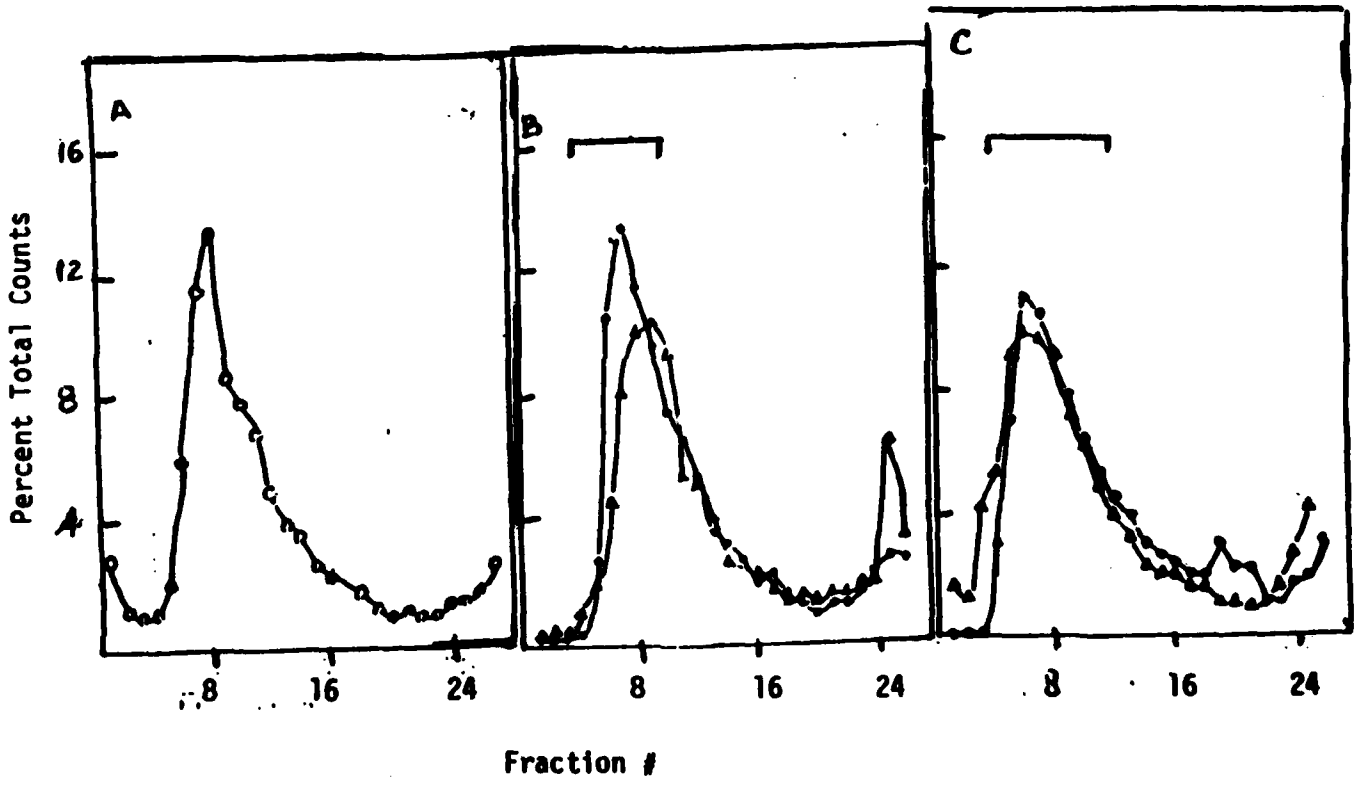

Figure 3. Alkaline sucrose gradient sedimentation profile of DNA from randomly proliferating human foreskin fibroblast cells treated with A) control O-O B) 1,1-DMH for 12 hrs at 0.026mM ▲—▲; 0.5mM ●—● and C) 1,2-DMH for 12 hrs at 0.026mM ●—● 0.166mM ▲—▲. Bracket indicates the position of DNA from untreated cells.

