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CHROMATOGRAPHIC ANALYSIS OF ENZYME DIGESTS OF DEOXYRIBONUCLEIC ACID AND DEOXYRIBONUCLEIC ACID METHYLATED BY THE CARCINOGENS DIMETHYLNITROSAMINE AND METHYL METHANESULPHONATE

BY

KATHLEEN F. HEDRICK SHUTTLEWORTH

A Thesis

Submitted to the Faculty of Graduate Studies through the Department of Chemistry in Partial Fulfillment of the Requirements for the Degree of Master of Science at The University of Windsor

Windsor, Ontario

1978

ABSTRACT

Several different deoxyribonucleic acid (DNA) solutions are enzymatically digested to the deoxyribonucleoside level. Each enzyme digest is then subjected to Sephadex LH-20 column chromatography employing a methanol:water gradient elution system. The samples of interest are the enzyme digests of rat liver DNA which has been reacted with the carcinogen dimethylnitrosamine (DMNA) *in vivo* and of calf thymus DNA which has been reacted with the carcinogen methyl methanesulphonate (MMS) *in vitro*. Peaks which are resolved from the column chromatography of an enzyme digest of normal rat liver DNA are identified by thin layer chromatography and spectral characteristics. Dowex 50W-X4 column chromatography which employs an ammonium formate elution buffer is used as a further supportive means of identification of peaks from the Sephadex LH-20 column chromatography. Discussion is presented as to the validity of the Dowex 50W-X4 column chromatography as a means of identification of peaks. Some suggestions are made to explain the differences seen in the Sephadex LH-20 chromatographic elution profiles of the methylated DNA samples.

ACKNOWLEDGEMENTS

First, I would like to acknowledge my faith in the Lord without whose grace and guidance this project could not be completed.

I would like to thank my advisor Dr. D.E. Schmidt Jr. for his perseverance and guidance. I must also thank Dr. D.S.R. Sarma for the samples of rat liver DNA methylated by ^{3}H-dimethylnitrosamine *in vivo* and calf thymus DNA methylated by methyl methanesulphonate *in vitro*. Thanks are also extended to Dr. K.E. Taylor, Dr. A.H. Warner and Dr. G.W. Wood for their help.

I wish to thank my fellow graduate students, particularly Ms. M.A. Wrobel, Ms. W. Pu and Mr. R. Ewing for their constant encouragement and assistance.

I wish to acknowledge the financial support of the Ontario government in the form of the Ontario Graduate Scholarship as well as teaching and research assistantships from the Department of Chemistry, the University of Windsor.

Lastly, I would like to acknowledge the loving support of my husband Dave during my entire course of graduate studies.

To Dave

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ABBREVIATIONS

B.S.A.	bovine serum albumin
cpm	counts per minute
dAdo	deoxyadenosine
dCyd	deoxycytidine
dGuo	deoxyguanosine
DEAE	diethylaminoethyl
DMNA	dimethylnitrosamine
DNA	deoxyribonucleic acid
DNase I	deoxyribonuclease I
dpm	disintegrations per minute
dThd	deoxythymidine
FDH	formaldehyde dehydrogenase
f.d.m.s.	field desorption mass spectrometry
GSH	reduced glutathione
MMS	methyl methanesulphonate
NAD	nicotinamide adenine dinucleotide
O.D.	optical density
PAH	polycyclic aromatic hydrocarbon
PEI	polyethyleneimine
R_f	$\frac{\text{distance spot travels}}{\text{distance solvent front travels}}$ (T.L.C.)
RNA	ribonucleic acid
RNase T_1	ribonuclease T_1
T_m	temperature of hyperchromic shift in DNA

ABBREVIATIONS

T.L.C. thin layer chromatography

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CHAPTER I

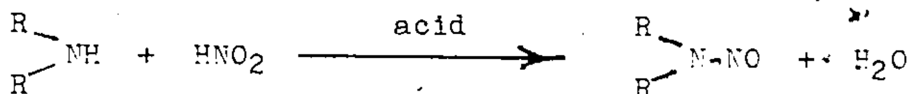
INTRODUCTION

The topic of cancer research is always one of great interest as it deals with the matter of human life or death. This chapter deals with some of the history of cancer research and also gives a brief description of the chromatographic system which was used to study enzyme digests of rat liver DNA which had been reacted with the carcinogen dimethylnitrosamine *in vivo* and calf thymus DNA which had been reacted with the carcinogen methyl methanesulphonate *in vitro*. The other DNA samples studied were calf thymus, human placenta and salmon sperm DNA. These samples acted as controls for the experiment.

The first class of carcinogenic agents studied in history were the polycyclic aromatic hydrocarbons (PAHs). PAHs are present in tar, tobacco smoke, polluted city air and charcoal-broiled or smoked foods(1). Early research dealing with the carcinogenicity of these compounds was done by Yamagiwa *et al*. who achieved experimental induction of cancer by applying a coal distillate to rabbits' ears(2). In 1932, Cook *et al*. showed that tumours developed at the site of treatment in mouse skin with 1:2-benzpyrene and some of its derivatives(3,4). Cook *et al*. were able to isolate cancer producing compounds from coal tar in pure form(5). From these initial findings, much has been done to investigate the manner in which PAHs induce cancer.

One theory is that the DNA is modified by the carcinogen. Carcinogenic PAHs have been shown to become covalently bound to nucleic acids in mouse skin(6,7). This binding has been shown to occur at specific reactive sites in DNA(8,9,10,11). Some of these sites are shown in Figure 1. Experiments have shown that the carcinogen is first metabolized to an active form called the "ultimate carcinogen" before becoming covalently bound to DNA(9,12,13).

In this research, rat liver DNA had been allowed to react with the carcinogen dimethylnitrosamine (DMNA) in vivo. DMNA acts specifically in the rat liver, kidney and lung tissue as a carcinogenic agent(14). DMNA is not usually ingested by an individual. However, nitrosamines are easily synthesized under acid conditions by mixing secondary amines with nitrous acid (1) as shown in the reaction below:



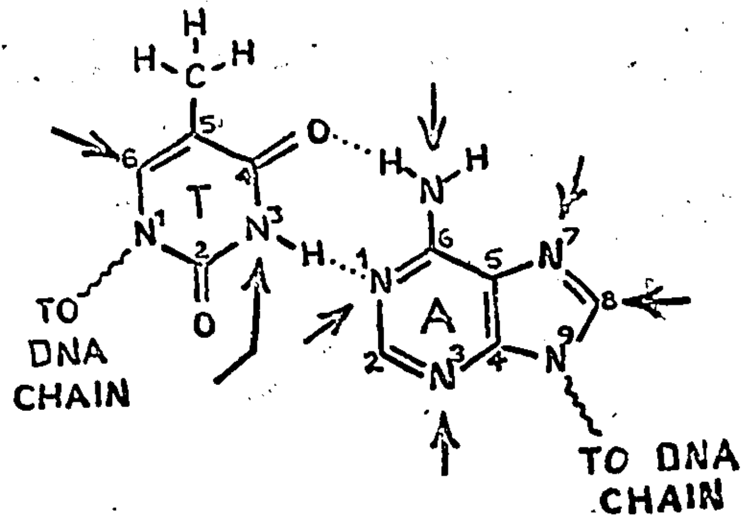
If the R group is made a methyl group, then dimethylnitrosamine will be the substance synthesized. The stomach presents a perfect acidic environment for the reaction of amines with nitrite salts. The amines could come from ingested food and some drugs such as piperazine and the nitrite salts from sausages and other preserved meat. Nitrites are also generated from nitrates by certain bacteria in the intestinal flora. Humans may therefore be exposed to DMNA indirectly.

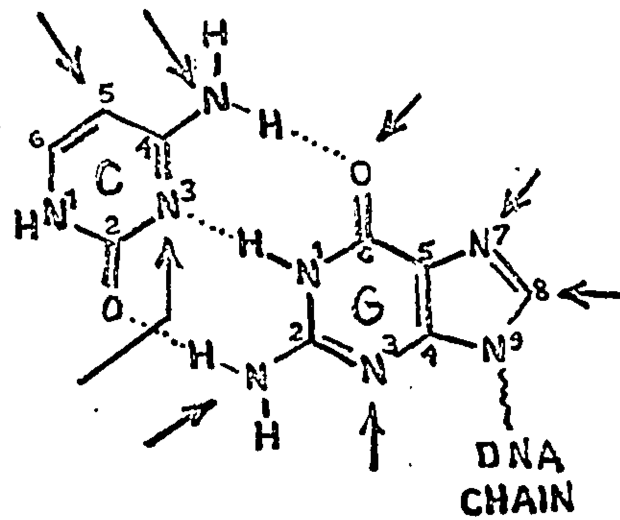
3

Figure 1. Possible Reaction Sites in DNA Components (12). Arrows indicate possible sites of hydrocarbon attachment.

FIGURE 1

Possible Reaction Sites in DNA Components

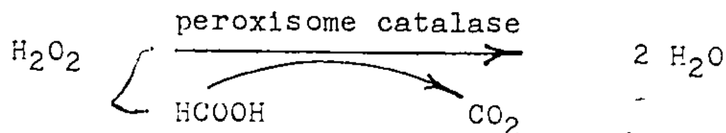




Enzymatic activation of DMNA has been found to be necessary to cause the methylation of DNA(8). The first step in activation is hydroxylation of DMNA by means of hydroxylase enzymes found in normal liver cells. The total activation reaction is shown in Figure 2.

A formaldehyde by-product is formed when the hydroxylated derivative decomposes to monomethylnitrosamine. When ^{14}C-DMNA was administered to rats, of the ^{14}C-label, 7% appeared as products in the urine, 40% as expired carbon dioxide and the rest was evenly distributed in the tissues (15). The products in the urine would probably be formaldehyde and formic acid. Formaldehyde forms a complex with reduced glutathione (GSH). Glutathione is a sulfhydryl compound utilized in biological oxidations. Using nicotinamide adenine dinucleotide (NAD^+) as the oxidizing agent, formaldehyde dehydrogenase (FDH) acts on the afore-mentioned complex to produce formic acid. A proposed mechanism is shown in Figure 3 (16). The formic acid is then excreted in the urine.

Formaldehyde oxidation may not stop at the formic acid level but may go on to the carbon dioxide level by means of reaction of formic acid with hydrogen peroxide by the mechanism shown below (17):



This reaction takes place in the peroxisomes which are found in rat liver cells, the location of DMNA activation.

Figure 2. The DMNA Activation Reaction.

(Adapted from Irving (8) p.206.)

FIGURE 2

The DMNA Activation Reaction

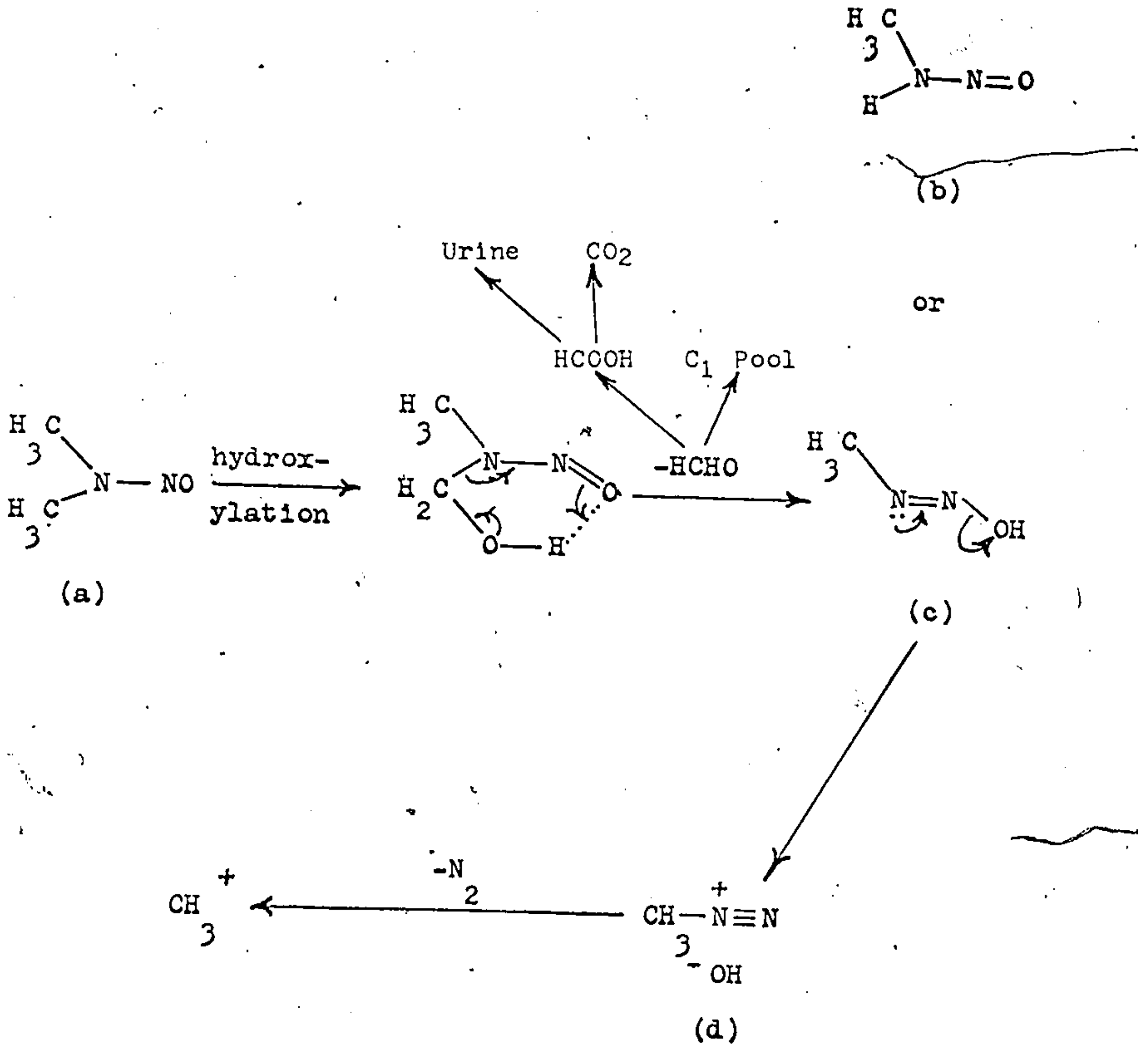
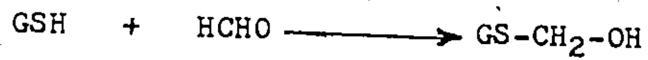
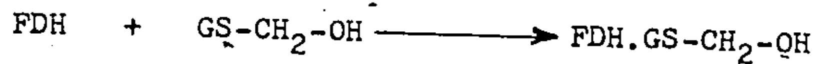


