THE EFFECT OF DIAZOMETHANE ON TOBACCO MOSAIC VIRUS RIBONUCLEIC ACID

by

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ABSTRACT

Previous work had shown that treatment of RNA with diazomethane under aqueous conditions resulted in chain breakage as well as methylation. It was postulated that chain breakage would be averted if the reaction was carried out under non-aqueous conditions. The effect of diazomethane on chain breakage was studied using ribosomal RNA as a model on which studies with TMV-RNA were based. It was found, using an essentially non-aqueous system, with both types of RNA the maximum diazomethane concentration that would yield intact chains was 0.006M. Although the extent of methylation has not been determined, data from independent studies indicates that concentrations found to yield intact chains are sufficient to result in significant methylation of the nucleotides, thereby allowing this system to be useful for altering nucleotide chains in an effort to establish nucleotide sequences.

INTRODUCTION

This thesis constitutes a report on a study of the reaction of diazomethane with ribonucleic acid in a non-aqueous medium. The purpose of this investigation was to determine if it is possible to methylate the purine and pyrimidine bases of intact ribonucleic acid without causing chain breakage. Methylated purines and pyrimidines have been isolated from transfer ribonucleic acids (Dunn and Smith 1955, Littlefield and Dunn 1958, and Adler, Weissman, and Gutman 1958). Subsequently, methylated bases have been studied in an effort to: (1) correlate mutagenesis with methylation, (2) study transfer ribonucleic acid (RNA) which is known to contain methylated bases, and (3) alter nucleotide bases in nucleic acids in an effort to selectively inhibit the action of specific nucleases and thereby provide a useful tool for studying nucleotide sequences (Lawley 1961, Brimacombe *et al.* 1965, and Holley *et al.* 1965).

Mutagenesis has been studied using ethyl methanesulfonate (Loveless 1959) and methyl methanesulfonate (Fahmy and Fahmy 1961 and Ludlum, Warner, and Wahba 1964) as alkylating agents. Studies on transfer RNA have involved investigation of enzymatic methylation (Fleissner and Borek 1963 and Borek 1963). It has been shown that purine and

pyrimidine bases are methylated after they are formed into polynucleotides and that the methyl donor is S-adenosyl methinonine (Mandel and Borek 1961). It is interesting that S-adenosyl methinonine and diazomethane are similar inasmuch as both compounds are capable of acting as nucleophilic or electrophilic species depending on the specific situation. Studies of diazomethane under conditions where it acts as a nucleophile or an electrophile should result in a better understanding of enzymatic methylation.

Holley was able to determine the nucleotide sequence of alanine transfer RNA because of the altered enzymatic specificity that the anomalous bases confer on this particular nucleic acid molecule (Holley et al. 1965). Two of the key anomalous bases were 1-methyl guanosine and 1-methyl inosine. It is not possible to extend this work to most other types of nucleic acid molecules because they do not contain these anomalous bases. Therefore the development of a system which could selectively methylate the bases is highly desirable.

Various alkylating agents have been studied for their effects on nucleic acids or components of nucleic acids. Lawley and Brooks (1963) reacted methyl methanesulfonate with ribosylpolynucleotides and found that the N-7 position of guanine is the most reactive site towards this reagent. Brookes and Lawley (1962) also report that

dimethyl sulfate will methylate cytidine in the N-3 position and cytosine in the N-3 and N-1 positions. One could question the authenticity of the methylation at N-1 because dimethyl sulfate is an electrophilic alkylating agent and therefore would not be expected to methylate at the N-1 position of cytosine. Broom et al. (1964) reported the methylation of guanosine in both the N-1 and N-7 positions using methyl iodide as the alkylating agent. Evidence for these methylated products was based on R_f values and ultraviolet absorption spectra. This paper is subject to question because methyl iodide is an electrophile and would be expected to methylate only at N-7.

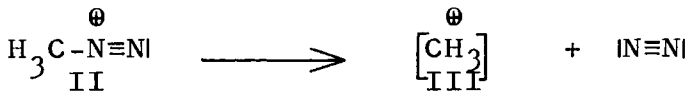
Haines has reported (Haines, Reese, and Todd 1962) the formation of 7-methyl guanosine when guanosine was treated with diazomethane under aqueous conditions. Further investigation of the reaction of diazomethane (Haines, Reese, and Todd 1964) revealed that the nucleosides reacted in the following order in aqueous solutions: uridine and guanosine >> cytidine > adenosine. Kriek and Emmelot (1963) have reported that diazomethane reacts with guanine at both N-1 and N-7 in aqueous media but only at N-1 in non-aqueous media. It was also observed that breakdown of RNA chains occurred after treatment of the RNA with diazomethane under aqueous conditions. This observation was of interest because in order to study the effect of methylation on mutagenesis, transfer RNA, and altered

enzyme specificity, it is desirable that the nucleic acid molecule remain intact.

A consideration of the mechanisms of methylation and chain breakage of RNA using diazomethane under aqueous conditions suggested a method for methylating RNA without extensive breakdown of the chains. Diazomethane is a resonance hybrid and may be written as Ia and Ib (Huisgen 1955).

