An Approach to the Methylation of Polynucleotides*

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ABSTRACT: A systematic study of the action of methylating agents on nucleic acid derivatives has been extended to include dinucleoside phosphates and homopolyribonucleotides. Adenylyl-(3′→5′)-uridine was methylated selectively on its adenine and uracil residues by dimethyl sulfate and diazomethane, respectively, without esterification of the internucleotidic linkage in either instance. The thymine residues of thymidylyl-(3′→5′)-thymidine could be completely methylated by diazomethane. Polyadenylic and polycytidylic acids were both methylated by dimethyl sulfate on almost half of their base residues, without detectable degradation. Methylation of cytosine residues of polycytidylic acid brought about localized resistance to digestion by pancreatic ribonuclease. The uracil residues of polyuridylic acid were extensively methylated by diazomethane, but there was some indication of polymeric breakdown.

The study of the methylation of nucleic acids (for important references, see Lawley, 1961; Lawley and Brookes, 1963; Broom *et al.*, 1964) has attracted much attention in the past few years. The process of *chemical* methylation is complex as nucleic acids are polyfunctional molecules, which may in principle be methylated on their base residues (and in more than one place on some of these), on their phosphodiester internucleotidic linkages, and, in the case of ribonucleic acids, even on the 2′-hydroxyl groups of the sugar residues. Some of the earlier workers in this field conducted methylation studies on nucleic acids before the behavior of the individual base and phosphodiester functions had been investigated, and accordingly their results (see Lawley, 1961) were often difficult to interpret. More recently, considerable progress has been made by several groups (Lawley and Brookes, 1963, and earlier references; Haines *et al.*, 1962, 1964; Broom *et al.*, 1964) who, before proceeding to such complex systems, first examined the effect of alkylating agents on simple ribo- and deoxyribonucleosides. This work has led to a knowledge of the relative reactivity of the common purine and pyrimidine bases, and of their principal sites of alkylation. In much of the reported work, the alkylating agents used have been esters of strong acids, for example, dimethyl sulfate and methyl methanesulfonate. We have used dimethyl sulfate in some of our experiments (Griffin and Reese, 1963; Haines *et al.*, 1964), but have also made a systematic study of the action of diazomethane as a methylating agent (Haines *et al.*, 1962, 1964). As diazomethane differs from the methyl esters of strong acids in its reaction with bases, it exhibits, not surprisingly, a different order of reactivity toward nucleosides (Haines *et al.*, 1964).

In addition to their intrinsic importance, these initial studies with nucleosides seem to constitute the most satisfactory approach to the problem of nucleic acid alkylation. However, the results obtained with simple nucleosides cannot be expected to lead directly to a full understanding of the methylation of polynucleotides since, in the latter, macromolecular features and especially possible secondary structures might well be involved. This point has been supported by some recent experiments on the methylation of DNA (Lawley and Brookes, 1963; Kriek and Emmelot, 1964).

It was then necessary to investigate the comparative susceptibilities of the bases and phosphodiester functions to methylation. The literature is often most obscure and confusing on this fundamental aspect of the problem of nucleic acid alkylation (see Lawley, 1961). We approached this matter by examining (Haines *et al.*, 1964) the methylation of the readily available 5′-mononucleotides. Although the latter are monoalkyl phosphates, and thus apparently unsuitable models for nucleic acids, these studies gave a strong indication that dialkyl phosphates and hence nucleic acids were unlikely to be esterified by dimethyl sulfate or diazomethane in neutral aqueous solution. If this were so, then methylation of the base residues of nucleic acids was unlikely to be accompanied by degradation due to the hydrolysis of comparatively labile phosphotriester groups.