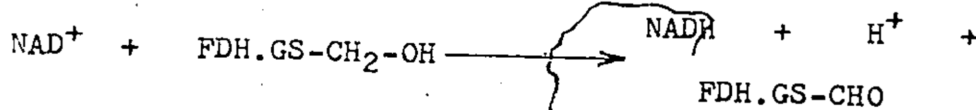
Figure 3. Formaldehyde Metabolism (16).

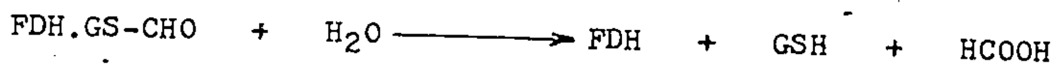
FIGURE 3

Formaldehyde Metabolism









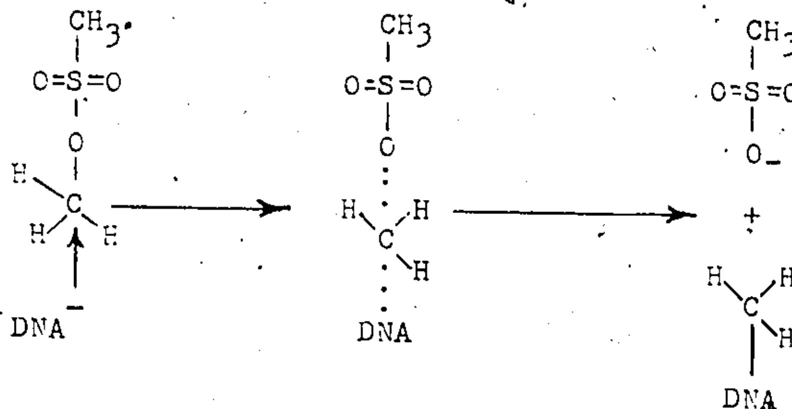
The carbon dioxide so formed could then be expired from the rat and represent the 40% of the ^{14}C-label previously mentioned.

The other 53% of the ^{14}C-label was found to be generally distributed in the tissues. This could be due to the fact that formaldehyde can be incorporated in to the C_1 metabolic pool of the rat. Some formaldehyde also binds to amino groups in purine and pyrimidine bases, nucleosides and nucleotides(18). The measurements of ^{14}C distribution in the rat were done eight hours after the administration of the ^{14}C-DMNA.

Reconsidering the activation reaction, it is seen that a carbonium ion is generated. This electrophile could react with any cellular nucleophile such as DNA, RNA and/or protein. Enzyme digests of DNA from rats treated with DMNA _in vivo_ seemed to indicate that methylation had occurred at the N-7 and O-6 positions in guanine and at the N-3 and O-4 positions in thymine(19). It is thought that this alkylation might be responsible for the initiation of tumours because the alkylated derivatives could serve as miscoding bases during DNA replication(19). The varying capability of cells in different tissues to repair this DNA damage might account for the tissue specificity observed with dialkylnitrosamines(19).

Calf thymus DNA was methylated _in vitro_ by the carcinogen methyl methanesulphonate (MMS). The DNA nucleophile is thought to react with MMS in an S_N2 (nucleo-

philic substitution) reaction (20) as shown below (adapted from (21)):



If the MMS were administered in vivo, the methanesulphonic acid by-product would be excreted in the urine.

Methyl methanesulphonate has been shown to induce tumours in rat kidney and brain(19). This type of direct-acting alkylating carcinogen is important to humans since it is a highly reactive chemical used in chemical laboratories as an intermediate in organic syntheses. Therefore, people working in these laboratories would be exposed to this carcinogen. The main site of alkylation by this compound in vitro and in vivo seem to include the N-1, N-3 and N-7 positions in adenine as well as the N-3 and N-7 position in guanine(22). A small proportion of the methylation products in DNA are derived from reaction on the O-6 and C-3 positions of guanine(20,22).

In this research, a chromatographic system was investigated which Baird and Brookes (23) described for the isolation of 7-methylbenz(a)anthracene-^{3}H-DNA products as deoxyribonucleosides using a water:methanol gradient

elution technique from a Sephadex LH-20 column(23,24). Unfortunately, no technique then available was sensitive enough to determine the structure of the DNA-bound hydrocarbon. With the advent of field desorption mass spectrometry (f.d.m.s.), a means was provided which could be capable of determining the previously mentioned structure. The f.d.m.s. technique produces more intense molecular ions and less fragmentation than the usual mass spectrometric techniques. It is not necessary to volatilize the sample and for this reason a great many molecules with low volatility and/or low thermal stability, such as deoxyribonucleosides, are amenable to f.d.m.s. analysis.

Preliminary work showed that a variety of underivatized methylated purine and pyrimidine bases; oxy- and deoxyribonucleosides and their analogues and nucleotides were amenable to f.d.m.s. analysis(25). The most notable feature in their f.d.m.s. analysis is the presence of an intense M or M+H ion. The major fragmentation process in nucleoside spectra involves the rupture of the glycosidic bond leading to the formation of the constituent sugar and base ions. Samples obtained from the Baird and Brookes Sephadex LH-20 column chromatography of an enzyme digest of calf thymus DNA had been analyzed by f.d.m.s. analysis by Lau(25). Detection of dGuo in one of the eluted peaks was accomplished with 2 X 10^{-9} moles of the deoxyribonucleoside. This work seemed to be promising as the detection level was in a range in which the DNA-bound carcinogen products were

suspected to be found. If the DNA-bound carcinogen products could be detected by f.d.m.s. analysis, then their structures could be determined.

The following work examined the resolution of the Sephadex LH-20 column chromatographic system for enzyme digests of DNA which had been reacted with alkylating carcinogens both in vivo and in vitro. It was hoped that this system would be capable of resolving the methylated nucleosides from the non-methylated nucleosides. If this resolution were possible, then in a future work, the resolved nucleosides could be subjected to f.d.m.s. analysis in order to elucidate the position of methylation in the DNA molecule. The Baird and Brookes chromatographic system has also been shown to be capable of resolving DNA-bound hydrocarbon products from normal nucleosides(23,24) so that another future application would be the analysis of enzyme digests of DNA samples which had been reacted with PAHs both in vivo and in vitro.

CHAPTER II

EXPERIMENTAL

A. METHODS AND MATERIALS

1) Materials

The following materials were commercially available: P.C.S. scintillation cocktail (Amersham/Searle); sodium dihydrogen phosphate (AnalaR); sodium citrate, sodium acetate, perchloric acid (J.T. Baker Chemical Co.); yeast RNA (B.D.H.); human placenta DNA, bovine serum albumin (Calbiochem.); 95% ethanol (Consolidated Alcohols Ltd.); sodium p-aminosalicylate, liquified phenol, m-cresol, sodium chloride, diethyl ether, 2-ethoxyethanol, isopropanol, phosphorous pentoxide, sodium hydroxide, magnesium chloride, methanol (spectra-analyzed), ammonium formate, Millipore filters, butanol, formic acid, lithium chloride (Fisher Scientific Co. Ltd.); ammonium hydroxide (Mallinckrodt Chemical Works); ribonuclease T_1, deoxyribonuclease I, phosphodiesterase, alkaline phosphatase, calf thymus DNA, salmon sperm DNA, 2'-deoxyadenosine, 2'-deoxycytidine, 2'-deoxyguanosine, 2'-deoxythymidine, Coomassie Brilliant Blue G250 dye, Sephadex LH-20 resin, Dowex 50W-X4 200-400 mesh resin (Sigma Chemical Co.).

The sample of rat liver DNA methylated by the carcinogen DMNA *in vivo* and the sample of calf thymus DNA methylated by the carcinogen MMS *in vitro* were both obtained from the laboratory of Dr. D.S. Sarma who is now associated with the Department of Pathology, University of Toronto

School of Medicine, Toronto, Ontario, Canada.

2) DNA Isolation from Rat Liver

Wistar rats were obtained from the Department of Psychology, University of Windsor. The adult rats were kept on a standard diet.

DNA was isolated by a method designed by Kirby(26) with some modifications.

Step 1

Four rats were decapitated and the livers quickly removed and weighed. The livers were chopped on a glass plate with a sharp knife. A 10 ml portion of ice-cold 6% (w/v) sodium p-aminosalicylate solution was added for each gram of chopped liver. The crude suspension was homogenized in a Waring blender at 4°C for 1 min.

One portion of the crude homogenate was mixed with one portion of a mixture composed of phenol, water and m-cresol in a ratio 10:1.1:1.4 (v/v/v). The resultant emulsion was stirred for 1 h at room temperature. The emulsion was broken by centrifugation at 11,700 x g (G.S.A. rotor) in a Sorvall RC2-B centrifuge for 20-30 min. The aqueous layer was extracted with the phenol-cresol mixture for a second 15 min time interval in like manner. The aqueous layer was siphoned off into a beaker and chilled to 0°C.

Step 2

Two portions of ice-cold 95% ethanol were added dropwise to one portion of the chilled aqueous layer.

After this addition was completed, the solution was allowed to stand for 1 h at 0°C. The resultant stringy precipitate was rolled out on a glass rod and was then dissolved in a 20 ml portion of an ice-cold 0.015 M sodium chloride-0.0015 M sodium citrate solution pH 7.0. This solution was made 1.5 M in sodium chloride in order to promote selective ribonucleic acid (RNA) precipitation and was left at 4°C overnight.

Step 3

The next day, the solution was centrifuged for 10 min at 37,000 x g (SS-34 rotor) in a Sorvall RC2-B centrifuge refrigerated to 0°C. Two portions of ice-cold 95% ethanol were added dropwise to one portion of the supernatant. After this addition was completed, the solution was allowed to stand for 1 h at 0°C. The resultant stringy precipitate was rolled out on a glass rod and then was dissolved in a 50 ml portion of a 0.015 M sodium chloride-0.0015 M sodium citrate solution pH 7.0 by gentle shaking in a Precision Scientific Co. Dubnoff metabolic shaking incubator at 4°C overnight.

Step 4

The resultant opalescent solution was centrifuged at 160,000 x g (A-170 rotor) on an International preparative ultracentrifuge Model B-60 for 1 h to sediment any glycogen that had precipitated overnight. A solution composed of a 1.5 ml portion of a 0.1 M sodium chloride-

0.01 M sodium acetate solution pH 5.0 and an aliquot of a 2.7 M ammonium sulphate suspension of ribonuclease T_1 containing 12,000 units was added to the clear supernatant. This solution had previously been heated to 80°C for 10 min in order to remove any contaminating deoxyribonuclease activity. The enzyme digest was incubated at 37°C for 30 min.

Step 5

A 100 ml volume of phenol was extracted with three 100 ml portions of a 0.15 M sodium chloride-0.015 M sodium citrate solution pH 7.0 by means of a separatory funnel. A 50 ml portion of the extracted phenol was mixed with the ribonuclease T_1 digest solution. The emulsion was stirred for 20 min at room temperature in order to extract the protein of the enzyme into the phenol layer. The emulsion was broken by centrifugation at 37,000 x g (SS-34 rotor) in a Sorvall RC2-B centrifuge for 10 min. The process of phenol extraction on the aqueous layer was repeated a second time in like manner. The aqueous layer was then extracted in a separatory funnel with an equal volume of diethyl ether in order to remove any traces of phenol. Nitrogen gas was bubbled through the aqueous layer in order to drive off any traces of diethyl ether. The solution was then chilled to 0°C.

