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Assay for 5-methylcytosine in the DNA of Dictyostelium mucoroides at two different stages in its life cycle.

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ASSAY FOR 5-METHYLCYTOSINE IN THE DNA OF *DICTYOSTELIUM MUCOROIDES* AT TWO DIFFERENT STAGES IN ITS LIFE CYCLE

A Thesis

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In Partial Fulfillment

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Master of Arts

by

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THESIS ACCEPTANCE

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INTRODUCTION

The Acrasiales, or cellular slime molds, are not true molds at all. They are a unique group of organisms that exhibit characteristics of both the fungi and the protozoa. The cellular slime molds are more than a taxonomic enigma, however. One genus, Dictyostelium, has been extensively studied as a model of multicellular development.

During the vegetative portion of its life cycle, D. mucoroides, a typical member of the genus, exists as a unicellular haploid organism. Amoebae feed on bacteria and reproduce asexually by fission. Depending on environmental conditions, depletion of the food source initiates one of two developmental pathways. The sexual cycle, which culminates in macrocyst formation, is favored by conditions of darkness, excess moisture, temperatures above 25°C, and the absence of phosphate. Under these conditions, starved amoebae form loose aggregates. Two cells in the aggregate fuse to form a diploid giant cell that ingests the remaining amoebae. During this time a multilayered wall forms around the mass of amoebae and the giant cell undergoes meiosis. After a period of dormancy, the macrocyst germinates and haploid amoebae are again produced (Nickerson and Raper, 1973).

Under other developmental conditions, starvation

initiates the asexual cycle which culminates in sorocarp formation. The first phase of this cycle is the cyclic-AMP mediated aggregation of the amoebae. Once tight cell contacts are established, the amoebae form a multicellular asyncidium called the slug or pseudoplasmodium stage (Blumberg et al., 1982). The slug has a defined anterior-posterior axis and is capable of movement.

In response to a high ionic strength medium or overhead light, culmination begins. While the posterior cells of the pseudoplasmodium continue to migrate, the anterior cells stop. This results in the rounding up of the pseudoplasmodium. The cell mass assumes a vertical position as the cells that were at the anterior of the pseudoplasmodium become vacuolated and push toward the base of the cell mass. While doing so they synthesize and become surrounded by cellulose, thus forming the stalk of the sorocarp. The cells that once formed the posterior of the slug are now at the apex of the stalk. These cells produce cellulose walls and develop into spores (Farmer, 1980). This entire cycle from aggregation through fruiting body formation occurs in the absence of cell growth, cell division, or DNA synthesis (Ashworth, 1971).

The differences in these two cell types are detectable in the slug stage. Ultrastructure studies have shown that the posterior cells contain a prespore vacoule not found

in the anterior cells (Garrod and Ashworth, 1973). The anterior and posterior cells also differ in their density and enzyme composition (Ashworth and Dee, 1975). In addition, enzymes unique to specific stages of development have been found in D. discoideum, a related member of this genus (Loomis, 1975). Also, it appears that there are at least eighty developmentally regulated polypeptides in this organism (Lodish et al., 1982). These cytological and biochemical differences are presumed reflections of differential gene expression (Firtel, 1972). The regulatory mechanisms responsible for these differences are not known. One possible explanation involves the methylation of the cytosine residues in the DNA.

5-Methylcytosine is the most prevalent modified base in eukaryotic DNA. It comprises from 0.03 to 8 mo% of the cytosine residues in animal DNA and up to 50 mol% of the cytosine residues in higher plants (Doerfler, 1983). Cytosine methylation occurs after DNA replication with the methylation pattern of the parental strand serving as a template for the methylation of the newly synthesized strand. Thus, methylation patterns are inheritable (Razin and Riggs, 1980). DNA methylation is associated with gene inactivation (Ehrlich and Wang, 1981), although its mechanism of action is not known.

In some organisms this inactivation appears to alter

development. For example, only the unmethylated sequences of mouse ribosomal DNA are transcriptionally active (Bird et al., 1981). In hepatocellular carcinomas, hypomethylation appears associated with the malignant transformation of cells (Lapeyre and Becker, 1979). Hypomethylation of DNA has also been observed during the differentiation of Friend erythroleukemia cells (Christman et al., 1977). Finally, cytosine methylation appears associated with the inactivation of some X-chromosome linked functions in female mammals. One of the X-chromosomes is inactivated in the somatic cells of the female mouse early in development. It has been indirectly shown that the gene for hypoxanthine-guanine phosphoribosyl transferase (HGPRTase) on this chromosome is inactive only while cytosine residues are methylated. If a cytosine analog that cannot be methylated is incorporated into the DNA, the gene is transcribed (Mohandes et al., 1981). This work with mouse somatic cells suggests that 5-methylcytosine may be associated with gene expression during an organism's development.

The purpose of this investigation was to determine whether or not there are quantitative differences in 5-methylcytosine content during two different stages of the life cycle of Dictyostelium mucoroides.

MATERIALS AND METHODS

Vegetative Culture

Amoebae of Dictyostelium mucoroides, strain DM-7, were grown in 250 Erlenmeyer flasks in 40 ml of glucose-yeast-peptone broth (0.5 g yeast extract, 5.0 g peptone, 5.0 g glucose, 2.25 g KH_2PO_4, and 0.5 g $MgSO_4 \cdot 7H_2O$ in 1.0 l of distilled H_2O). Each flask was first inoculated with a loopful of Eschericia coli B/r and then a spore suspension of DM-7 so that the final spore concentration was approximately 2.8×10^4 spores/ml. Cultures were incubated at 23°C in gyratory water baths. Amoebae were harvested in late exponential phase at an average concentration of 9×10^6 cells/ml. The contents of 27 flasks were considered to be one sample.

Development of Amoebae

The amoebae of vegetative samples were washed and concentrated by centrifugation in 0.025 M phosphate buffer, pH 6.5, at 1000 rpm (approx. 200 x g) in 50 ml tubes in a Sorval HL-4 rotor for 10 min.

Samples to be incubated under conditions favoring the formation of macrocysts were washed and concentrated in Bonner's Salt Solution (BSS) (Bonner, 1953) with streptomycin (500 ug/ml). The pellet was resuspended in 8 ml of BSS and dispensed onto non-nutrient agar plates (15 g agar

and 1 l of distilled water) at an average concentration of 9.53×10^8 amoebae/plate. Each plate was flooded with an additional 10 ml of BSS. Plates were incubated in the dark at 23°C for 3 or 6 hrs. One plate from each sample remained in the appropriate incubator for one week as a developmental control. After the prescribed incubation time, cells were scraped from the plates, washed with BSS, and concentrated by centrifugation.

DNA Isolation

The DNA isolation procedure was adapted from Pederson (1977). The pellet of washed cells was resuspended in cold lysis buffer at a concentration not exceeding 5×10^8 cells/ml. Lysis buffer was 5 mM magnesium acetate, 10% sucrose, 50 mM HEPES (pH 8.0), and 0.5% (v/v) Triton X-100. The cell suspension was placed in a pre-chilled tube and vortexed at ambient temperature for 15 sec. The lysate was transferred to an ice-cold Dounce homogenizer and with the loose fitting pestle give 4 to 5 strokes. The lysate was again transferred to a pre-chilled tube and centrifuged at 1500 rpm (approx. 270 x g) for 4 min in a Sorvall SS-34 rotor. All centrifugations using the SS-34 rotor were done at 4°C. The presence of nuclei was initially confirmed by phase contrast microscopy.

Nuclei were pelleted from the supernatant by centrifugation at 4000 rpm (approx. 1930 x g) in the SS-34 rotor

for 10 min. The pellet was washed twice using lysis buffer without Triton X-100, 25 ml for each 10^{10} cells in the initial sample. After the second wash nuclei were resuspended in 5 ml of TE buffer, (10 mM Tris-HCl and 1 mM EDTA, pH 8.0), placed in an ice-water bath and sonicated for 10 s at a setting of 50 using the intermediate tip of a Model 16-850 Virsonic cell disrupter (Virtis Co.). The suspension of disrupted nuclei was then layered on 18 ml of 30% (w/v) sucrose in buffer (0.01 M KCl, 0.01 M magnesium acetate, and 0.03 M HEPES, pH 8.0) and centrifuged at 5800 rpm (approx. 4060 x g) for 20 min in the SS-34 rotor. Chromatin was recovered from the supernatant-30% sucrose interface and frozen if not immediately concentrated.