In the present report, these conclusions are extended further. An examination of the effect of methylating agents, first on dinucleoside phosphates, which contain the reactive functions of nucleic acids but not their macromolecular features, and second on homopolyribonucleotides, is discussed. The latter serve as useful models for natural polynucleotides, and the presence of only one purine or pyrimidine base greatly simplifies the analysis of the products. Since this work was com-

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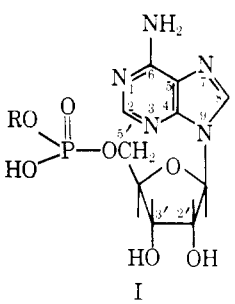
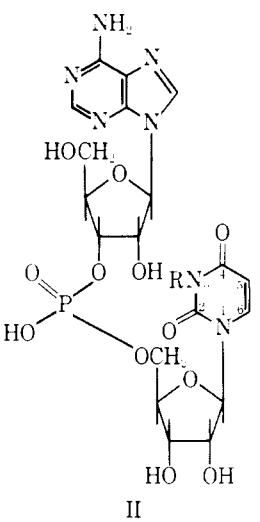
pleted, a study of the methylation of polyadenylic acid (poly-A) has been reported (Ludlum *et al.*, 1964).

Methylation with Dimethyl Sulfate. The order of reactivity of the principal ribonucleosides toward methyl esters of strong acids (Lawley, 1961) has been found to be guanosine, adenosine, and cytidine, with uridine virtually inert. A corresponding order has been found for deoxyribonucleosides (with thymine taking the place of uracil), and also for bases as they occur in nucleic acids, except that the sites of methylation of the latter bases were not invariably the same as those[1] of simple nucleosides (Lawley and Brookes, 1963). Evidence has been obtained which suggests that dialkyl phosphates and hence internucleotidic phosphodiester functions would be resistant to esterification by dimethyl sulfate. In the reaction between the latter and adenosine 5′-phosphate (I, R = H) at pH 4.5–5.0, only monoesterification was observed (Griffin and Reese, 1963), and the rate of this reaction was slower even than N-1 methylation. The dialkyl phosphates, adenosine 5′-methyl hydrogen phosphate (I, R = Me) and its N-1 methyl derivative, were both formed and neither showed a tendency to be esterified further. In the pH range 4.5–5.0, a monoalkyl phosphate is present largely as its monoanion, which is believed to be considerably less nucleophilic than the dianion predominating above pH 6. Nevertheless, the concentration of dianion at the lower pH would be sufficient to account for the observed partial monoesterification. The anion of a dialkyl phosphate would also be expected to be only weakly nucleophilic and thus resistant to esterification. This is in accord with the experimental observations discussed above.



I



II

Although the results of the methylation of adenosine 5′-phosphate, and of cytidine 5′-phosphate (Haines *et al.*, 1964) made it seem likely that the internucleotidic phosphodiester linkages of nucleic acids would be resistant to esterification in aqueous solution, it was considered necessary to seek further confirmation of this important point. Therefore we examined the methylation of adenylyl-(3′→5′)-uridine[2] (ApU; II, R = H), which is a dialkyl phosphate and hence a suitable model for a polynucleotide. When an aqueous solution of ApU (II, R = H) (Griffin and Reese, 1964) was treated with a large excess of dimethyl sulfate at 20° and pH 7, the sole product (obtained in 80% yield) was MeApU, which was separated from unchanged ApU by anion-exchange chromatography. No esterification or degradation was observed. This experiment confirmed that dialkyl phosphate anions were resistant to attack by dimethyl sulfate, and also showed the complete selectivity of this reagent for the adenine residue of ApU. The MeApU was completely degraded by spleen phosphodiesterase into 1-methyladenosine 3′-phosphate and uridine; it was hydrolyzed into N^6-methyladenosine (2′)3′-phosphates and uridine by aqueous alkali.