Step 6

A 2.0 g sample of solid sodium acetate was added to the solution in order to maintain any traces of glycogen in solution and to help promote DNA precipitation in the next addition. Two volumes of ice-cold 2-ethoxyethanol were added dropwise to one volume of the solution. After this addition was completed, the solution was allowed to set for 1 h at 0°C. The stringy precipitate was rolled out on a glass rod and then was mixed with a 5.0 ml portion of a 0.015 M sodium chloride-0.0015 M sodium citrate solution pH 7.0. The mixture was gently shaken overnight at 4°C as mentioned in Step 3.

Step 7

DNA was precipitated by the dropwise addition of an ice-cold 5.4 ml volume of isopropanol. After this addition was completed, the solution was allowed to set at 0°C for 1 h. The DNA fibres were then wound on a glass rod and washed consecutively with 10 ml portions of ice-cold: 75% ethanol-1% sodium chloride; 75% ethanol and 95% ethanol in order to remove any contaminating substances which would be adhering to the surface of the fibres. The fibres were placed on a watch glass _in vacuo_ over phosphorous pentoxide in order to dry.

Step 8

After drying, the fibres were weighed and the yield was calculated in terms of mg of DNA per gm of liver. Analysis was made as to the content of RNA and protein and the T_m value was measured as outlined in the Methods 3c).

3) Evaluation of DNA Purity

The types of DNA tested were calf thymus, human placenta, rat liver and salmon sperm.

a) RNA Content

A 10 unit aliquot of a DNA solution was made 0.3 N in sodium hydroxide. This solution was then heated for 2 h at 50°C. The optical density (O.D.) was then measured at 260 nm in a Beckman Acta MVI spectrophotometer on a 60µl sample of the incubated solution. The aliquot was added to a 1.5 ml portion of a 0.05 M sodium dihydrogen phosphate solution pH 5.0.

A 0.05 ml ice-cold portion of concentrated (70%) perchloric acid solution was added to each millilitre of incubated solution and DNA precipitation was allowed to proceed at 0°C for 1/2 h. The solution was then filtered through a Millipore filter (pore size 0.45µ). The filter was washed twice with 1.0 ml portions of a 0.5 N solution of ice-cold perchloric acid. The O.D. was then measured on the filtrate as mentioned before. A percentage of the RNA content was calculated by the following equation:

$$\%RNA = \frac{O.D._{260nm} \text{ (of the filtrate after perchloric acid treatment)}}{O.D._{260nm} \text{ (of the DNA solution before perchloric acid treatment)}} \times \text{dilution factor} \times 100$$

b) Protein Content

Protein content was determined by a method outlined by Sedmak and Grossberg (27). This method uses the Coomassie Brilliant Blue G250 dye and is specific for polypeptides and proteins of molecular weights greater than 3000. This method was chosen due to its sensitivity (<1.0 μg of albumin). The Lowry protein assay would not be suitable if the bases adenine, cytosine, guanine and/or thymine were present because the Folin reagent binds to these species especially to guanine (28). Although only polynucleotides were generated by DNase I digestion of the DNA samples, the possibility still existed that some nucleic acid bases could be present in solution. These species do not interfere with the Coomassie Blue technique.

Standard concentrations of bovine serum albumin (B.S.A.) were prepared using a 0.01 M sodium dihydrogen phosphate-0.01 M magnesium chloride buffer pH 6.0. A 1.5 ml aliquot of each of the concentrations of B.S.A. solution was mixed with a 1.5 ml portion of a 0.06% solution of the Coomassie Blue dye prepared in 3% perchloric acid. The blue colour was allowed to develop for 8 minutes. An absorbance reading was measured at 620 nm, the wavelength of maximum absorbance of the dye:protein complex. An absorbance reading was also measured at 465 nm, the wavelength of maximum absorbance of the leuko form of the dye. A ratio was calculated for each of the B.S.A. concentrations as follows:

$$\text{Ratio} = \frac{\text{absorbance at 620 nm}}{\text{absorbance at 465 nm}}$$

From this ratio was subtracted the following ratio:

$$\frac{\text{absorbance of the buffer blank + dye at 620 nm}}{\text{absorbance of the buffer blank + dye at 465 nm}} .$$

This subtraction was necessary as the buffer itself reacted with the dye. The corrected ratios and their corresponding protein concentrations were subjected to least squares analysis in order to determine constants to be used in the determination of any unknown protein concentrations.

A 50 unit aliquot of DNase I activity was added to each of the following samples and the volume was brought to 1.5 millilitres with the addition of buffer:

a) an empty test tube in order to determine the contribution of the enzyme to the protein assay.

b) an aliquot of the buffer solution containing a 7.5μg portion of B.S.A. protein in order to determine if the DNase I solution contained any proteolytic activity.

c) aliquots of the buffer solution containing 700μg portions of each of the DNA samples to be assayed.

A 7.5μg portion of B.S.A. protein was added to a 1.5 ml volume of buffer. This sample was compared with the sample containing B.S.A. protein and DNase, (b), in order to check for any proteolytic activity in the DNase I solution. A 700μg portion of commercial calf thymus DNA was brought to 1.5 millilitres with the addition of buffer in order to provide a sample of intact DNA with which the Coomassie Blue dye could react. The dye and DNA complex was expected to precipitate from solution (27). A 1.5 ml volume of the buffer was measured out as a buffer blank. The afore-mentioned

samples were allowed to stand for 2 h at room temperature to allow DNase digestion of DNA. After 2 h, all the solutions were assayed by the Coomassie Blue technique. The ratio for the buffer as well as for the DNase was subtracted from the appropriate samples. Using the corrected ratio and the standard protein data, it was possible to determine the amount of protein in each sample.

The following calculation was done to obtain the percent protein content in DNA:

$$\frac{\text{Protein Concentration } \mu g/ml \text{ (Least Squares Analysis)} \times 1.5 \text{ ml}}{700 \mu g \text{ DNA}} \times 100$$

c) T_m Measurement

The T_m values for the isolated rat liver DNA and the rat liver DNA methylated *in vivo* by DMNA were measured following a method outlined by Irving (8). A 0.6 unit aliquot of DNA was mixed into a 3 ml portion of a 0.015 M sodium chloride-0.0015 M sodium citrate solution pH 7.0. The temperature of the solution was increased at a rate of about 0.5°C/min. The optical density (O.D.) was noted at 254 nm at 1°C intervals until the temperature was 10°C away from the T_m value at which point the O.D. was noted at 0.5°C intervals. The data were plotted on a graph with the O.D. values on the ordinate axis and the temperature in°C on the abscissa axis.

The half-height of the curve was used to determine the T_m value. The low plateau O.D. value was subtracted from the high plateau O.D. value and the difference was divided by 2. The number obtained was then added to the low plateau O.D. value and the corresponding temperature, T_m value, was interpolated from the graph.

The T_m values for calf thymus, salmon sperm and human DNA were measured following the method of Marmur & Doty (29). The only difference in the method was the incubation medium. In this case, a 0.15 M sodium chloride-0.015 M sodium citrate solution pH 7.0 was used. The data were measured and plotted and calculations made as before.

4) Purification of Human Placenta DNA

The DNA was dissolved in a minimal quantity of 0.015 M sodium chloride-0.0015 M sodium citrate solution pH 7.0. The solution was then treated as described in the Methods 2), steps 4, 5 & 6 making allowances for the volume of solution used. Due to the low quantity of DNA, the stringy precipitate was not rolled out on a glass rod, but was filtered by suction on a Buchner funnel. The filter paper disc was soaked in a 10 ml portion of a 0.01 M magnesium chloride solution pH 6.0 overnight.

The filter paper was removed the next day and the solution was again filtered by suction to remove any paper fibres. The O.D. at 254 nm was measured on an aliquot of the solution and the units of DNA were calculated. The solution was tested for purity as described in the Methods 3) a, b & c.

5) Preparation for Chromatography

a) Sample Dissolution and Dialysis

A 4.0 mg sample of DNA was dissolved in a 4.0 ml portion of a 0.01 M magnesium chloride solution pH 6.0 overnight. The next day, the total number of units of DNA were calculated. The assumption was made that the only absorbing species present was intact DNA. If there were any bases, nucleotides, nucleosides and/or DNA fragments present, they would pass through the dialysis tubing in the next step and be noted as a decrease in O.D. at 254 nm.

The sodium ion interferes with any f.d.m.s. analysis of nucleosides (25) so that in preparation for future work, this problem had to be minimized. Dialysis of the DNA solutions versus a solution containing no sodium helps to reduce the amount of sodium present in the DNA sample. The DNA solutions were placed in dialysis tubing and each sample was dialyzed against a 1.0 L volume of a 0.01 M magnesium chloride solution pH 6.0 overnight.

Tris buffer also interferes with f.d.m.s. analysis of nucleosides (25). For this reason, a tris buffer solution was not employed as the digestion medium. The pH of the solution was maintained as described in the Methods 5b).

b) Enzyme Digestion

The method of Baird & Brookes (23) was followed with some modifications by Hall (30). This method hydrolyzes intact DNA to 2'-deoxyribonucleosides.

A 1600 unit sample of the endonuclease, deoxyribonuclease I (EC. 3.1.4.5) was added to the dialyzed solution and the digestion was continued for 1 h at 25°C. The pH was monitored on a Radiometer pH meter 26 and was maintained at pH 6.0 by the addition of dilute ammonium hydroxide solution.

Then, the pH was adjusted to 8.6 by the addition of dilute ammonium hydroxide solution. A 0.5 unit aliquot of the exonuclease, phosphodiesterase (EC. 3.1.4.1) and an 11 unit aliquot of a 2.5 M ammonium sulphate suspension of the hydrolase, alkaline phosphatase (EC.3.1.3.1) were added simultaneously and digestion was continued for 8 h at 37°C. The pH was monitored and maintained at 8.6 as described previously. The solution was then frozen.

The next day, a second aliquot of 0.4 ml of a 0.01M solution of magnesium chloride pH 8.6 was added. Then, a second 0.157 unit aliquot of phosphodiesterase and a 2.17 unit aliquot of alkaline phosphatase were added and digestion was continued for 8 more h at 37°C. The pH was monitored and maintained at 8.6 as described previously.

The digest was then centrifuged at 37,000 x g (SS-34 rotor) on a Sorvall RC2-B centrifuge for 10 min to sediment any protein that had precipitated. The O.D. was measured at 254 nm on an aliquot of the supernatant and the total units of deoxyribonucleosides present were calcultaed. This calculation makes the assumption that all the intact DNA has been completely digested to deoxyribonucleosides. This may not be the case if the DNA is not completely hydrolyzed.

6) Sephadex LH-20 Column Chromatography

The enzyme digests were then concentrated to a volume of about 1 to 3 ml by means of lyophilization or flash evaporation at 30°C. The O.D. was then measured at 254 nm on an aliquot of the concentrated solution and the total units of deoxyribonucleosides present were calculated.

A 35 g sample of the Sephadex LH-20 resin was washed several times with distilled deionized water. The resin was then washed 3 times with 30% methanol and allowed to swell overnight in 30% methanol. The excess methanol was drained off the next day and a thick slurry of the resin was slowly poured down the sides of a 90 x 1.5 cm Pharmacia column. The resin was allowed to settle and then a 1.0 L portion of 30% methanol was pumped through the column by means of a Pharmacia P-3 peristaltic pump at a rate of about 0.5 ml/min.

The concentrated enzyme digest was made 30% in methanol and was applied to the top of the resin bed and allowed to drain into the resin. The column was then filled with 30% methanol and connected to the pump. A convex exponential gradient of 30%-93% methanol was created by pumping from a flask containing a 500 ml portion of 100% methanol (limit buffer) to a mixing flask containing a 500 ml portion of 30% methanol. This method was used to replace the Baird & Brookes method (23) of using a glass siphon to transfer from the limit buffer vessel to the mixing buffer vessel. The gradient generated by the pump was essentially

linear in the elution region of the deoxyribonucleosides as calculated by the following equation (31):

$$C = C_0\left[1-\exp(-tR/V_0)\right] + 30\%$$

where: C = the concentration of the elution buffer at time t (% methanol)
t = time in min
C_0= the concentration of the limit buffer = 100% methanol
R = rate of flow into the mixing buffer = total flow rate out of the mixing buffer = 0.5 ml/min
V_0= the initial volume of the mixing buffer = 500 ml

The solution was then pumped from the mixing flask to the column at a rate of about 0.5 ml/min. Fractions of 4.5 ml volume were collected from the time of sample application by means of an ISCO Golden Retriever fraction collector. After 450-612 millilitres had been collected, the pump was shut off and the O.D. values of each fraction were measured at 254 nm on a Beckman Acta MVI spectrophotometer.