Concentration of Nucleic Acids

Chromatin recovered from the supernatant-30% sucrose interface was concentrated by ethanol precipitation (Maniatis et al., 1982). The DNA solution was made 0.1 M in NaCl. Two volumes of ice-cold 95% ethanol were added and the samples were immediately placed at -70°C for 6 hrs. DNA was recovered by sedimentation centrifugation at 15,000 rpm (approx. 27,100 x g) in the SS-34 rotor for 30 min. The supernatant was drawn off and discarded. The tubes were inverted on absorbent toweling for approximately 10 min and were then subjected to two minutes of vacuum desiccation.

DNA Quantitation

Each sample was resuspended in 3 ml of cold TE buffer for quantitation. The concentration and purity of recovered DNA was estimated using a Model 634 Spectrophotometer (Varian Techtron). Absorbance was calculated from % transmittance at 260 and 280 nm using a slit width of 2 nm. Salmon sperm DNA was used as the standard. The solutions were then adjusted to a final concentration of 0.01 M $MgSO_4$ and subjected to a second ethanol precipitation.

Preparation of DNA for Separation by Reverse-phase HPLC

Recovered DNA was enzymatically digested and resolved into deoxyribonucleoside 5'-monophosphates (dNMP's) according to the method of Christman (1982). The DNA sample was resuspended in 0.25 ml cold TE buffer. For every 25 ug of DNA in the sample approximately 28.5 ug DNase I (2105 Kunitz U/mg) was added. The mixture was incubated for 2 hrs at 37°C. Next, 116 ug of snake venom phosphodiesterase (31 U/mg) was added and the mixtures were incubated for another 2 hrs. Prior to injection onto the column each sample was centrifuged for 5 min at 1000 rpm (approx. 200 x g) in the HL-4 rotor to remove insoluble material.

High Performance Liquid Chromatography

High performance liquid chromatography was performed using a Beckman Model 112 pump, a Model 210 sample injec-

tion valve with a 20 ul sample loop, and a Model 160 fixed wavelength UV detector with a 254 nm filter (Beckman Instr. Inc.). The data handling system was a 3390A reporting integrator (Hewlett Packard). Separations were performed on a reversed-phase Ultrasil ODS column 4.6 mm i.d. x 250 mm (Beckman Instr. Inc.). The elution solvent was 0.1 M sodium phosphate buffer, pH 5.8. The column storage solution was 0.05% sodium azide. All HPLC buffers were prepared with glass distilled H_2O and were filtered through a 0.45 um Durapore filter (Millipore Corp.) and degassed before use. Salmon sperm DNA was digested to dNMP's and then separated by reverse-phase HPLC as a control. Chromatogram peaks from experimental samples were identified by comparison with chromatograms of individual standards, mixtures of standards, and by peak amplification. Effective concentration of dNMP standards was determined using a Gilford Model 252 Photometer set at 254 nm.

Chemicals

Snake venom phosphodiesterase and thymidine 5'-monophosphate were purchased from United States Biochemical Corp. DNase I and all other standards were obtained from Sigma Biochemical Corp.

RESULTS

Vegetative Culture

For the isolation of Dictyostelium DNA, the maximum cell density that still gives acceptable nuclear purity is 5 x 10^6 cells/ml (Pederson, 1977). The growth curve in Figure 1 shows that DM-7 reached this cell density, under the growth conditions used in this investigation, approximately 66 hours after inoculation.

Purity of Isolated DNA

The purity and concentration of the experimentally isolated DM-7 chromatin was estimated from ultraviolet absorbance readings at 260 nm and 280 nm. Table I shows that the A_{260}:A_{280} ratios for these samples ranged from 1.30 to 1.61.

High Pressure Liquid Chromatography of dNMP's

Figure 2 is a typical chromatogram of vegetative D. mucoroides DNA digested to 5'dNMP's and separated by reverse-phase HPLC. Five peaks were consistently observed. Preliminary identification of four of the peaks was made by comparison with chromatograms of individual dNMP standards and mixtures of these standards, Figure 3. A comparison of Figures 2 and 3 suggests the following: the peak at 5.02 min was dCMP, the peak at 14.20 min was dTMP, the peak at

Figure 1. Growth curve for *D. mucoroides* in GYP broth at 23°C.

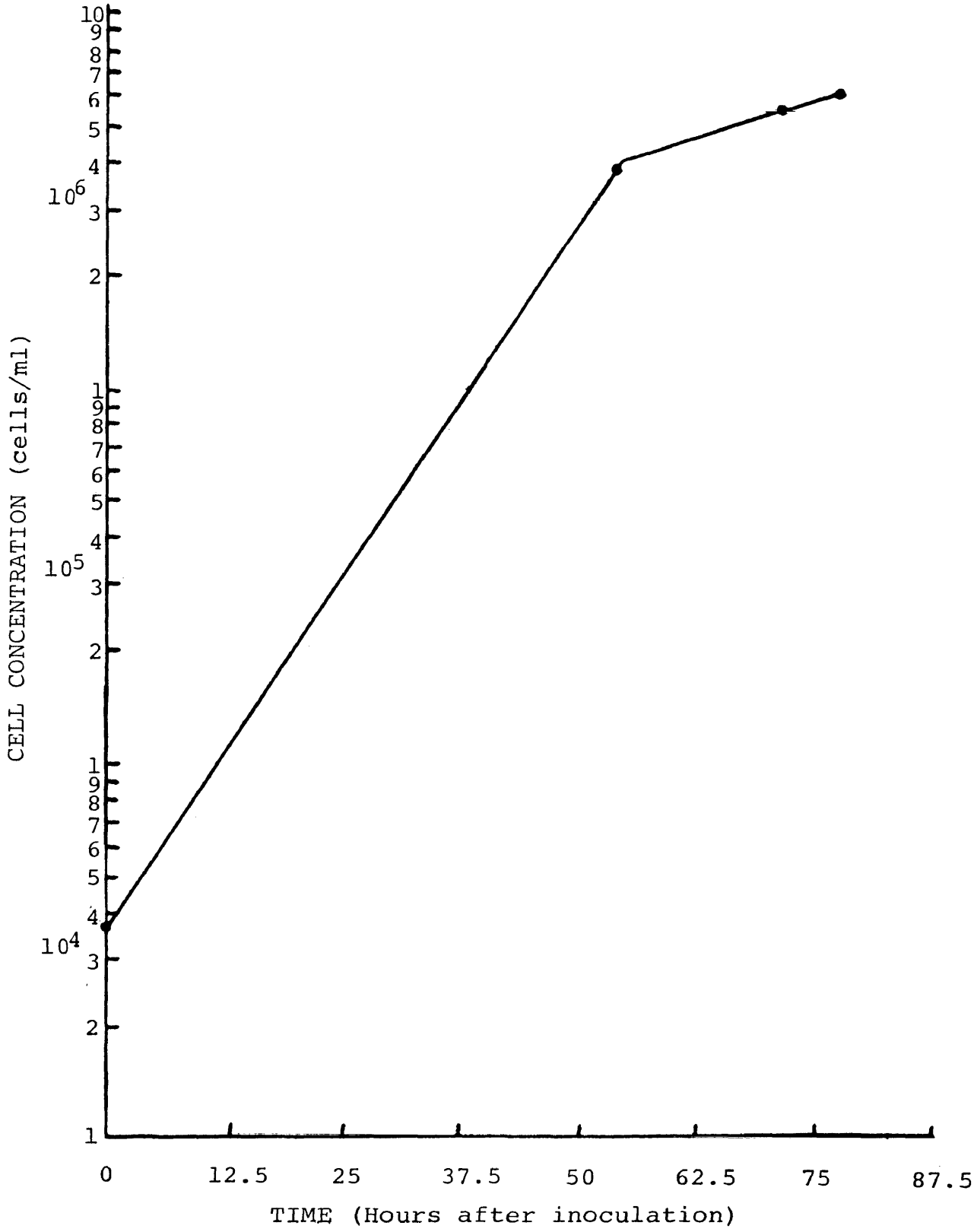

CELL CONCENTRATION (cells/ml)
10
9
8
7
6
5
4
10^6
3
2
1
9
8
7
6
5
4
10^5
3
2
1
9
8
7
6
5
4
10^4
3
2
1
0
12.5
25
37.5
50
62.5
75
87.5
TIME (Hours after inoculation)

Table I. Estimated concentration and purity of *D. mucoroides* DNA prior to ethanol precipitation.

Stage of Life Cycle	Sample Number	Estimated DNA Concentration (ug/ml)[1]	Absorbance Ratio 260/280
Vegetative amoebae	S8	153	1.30
	S9	58	1.41
Developing macrocysts (3 hours)	S10	48	1.59
	S11	17	1.61
Developing macrocysts (6 hours)	S12	27	1.58
	S15	39	1.57

[1]DNA concentration determined by absorbance at 260 nm.