Ia Ib

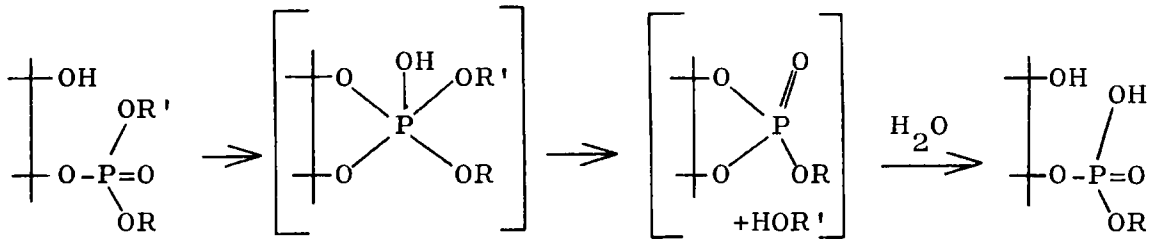
In an aqueous solution diazomethane may add a proton to yield the very unstable diazonium ion II which may subsequently decompose to the carbonium ion III and free nitrogen.



II III

The carbonium ion III is a highly reactive electrophilic alkylating reagent which can react with water to form methanol or with RNA phosphate diesters and lead to chain breakage. In contrast to this, in ether solution diazomethane reacts as a nucleophilic reagent because the formation of the diazonium ion is prevented. Thus in water the most nucleophilic site on a base is substituted whereas in ether the site containing the most acidic proton is substituted.

Todd and co-workers (Brown, Magrath, and Todd 1955) have proposed the following mechanism for the hydrolysis of nucleotide triesters.



This mechanism also applies to triesters formed by alkylation of the phosphodiester linkages in nucleic acids. If R represents CH_3 in the above mechanism and R' represents the continuation of the nucleotide chain, the above mechanism would result in chain breakage. In the nucleic acid chains the diphosphate ester exists in the dissociated form and would therefore be nucleophilic. If diazomethane is prevented from forming a carbonium ion, esterification could not occur and consequently the cleavage reaction as proposed by Brown et al. would be averted. With this hypothesis, studies were initiated on the effect of diazomethane on RNA under non-aqueous conditions.

MATERIAL AND METHODS

Preparation of the Ribonucleic Acid

Leaves of Nicotiana tabacum L. var. Xanthi-nc were homogenized in 0.067M phosphate at pH 7.0 and extracted three times with phenol (Gierer and Schramm 1956). The RNA from the aqueous layer was precipitated with cold 95% ethanol. These preparations were composed primarily of ribosomal RNA and were used for the preliminary investigations. Tobacco mosaic virus ribonucleic acid (TMV-RNA), isolated in the same manner, served as the RNA source for the studies with diazomethane. The RNA was dissolved in a minimal volume of aqueous medium to minimize the amount of water in the reaction mixtures. Usually concentrations of RNA ranged from 4 to 6 mg/ml.

Preparation of Diazomethane

Method A. Bis-N-methyl-N-nitrosoterepthalamide (du Pont EXR-101) (17.5 gm, 0.07 moles) was suspended in 500 ml of cold anesthetic grade ether in a 1000 ml distillation flask equipped with a mechanical stirrer. Hydrolysis was accomplished using 105 ml of a 40% sodium hydroxide solution. The reaction was kept at 40-45°C with concomitant stirring of the reactants resulting in co-distillation of the ether-diazomethane. A second distillation flask,

cooled in an ice bath, was used as a receiver. Distillation was continued until the distillate was no longer yellow. The concentration was determined by titration (Arndt 1943).

Method B. One gram N-methyl-N-nitroso-urea was suspended in 50 ml cold anesthetic grade ether. Hydrolysis was accomplished by addition of 2 ml of 40% sodium hydroxide solution and stirring for 30 minutes (Redemann et al. 1955). The ether layer was drawn off and the concentration of diazomethane was determined by titration. Freshly-opened anesthetic grade ethyl ether U.S.P. was used throughout this investigation for all diazomethane preparations.

Sucrose Gradients

A one molar sucrose (Merck, reagent grade) solution was prepared in 0.002M potassium phosphate buffer at pH 7.4. From the stock solution a series of concentrations was prepared in 0.1M decrements beginning with 1M and ending with 0.2M. These were each layered into cellulose nitrate tubes (Spinco 39SW, 1/2"dia. X2") in 0.5 ml fractions. In order to effect a linear gradient, the tubes were held at 4°C for 6-8 hours before use. Next a 0.2 ml sample containing 0.2 mg of RNA was layered on the gradient and centrifuged at 39,000 r.p.m. (175,000 x G) for the appropriate time at 3-5°C. Two-drop fractions were then collected by piercing the bottom of the tube with a 24 gauge hypodermic needle.

These fractions were then diluted with 2.6 ml H_2O and the optical density at 260 mμ was determined (Brakke 1960).