A 4.0 ml portion of the 0.01 M magnesium chloride solution pH 6.0 containing the same quantities of the three enzymes used in the Methods 5b) was flash evaporated to one millilitre, made 30% in methanol and applied to the column. This solution served as a blank to determine the contribution of the enzymes to the observed O.D. of the eluted deoxyribonucleosides. Any contribution of the enzyme blank was subtracted from the observed O.D. for the species eluted during the column chromatography of the enzyme digests of DNA. The total units of the eluted species from the chromatography were calculated from the corrected O.D.

values. The corrected O.D. values were plotted on the ordinate axis and the elution volume in millilitres from the column were plotted on the abscissa axis.

A 3.0 ml portion of the 0.01 M magnesium chloride solution pH 6.0 containing 54 units of intact commercial calf thymus DNA was made 30% in methanol and applied to the column. It was assumed that this sample would give some information regarding the elution of incompletely digested DNA from the column.

A 1.0 ml portion of a 0.01 M magnesium chloride solution pH 6.0 containing 20.2 units of intact yeast RNA was made 30% in methanol and applied to the column. It was assumed that this sample would give some information regarding the elution of any contaminating RNA from the column.

A 5.0 ml portion of the 0.01 M magnesium chloride solution pH 6.0 containing 0.1 mg of 5-methyl-2'-deoxycytidine was made 30% in methanol and applied to the column. It was assumed that this sample would give some information as to the resolution of the column for methylated dCyd in comparison to non-methylated dCyd.

The fractions containing the species eluted from the Sephadex LH-20 column chromatography of the digests of DNA were pooled according to those tubes which contained the maximum absorption at 254 nm. The volumes of each of the pooled fractions were divided into two parts. One part was subjected to Dowex 50W-X4 column chromatography. The second

part was studied only in the case of the digest of the isolated rat liver DNA for which spectral ratios were determined and thin layer chromatographs analyzed as to the identification of the eluted species. The second part of the other DNA samples was saved for future f.d.m.s. analysis provided that the Sephadex LH-20 column chromatography was found to be suitable.

A comparison of the elution profile of the digest of the calf thymus DNA methylated by MMS *in vitro* to the elution profile of the digest of commercial calf thymus DNA was made in order to determine the ability of the column to resolve these species. A comparison of the elution profiles of digests of isolated rat liver DNA and rat liver DNA methylated by ^{3}H-DMNA *in vivo* was made for a similar reason. Salmon sperm and human placenta DNA digests provided other sources of species derived from DNA which had not been reacted with carcinogens.

7) Identification of Species Eluted from Sephadex LH-20 Column Chromatography

a) Thin Layer Chromatography

This technique was used to identify the species eluted from the Sephadex LH-20 column chromatography of an enzyme digest of isolated rat liver DNA. The conclusions reached from this particular thin layer chromatography (T.L.C.) were extended so as to make some predictions regarding the nature of the species eluted from the Sephadex LH-20 column chromatography of the other DNA digests.

The plates used for the T.L.C. were commercially prepared by Macherey-Nagel & Co. and were 20 cm x 20 cm plastic sheets coated with absorbent to a uniform thickness of 0.1 mm. These sheets were placed in glass tanks (27.4 cm x 7.1 cm x 24.1 cm) in which the solvent atmosphere had been allowed to equilibrate for 24 h. This preliminary step eluted any impurities that may have been on the sheets. The specific thin layer systems used were:

1) DEAE-Cellulose plates run in butanol:ammonium hydroxide (4:1) (32).

2) DEAE-Cellulose plates run in butanol:formic acid: water (60:30:10) (33).

3) PEI-Cellulose plates run in 0.025 M lithium chloride (34).

These three systems represented three different pH ranges which were basic, acidic and neutral respectively. The plates were allowed to develop in an ascending manner until the solvent front had reached the top of the plate. The solvent was evaporated from the sheets, which had been removed from the tanks, by the gentle warm air of a hair

dryer. The sheets were then viewed under ultraviolet light of 260 nm in a darkened room in order to check for any impurities that still may have been left on the plates.

Most of the second part of the volume of the species eluted from the Sephadex LH-20 column chromatography of the digest of isolated rat liver DNA was concentrated to a volume of about 100μl by flash evaporation at 30°C. A capillary tube was used to spot each of these species as well as standard deoxyribonucleosides onto the thin layer sheets. The sheets were then allowed to develop as described previously until the solvent front had advanced 15 cm. The sheets were removed from the developing tank, dried and viewed under ultraviolet light. The spots were circled and the R_f values were determined for each of the species run on the chromatogram. The R_f values for the standard deoxyribonucleosides were compared with the R_f values of the eluted species and an identity was assigned to the eluted species. The volumes of the identified species eluted from the Sephadex LH-20 column chromatography of the digest of isolated rat liver DNA were compared to the volumes of the species eluted from the Sephadex LH-20 column chromatography of the digests of the other DNA samples. Some suggestions were then made regarding the nature of the unknown eluted species.

b) Spectral Ratios Determination

An aliquot of each of the pooled fractions obtained from the Sephadex LH-20 column chromatography of the digest of isolated rat liver DNA was taken for spectral ratios determination. Each of these samples was divided into three parts and each part was titrated to a basic, acidic or neutral pH. The optical density was measured at 230, 240, 250, 260, 270, 280 and 290 nm. A ratio was determined as follows: Spectral Ratio = $\frac{\text{O.D. (at a particular wavelength)}}{\text{O.D.}_{260\ nm}}$

The spectral ratios were also determined for solutions of standard deoxyribonucleosides in like manner. Spectral ratios were obtained from the Handbook of Biochemistry (35) for the standard deoxyribonucleosides also. The eluted species were identified by comparing their spectral ratios with those obtained for the standard deoxyribonucleosides (by measurement and from the Handbook). The possible identities of the species eluted from the Sephadex LH-20 column chromatography of the digests of the other DNA samples were assigned by comparing elution volumes to those obtained for the isolated rat liver DNA.

c) Dowex 50W-X4 Column Chromatography

The first part of the volumes of each of the species eluted from the Sephadex LH-20 column chromatography of the digests of each DNA sample was concentrated to a 1-3 ml volume by lyophilization or flash evaporation at 30°C.

A 40 gram sample of Dowex 50W-X4 400 mesh resin was washed several times with distilled deionized water. The resin was then washed 3 times with a 0.3 M ammonium formate solution pH 8.9 and allowed to stand overnight in this buffer. The excess ammonium formate was drained off the next day and a thick slurry of the resin was slowly poured down the sides of a 90 x 1.5 cm Pharmacia column. The resin was allowed to settle and then a 1.0 L portion of a 0.3 M ammonium formate solution pH 8.9 was pumped through the column as mentioned in the Methods 6) at a rate of about 0.5 ml/min.

Then, one of the concentrated species from the Sephadex LH-20 column chromatography was applied to the column and samples of 6.4 ml volume were eluted by means of pumping 0.3 M ammonium formate solution pH 8.9 through the column as mentioned in the Methods 6) at a rate of about 0.5 ml/min. The buffer was changed to 1.0 M ammonium formate solution pH 8.9 after 640 millilitres had been eluted from the column only for those samples which were obtained from the Sephadex LH-20 column chromatography of the enzyme digests of methylated DNA samples. Some of the methylated derivatives of deoxyribonucleosides are eluted by this buffer (36).

Aqueous solutions of dAdo, dCyd, dGuo and dThd were applied in turn to the Dowex column and the chromatography was carried out as before. This procedure yielded the elution volumes of each of the standard deoxyribonucleosides from the Dowex column. A 7.0 ml volume of water containing a total of 140μg of a mixture of the standard deoxyribonucleosides was applied to the Dowex column and the chromatography was carried out as before in order to determine the resolution of the Dowex column for a mixture of all four deoxyribonucleosides.

A comparison was made between the elution volumes from the Dowex 50W-X4 column of solutions of standard deoxyribonucleosides and the elution volumes from the Dowex 50W-X4 column of each of the species obtained from the Sephadex LH-20 column chromatography of the digests of the DNA samples. It was hoped that this comparison would give some information as to the possible identity of the species eluted from the Sephadex LH-20 column. This information would be supplementary to that obtained from the T.L.C. analysis and spectral ratios determinations.

Other samples run on the Dowex 50W-X4 column were:

1) A 30% methanol:water blank (in order to determine the background absorption coming off the Dowex column).

2) A solution containing the standard bases adenine, cytosine, guanine and thymine (in order to determine if the Dowex column could resolve these species from their corresponding deoxyribonucleosides).

3) A solution containing 5-methyl-2'-deoxycytidine by itself (in order to determine the elution volume of

this methylated derivative from the Dowex column).

4) A solution containing deoxyadenosine, 1-methyl-2'-deoxyadenosine, deoxycytidine and 5-methyl-2'-deoxycytidine (in order to determine the ability of the Dowex column to resolve methylated and non-methylated deoxyribonucleosides).

8) Liquid Scintillation Counting

a) Scintillation Cocktail Counting Efficiency

The scintillation cocktail, P.C.S., by Amersham/ Searle, was tested for efficiency in counting ^{3}H-inulin. This compound was used to check counting efficiency as no ^{3}H-labelled deoxyribonucleosides of known specific activity were available. It was assumed that the counting efficiency would be similar for ^{3}H-labelled deoxyribonucleosides. The inulin sample had a known specific activity of 5.58×10^{5} disintegrations per minute (dpm)/1.0 μl sample. A 1.0 μl sample of inulin was mixed with a 10.0 ml aliquot of the cocktail and was counted for 0.1 min in a Beckman LS 3150P liquid scintillation counter set to monitor the ^{3}H-label in counts per minute (cpm). The percent efficiency of the cocktail was equal to the (cpm/dpm) x 100.

b) Preparation for Chromatography

The only sample which was ^{3}H-labelled was the rat liver DNA which had been methylated by ^{3}H-DMNA *in vivo*. This sample was dialyzed as in the Methods 5a). The dialyzing medium was monitored before and after dialysis for any ^{3}H-label. If any small ^{3}H-labelled species were present in this sample of DNA, they would have passed through the dialysis tubing to the medium. This sample was digested and concentrated as in the Methods 5b).

c) Sephadex LH-20 Column Chromatography

The chromatography was carried out as in the Methods 6. The fractions were monitored for optical density

at 254 nm and for cpm. A 1.0 ml aliquot of each of the fractions was mixed with a 10.0 ml aliquot of the cocktail. The samples were then counted for 10.0 min as in the Methods 8a). The values in O.D.$_{254\ nm}$ and in cpm were plotted on the ordinate axis and the elution volume in millilitres from the column were plotted on the abscissa axis. The elution profile of the ^{3}H-labelled rat liver DNA digest from the Sephadex column as measured by O.D.$_{254\ nm}$ was compared to the elution profile as measured by cpm in order to determine the ability of the column to resolve the species which were derived from the site of reaction of the ^{3}H-DMNA on the rat liver DNA *in vivo* from those species which were derived from unreacted sites on the same DNA molecule.

d) Dowex 50W-X4 Column Chromatography

Half of the pooled fractions from the Sephadex LH-20 column chromatography of the digest of rat liver DNA methylated by ^{3}H-DMNA *in vivo* were subjected to Dowex 50W-X4 column chromatography as in the Methods 7c). A 1.0 ml aliquot of each of the Dowex fractions was mixed with a 10.0 ml aliquot of the cocktail and the samples were counted for 10.0 min as in the Methods 8a). The O.D.$_{254\ nm}$ was also measured for these fractions.

B. RESULTS

a) Isolation of DNA

DNA was isolated from rat liver tissue. It was first necessary to test the Coomassie Blue method (27) of determination of protein as to its applicability in assaying protein in the presence of DNA. These data are shown in Table I. DNase I, the enzyme used to break down the DNA to polynucleotide level, was thought to contain some protease activity. If protease activity were present, then measurement of protein would yield false low readings as Coomassie Blue dye can only detect polypeptides and proteins of molecular weight greater than three thousand (27). Comparison was made, as seen in Table I, between the sample containing B.S.A. and the sample containing B.S.A. of the same concentration incubated in the presence of DNase I. The percent difference between the two protein concentrations calculated from standard data was found to be 6% which is within limits of experimental error. It was, therefore, assumed that no protease activity was present in the sample of DNase I. The standard curve for protein yielded a correlation coefficient of 0.9985 and a y-intercept of 2×10^{-2} indicating that the data are valid for the calculation of unknown protein concentrations within the range of 1.0-20.0μg/ml.

Figure 4 shows the curve obtained in the measurement of the T_m of the isolated rat liver DNA. The T_m value as well as other characteristics of the isolated rat liver DNA are shown in Table II. Due to the small amount of

Table I. Protein Determination. The details of the measurement and calculation of the data shown in this table are explained in the Methods 3b).