Figure 2. Separation of dNMP's from vegetative amoebae of D. mucoroides DNA.
Separations were performed on a Beckman reverse-phase Ultrasil ODS column.
Flow rate=1 ml/min.
Buffer=0.1 M sodium phosphate, pH 5.8
0.01 AUFS (Absorbance units full scale)

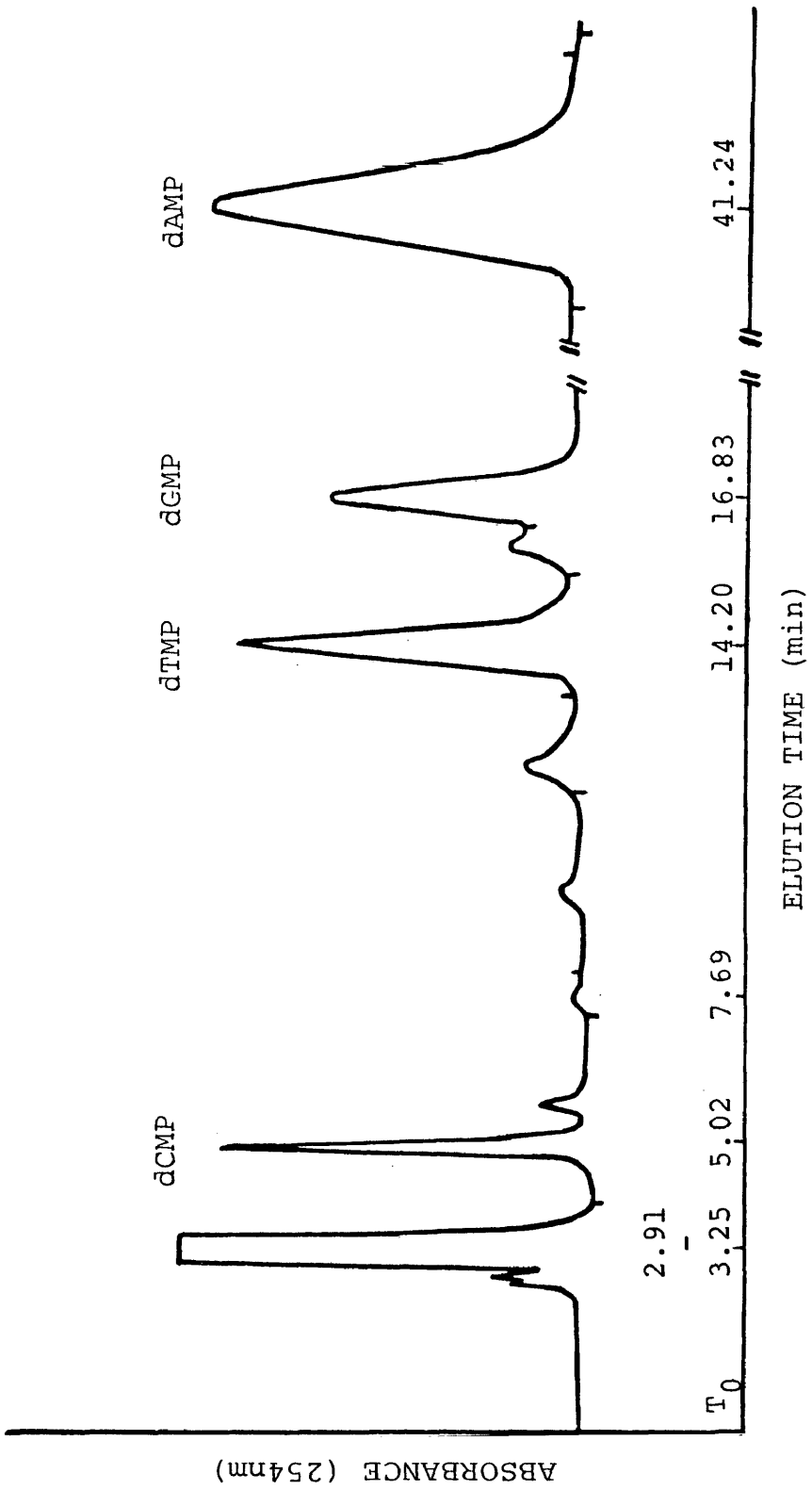
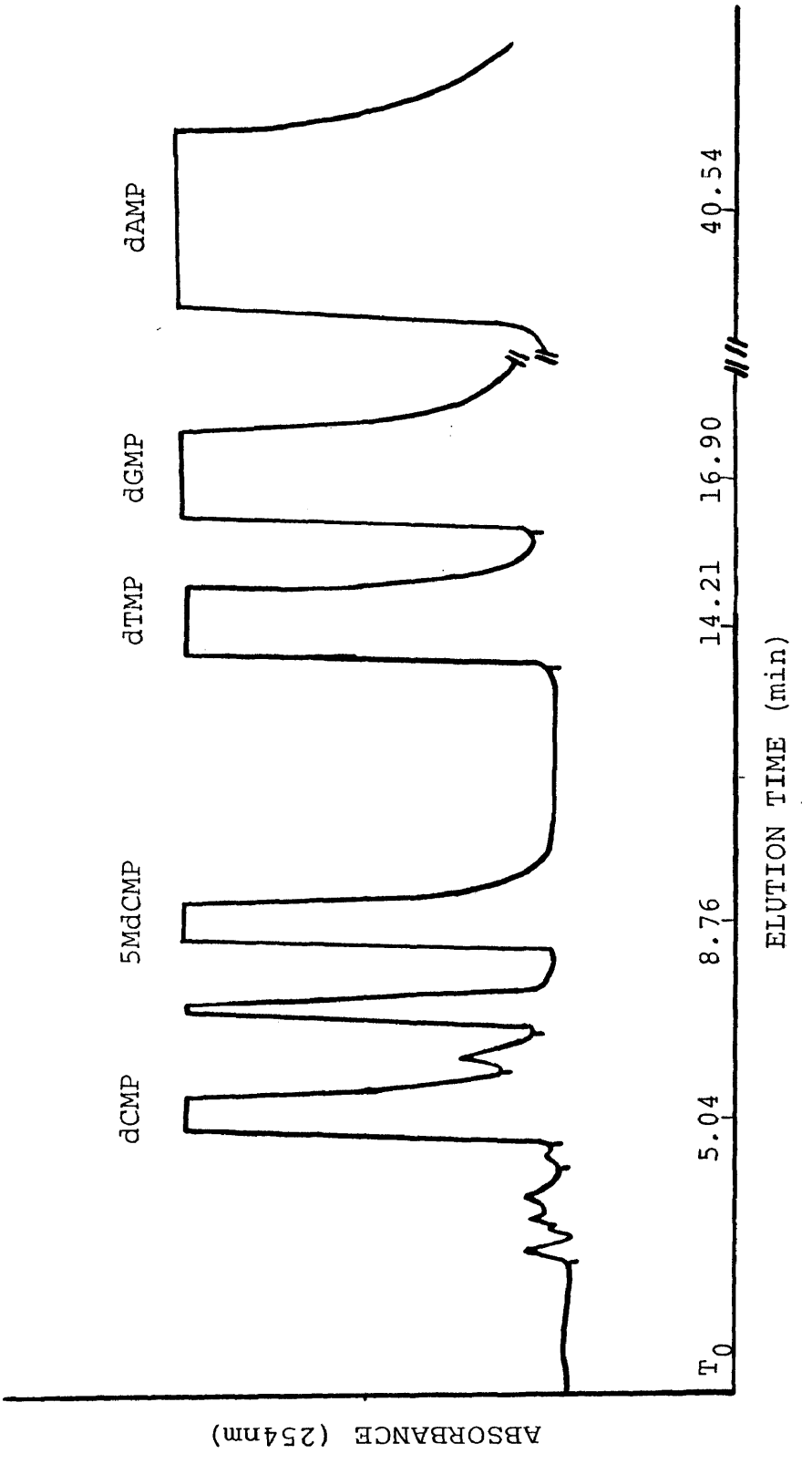

dCMP
dTMP
dGMP
dAMP
ABSORBANCE (254nm)
T0
2.91
3.25
5.02
7.69
14.20
16.83
41.24
ELUTION TIME (min)

Figure 3. Separation of a mixture of five dNMP standards. Separations were performed using a Beckman reverse-phase Ultrasil ODS column.
Flow rate=1 ml/min.
Buffer= 0.1 M sodium phosphate, pH 5.8
0.01 AUFS


dCMP
5MdCMP
dTMP
dGMP
dAMP
T0
5.04
8.76
14.21
16.90
40.54
ELUTION TIME (min)
ABSORBANCE (254nm)

16.83 min was dGMP, and the peak at 41.24 min was dAMP.