In order to determine the possible effect of macromolecular structure on the course of methylation, the action of dimethyl sulfate on two high molecular weight homopolyribonucleotides, poly-A and poly-C, was investigated. In the first experiment, poly-A in aqueous solution was treated with an excess (approximately 45 equiv) of dimethyl sulfate at 20° while alkali was automatically added to maintain the pH at 7.5. As no dialyzable nucleotidic material was detected in the products, it was assumed that there had been no appreciable breakdown of polymer. This was supported by the observation that the sedimentation constant of the product was greater than that of the original poly-A.

An estimate of the relative proportions of the (2′)3′-phosphates of adenosine and 1-methyladenosine, obtained by acidic hydrolysis[3] of the methylated polymer, indicated that 45% of the adenine residues had been methylated. The methylated and unmethylated nucleotides were readily separated by paper electrophoresis and estimated spectrophotometrically. This assay procedure was also adopted for the other methylated polymers, discussed below. The methylated poly-A was also submitted to alkaline hydrolysis and converted into a mixture of the (2′)3′-phosphates of adenosine and N^6-methyladenosine (Brookes and Lawley, 1960; Griffin and Reese, 1963). The methylated and unmethylated fractions, easily separable by paper chromatography were, as before, estimated spectrophotometrically. The results of this assay indicated that the polymer was 42% methylated.

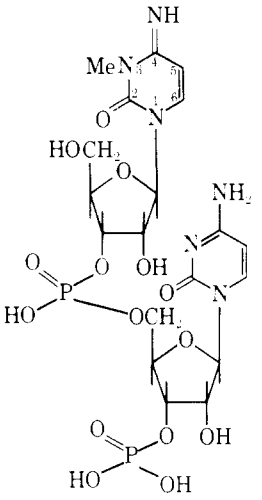
In a second experiment with poly-A, a much larger excess (approximately 300 equiv) of dimethyl sulfate was used. Examination of the acid hydrolysate suggested that 76% of the adenine residues had been methylated. Unfortunately, however, a small amount of polymer degradation had occurred, as was evidenced

[1] *I.e.*, N-7 for guanine, N-1 for adenine, and N-3 for cytosine.

[2] The abbreviations which are used subsequently for the dinucleoside phosphates under discussion are: adenylyl-(3′→5′)-uridine, ApU; 1-methyladenylyl-(3′→5′)-uridine, MeApU; adenylyl-(3′→5′)-3-methyluridine, ApMeU; thymidylyl-(3′→5′)-thymidine, TpT; 3-methylthymidylyl-(3′→5′)-3-methylthymidine, MeTpMeT.

[3] About 10% of the material was degraded to a mixture of adenine and 1-methyladenine under the conditions used.

by the appearance of dialyzable nucleotides among the products.

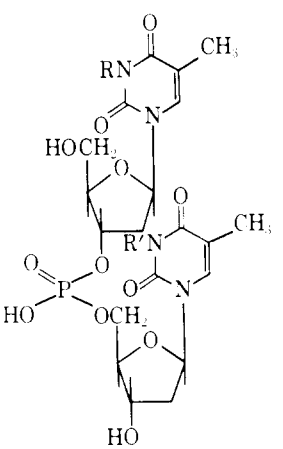


III

Poly-C was treated with an excess (approximately 50 equiv) of dimethyl sulfate at 20° and pH 8. No dialyzable nucleotidic material was obtained and, as was observed with poly-A, the sedimentation constant of the polymer had increased after methylation. The methylated polymer was degraded by acid hydrolysis and estimated to contain 46% of 3-methylcytosine residues. In a second experiment, a smaller excess of dimethyl sulfate was used and only 15% of the cytosine residues were methylated. This material was incubated with pancreatic ribonuclease under conditions which were more than sufficient to degrade poly-C completely to cytidine 3′-phosphate. Two products were obtained in the approximate proportions of 5:1; they were identified as cytidine 3′-phosphate and the dinucleotide III, respectively. Thus the hydrolysis of esters of 3-methylcytidine 3′-phosphate, unlike the corresponding derivatives of 4-methyl- and 4,4-dimethylcytidine (Brimacombe, 1964) but like those of 3-methyluridine (Szer and Shugar, 1961), is not catalyzed by pancreatic ribonuclease. This observation could possibly be exploited in the determination of the base sequence of RNA.