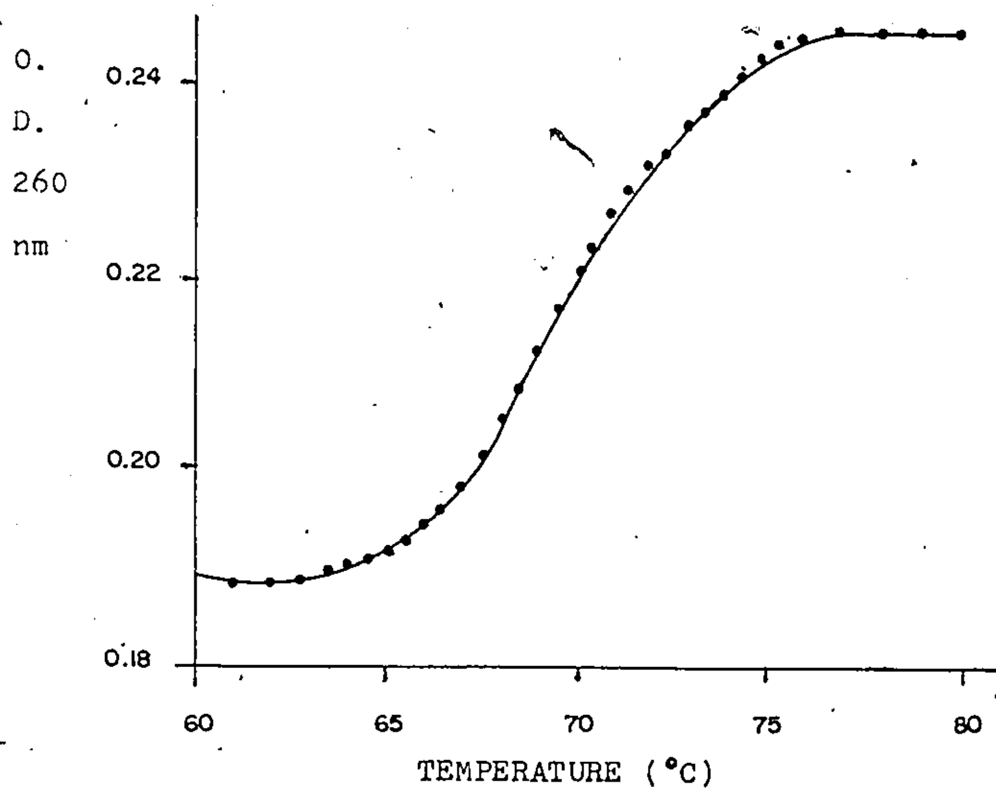
TABLE I

Protein Determination

Solution	Ratio $\frac{O.D._{620nm}}{O.D._{465nm}}$	Ratio Corrected (for buffer and DNase contribution)	Protein Concentration (μg/ml)
DNase Digest (Calf DNA)	0.599	0.020	0.000
DNase Digest (Human DNA)	0.574	0.000	0.000
DNase Digest (Salmon DNA)	0.666	0.087	0.957
DNase Digest (Rat DNA)	0.597	0.018	0.000
7.5μg B.S.A. + DNase	0.950	0.371	5.014
DNase	0.579	0.102	1.171
7.5μg B.S.A.	0.870	0.393	5.329
Calf DNA	DNA precipitated out	-	-
Buffer	0.477	0.000	0.000

Figure 4. T_m Determination for Rat Liver DNA. The details of this measurement and calculations are explained in the Methods 3b).

FIGURE 4



45

Table II. DNA Purity. The details of the measurement and calculations of the data shown in this table are explained in the Methods 2) step 8 and 3) a, b and c.

TABLE II

DNA Purity

Type of DNA	Yield	RNA %	Protein %	T_m (exp.)	T_m (lit.)*
Commercial Calf Thymus	-	0.0	0.000	-	-
Commercial Human Placenta	-	13.7 (before purif.)	-	-	-
	-	1.0 (after purif.)	0.000	-	-
Commercial Salmon Sperm	-	0.0	0.205	-	-
Isolated Rat Liver	1.0 mg DNA / gram liver	0.0	0.000	69.5°C ±0.5°C	69.7°C ±0.1°C
Rat Liver (DMNA treated)	-	19.6	-	-	-
Calf Thymus (MMS treated)	-	-	-	-	-

* see Irving's paper (8).

sample of methylated DNA, the following tests were not done:

i) protein and T_m analysis of both methylated rat liver and methylated calf thymus DNA.

ii) RNA analysis of the methylated calf thymus DNA.

T_m values were not measured for normal calf, human and salmon DNA samples due to mechanical difficulties with the circulating hot water bath.

b) Human Placenta DNA Purification

The human DNA sample was found to contain a high percentage of RNA contamination as is shown in Table II. This sample was purified as described in the Methods 4) and the RNA content was lowered as shown in Table II. The rat liver DNA methylated by DMNA *in vivo* was not purified due to the small amount of sample.

c) Preparation for Chromatography

The samples were dissolved and enzymatically digested as described in the Methods 5b). The total units before and after digestion are shown in Table III. The hyperchromic shift was observed as is expected when DNA is hydrolyzed by enzymes. The total units after digestion were not measured for the human placental DNA and the commercial calf thymus DNA. The volume of the resultant digest was concentrated except in the case of the calf thymus DNA methylated by MMS *in vitro*. This procedure was not necessary in this case because the volume was small after digestion and therefore suitable for subsequent Sephadex LH-20 column chromatography. The units of calf thymus DNA (MMS treated) were not measured prior to digestion.

Table III. Total Units of Absorption at 254 nm.

TABLE III

Total Units of Absorption at 254 nm

TYPE OF DNA	UNITS BEFORE DIGESTION	UNITS* AFTER DIGESTION	UNITS AFTER SEPHADEX LH-20 CHROMATOGRAPHY
Commercial Calf Thymus	73.2	-	102.4*
Commercial Salmon Sperm	45.6	66.3	66.8
Commercial Human Placenta	21.4	-	31.5*
Isolated Rat Liver	22.6	29.2	29.2
Rat Liver (DMNA treated)	17.4	19.4	19.1
Calf Thymus (MMS treated)	-	99.5	92.6

*These values are an indication of the hyperchromic shift which occurs when DNA undergoes hydrolysis.

d) Sephadex LH-20 Column Chromatography

The concentrated samples were then applied to a Sephadex LH-20 column. A 30% methanol:water blank alone did not elute any absorbing species from the column. The magnesium chloride buffer blank, to which the three enzymes used in the digestion of DNA were added, was adjusted to contain 30% methanol. This solution of enzymes contributed significantly to the elution profile as shown in Table IV. All statements regarding the total absorbance units at 254 nm have been corrected so as to exclude the enzyme contribution. Table III shows the total units recovered after chromatography.

Figure 5 shows the elution profile of enzyme digests of commercial calf thymus DNA and calf thymus DNA methylated by MMS _in vitro_. Figure 6 shows the elution profile of the enzyme digests of isolated rat liver DNA and rat liver DNA methylated by DMNA _in vivo_. Figure 7 shows the elution profile of an enzyme digest of rat liver DNA methylated by DMNA as measured by optical density measurements at 254 nm and by radioactive counting of the ^{3}H-label as cpm (^{3}H-DMNA was used to achieve methylation of the rat liver DNA).

Table IV compares the regions of elution from the Sephadex LH-20 column chromatography of the enzyme digests. Peaks occurred in several reproducible regions of elution volumes and were assigned Roman numerals as follows: Ia = 4.5 ml; Ib = 40.5-49.5 ml; Ic = 54.0-58.5 ml; Id = 63.0-81.0 ml; Ie = 90.0 ml; II = 108.0-117.0 ml; III = 126.0-139.5 ml; IV = 148.5-162.0 ml and V = 171.0-198.0 ml.

Figure 5. Sephadex LH-20 Column Chromatography of Enzyme Digests of: 1)Commercial Calf Thymus DNA •.........•
2)Calf Thymus DNA (MMS treated) •———•

FIGURE 5

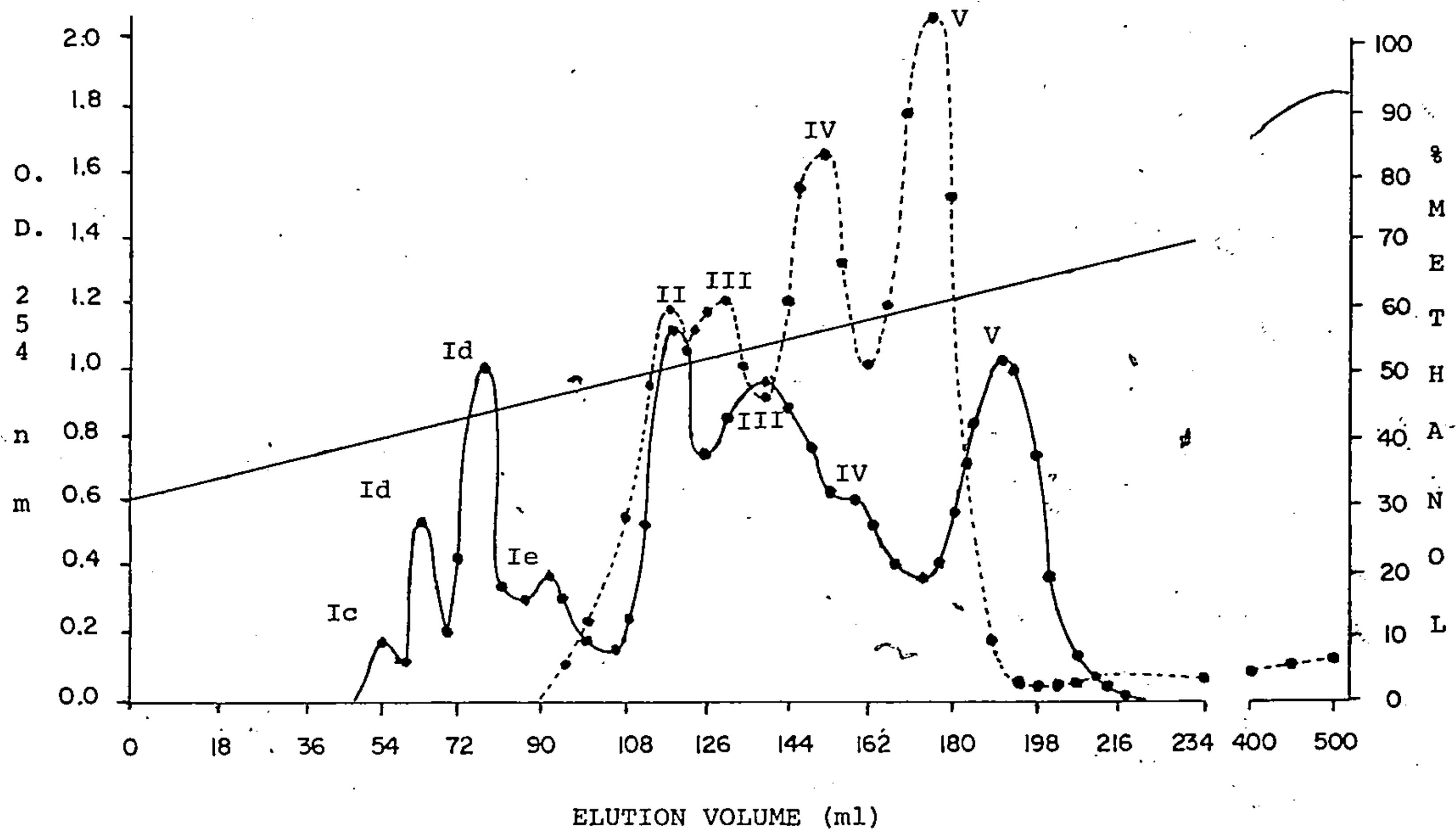


Figure 6. Sephadex LH-20 Column Chromatography of Enzyme Digests of: 1) Isolated Rat Liver DNA •-------•

2) Rat Liver DNA (DMNA treated) •——•

(O.D.$_{254}$ nm)