Conclusive identification of the four major dNMP peaks in the experimental samples was made by adding a known amount of each of the dNMP standards to an experimental sample. If the peak of a known compound exactly amplified a peak in the experimental sample, I assumed that the compounds were the same. If a known standard did not amplify an experimental peak I assumed that the compound either was not present or not detectable in the experimental sample.

The double peak at 2.91 to 3.25 min was characteristic of every enzymatically digested sample: salmon sperm DNA control as well as experimentally isolated DM-7 DNA. A comparison with chromatograms of TE buffer, DNase I, and phosphodiesterase indicated that these three substances produced the peak at approximately 3.00 min. In this same manner the peak at 7.69 min was also identified as a solvent peak.

The DNA from DM-7 cells incubated for 3 or 6 hours under conditions favoring macrocyst development was also isolated, enzymatically digested to 5'dNMP's, and separated by reverse-phase HPLC. Figure 4 is a typical chromatogram from the three hour sample. Figure 5 is a representative chromatogram from the six hour sample. Once again peaks were identified by comparisons with chromatograms of in-

Figure 4. Separation of dNMP's from D. mucoroides DNA three hours into macrocyst development. Separations were performed on a Beckman reverse-phase Ultrasil ODS column. Flow rate=1 ml/min. Buffer=0.1 M sodium phosphate, pH 5.8 0.01 AUFS

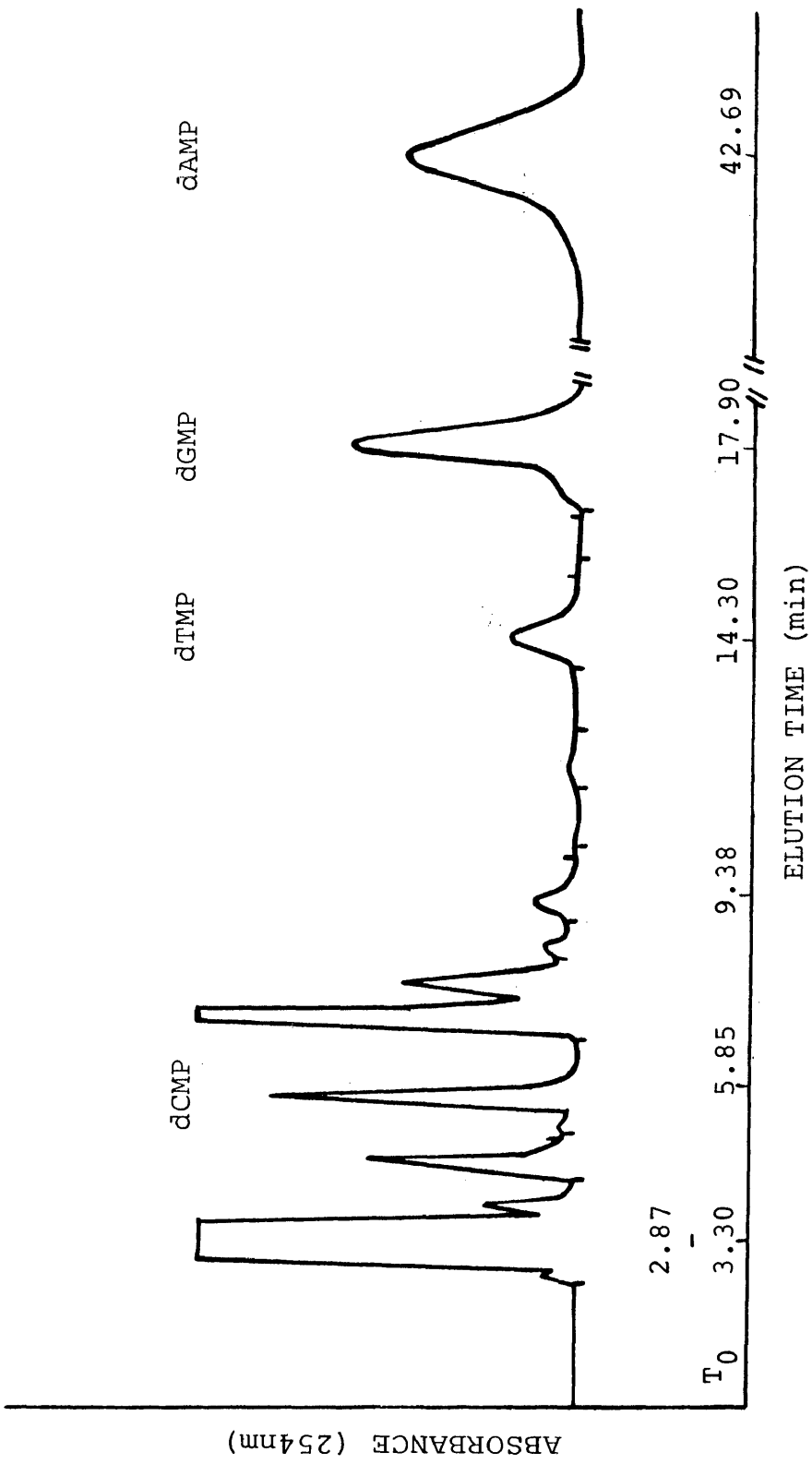
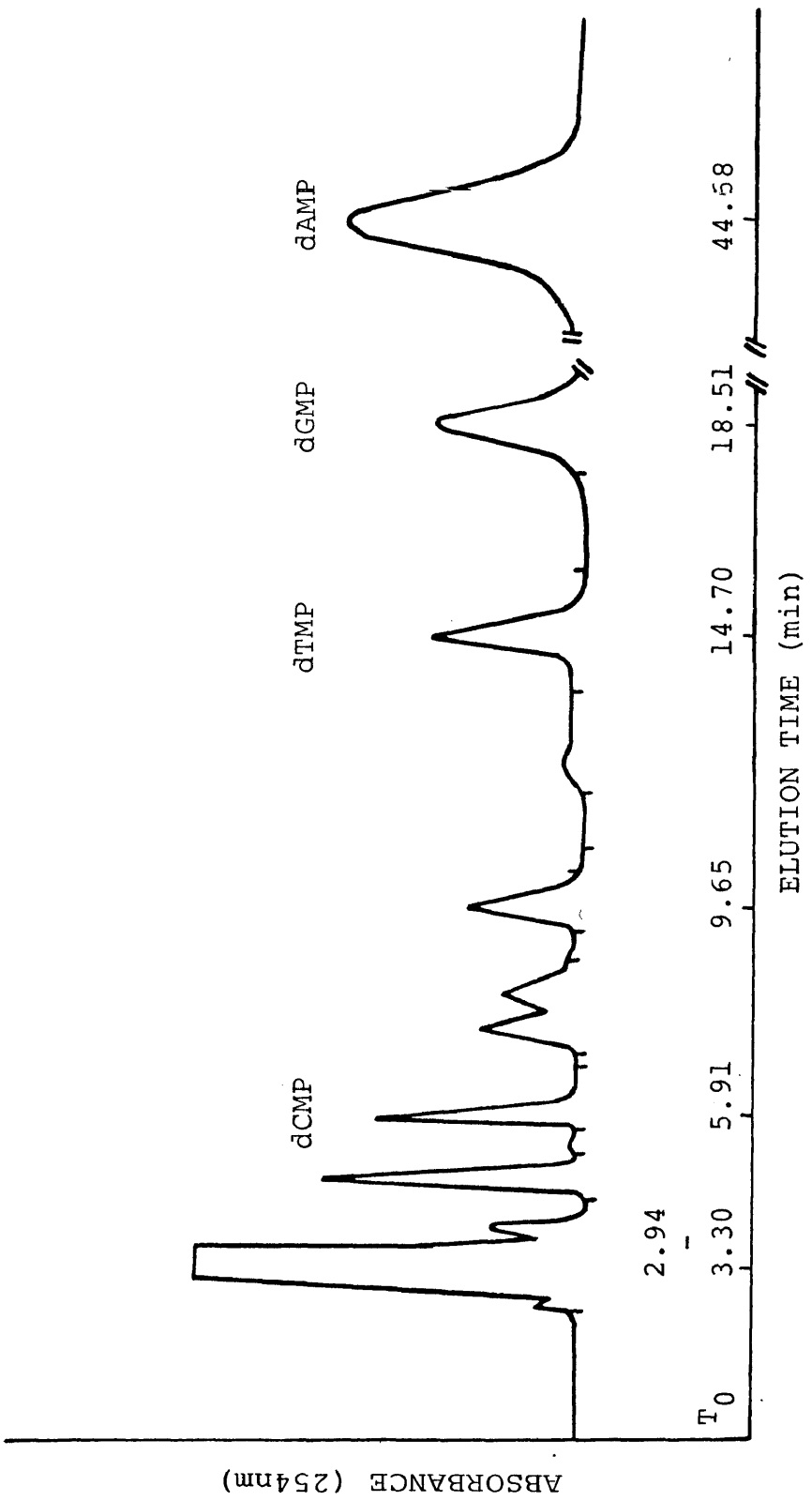