Reaction of RNA with Diazomethane

An aliquot of aqueous RNA (1 mg) was added to 50 ml of ethereal diazomethane solution and after 30 minutes the reaction was quenched by the addition of 25 ml water. When destruction of the diazomethane was completed, as indicated by a loss of the characteristic yellow color, the aqueous layer was removed and lyophilized to dryness. An estimate of the chain breakage was obtained by dissolving the residue in 1 ml of water and subjecting an aliquot to sucrose density gradient centrifugation.

Column Chromatography of nucleotides

Dowex 1, X-10, 200-400 mesh was used in the formate form. To convert the resin from the chloride to the formate form, the extremes of fine and coarse were first removed by sedimentation and the remainder then packed in a column 2 cm X 40 cm and washed with 3 M sodium formate until chloride ion could no longer be detected in the eluate. The resin was then washed with 5-7 bed volumes of 1 M sodium formate in 6 N formic acid. This served as stock resin. The stock resin was then packed under air pressure in approximately 2 cm increments in a column 0.8 cm in diameter to a total bed height of 34 cm. After the resin was washed with approximately two bed volumes of

90% formic acid followed by 500-1000 ml of water, the sample of hydrolyzed RNA (at pH 10-11) was introduced onto the column in 2 ml of solution. This was washed with 10 ml water and then eluted with a 0-2.5N gradient of formic acid using a 500 ml mixing flask to effect the gradient. With this system, nucleosides were eluted first followed by the 2' and 3' phosphates of cytidylic acid at 80 ml of eluate, adenylic acid at 144 ml, guanylic acid at 480 ml and uridylic acid at 640 ml of eluate (Hurlbert et al. 1954).

RESULTS AND DISCUSSION

Chain Breakage of RNA Resulting From Reaction With Diazomethane

A study was made of the effect of diazomethane on RNA chain breakage. Tobacco leaf RNA rather than viral RNA was selected as a substrate for the preliminary investigations because of the relative ease with which it could be obtained. Solutions containing 50 ml of 0.05M, 0.02M, 0.01M, 0.002M, and 0.001M diazomethane in ether were each stirred with 1.1 mg of leaf RNA contained in 0.2 ml water for 30 minutes at 0°C. As a control, 1.1 mg leaf RNA in 0.2 ml water was treated with 50 ml ether under the same conditions. The reaction was quenched by the addition of 25 ml water, after which the aqueous layer was separated and lyophilized. A white residue was obtained which was dissolved in 1 ml water and subjected to analysis by sucrose density gradient centrifugation. Figure 1 shows the sucrose density gradient profile obtained after 5 hours of centrifugation. As can be seen in figure 1, curve A, when no diazomethane was used a prominent peak of higher molecular weight material was observed in fractions 20-30. This corresponded quite well with the results obtained from an untreated sample of the RNA and was taken as a reference. Furthermore, the correspondence between profiles obtained