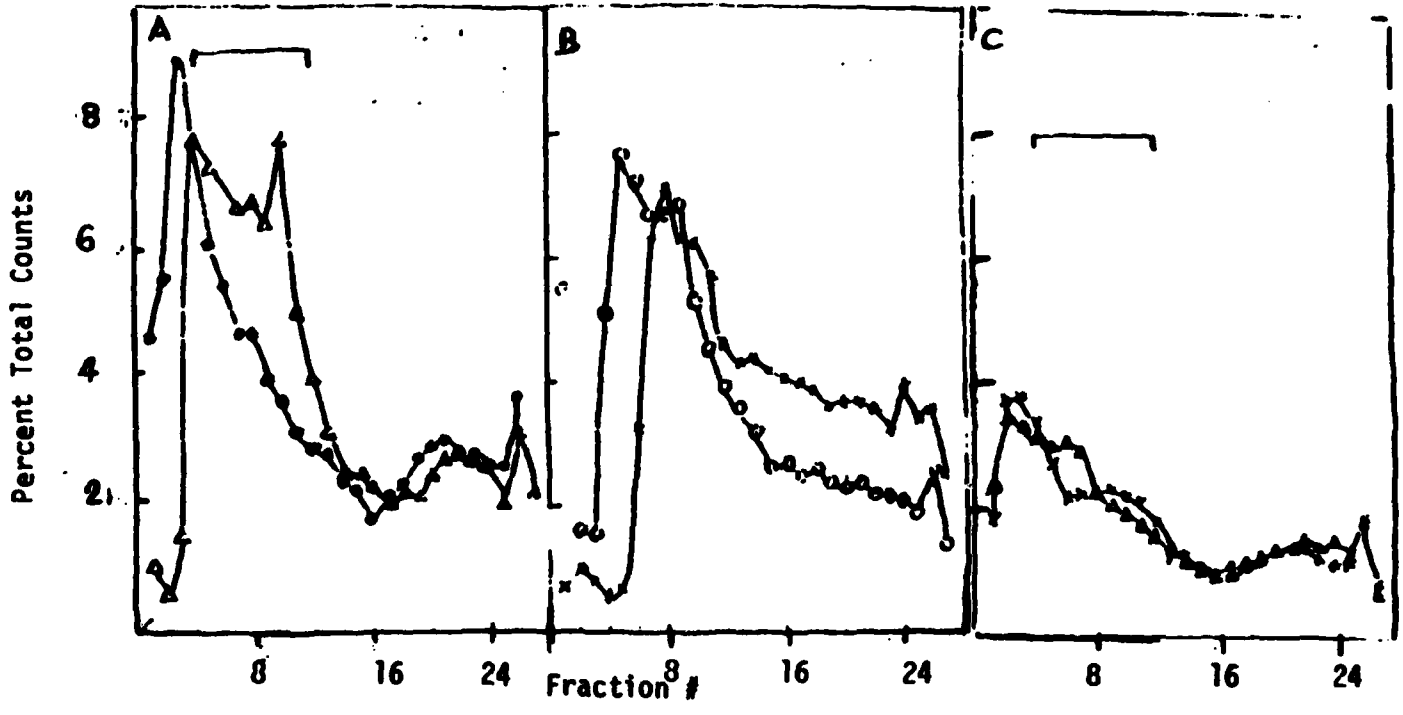

Figure 4. Alkaline sucrose gradient sedimentation profile of DNA from synchronized human foreskin fibroblast cells treated 10 hrs after release from synchrony with A) 1,1-DMH 0.5mM ●—●; 5.0mM△—△ B) 1,1-DMH 50mM x—x; control o—o and C) 1,2 DMH 0.5mM △—△ and 5.0mM x---x for 12 hrs. Bracket indicates the position of undamaged DNA from control cells.