ABSORBANCE (254nm)
dCMP
dTMP
dGMP
dAMP
T_0
2.87
3.30
5.85
9.38
14.30
17.90
42.69
ELUTION TIME (min)

Figure 5. Separation of dNMP's from D. mucoroides DNA six hours into macrocyst development. Separations were performed using a Beckman reverse-phase Ultrasil ODS column. Flow rate=1 ml/min. Buffer=0.1 M sodium phosphate, pH 5.8 0.01 AUFS


ABSORBANCE (254nm)
T_0
2.94
3.30
dCMP
5.91
9.65
dTMP
14.70
dGMP
18.51
dAMP
44.58
ELUTION TIME (min)

dividual standards, mixtures of standards, and by peak amplification. In this way the peaks at 5.85 and 5.91 min were identified as dCMP, the peaks at 14.30 and 14.70 min were identified as dTMP, the peaks at 17.90 and 18.51 min were identified as dGMP, and the peaks at 42.69 and 44.68 min were identified as dAMP.

Chromatograms from vegetative samples and samples 3 hours and 6 hours into macrocyst development consistently exhibited peaks at 9.35 to 9.40 min. The retention time for 5mdCMP was 9.30 min. However, co-injecting the 5mdCMP standard and D. mucoroides DNA demonstrated that this peak was not 5-methylcytosine. Figure 6 is an enlargement of this chromatogram showing the distinctness of these two peaks. Therefore, 5mdCMP was not detected in any of the samples examined. By calibrating the chromatograms and determining the smallest peak detectable at the 9 to 10 min elution time of 5-methylcytosine, I calculated that there was less than 0.82 mol% of 5mdCMP in these samples.

Because of the possibility of RNA nucleotide contamination, five ribonucleotide 5'-monophosphate standards were injected individually and in mixtures. A minor deoxyribonucleotide base, dUMP, was also run at this time. Figure 7 is a typical chromatogram of a mixture of these dNMP's. CMP and UMP eluted first with retention times of 3.82 and 4.71 min respectively. GMP, dUMP, and IMP were not clearly

Figure 6. Detail of peak amplification with 5mdCMP.

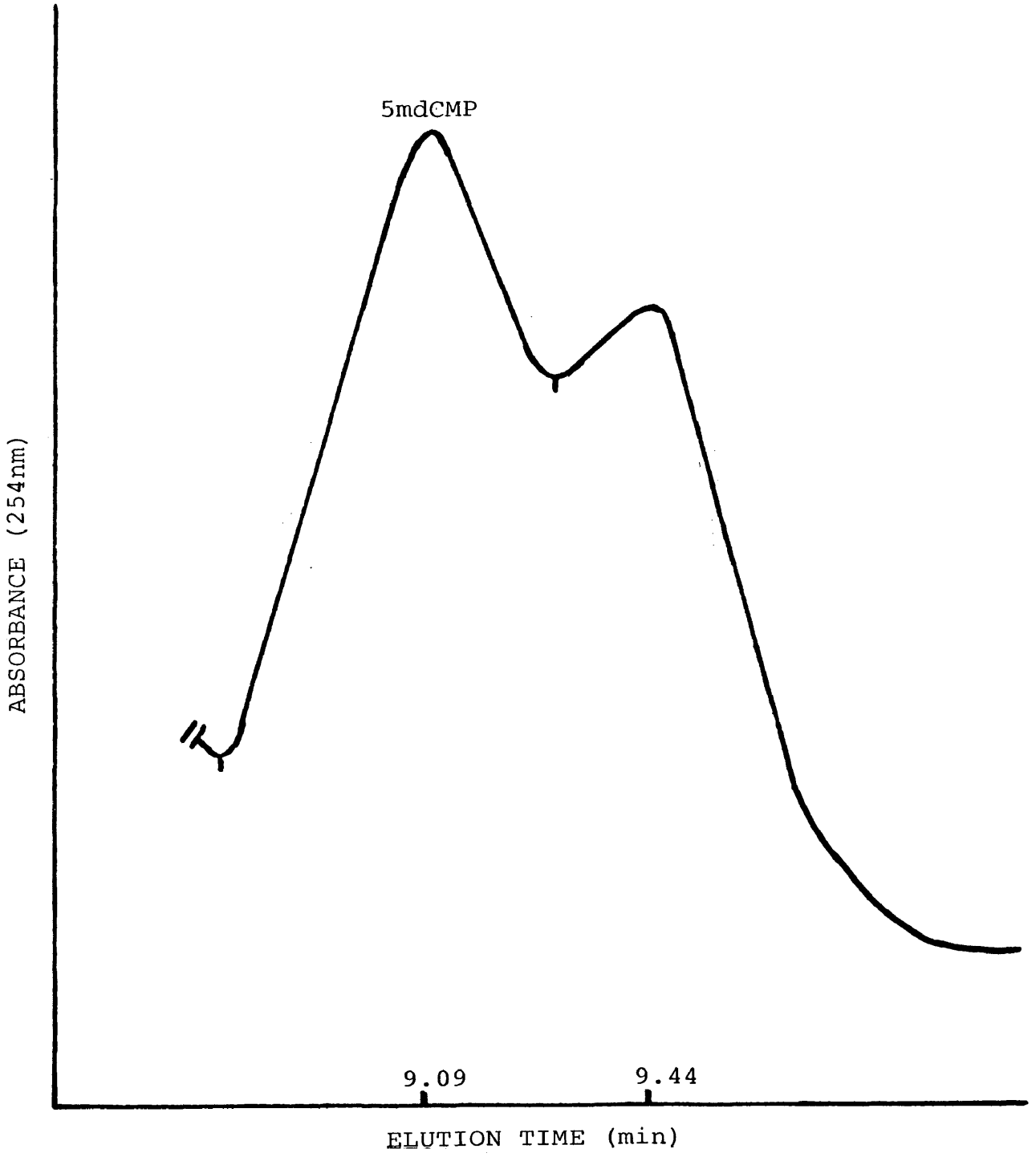
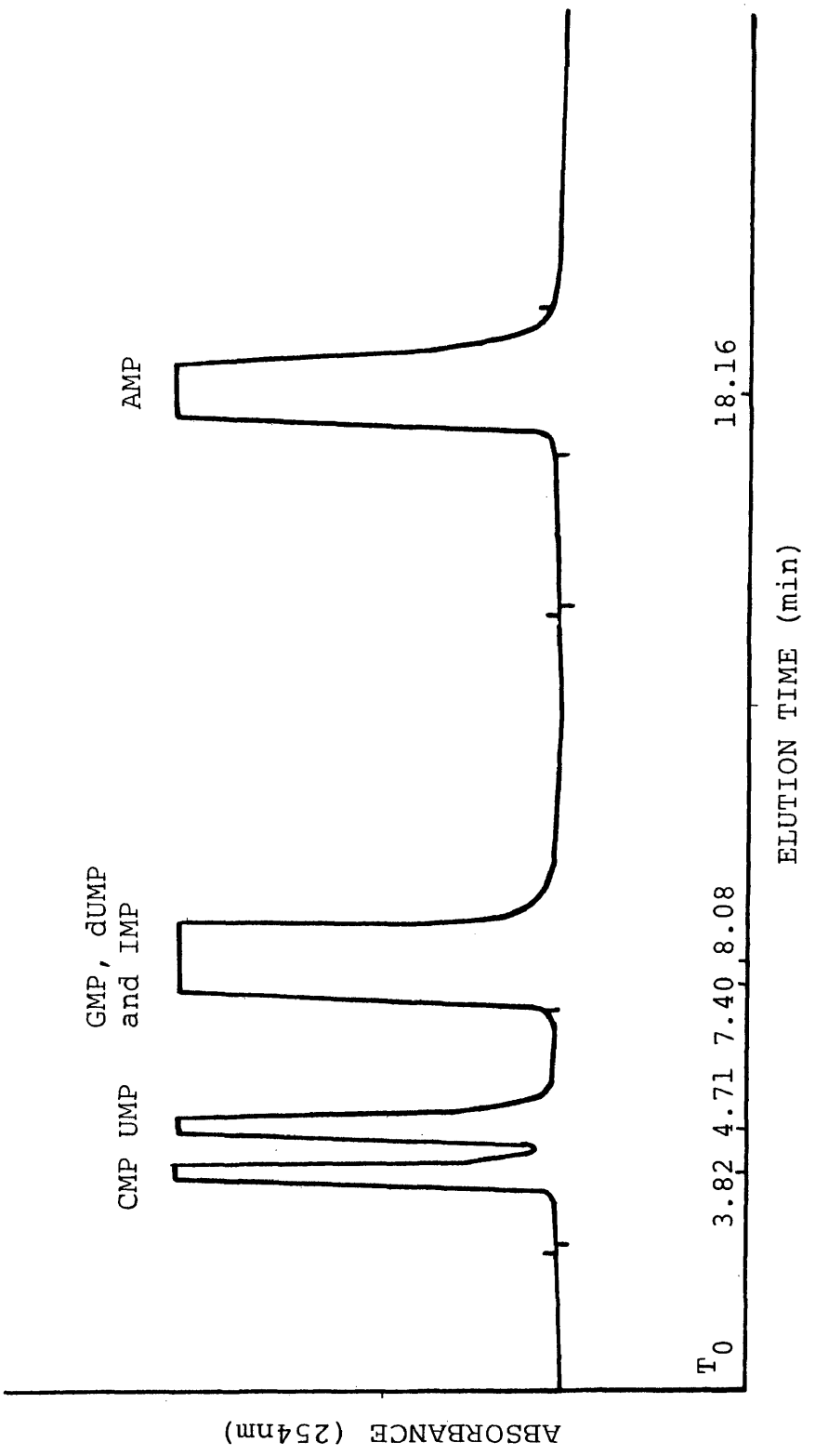