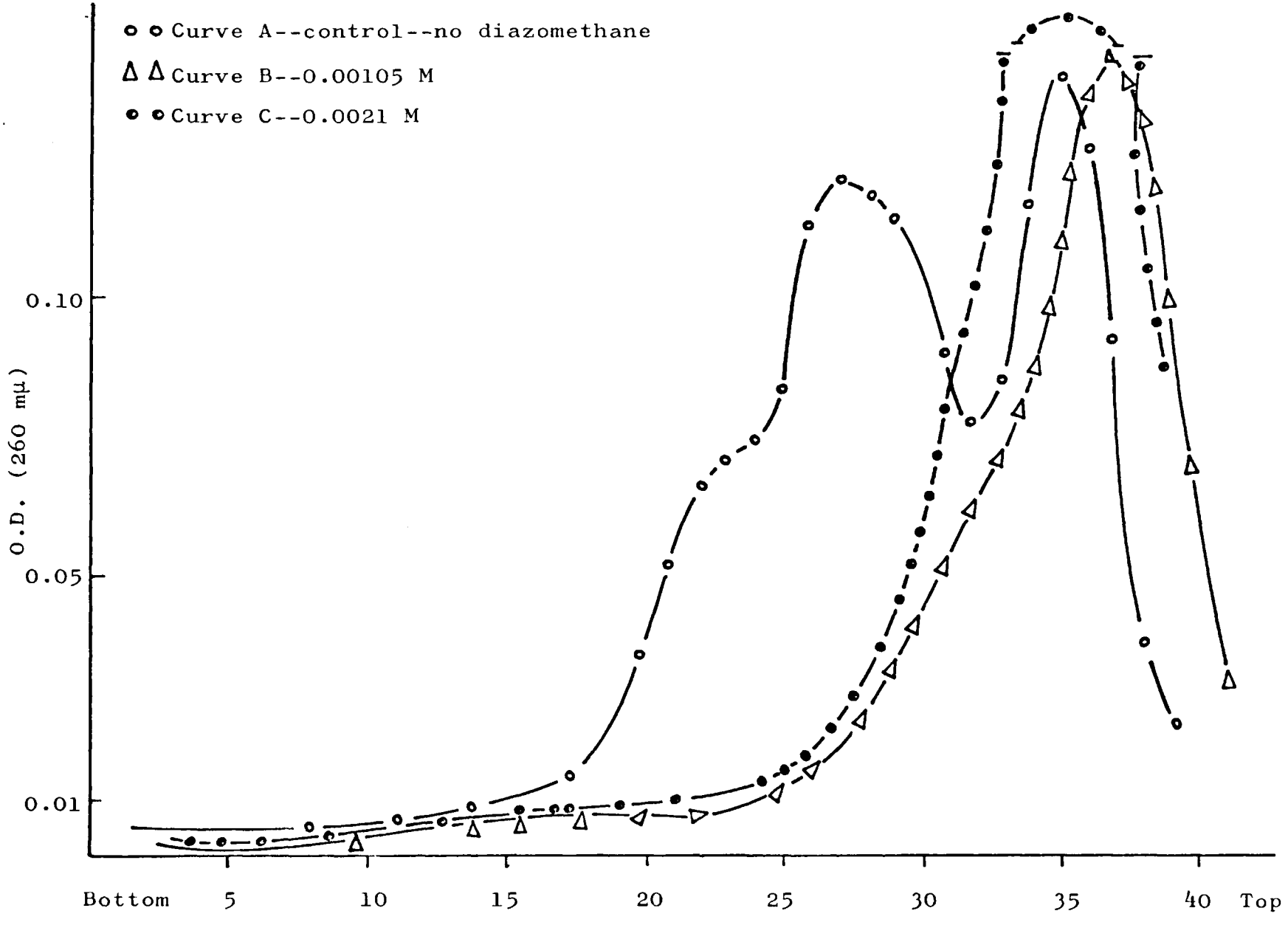


Figure 1(a). Sucrose density gradient profiles of tobacco leaf RNA with five hours centrifugation

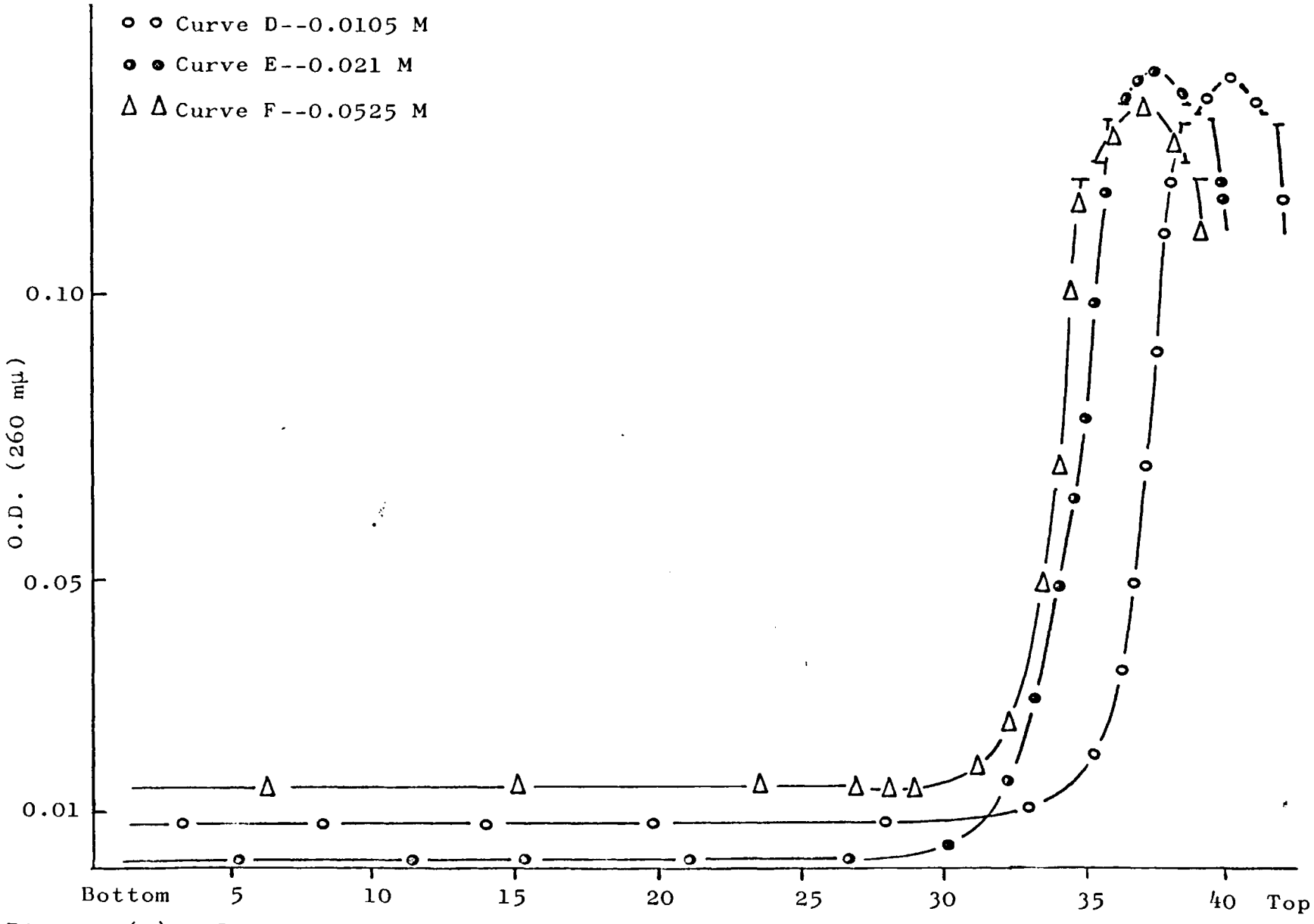


Figure 1(b). Sucrose density gradient profiles of tobacco leaf RNA with five hours centrifugation