FIGURE 6

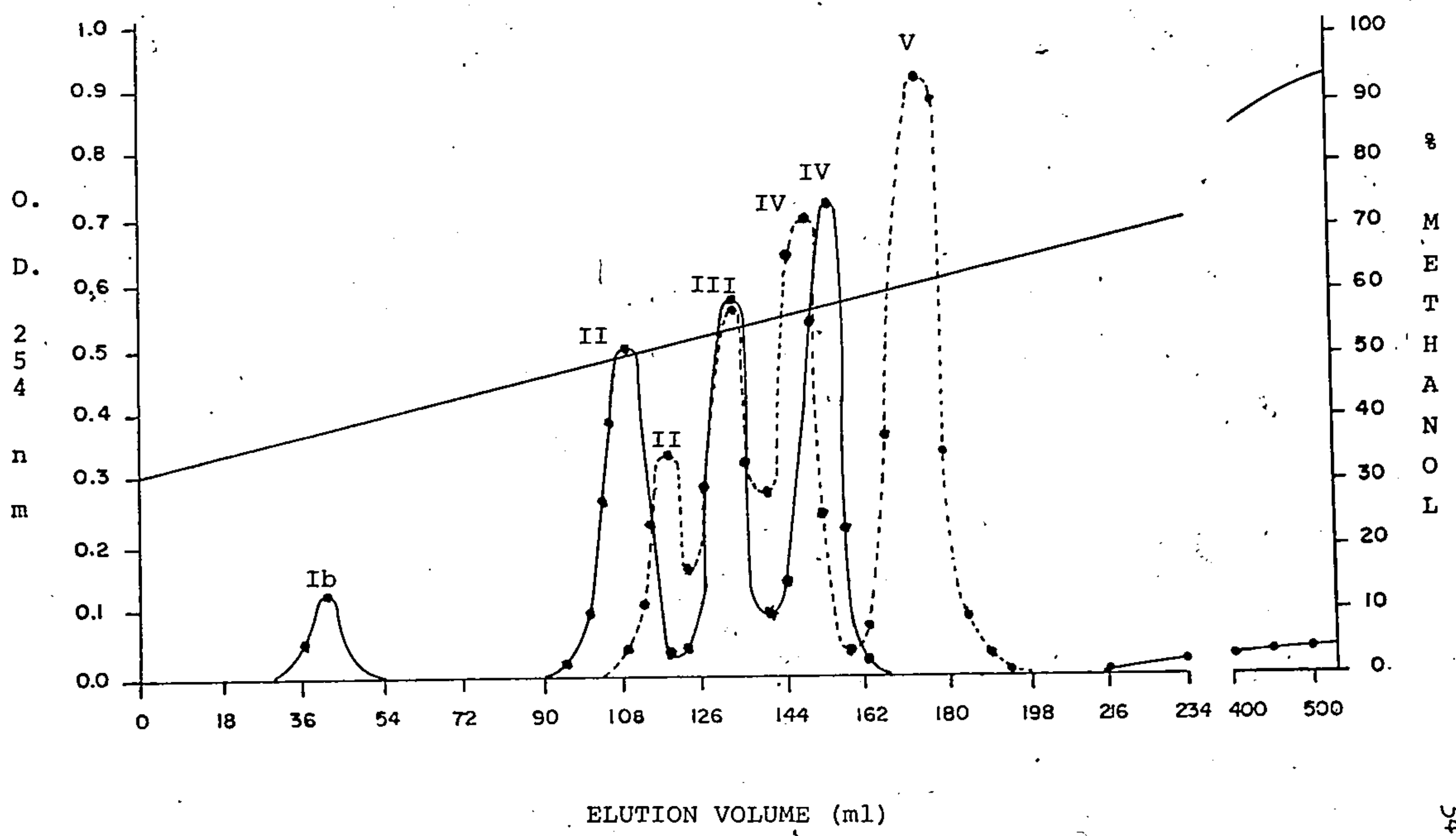


Figure 7. Sephadex LH-20 Column Chromatography of Enzyme Digests of: 1) Rat Liver DNA (DMNA treated) (O.D.$_{254\ nm}$) •———•

2) Rat Liver DNA (DMNA treated) (cpm) •-------•

FIGURE 7

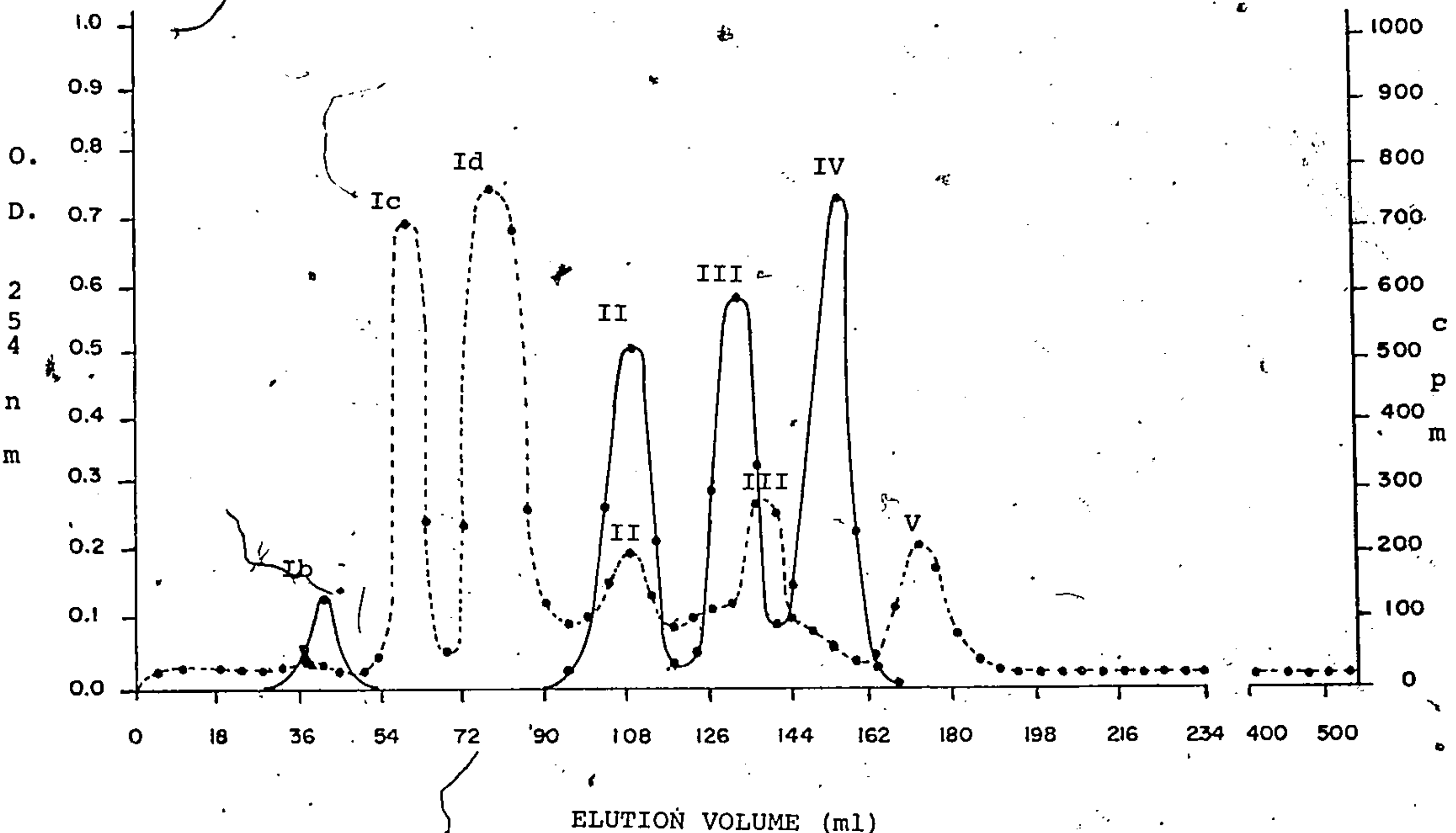


Table IV. Summary of Sephadex LH-20 Column Chromatography. The details of the measurement of the data shown in this table are explained in the Methods 6).

TABLE IV

SAMPLE	Elution Volume (ml) of Maximum Absorption (254 nm) / Total Units (254 nm)								
	I					II	III	IV	V
	a	b	c	d	e				
Enzyme-Magnesium Chloride Blank	4.5 / 0.3	49.5 / 2.5	58.5 / 2.5	72.0 / 5.3	90.0 / 1.2	Minimal contribution showing tailing as elution volume increases.			
Commercial Calf Thymus DNA Digest	- / -	- / -	- / -	- / -	- / -	117.0 / 14.7	13[illegible].5 / 29.6	153.0 / 20.3	175.5 / 37.8
Calf Thymus DNA (MMS treated) Digest	- / -	- / -	54.0 / 0.8	63.0 76.5 / 2.7 8.6	90.0 / 5.2	117.0 / 16.9	139.5 / 22.1	162 / 13.7	198 / 22.6
Commercial Calf Thymus Intact DNA	- / -	49.5 / 46.9	- / -	- / -	- / -	- / -	- / -	- / -	- / -
Commercial Yeast Intact RNA	- / -	45 / 6.0	- / -	58.5 / 15.4	- / -	- / -	- / -	162 / 0.1	180 / 0.1
Commercial 5-methyl-2'-deoxycytidine	- / -	- / -	- / -	- / -	- / -	117 / 5.5	- / -	- / -	- / -

TABLE IV CONT'D

SAMPLE	Elution Volume (ml) of Maximum Absorption (254 nm) / Units (254 nm) or Units (cpm)								
	I					II	III	IV	V
	a	b	c	d	e				
Rat Liver DNA (DMNA treated) Digest (O.D.254 nm)	- / -	40.5 / 0.6	- / -	- / -	- / -	108.0 / 5.0	130.5 / 6.6	153.0 / 6.9	- / -
Rat Liver DNA (DMNA treated) Digest (cpm)	- / -	- / -	58.5 / 4243	76.5 / 9241	- / -	108.0 / 2197	135 / 2488	- / -	171.0 / 2665
Isolated Rat Liver DNA Digest	- / -	- / -	- / -	- / -	- / -	117 / 3.1	130.5 / 6.5	148.5 / 7.4	171 / 12.2
Commercial Salmon DNA Digest	- / -	45 / 1.4	54 / 6.0	- / -	- / -	- / -	126 / 19.2	148.5 / 18	171 / 22.2
Commercial Human DNA Digest	- / -	- / -	54.0 / 0.5	- / -	- / -	117 / 4.2	126 / 5.4	148.5 / 8.0	171 / 13.4

Other data shown in Table IV are the elution volumes of commercial calf thymus intact DNA, commercial yeast intact RNA and 5-methyl-2'-deoxycytidine. The elution of calf thymus intact DNA was measured in order to determine the contribution of any DNA which had not been completely digested by enzymes. The elution volume of the intact yeast RNA was measured to determine the contribution of any contaminating RNA to the elution profile of deoxyribonucleosides. Commercially prepared 5-methyl-2'-deoxycytidine was used as a standard methylated deoxyribonucleoside. The elution volume of this compound from the column would indicate some information regarding the resolution of the column for methylated deoxyribonucleosides in relation to the non-methylated ones which arise from the digestion of methylated as well as normal DNA samples.

e) Identification of Peaks in the Elution Regions

i) Thin Layer Chromatography

Thin layer chromatograms were run for each of the peaks in each region from the Sephadex LH-20 column chromatography of an enzyme digest of the isolated rat liver DNA. The chromatography of standard deoxyribonucleosides with each unknown facilitated the identification in that the R_f values could be directly compared. The various R_f values are shown in Table V. Any material eluting from 4.5 ml to 90.0 ml was thought to contain the enzymes as is seen in Table IV and was not subjected to thin layer chromatography.

Table V. Thin Layer Chromatography.

The measurement and calculation of the data in this table are explained in the Methods 7a). Peaks were obtained from the Sephadex LH-20 column chromatography of a normal rat liver DNA digest.

TABLE V

Thin Layer Chromatography

System	Peak	R_f	R_f Values for Standards			
			dAdo	dCyd	dGuo	dThd
Butanol:Ammonium Hydroxide (4:1) DEAE-Cellulose (pH > 7.0)	II	.088	.176	.088	.061	.332
	III	.301	.161	.088	.068	.297
	IV	.062	.210	.122	.072	.404
	V	.327	.320	.192	.107	.453
Butanol:Formic Acid: Water (60:30:10) DEAE-Cellulose (pH < 7.0)	II	.114	.135	.111	.104*	.458
	III	.449	.139	.109	.115*	.446
	IV	.122*	.143	.116	.119*	.493
	V	.171	.158	.114	.113*	.503
0.025 Lithium Chloride PEI-Cellulose (pH = 7.0)	II	.753	.507	.753	.521	.809
	III	.797	.532	.823	.574	.805
	IV	.521	.517	.771	.536	.838
	V	.516	.488	.722	.487	.766

*These spots gave a special light purple fluorescence.

ii) Spectral Ratios Determination

The spectral ratios were determined for each of the peaks in each of the regions obtained from the Sephadex LH-20 column chromatography of an enzyme digest of isolated rat liver DNA. The data obtained are shown in Table VI along with spectral ratio data obtained from measurements done on solutions of standard deoxyribonucleosides and from the Handbook of Biochemistry (35). Any material eluting from 4.5 ml to 90.0 ml was thought to contain the enzymes as seen in Table IV and was not analyzed for spectral ratios.

iii) Dowex 50W-X4 Column Chromatography

Figure 8 shows the elution profile of standard deoxyribonucleosides from the Dowex 50W-X4 column superimposed on which is the elution profile of a 30% methanol: water blank. No data are shown for the elution volumes following the changeover from 0.3 M to 1.0 M ammonium formate buffer pH 8.9. No absorbing substances were eluted from the column following this changeover.