5mdCMP
ABSORBANCE (254nm)
9.09
9.44
ELUTION TIME (min)

Figure 7. Separation of ribonucleotide 5'-monophosphate standards. Separations were performed on a Beckman reverse-phase Ultrasil ODS column.
Flow rate=1 ml/min.
Buffer=0.1 M sodium phophate, pH 5.8
0.1 AUFS


ABSORBANCE (254nm)
T_0
3.82
4.71
7.40
8.08
18.16
ELUTION TIME (min)
CMP
UMP
GMP, dUMP and IMP
AMP

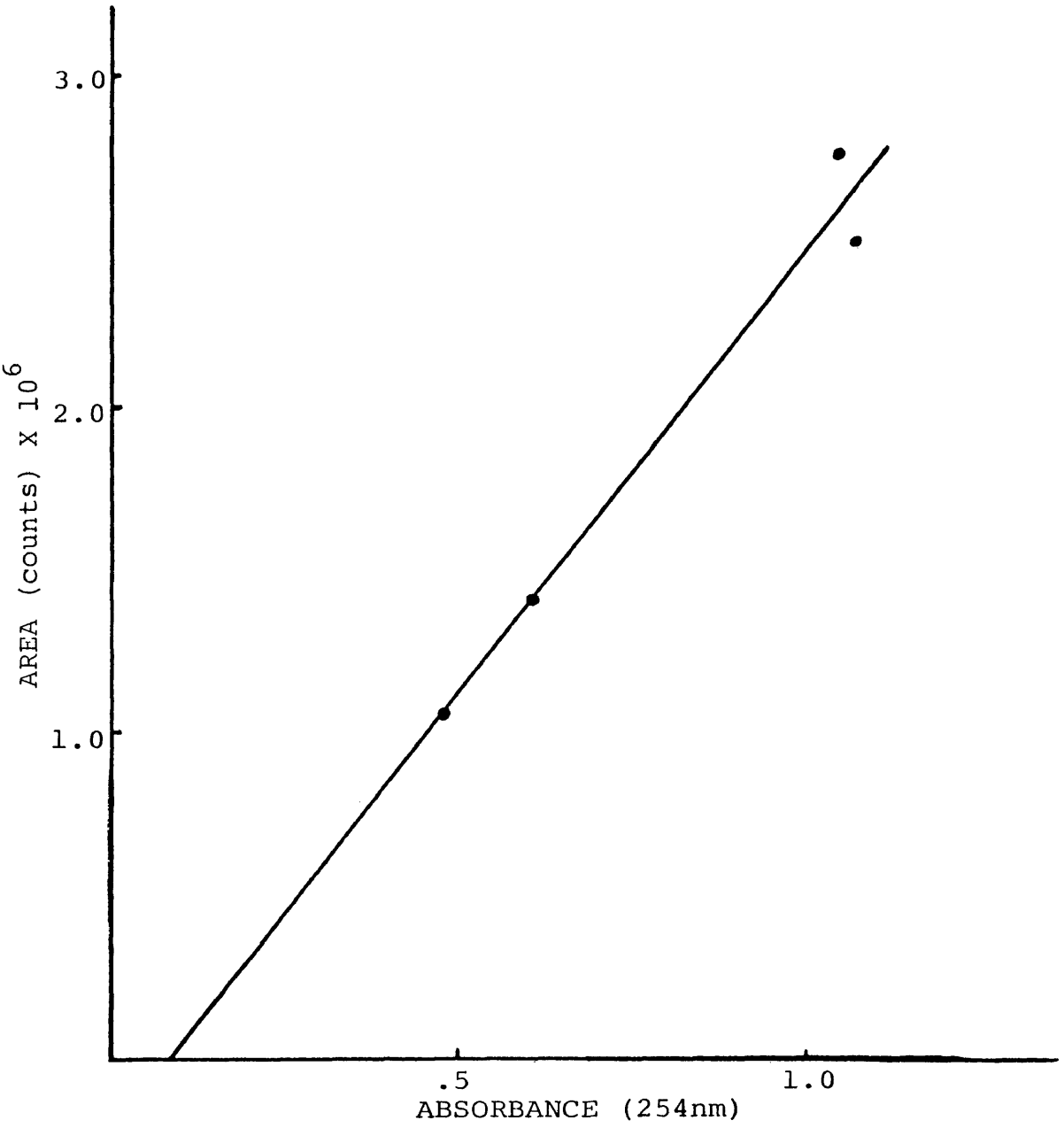
separated from one another and co-eluted from 7.40 to 8.08 min. AMP was retained on the column the longest and finally eluted at 18.16 min.

dNMP Composition

Absorbance is related to concentration by the equation $A=cb\epsilon$: A=absorbance, b=pathlength of the cuvette, c=concentration of the sample in moles, and ϵ is the molar extinction coefficient of the substance in question (Clark and Switzer, 1977). Clearly, absorbance is directly proportional to concentration. However, the data supplied by the integrator were reported as area, not absorbance. Thus, it was necessary to confirm the relationship between absorbance and area before determining dNMP composition.

First, absorbance of the four major dNMPs was read in a spectrophotometer separate from the HPLC apparatus. Then, the standards were injected onto the column. A plot of spectrophotometer absorbance vs HPLC integrator area appears in Figure 8. This graph shows that absorbance was directly porportional to area. The R square statistic was equal to .974, indicating that there was a significant correlation between the two variables. Since area was shown to be directly proportional to absorbance, and $A=cb\epsilon$; it was possible to conclude that area was related to $cb\epsilon$ by some proportionality constant, k. Thus, $area=kcb\epsilon$ and dNMP concentration could now be calculated from the HPLC

Figure 8. Confirmation of the relationship between area, as reported by the 3390A integrator, and ultraviolet absorbance of four dNMP standards.


3.0
2.0
1.0
AREA (counts) X 10^6
.5
1.0
ABSORBANCE (254nm)

integrator data.

Table II is a comparison of the relative dNMP composition of D. mucoroides DNA at two different stages in the organism's life cycle. From these data it appeared that there was as much variation in dNMP composition within a particular stage in development as there was between two different stages in development. For example, percent dCMP in the samples three hours into macrocyst development ranged from 8.92 to 15.76; a difference of 6.4%. The mean percent dCMP of samples three hours into macrocyst development was 12.27, compared to 8.46 for the samples 6 hours into macrocyst development; a difference of 3.81%. This type of variation was not unique to dCMP. A one-way analysis of variance for each dNMP confirmed that the variation observed within a particular state in development was greater than or equal to the variation observed between different stages in development.

In spite of these variations, the data did show some consistencies. The mean values showed that dCMP always comprised the smallest fraction of the total dNMP content, followed by dTMP, dGMP, and dAMP. Although approximately equal percentages of dCMP and dGMP, and dTMP and dAMP would be expected, this was not observed. The purine concentrations were at least 2 to 2.5 times the concentration of their complementary pyrimidines.