from untreated RNA and that obtained from the control treatment indicated that the manipulations of the procedures do not in themselves cause chain breakage. Figure 1 curve B (0.001M diazomethane) lacked this prominent peak of high molecular weight material but did contain another component as indicated by a slight shoulder in tubes 28-34. Figure 1 curve C (0.002M diazomethane) did not contain this shoulder and thus was interpreted as an indication that this treatment caused a considerable amount of chain breakage. Curves D, E, and F in figure 1 indicated an even greater degree of chain breakage as shown by the increased concentration of sample at the top of the gradient tube. These results suggested that perhaps chain breakage was dependent on diazomethane concentration as evidenced by a decreased amount of higher molecular weight material with increasing concentrations of diazomethane.

As a shoulder appeared in fractions 21-24 of the high molecular weight peak of curve A figure 1, it was postulated that previous gradient runs were not carried out for a long enough time to resolve the RNA sample into the three components shown by Sänger and Knight (1963) when total RNA from a tobacco leaf was subjected to sucrose density gradient centrifugation. In order to test this postulation, 1 mg leaf RNA contained in 0.22 ml water was reacted with 50 ml ether containing a graded series of diazomethane concentrations between 0 and 0.05M (Table 1

Table 1. The Relationship of Diazomethane Concentration to RNA Chain Survival

Experiment	A		B		C		D		E	
RNA Source	Tobacco leaf		Tobacco leaf		TMV		TMV		TMV	
RNA Conc.	1 mg in 0.22 ml H_2O		1 mg in 0.22 ml H_2O		0.95 mg in 0.22 ml H_2O		1.05 mg in 0.28 ml pH 7.0 buffer		1.05 mg in 0.28 ml pH 8.6 buffer	
	DAM[1] conc. $\times 10^{-2}$	% Chain survival	DAM[1] conc. $\times 10^{-2}$	% Chain survival	DAM[2] conc. $\times 10^{-2}$	% Chain survival	DAM[2] conc. $\times 10^{-2}$	% Chain survival	DAM[2] conc. $\times 10^{-2}$	% Chain survival
	5 M	0	1 M	0	2.4 M	0	1 M	0	1 M	0
	2 M	0	0.8 M	0	1.6 M	0	0.6 M	50%	0.6 M	0
	1 M	0	0.6 M	60%	8.2 M	0	0.2 M	75%	0.2 M	30%
	0.2 M	80%	0.4 M	60%	1.6 M	70%	ether control	100%	ether control	100%
	ether control	100%	0.2 M	80%	ether control	100%				
			ether control	100%						

All reactions were carried out at 0°C for 30 minutes. Sucrose density gradient centrifugation time was six hours.

[1]Diazomethane prepared according to method A.

[2]Diazomethane prepared according to method B.

experiment A). The reaction was allowed to proceed for 30 minutes at 0°C before quenching and lyophilization of the aqueous layer. The white residue obtained was dissolved in 1 ml water and analyzed by sucrose density gradient centrifugation. Figure 2 presents the profile obtained after treatment with 0, 0.002M, and 0.01M diazomethane. It can be seen in curve A that six hours centrifugation resolves the RNA into three peaks. The two peaks appearing in tubes 8 to 17 and 18 to 24 consist of ribosomal RNA (Ingram 1966) and represent the high molecular weight RNA under study. Tubes 29 to 39 contain the soluble or transfer RNA and constitute low molecular weight material. The effect of diazomethane on the integrity of the ribosomal RNA was measured by the disappearance of 260 mμ absorption of the two ribosomal RNA species and the increase in absorption towards the top of the gradient tubes. The three higher concentrations of diazomethane used in this experiment resulted in a complete loss of high molecular weight RNA. Only with the lowest concentration of diazomethane (0.002M) was much of the ribosomal RNA preserved intact. Even in this case, however, there is an indication of some chain breakage as revealed by the proportion of total absorption contributed by ribosomal RNA. In the sample treated with no diazomethane, 70% of the absorption in the gradient tube is contributed by ribosomal RNA, whereas in the sample treated with 0.002M diazomethane this