An attempt was made to decrease the peak seen between elution volumes 25.6 and 70.4 ml from the Dowex column. It was thought that this peak could be the result of some of the sample not having an opportunity to exchange on the resin and thereby being eluted from the column early in the chromatography. The sample was applied to a column whose resin had been equilibrated in a 0.03 M ammonium formate buffer pH 8.9. A 50.0 ml portion of this lower ionic strength buffer was pumped through the column and

Table VI. Spectral Ratios Data.

Measurement and calculation of the data in this table are explained in the Methods 7b).

$$\text{Spectral Ratio} = \frac{\text{O.D. (at a particular wavelength)}}{\text{O.D.}_{260\ \text{nm}}}$$

Regions were obtained from the Sephadex LH-20 column chromatography of a normal rat liver DNA digest.

TABLE VI

SPECIES	Spectral Ratios (pH < 7.0)						λ (nm)		Spectral Ratios (pH = 7.0)						λ (nm)		Spectral Ratios (pH > 7.0)						λ (nm)	
	230 nm	240 nm	250 nm	270 nm	280 nm	290 nm	MAX	MIN	230 nm	240 nm	250 nm	270 nm	280 nm	290 nm	MAX	MIN	230 nm	240 nm	250 nm	270 nm	280 nm	290 nm	MAX	MIN
Region II	1·17	·65	·67	1·53	1·81	1·38	280	244	1·29	1·05	·91	1·18	·97	·39	271	252	1·42	1·09	·92	1·18	·95	·38	271	250
dCyd (expt.)	·61	·42	·45	1·66	1·90	1·50	280	241	1·07	·95	·84	1·20	·96	·29	271	250	1·12	·96	·85	1·21	·94	·29	271	250
dCyd (Hand.)	–	·42	–	–	2·15	1·61	280	241	–	–	·83	–	·97	·31	271	250	–	–	·83	–	·97	·31	271	250
Region III	·43	·37	·66	1·10	·80	·32	268	235	·44	·41	·68	1·08	·75	·26	267	235	1·47	·88	·81	1·06	·72	·25	268	246
dThd (expt.)	·33	·32	·64	1·09	·75	·25	267	235	·32	·33	·65	1·06	·70	·21	267	235	1·18	·76	·74	1·05	·65	.16	267	246
dThd (hand.)	·33	·34	·65	1·06	·70	·22	267	235	·34	·33	·65	1·08	·74	·24	267	235	1·25	·79	·77	1·06	·68	.19	267	246
Region IV	·33	·56	·93	·78	·71	·50	256	229	·44	·78	1·10	·83	·68	·29	254	225	·62	·61	·88	·98	·65	·15	260	234
dGuo (expt.)	·27	·60	1·0	·76	·70	·48	255	227	·38	·81	1·14	·83	·68	·27	254	223	·43	·56	·89	·98	·62	·11	260	230
dGuo (Hand.)	·26	·60	1·0	·84	·69	·47	255	232	·38	·81	1·16	·75	·68	·27	254	223	·40	·55	·87	·98	·61	·09	260	230

TABLE VI CONT'D

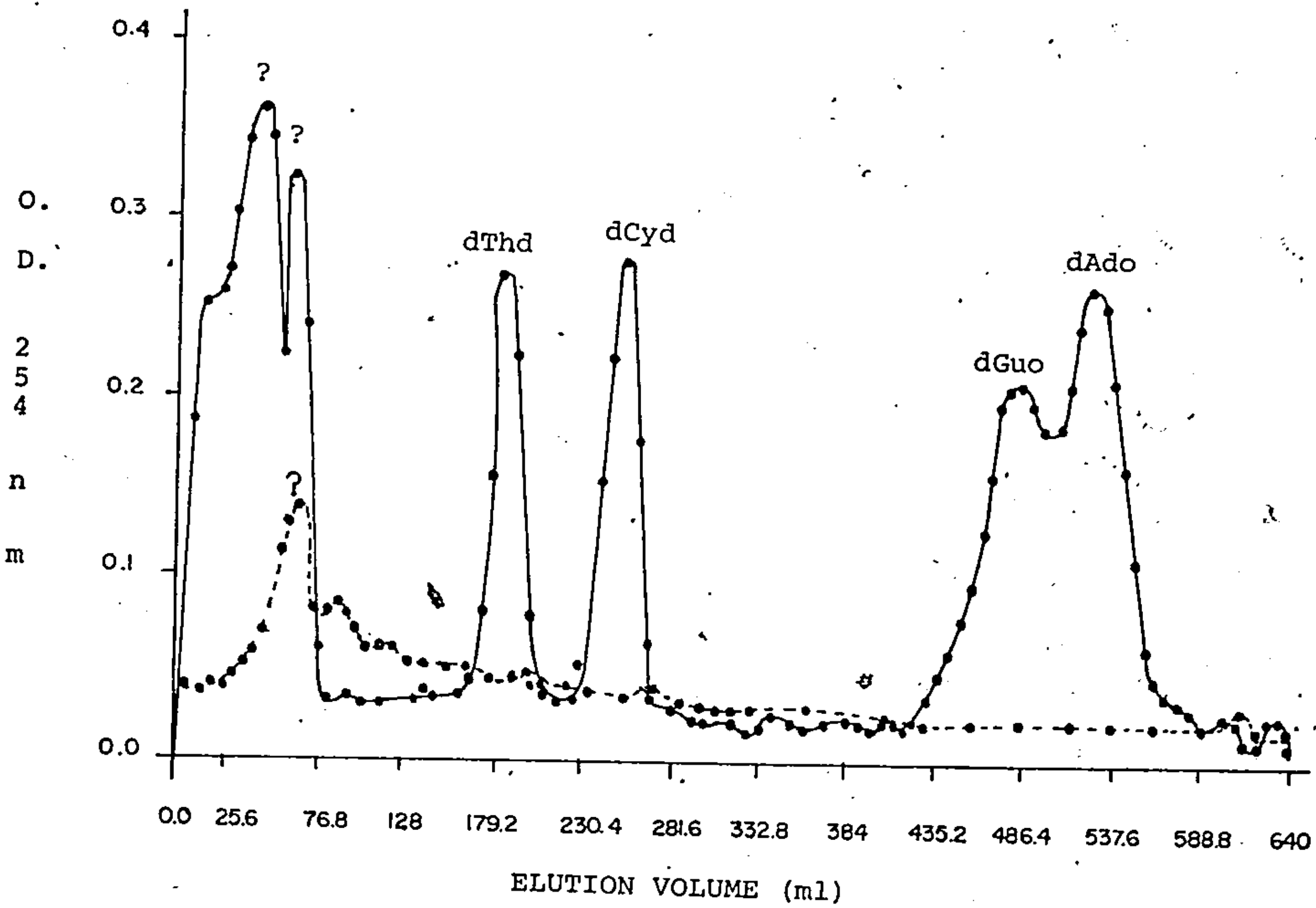
SPECIES	Spectral Ratios (pH<7.0)						λ (nm)		Spectral Ratios (pH = 7.0)						λ (nm)		Spectral Ratios (pH>7.0)						λ (nm)	
	230 nm	240 nm	250 nm	270 nm	280 nm	290 nm	MAX	MIN	230 nm	240 nm	250 nm	270 nm	280 nm	290 nm	MAX	MIN	230 nm	240 nm	250 nm	270 nm	280 nm	290 nm	MAX	MIN
Region V	.29	.45	.82	.74	.28	.08	258	230	.22	.40	.77	.72	.21	.03	260	226	.55	.55	.81	.73	.24	.07	260	236
dAdo (exp't)	.23	.48	.88	.79	.26	.04	258	230	.20	.41	.78	.70	.16	.01	260	226	.30	.43	.80	.69	.18	.04	260	234
dAdo (Hand.)	-	-	.83	-	.24	-	258	228	-	-	.79	-	.15	<.01	260	225	-	-	-	-	-	-	261	-

Figure 8. Dowex 50W-X4 Column Chromatography of:

1) 30% Methanol:Water Blank ●··········●

2) Standard Deoxyribonucleosides ●————●

FIGURE 8



the effluent was monitored at 254 nm. The buffer was then changed to a 0.3 M ammonium formate buffer pH 8.9 and the chromatography was continued using this higher ionic strength buffer. This technique did not reduce the size of the previously mentioned peak and was not used in subsequent chromatography. This unknown peak is not mentioned in Table IV.

The peaks eluted from the Dowex 50W-X4 column chromatography of unknowns were compared with the elution volumes of standard deoxyribonucleosides. The validity of this extrapolation is described in the Discussion. A summary of the Dowex 50W-X4 column chromatography is given in Table VII.

Table VII also gives the elution volumes of the commercial bases adenine, cytosine, guanine and thymine. These bases were studied in order to determine if the Dowex column was able to distinguish between the deoxyribonucleosides and their corresponding bases. These bases could be present in applied samples if the deoxyribonucleosides have been broken down somehow during enzyme hydrolysis. The elution volumes of 5-methyl-2'-deoxycytidine alone and 5-methyl-2'-deoxycytidine run with 2'-deoxycytidine, 2'-deoxyadenosine and 1-methyl-2'-deoxyadenosine as a group are also shown in Table VII. The methylated and non-methylated deoxyribonucleoside elution profiles were studied in order to determine if the Dowex column was able to distinguish between the two species.

Table VII. Summary of Dowex 50W-X4 Column Chromatography

Measurement of the data in this table is explained in the Methods 6c).

TABLE VII

Summary of Dowex 50W-X4 Column Chromatography

Sample	Peak	Elution Vol (Sephadex)	Elution Vol (Dowex)	Nucleo-side	Elution Vol (Dowex)
Commercial Calf Thymus DNA Digest	II	117.0	236.8	dCyd	256.0
	III	130.5	172.8	dThd	185.6
	IV	153.0	435.2	dGuo	467.2
	V	175.5	512.0	dAdo	524.8
Calf Thymus DNA(MMS treated) Digest	-	-	-	-	-
Rat Liver DNA(DMNA treated) (O.D. 254 nm) Digest	II	108.0	179.2	dThd	185.6
	III	130.5	467.2	dGuo	467.2
	IV	153.0	492.8 512.0	dGuo dAdo	467.2 524.8
Rat Liver DNA(DMNA treated) (cpm) Digest	-	-	-	-	-
Isolated Rat Liver DNA Digest	II	117.0	256.0	dCyd	256.0
	III	130.5	192.0	dThd	185.6
	IV	148.5	473.6	dGuo	467.2
	V	171.0	499.2	dAdo	524.8

TABLE VII

Summary of Dowex 50W-X4 Column Chromatography Cont'd

Sample	Peak	Elution Vol (Sephadex)	Elution Vol (Dowex)	Nucleoside	Elution Vol (Dowex)
Commercial Salmon Sperm DNA Digest	III	126.0	185.6 256.0	dThd dCyd	185.6 256.0
	IV	148.5	467.2	dGuo	467.2
	V	171.0	537.6	dAdo	524.8
Commercial Human DNA Digest	II	117.0	243.2	dCyd	256.0
	III	126.0	147.2	dThd	185.6
	IV	148.5	467.2	dGuo	467.2
	V	171.0	537.6	dAdo	524.8
Commercial Bases (Adenine Cytosine Guanine Thymine)	-	-	230.4	dThd	185.6
	-	-	256.0	dCyd	256.0
	-	-	480.0	dGuo dAdo	467.2 524.8
dCyd & 5-CH_3dCyd & dAdo & 1-CH_3dAdo	-	-	224.0	dCyd 5-CH_3 dCyd	256.0 217.6
	-	-	480.0	dAdo 1-CH_3 dAdo	524.8 -
30% Methanol: Water	-	-	70.4	-	?

Dowex 50W-X4 column chromatography was not applied to the fractions from the Sephadex LH-20 column chromatography of the enzyme digest of calf thymus DNA methylated by MMS *in vitro*. The samples from the Sephadex LH -20 column chromatography of the enzyme digest of the rat liver DNA methylated by DMNA *in vivo* were subjected to Dowex chromatography. The data obtained from this chromatography are shown in Table VII.

f) Liquid Scintillation Counting

The percent efficiency of the scintillation cocktail in counting the ^3H-label, using ^3H-inulin as a standard, was found to be 50.7% for a 1.0 μl sample. No quench correction was made to account for the fact that the 1.0 ml samples of chromatographic fractions taken existed in methanol:water mixtures of varying proportions. No change was observed in the total counts per minute in the dialysing medium before and after dialysis which indicates that no small fragments were present in the intact sample. Figure 7 shows the elution profile from the Sephadex LH-20 column of the digest of the afore-mentioned DNA sample (as measured by $O.D._{254\ nm}$ and by cpm). The data are summarized in Table IV. The fractions from the Sephadex chromatography of the afore-mentioned DNA digest which showed high cpm values were not subjected to Dowex 50W-X4 column chromatography. In this case, only the fractions showing the maximum O.D. at 254 nm were subjected to Dowex chromatography. The data (as measured by $O.D._{254\ nm}$) are shown in Table VII.