Table II. dNMP composition of D. mucoroides, DM-7

Stage of Life Cycle	Mole percent dCMP	dTMP	dGMP	dAMP
Vegetative amoebae	12.25	18.62	14.85	54.29
	13.20	19.20	25.52	42.08
Mean	12.73	18.91	20.19	48.19
Developing macrocysts (3 hours)	14.49	13.39	34.37	37.76
	14.09	12.60	29.08	44.24
	15.76	13.76	33.45	37.03
	10.11	13.63	31.12	45.15
	10.25	18.61	24.74	47.40
	8.92	21.02	25.57	44.49
Mean	12.27	15.50	29.56	42.68
Developing macrocysts (6 hours)	7.65	17.49	29.05	45.82
	8.33	24.85	15.33	51.48
	9.18	12.35	36.20	42.27
	8.67	21.41	17.75	52.16
Mean	8.46	19.03	24.58	47.93

DISCUSSION

In these investigations, 5-methylcytosine was not detected in the nuclear DNA of Dictyostelium mucoroides. Both vegetative amoebae and amoebae allowed to develop under conditions conducive to macrocyst formation contained less than 0.82 mol% 5mdCMP.

The DNA from D. mucoroides nuclei was purified, enzymatically digested to deoxyribonucleosides, and analyzed by reverse-phase HPLC. A peak corresponding to each of the four major deoxyribonucleosides was identified from each sample. Dictyostelium nuclei contain DNA, RNA, and protein in a ratio of 1:3.4:2.2 (Jacobson and Lodish, 1975). Therefore, to eliminate the possibility of peak misidentification, the influence of contaminants was considered.

The A_{260}:A_{280} ratio of pure double-stranded DNA is typically between 1.65 and 1.85. Lower values can indicate the presence of protein (Schleif and Wensink, 1981). The A_{260}:A_{280} ratios for the experimentally isolated DM-7 DNA ranged from 1.30 to 1.61. This compares to a literature value of 1.44 for Dictyostelium chromatin (Pederson, 1977). Dictyostelium nuclei contain both histone and non-histone proteins (Pederson, 1977). These A_{260}:A_{280} ratios suggest that protein was present in the experimental samples. However, histone proteins have only minimal absorbance at 260

nm (Bonner et al., 1968). Ultraviolet absorption for these experiments was performed at 254 nm. Further, individual runs of two enzymes, phosphodiesterase and DNase I, demonstrated that these proteins eluted at 2.91 to 3.30 min. Thus, even if histone and/or non-histone proteins were present in the DNA samples, it seemed unlikely that they interfered with the elution or detection of the deoxyribonucleosides.

The possibility of RNA contamination also appeared unlikely. The retention times of five ribonucleotide standards were determined and shown to be clearly distinct from the dNMP's. This confirmed the elution pattern and sequence for ribonucleotides as determined by Christman (1977). Cleary even if individual ribonucleotides were present in the DNA samples, these compounds did not interfer with the conclusive identification of the peaks in question.

A peak with approximately the same elution time as the 5mdCMP standard was consistently observed in the chromatograms of all DM-7 samples. Although this compound was not positively identified, it was conclusively shown not to be due to 5mdCMP. Thus, neither DNA from vegetative cells nor DNA from cells 3 or 6 hours into macrocyst development exhibited detectable levels of 5mdCMP. These data are consistent with the results of restriction enzyme analysis of

Dictyostelium discoideum DNA (Loomis, 1982). Kindle and Firtel (1978) and Kimmel and Firtel (1979) used Sau 3A and MboI to probe for adenine methylation, and HpaII and MspI to probe for cytosine methylation. They observed no evidence of methylation in digests of total genomic DNA, rRNA cistrons, or actin sequences.

Since the G+C content of Dictyostelium nuclear DNA is only 22% (Sussman and Raynor, 1971; Firtel and Bonner, 1972), one might anticipate a correspondingly reduced percentage of 5mdCMP. A correlation coefficient was determined for mol% G+C and mol% 5mdCMP for 123 organisms whose base compositions were listed in the Handbook of Biochemistry and Molecular Biology (Shapiro, 1976). Only organisms reported to contain 5mdCMP were used in the analysis. From this sampling Pearsons r was found to be -.3396. This indicates that there is a statistically significant inverse correlation between G+C content and the percentage of cytosine residues methylated.

My investigations also showed that the relative dNMP composition of D. mucoroides DNA did not change as the commitment to macrocyst development began. It is also evident from Table II that the published G+C value of 22% (Susman and Raynor, 1971; Firtel and Bonner, 1972) was not supported by these data. At this time it is not clear why there was an underrepresentation of the pyrimidines

and an overrepresentation of dAMP. Nuclei were separated from cells prior to lysis so that cytoplasmic nucleotide pools would not be a factor.

It could be argued that the enzymatic digestion of DNA to dNMP's and separation by reverse-phase HPLC might not yield composition figures comparable to those obtained using buoyant density centrifugation. However, my base composition figure for salmon sperm DNA very closely approximated that in the literature (Table III). In addition, experiments by Kuo, McCune, and Gerke (1980) on DNA from a variety of sources indicate that enzyme digestion-HPLC separation is a valid method for base determination.

One explanation for this disparate base composition data involves the histone and non-histone proteins. Recall that the A_{260}:A_{280} ratios for the experimental samples were similar to those of Dictyostelium chromatin. Although it was shown that protein did not interfer with the elution of the dNMP's, it could have interfered with the enzymatic digestion of the DNA. If this did happen it seems clear from these data that cytosine and thymine were more protected from enzymatic action than guanine and adenine. Possibly this type of protection extends to other types of DNA-enzyme interactions as well.

The implications of my investigations are twofold: either 5mdCMP is present but was not detected, or 5mdCMP

Table III. Comparison of experimentally determined dNMP composition and literature value base composition of salmon sperm DNA.

Source	Percent of total dCMP	dTMP	dGMP	dAMP
Experimental	20.49	24.94	25.49	29.26
Literature[1]	20.4	29.1	20.8	29.7
	20.6	30.4	21.5	27.6
	21.6	27.1	22.4	28.9

[1]Shapiro, 1976.

is not present in Dictyostelium DNA. Although the physiological significance of cytosine methylation is not known, it seems reasonable to assume that cytosine methylation is associated with the events of transcription. Most 5mdCMP is found as 5mCpG (Erlich and Wang, 1981). With a comparatively low G+C ratio, methylation of a few crucial cytosine residues could have a developmental impact on this organism.

If 5mdCMP is not involved in developmental gene expression in Dictyostelium, then other forms of transcriptional modulation must be considered. Britten and Davidson (1969) have proposed a model of gene regulation in which multiple integrator and receptor sites could control the expression of functionally related structural genes during different stages of an organism's development. Approximately 50% of the Dictyostelium genome consists of single copy DNA interspersed with short repeated sequences (Firtel and Kindle, 1975). Also, Zuker and Lodish (1981) have hybridized two families of developmentally regulated mRNA to plasmid clones of D. discoideum DNA that contained repeat sequences. The cloned DNA specifically hybridized to a species of mRNA that is induced at 5.5 hours of development and to another species that is induced between 5.5 and 15 hours of development. These findings support the Britten-Davidson model of transcriptional control.

Ribosomal protein synthesis also appears to be under transcriptional control in D. discoideum. One of the first effects of amoebae starvation is a reduction in the size and amount of free and membrane-bound polysomes (Cardelli and Dimond, 1981). Jacobson and Lodish (1975) observed that rRNA is continually synthesized throughout differentiation. These observations suggest the possibility of a heterogeneous population of ribosomes in developing organisms. Experimental support for such a hypothesis was lacking until the work of Ramagopal and Ennis (1981, 1982). Their experiments involved two-dimensional polyacrylamide-gel electrophoresis of ribosomal proteins from various developmental stages of D. discoideum. They observed twelve unique ribosomal proteins in vegetative amoebae and in spores. Two of these proteins were found only in amoebae, three only in spores, and the remainder were present in both types of cells, but differed in their stoichiometry.

These results suggest that distinct populations of ribosomes are synthesized at different times in the development of D. discoideum. These differences are due to the protein component of the ribosome and not the RNA component. It has been postulated that differential ribosomal synthesis could be used to regulate protein synthesis during development via mRNA selection and translation of cell-specific polypeptides (Jacobson and Lodish, 1975; Ramagopal and

Ennis, 1981). Such a regulatory mechanism could explain the decrease in the initiation rate of protein synthesis observed immediately after starvation in D. discoideum (Cardelli and Dimond, 1981).