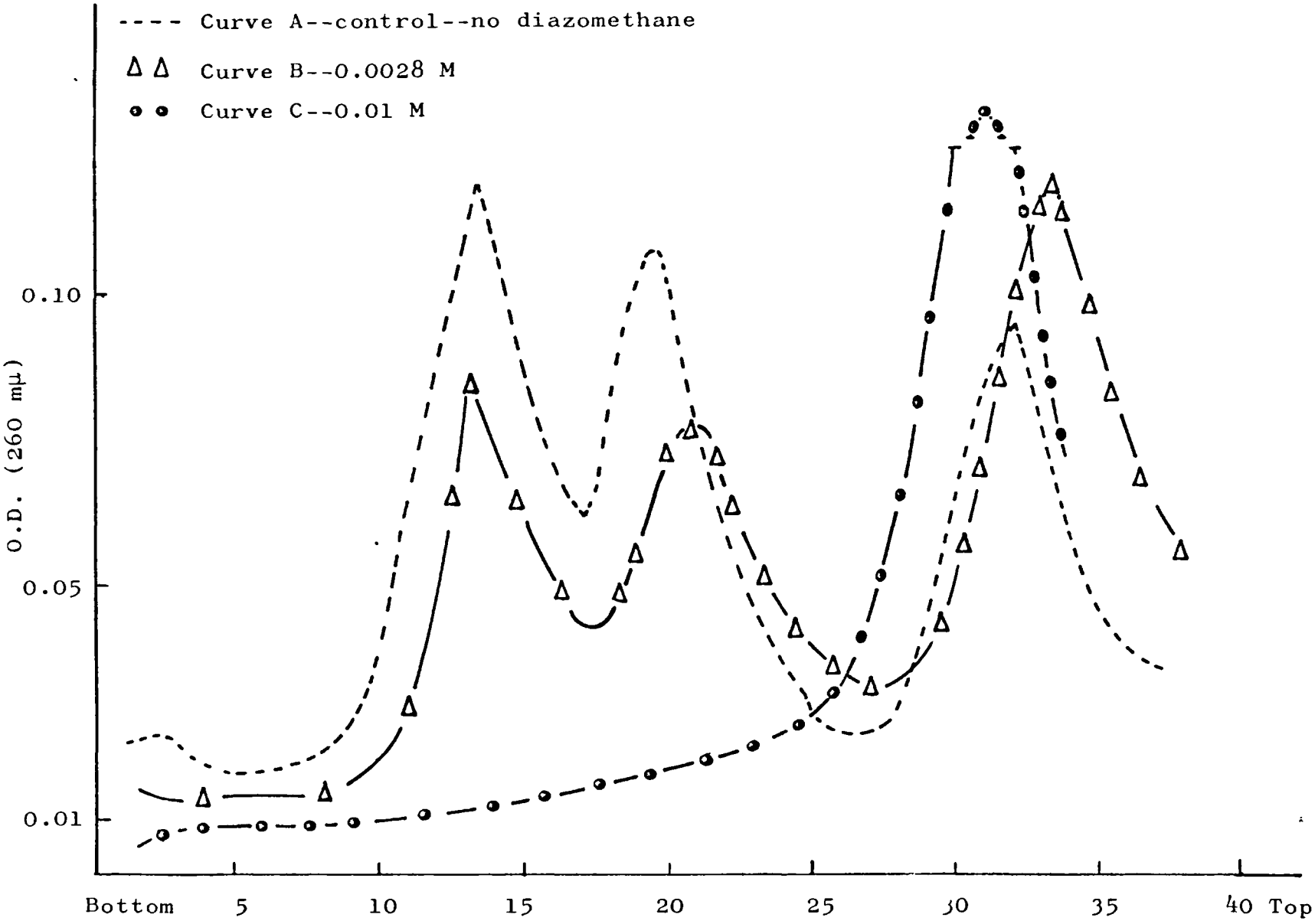


Figure 2. Sucrose density gradient profiles of tobacco leaf RNA with six hours centrifugation

value falls to 50% which corresponds to 80% survival of the ribosomal RNA when the control is taken as 100% survival. These values represent only approximations and are not intended to indicate absolute values. This experiment was repeated and extended to a narrower range of diazomethane concentrations consisting of 0.002M, 0.004M, 0.006M, 0.008M, and 0.01M diazomethane (Table 1, experiment B). Figure 3 is a graph of the results obtained with this dilution series where per cent survival of intact chains is plotted against diazomethane concentration.