CHAPTER III

DISCUSSION

This chapter will discuss the chromatography.

The isolation of DNA from rat liver was necessary in order to provide a non-methylated source of rat liver DNA to which the chromatography of the rat liver DNA methylated by ^{3}H-DMNA *in vivo* could be compared. Table II shows that the yield was 1.0 mg DNA/1.0 gram liver which is the expected yield as stated by Irving (8). There was no detectable RNA or protein contamination as seen in Table II so that the isolated rat liver DNA digest was suitable for chromatographic study. Table II shows the T_m value to be 69.5°C ±0.5°C which is within the range of the expected T_m found by Irving (8). The purity of the other DNA samples is shown in Table II. There was negligible protein and RNA except for the case of the rat liver DNA (DMNA treated) which had RNA contamination. Had there been a sufficient amount of sample, purification would have been carried out on this sample. It may be possible that the calf thymus DNA (MMS treated) was also contaminated with RNA. This point will be discussed later in relationship to chromatography.

The digested DNA samples were subjected to Sephadex LH-20 column chromatography using Baird & Brookes system (23). Figures 5-7 show the elution profile of the

methylated and non-methylated DNA digests that are of importance. The necessary data from these figures are summarized in Table IV. The total units recovered from the Sephadex LH-20 column chromatography show over 93% yield (Table III) which means that the material is being completely eluted from the column.

Each of the regions eluted from the Sephadex LH-20 column chromatography of the isolated rat liver DNA digest was subjected to thin layer chromatography. Examination of the data in Table V for the three T.L.C. systems, yielded a means by which an identity could be given to the species present in each region. Complementary to the T.L.C. analysis, was the spectral ratios analysis of the same species as seen in Table VI. There seems to be some discrepancy between the standard deoxyribonucleosides and the unknowns in the spectral ratios around 230 nm. This discrepancy is thought to be due to the tailing from the column of enzymes used in the digestion of the DNA (Table IV). Proteins have a higher extinction co-efficient than deoxyribonucleosides at 230 nm and therefore, the spectral ratios show a high positive reading. The discrepancy in the spectral ratios around 230 nm decreases as the regions are eluted from the column because the concentration of enzymes decreases as each region is eluted. The spectral ratios otherwise give positive evidence as to the identity of the species present in each region.

The Dowex 50W-X4 column chromatography (23) seemed to yield supporting evidence to the identification of the

regions eluted from the Sephadex LH-20 column chromatography (Table VII). The Dowex column chromatography on its own is not a good tool for identification for the following reasons: 1) An unidentifiable substance is always eluted from the column between 25.6 and 70.4 ml.

2) The column does not always resolve dGuo from dAdo (Table VII).

3) The elution volumes of a particular deoxyribonucleoside are not very reproducible (Table VII).

4) The column does not resolve deoxyribonucleosides from their corresponding bases (Table VII).

5) The column does not resolve deoxyribonucleosides from some of their methylated derivatives (Table VII) & (26).

Regions obtained from the Sephadex LH-20 column chromatography of the enzyme digest of isolated rat liver DNA were identified by T.L.C. (Table V), spectral ratios (Table VI) and Dowex 50W-X4 column chromatography (Table VII) as being: 1) Region II (elution volume = 117.0 ml) as dCyd

2) Region III (elution volume = 130.5 ml) as dThd

3) Region IV (elution volume = 148.5 ml) as dGuo

4) Region V (elution volume = 171.0 ml) as dAdo.

These data are in accordance with that found by Baird & Brookes (23) and Brookes, Jones and Amos (24).

In region I of the Sephadex LH-20 column chromatography, Baird & Brookes observed a peak which was due to the Tris buffer salt elution of small amounts of normal deoxyribonucleosides (23,24). Tris buffer was not used as the incubation solution during enzyme digestion for reasons discussed in the Methods 5a) and therefore, any peaks in region I of this work should not be compared to those

of Baird & Brookes.

According to Table IV, two species were present in region Id for the digest of calf thymus DNA (MMS treated). The species in the first peak (63.0 ml) could be intact RNA contamination as indicated by comparison with the peak seen in Id for commercial intact yeast RNA. The species in the second peak (76.5 ml) could be due to an excess amount of enzyme being present in the digest over the amount used in the blank because peak Id is seen in Table IV for the enzyme blank. All the values in Table IV have had the total units contributed by the enzymes deducted from the total units observed. The species eluted in Ic and Ie could also be due to the presence of excess enzymes.

The commercial calf thymus DNA digest did not show any peaks in regions Ia-e, indicating negligible RNA contamination and the possibility of complete digestion as no peak was seen in region Ib which could correspond to intact DNA. These ideas are supported by the evidence seen in Table II and the hyperchromic shift seen in Table III.

The isolated rat liver DNA digest did not show any peaks in regions Ia-e, indicating negligible RNA contamination and complete digestion as explained in the previous paragraph.

The rat liver DNA (DMNA treated) had a peak in region Ib as measured by O.D.$_{254\ nm}$. This would indicate the presence of intact DNA and therefore incomplete digestion which is in accordance with the data in Table III. This peak could also indicate RNA contamination which is in accordance with data in Table II. There were also peaks in regions Ic and Id as measured by cpm. The majority of the ^{3}H-label was in these peaks as seen in Table IV. It is also assumed that these peaks arise from RNA contamination which may also have been methylated by ^{3}H-DMNA *in vivo*. The amount of RNA that is labelled with ^{3}H is small, however, as no peaks appeared in these regions as measured by O.D.$_{254\ nm}$.

Human DNA and salmon DNA seem to have negligible RNA contamination (Table II) and seem to have undergone complete hydrolysis (Table III). Therefore, the peaks seen in region Ib and Ic for salmon DNA digest and region Ic for human DNA digest are probably due to the presence of an excess of enzymes in the digest over that used in the blank. This idea has been previously discussed in the case of the digest of calf thymus DNA (MMS treated).

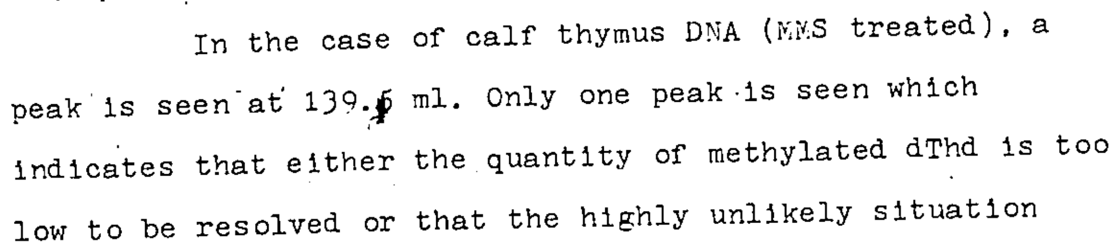
The peak at 117.0 ml in region II has been identified as dCyd for rat liver DNA.This peak is seen in all the normal DNA digests but salmon sperm DNA.Data from Table VII seems to support the idea that dCyd was co-eluted with dThd in region III in this particular run.

5-methyl-2'-deoxycytidine is also eluted at 117.0 ml in region II (Table IV).It seems that dCyd and its methylated derivative are not resolved on this column. In the case of

calf thymus DNA (MMS treated), it is therefore difficult to conclude whether or not methylation has occurred on the cytosine moiety. No methylation is thought to occur on the cytosine moiety by MMS in the cited literature (20,22). It is therefore highly unlikely that methylated dCyd is present in this peak.

Only one peak was eluted at 108.8 ml in region II for rat liver DNA (DMNA treated) as measured by O.D.$_{254}$ nm and by cpm. No methylation is thought to occur on cytosine by DMNA as cited in the literature (19). The presence of a total of 5.0 units (O.D.$_{254}$ nm) of material with 2,107 total counts per minute in this peak indicates that cytosine may have been methylated.

The peak seen at 130.5 ml in region III has been identified as dThd for the normal rat liver DNA. This peak (±4.5 ml) is seen for all the normal DNA digests. In the case of rat liver DNA (DMNA treated), a peak is seen at 130.5 ml as measured by O.D.$_{254}$ nm and is thought to be normal dThd. The presence of 2,488 total counts per minute at 135.0 ml indicates that thymine may have been methylated by DMNA. The literature (19) states that methylation is thought to occur on thymine. It is therefore likely that this peak does indeed contain methylated dThd.



In the case of calf thymus DNA (MMS treated), a peak is seen at 139.5 ml. Only one peak is seen which indicates that either the quantity of methylated dThd is too low to be resolved or that the highly unlikely situation

exists in which all the thymine residues have been methylated. Since methylation is thought not to occur on thymine by MMS as cited in the literature (20,22), it is more probable that this peak contains normal dThd.

The peak at 148.5 ml in region IV has been identified as dGuo for normal rat liver DNA. This peak (±4.5 ml) is seen for all the normal DNA digests. Only one peak was seen at 162.0 ml for calf thymus DNA (MMS treated) which indicates that the quantity of methylated dGuo is too low to be resolved or that the highly unlikely situation exists in which all the guanine residues have been methylated. Since MMS is thought to methylate guanine from the cited literature (20,22), this peak probably does contain some methylated dGuo. Some minor contribution from RNA contamination shows at 162.0 ml also.

The peak at 153.0 ml in region IV for rat liver DNA (DMNA treated) is thought to contain dGuo and dAdo as no peak was seen in region V as measured by $O.D._{254\ nm}$ and from evidence of resolution of this peak on the Dowex column as seen in Table VII. These two compounds may have co-eluted from the column. No ^3H-label was associated with region IV which indicates that no methylation of the guanine moiety seems to have occurred. The cited literature (19) states that methylation is thought to occur on guanine. Perhaps the peak in region V as measured by cpm is methylated dGuo which has been resolved from normal dGuo but not from normal dAdo.

The peak at 171.0 ml in region V has been identified as dAdo for normal rat liver DNA. This peak (±4.5 ml) showed in all the normal DNA digests. Only one peak was seen at 198.0 ml in the case of calf thymus DNA (MMS treated) which indicates that either the quantity of methylated dAdo is too low to be resolved or that the highly unlikely situation exists in which all the adenine residues have been methylated. The literature cited (20,22) states that MMS is thought to methylate adenine. It seems possible then that some methylated dAdo is present in this peak.

The peak seen at 171.0 ml in region V did not show for rat liver DNA (DMNA treated) as measured by O.D. 254 nm. This peak did contain 2,665 total cpm units of the ^{3}H-label which indicates that DMNA has methylated some species which is eluted in this region. A question is raised as to whether this compound is methylated dAdo or methylated dGuo or perhaps this peak is due to the presence of contaminating RNA which reacted with ^{3}H-DMNA in vivo. RNA shows some region V contribution (Table IV).

Resolution of the methylated from the non-methylated deoxyribonucleosides would be a helpful preliminary technique to f.d.m.s. analysis. In the case of the calf thymus DNA (MMS treated), this resolution was not detected. In the case of the rat liver DNA (DMNA treated), there was thought to be some methylated dCyd present but the column was not able to resolve methylated dCyd from normal dCyd. There was some minor resolution of a species

thought to be methylated dThd from normal dThd for the above-mentioned sample. The peak resolved at 171.0 ml for the above-mentioned sample may be composed of one or more different species. This inability of the column to resolve the methylated species is unfortunate. Some data from the Sephadex chromatography seem to indicate reaction of the methylating agents with bases in DNA which are not thought to be likely candidates for reaction (19, 20, 22). It might be possible to make adjustments to the Sephadex LH-20 column chromatographic system in order to improve the resolution. One suggestion would be to use a shallower linear methanol:water gradient.

In summary, it seems as if the Sephadex LH-20 column chromatographic system was not suitable for the resolution of methylated deoxyribonucleosides from their normal non-methylated counterparts. Even so, the non-resolved methylated and non-methylated species could be identified by f.d.m.s. analysis if there were a sufficient amount of each species present and if an assignable molecular ion were formed during f.d.m.s. analysis which would indicate the presence of some methylated species.

CHAPTER IV

SUMMARY

It has been shown that the Sephadex LH-20 column chromatographic system was not suitable for the resolution of methylated deoxyribonucleosides from their normal non-methylated counterparts. This conclusion was based on the chromatographic studies performed on the digests of: 1) rat liver DNA methylated by DMNA *in vivo* and 2) calf thymus DNA methylated by MMS *in vitro*. The suggestion was made that a shallower linear methanol:water gradient be used to improve the resolution of the Sephadex column for the above-mentioned species. Thin layer chromatography and spectral ratios determinations were used to identify peaks eluted from the Sephadex column. Dowex 50W-X4 column chromatography was found not to be a valid means of identification. Even though the methylated and non-methylated deoxyribonucleosides were not resolved on the Sephadex column, it still could be possible to identify the species by f.d.m.s. analysis. A sufficient amount of the sample must be present and an assignable molecular ion must be formed during the f.d.m.s. analysis which would indicate the presence of some methylated species.

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