The question of transcriptional control was also investigated by comparing the pattern of total protein synthesis in developing cells with stage specific mRNA isolates (Alton and Lodish, 1977). Proteins isolated during development were subjected to two-dimensional electrophoresis. Stage specific isolates of mRNA were translated in a wheat germ cell-free system and these proteins were then subjected to two-dimensional electrophoresis. Developmental changes were observed in the synthesis of 100 of the 400 detectable proteins isolated from developing cells. In most instances these fluctuations corresponded to observed changes in the translatable mRNA population.

The results of Alton and Lodish suggest that most of the protein-encoding mRNA in D. discoideum appears in the cytoplasm in translatable form just prior to the actual synthesis of its corresponding protein. There was, however, one constantly detected mRNA that did not appear to be translated until the late aggregation stage. While transcriptional control of protein synthesis is one explanation for these results, they also suggest that post-transcrip-

tional modification or degradation of mRNA may be involved.

SUMMARY

5-Methylcytosine is the most prevalent modified base found in eukaryotic DNA. Although its exact function is not yet known, methylation appears to be associated with gene inactivation. In some organisms this appears to alter development.

The DNA from the cellular slime mold Dictyostelium mucoroides was extracted, digested to deoxyribonucleoside 5'-monophosphates (dNMP's), and analyzed by reverse-phase high pressure liquid chromatography. Using this method, no significant difference in 5-methylcytosine content or dNMP composition was observed between vegetative amoebae, and amoebae incubated for three and six hours under conditions conducive to macrocyst formation. Under the conditions of these investigations, the concentration of 5-methylcytosine was determined to be less than 0.82 mol%.

LITERATURE CITED

Alton, T. and H. Lodish. 1977. Developmental changes in messenger RNAs and protein synthesis in Dictyostelium discoideum. Dev. Biol. 60:180-206.

Ashworth, J. M. 1971. Control mechanisms of growth and differentiation. Symp. Soc. Exp. Biol. 25:27-49.

Ashworth, J. M. and J. Dee. 1975. The Biology of Slime Moulds. Edward Arnold Publishers Limited, London.

Bird, A. P., M. H. Taggart, and C. A. Gehring. 1981. Methylated and unmethylated ribosomal RNA genes in the mouse. J. Mol. Biol. 152:1-17.

Blumberg, D. D., J. P. Margolskee, E. Barklis, S. N. Chung, N. S. Cohen, and H. F. Lodish. 1982. Specific cell-cell contacts are essential for induction of gene expression during differentiation of Dictyostelium discoideum. Proc. Natl. Acad. Sci. USA. 79:127-131.

Bonner, J. T. and E. B. Frascella. 1953. Variations in cell size during the development of the slime mold, Dictyostelium discoideum. Biol. Bull. 104:297-300.

Bonner, J, G. R. Chalkley, M. Dahmus, D. Fambrough, F. Fugimura, R. Huang, J. Huberman, R. Jensen, K. Marushige, H. Ohlenbusch, B. Olivera, and J. Widholm. 1968. Isolation and characterization of chromosomal nucleoproteins. In Methods in Enzymology. 12:3-65.

Britten, R. J. and E. H. Davidson. 1969. Gene regulation for higher cells: A theory. Science. 165:349-357.

Cardelli, J. A. and R. Dimond. 1981. Regulation of protein synthesis in Dictyostelium discoideum: effects of starvation and anoxia on initiation. Biochemistry. 20:7391-7398.

Christman, J. 1982. Separation of major and minor deoxyribonucleoside monophosphates by reverse-phase high-performance liquid chromatography: A simple method applicable to quantiation of methylated nucleotides in DNA. Anal. Biochem. 119:38-48.

Christman, J., P. Price, L. Pedrinan, and G. Acs. 1977. Correlation between hypomethylation of DNA and expression of globin genes in Friend erythroleukemia cells. Eur. J. Biochem. 81:53-61.

Clark, J. R. and R. Switzer. 1977. Experimental Biochemistry. 2nd ed. W. H. Freeman and Co., San Francisco.

Doerfler, W. 1983. DNA methylation and gene activity. Ann. Rev. Biochem. 52:93-124.

Ehrlich, M. and R. Wang. 1981. 5-Methylcytosine in eukaryotic DNA. Science. 212:1350-1357.

Farmer, John N. 1980. The Protozoa. The C. V. Mosby Co., St. Louis.

Firtel, R. A. 1972. Changes in the expression of single-copy DNA during development of the cellular slime mold Dictyostelium discoideum. J. Mol. Biol. 66:363-377.

Firtel, R. A. and J. T. Bonner. 1972. Characterization of the genome of the cellular slime mold Dictyostelium discoideum. J. Mol. Biol. 66:339-361.

Firtel, R. A. and K. Kindle. 1975. Structural organization of the genome of the cellular slime mold Dictyostelium discoideum: interspersion of repetetive and single-copy DNA sequences. Cell. 5:401-411.

Garrod, D. and J. M. Ashworth. 1973. Development of the cellular slime mould Dictyostelium discoideum. Symp. Soc. Gen. Micro. 23:407.

Jacobson, A. and H. F. Lodish. 1975. Genetic control of development of the cellular slime mold, Dictyostelium discoideum. Ann. Rev. Gen. 9:145-185.

Kimmel, A. and R. A. Firtel. 1979. A family of short, interspersed repeat sequences at the 5' end of a set of Dictyostelium single-copy mRNAs. Cell. 16:787-796.

Kindle, K. and R. A. Firtel. 1978. Identification and analysis of Dictyostelium actin genes, a family of moderately repeated genes. Cell. 15:763-778.

Kuo, K. C., R. McCune, and C. Gehrke. 1980. Quantitative reversed-phase high performance liquid chromatographic determination of major and modified deoxyribonucleosides in DNA. Nuc. Acids. Res. 8:4763-4775.

Lapeyre, J. and F. Becker. 1979. 5-Methylcytosine content of nuclear DNA during chemical hepatocarcinogensis and in carcinomas which result. Biochem. Biophys. Res. Comm. 87:698-705.

Lodish, H. F., D. D. Blumberg, R. Chisholm, S. Chung, A. Coloma, S. Landfear, E. Barklis, P. Lefebore, C. Zuker, and G. Mangiarotti. 1982. Control of Gene Expression in the Development of Dictyostelium discoideum. Wm. F. Loomis, ed. Academic Press, New York. pp. 326-352.

Loomis, W. F. 1975. Dictyostelium discoideum: A Developmental System. Academic Press, New York.

Loomis, W. F. 1982. The Development of Dictyostelium discoideum. Academic Press, New York.

Maniatis, T., E. F. Fritsch, and J. Sambrook. 1982. Molecular Cloning: A Laboratory Manual. Cold Spring Harbor Lab. Cold Spring Harbor, New York. pp. 458-460.

Mohandas, T., R. S. Sparkes, and L. Shapiro. 1981. Reactivation of an inactive human X chromosome: evidence for X inactivation by DNA methylation. Science. 211:393-396.

Nickerson, A. W. and K. B. Raper. 1973. Macrocysts in the life cycle of the Dictyosteliaceae. I. Formation of the macrocysts. Amer. J. Bot. 60:190-197.

Pederson, T. 1977. Isolation and characterization of chromatin from the cellular slime mold Dictyostelium discoideum. Biochemistry. 16:2771-2777.

Ramagopal, S. and H. Ennis. 1981. Regulation of synthesis of cell-specific ribosomal proteins during differentiation of Dictyostelium discoideum. Proc. Natl. Acad. Sci. USA. 78:3083-3087.

Ramagopal, S. and H. Ennis. 1982. Ribosomal protein synthesis during spore germination and vegetative growth in Dictyostelium discoideum. J. Biol. Chem. 257: 1025-1031.

Razin, A. and A. Riggs. 1980. DNA methylation and gene function. Science. 210:604-610.

Schleif, R. and P. Wensink. 1981. Practical Methods in Molecular Biology. Springer-Verlag, New York.

Shapiro, H. 1976. Distribution of Purines and Pyrimidines in Deoxyribonucleic acids. In Handbook of Biochemistry and Molecular Biology. 3rd. ed. Nucleic Acids. Vol. II. (Gerald D. Fasman, ed.) CRC Press, Cleveland.

Sussman, R. R. and E. P. Rayner. 1971. Physical characterization of deoxyribonucleic acid in Dictyostelium discoideum. Biochem. Biophys. 144:127-137.

Zuker, C. and H. Lodish. 1981. Repetitive DNA sequences cotranscribed with developmentally regulated Dictyostelium discoideum mRNAs. Proc. Natl. Acad. Sci. USA. 78:5386-5390.