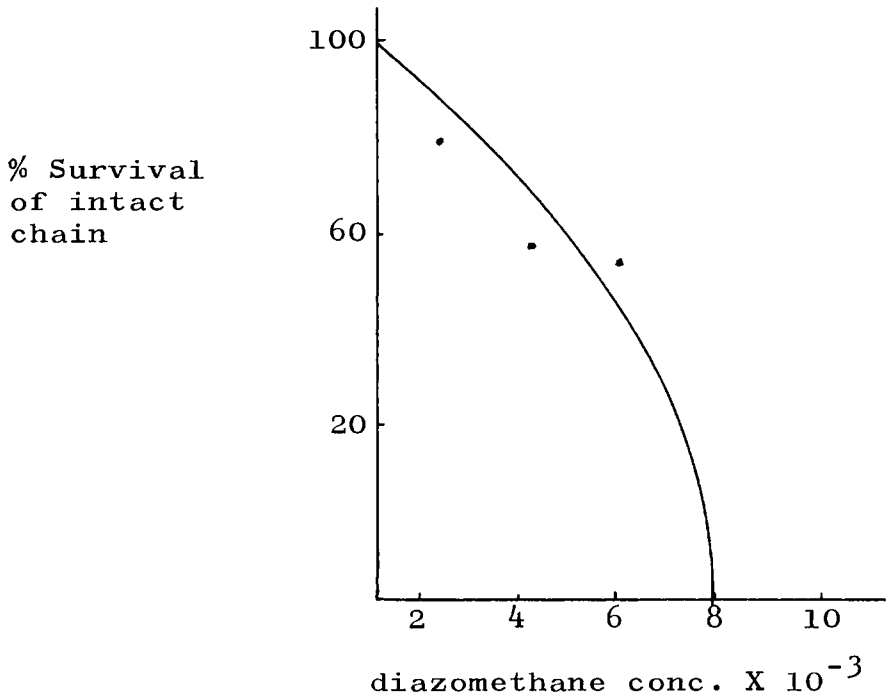


Figure 3. Relationship of chain survival of tobacco leaf RNA to diazomethane concentration

Using the total leaf RNA system as a model, work was directed towards the effect of the diazomethane on viral nucleic acid. Initial experiments with the viral RNA were carried out with 0.0018M diazomethane and 0.95 mg RNA in 0.22 ml solution. It was felt that five hours sucrose density gradient centrifugation time would be sufficient because of the more homogeneous composition of this RNA. Results of this work showed that the RNA remained too close to the top of the gradient tube to determine accurately if chain breakage was occurring. Subsequent experiments were carried out with a six hour centrifugation time. Since the leaf RNA had shown a diazomethane concentration dependence on chain breakage, it was necessary to see if the same was true for the viral RNA. A dilution series was prepared and tested (table 1, experiment C). Results showed that only the 0.0016M diazomethane treatment gave survival of intact chains which corresponded quite well with results obtained with the leaf RNA system. Interpretation of this result was carried out much the same way as was done for leaf RNA. A decrease in the viral RNA peak with the appearance of a new peak corresponding to low molecular weight material was interpreted as an indication of the extent of chain breakage. Results of the latter experiment encouraged further investigation of the effect of diazomethane concentration on chain breakage. Furthermore, the RNA solutions in all previous experiments were not buffered. RNA was therefore

dissolved in 0.067M phosphate buffer at pH 7.0 and a dilution series analogous to that of table 1 experiment B was prepared and tested (table 1, experiment D). The reaction was quenched with water as in previous experiments. As can be seen in the table, there was a good correlation of the results with viral RNA with those obtained from the analogous leaf RNA experiment. To investigate further the effect of pH on the reaction, the latter experiment was repeated with RNA dissolved in tris phosphate buffer at pH 8.6 (table 1, experiment E). Results from this treatment did not correspond to those obtained with the treatment at pH 7.0. The reason for the different results was difficult to ascertain and could have been due to a number of factors. It was therefore concluded that the unbuffered RNA reacted similarly to the RNA buffered at pH 7.0 and further work is necessary to establish the effect of other pH's on the reaction.

In summary, it has been established that high molecular weight RNA of two types, ribosomal and viral, is degraded by diazomethane in a non-aqueous medium under the conditions described. This degradation is slight with 2×10^{-3}M diazomathane and increases as the diazomethane concentration is increased until no or very little undegraded material remains with diazomethane concentration of 1×10^{-2}M.

The Methylation of RNA by Diazomethane

One of the primary objects of this investigation was to study the conditions under which high molecular weight RNA could be methylated by diazomethane without degradation. In section I the conditions for reacting RNA with diazomethane with minimal degradation were determined. The degree of methylation achieved under these conditions was then studied. This study is in its preliminary stages and although the degree of methylation under non-degradative conditions has not yet been determined, the techniques for doing this have been investigated. Independent studies by Marvel (unpublished data) have indicated that the sites of methylation by diazomethane under non-aqueous conditions are those shown in figure 4. The following considerations indicate that low concentrations of diazomethane which do not degrade the RNA may also result in methylation of the intact RNA. A sample of RNA (2.5 mg in 0.75 ml water) was treated in the usual way with 0.05M diazomethane; a condition which leads to degradation of RNA. The recovered product was hydrolyzed with 0.3N KOH for 18 hours at 37°C in order to hydrolyze the RNA fragments to the 2', 3' nucleotides. Column chromatography of the sample resulted in the expected cytidylic and adenylic acid peaks but the eluate which normally contained guanylic and uridylic acid contained a mixture of nucleotides which were not identified. It is probable that lack of characteristic uridylic