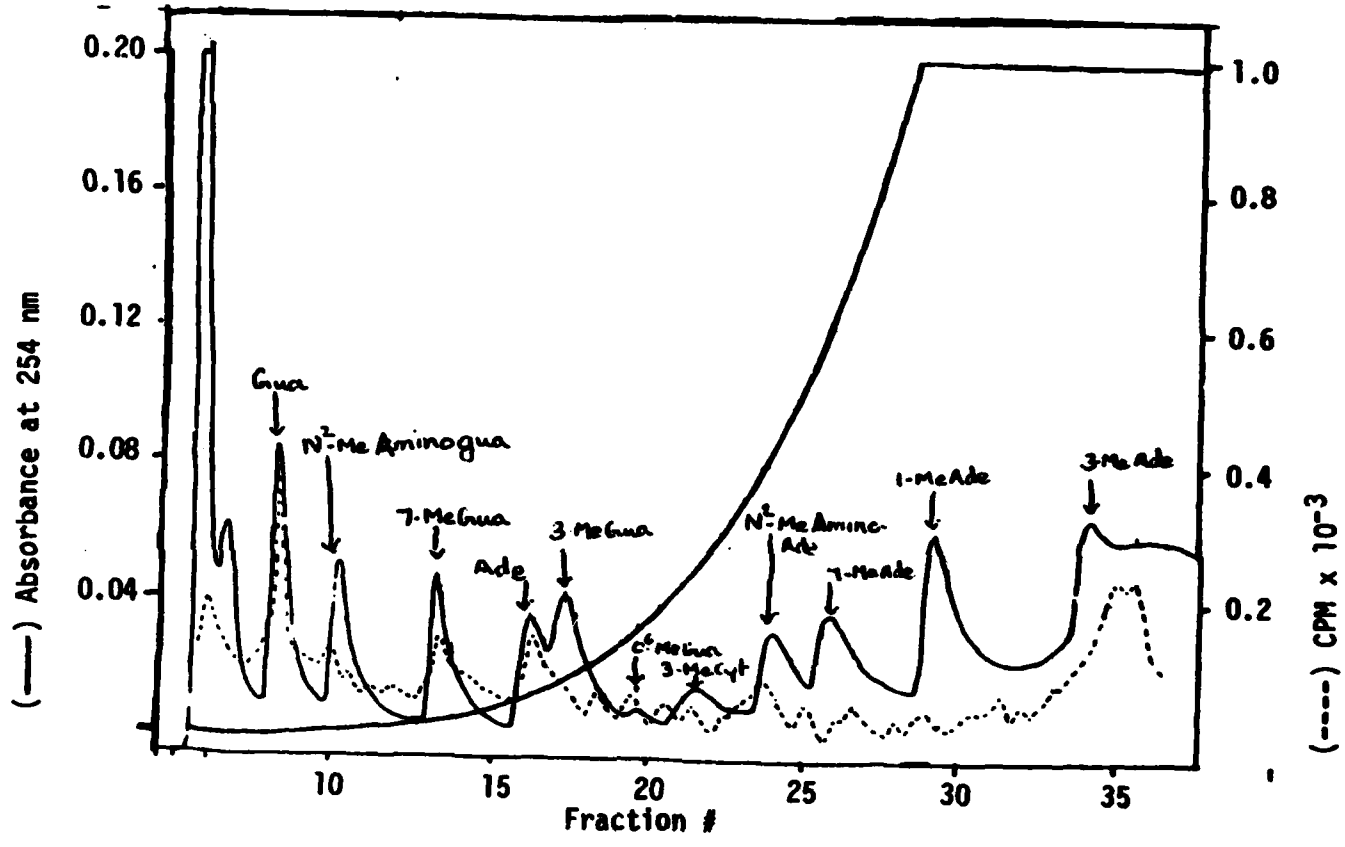

Figure 5. HPLC profile of acid hydrolyzed DNA from cells treated with MAMA at 4ug/ml. The sample was hydrolyzed in 0.1N HCl at 70°C for 30 min and chromatographed on a strong cation exchange column (Partisil 10SCX 9M in series with Partisil 10SCX). Bases were eluted at 3 ml/min with a buffer which changed from 20mM ammonium formate in 6% methanol pH 4.0 to 200mM ammonium formate in 8% methanol pH 4.0 over 30 min using a concave gradient exp. 5. in LDC system.(---) represents radioactive profile

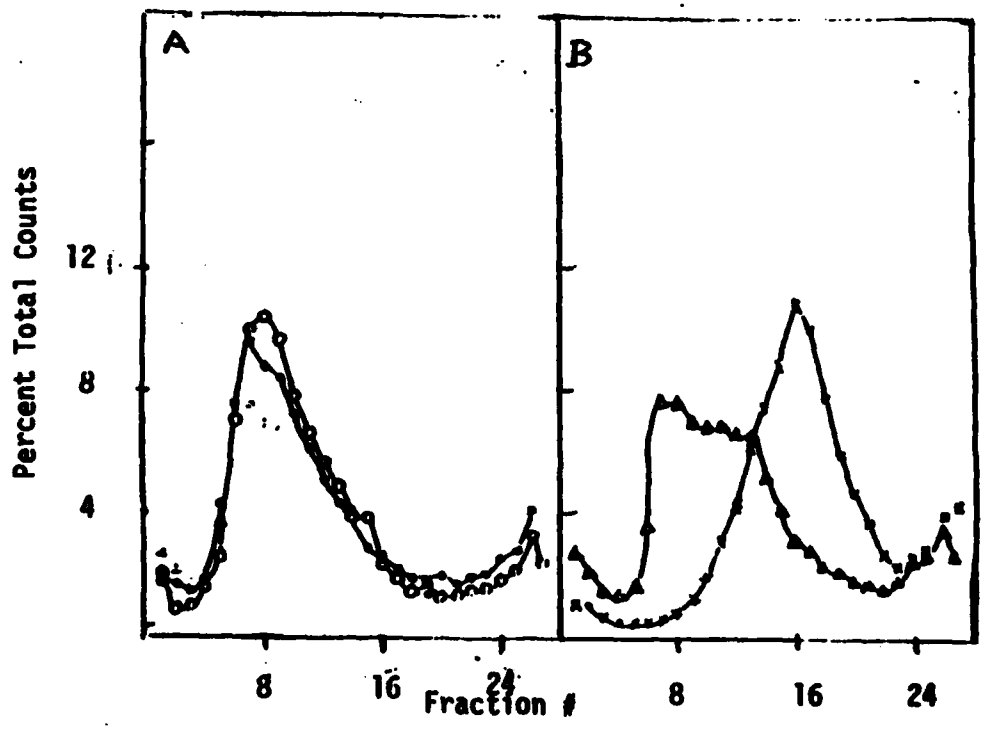

Figure 6. Alkaline sucrose gradient profile of DNA from synchronized cells treated 10 hrs after release with A) control o---o; MAMA 1.0ug/ml; B) MAMA 4ug/ml x---x; 10ug/ml x---y for 12 hrs.