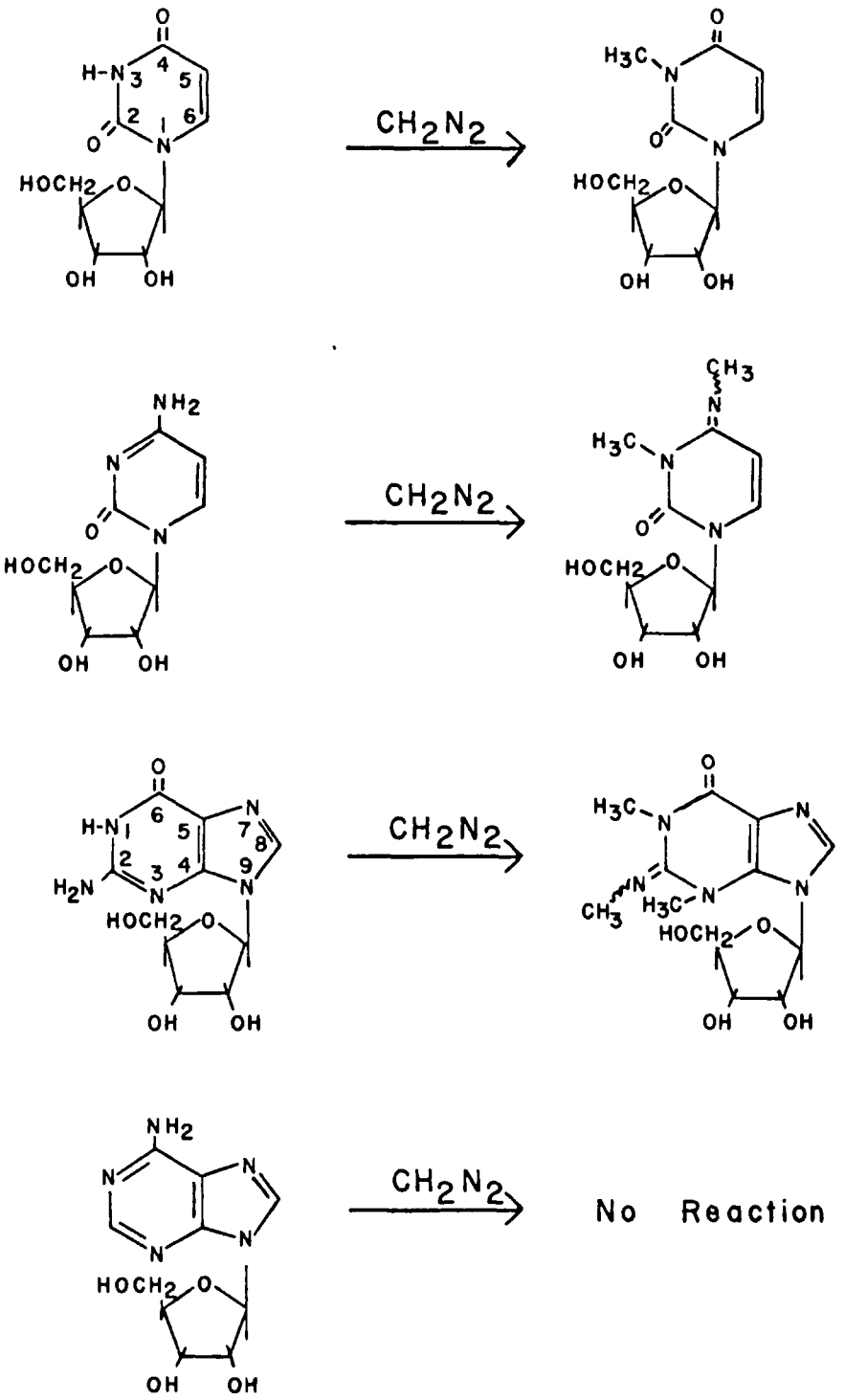


Figure 4. Postulated sites for methylation of nucleosides with diazomethane under non-aqueous conditions

acid and guanylic acid peaks results from the appearance of methylated nucleotides of guanosine and uridine. Although the chromatogram gave a characteristic early peak for cytidylic acid, it does not exclude the possibility that cytidylic acid was methylated for two reasons. First cytidylic is eluted early in the gradient therefore decreasing the probability that the column would separate methylated and unmethylated species (Marvel, unpublished data). Second cytosine is difficult to methylate with diazomethane (Haines, Reese, and Todd 1964) and therefore the amount of cytosine methylated would be expected to be small. Adenylic acid would also be expected to give a characteristic peak because adenine has no postulated sites for methylation. Chain breakage with 0.05M diazomethane was found in previous experiments to be extensive and therefore this does not supply direct evidence for methylation at concentrations of diazomethane that results in survival of intact chains. However, 0.05M diazomethane represents a 25 fold increase in concentration over 0.002M which does result in survival of intact chains. Therefore, if it was assumed that 0.05M diazomethane results in complete methylation of the postulated sites, 0.002M diazomethane would correspond to methylation of 4% of the nucleotides present which is 260 of the 6500 contained in TMV-RNA.

SUMMARY AND CONCLUSIONS

Chain breakage of tobacco leaf and TMV-RNA has been studied using diazomethane under essentially non-aqueous conditions. It was found that 0.006M was the maximum diazomethane concentration that would yield intact nucleic acid molecules. It was also determined that chain breakage was dependent on diazomethane concentration as shown by the decreasing breakage with decreasing diazomethane concentration. Methylated products of the reaction mixtures were not characterized, however, other work with aqueous solutions (Haines 1964) has shown that methylation can occur at these diazomethane concentrations especially with uridine and guanosine.

The data indicates that the bases methylated when a large excess of diazomethane is used are uridine and guanosine but not adenosine. Once the degree of methylation has been established, this system may prove suitable for the study of nucleotide sequences due to the increased resistance of the methylated nucleic acids to enzymatic hydrolysis.

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