Methylation with Diazomethane. The order of reactivity of ribonucleosides toward diazomethane (Haines *et al.*, 1964) was found to be: uridine and guanosine ≫ cytidine > adenosine. Surprisingly enough, the principal sites of methylation were the same as those for dimethyl sulfate. Although the rate of methylation of uridine was approximately equal to that of guanosine, thymidine was found to be much more resistant to methylation than 2′-deoxyguanosine. This effect, which is presumably due to the inductive properties of the 5-methyl group in the thymine residue, has been confirmed by examining the action of diazomethane on equimolecular mixtures of the 5′-methyl hydrogen phosphates of (i) uridine and guanosine and (ii) thymidine and 2′-deoxyguanosine. The results (see Experimental Section) indicate that the uridine derivative was methylated on N-3 at approximately the same rate as was the guanosine derivative on N-7, but, under similar conditions, the thymidine derivative remained unchanged while the deoxyguanosine derivative was largely methylated.

All methylation reactions with diazomethane were carried out by shaking neutral or buffered aqueous solutions of substrates with a large excess (in one or several portions) of ethereal diazomethane at 0°. When solutions of 5′-mononucleotides were treated in this way, the comparative rates of base methylation and esterification depended, as expected, on the nature of the base. Thus for uridine 5′-phosphate, base methylation occurred more rapidly than esterification, but the reverse was true for adenosine 5′-phosphate. However, in all cases, only monomethyl esters were obtained.[4] This resistance to phosphotriester formation was not unexpected on mechanistic grounds. An intermediate phosphodiester, such as I (R = Me), is a strong acid and hence the number of undissociated molecules at pH 7 would only be about 1 in 10^6. If, as has been postulated (Eistert, 1941), diazomethane reacts only with molecules of undissociated acids, then methylation of a dialkyl phosphate in neutral solution would not be favored. In this way the results obtained from the study of mononucleotides suggested that the reaction of diazomethane with polynucleotides would involve methylation of base residues without concomitant esterification of the internucleotidic linkages.



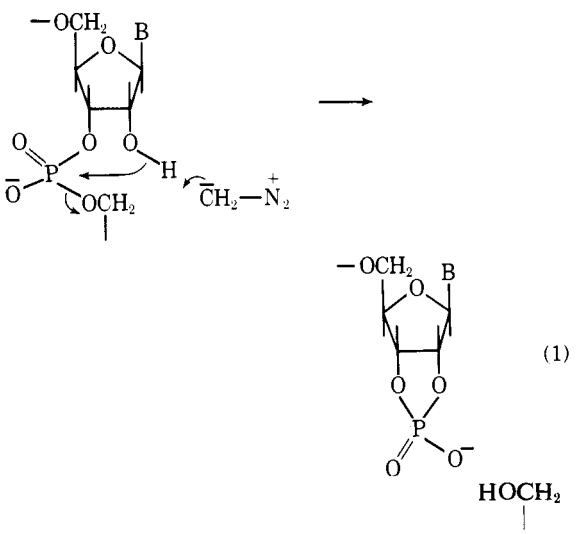
IV

As in the case of dimethyl sulfate, discussed above, it was decided to test this point further by examining the behavior of diazomethane toward dinucleoside phosphates. As a first example, the deoxynucleoside derivative TpT (IV, R = R′ = H) was treated with an approximately 250 molar excess of diazomethane. All the starting material was consumed and two products were detected by paper chromatography. From paper electrophoretic evidence (obtained at pH 10.5), these products were identified as MeTpMeT (IV, R = R′ = Me) and monomethyl TpT (IV, R = H, R′ = Me

[4] Traces perhaps of dimethyl esters were obtained when a several hundredfold excess of diazomethane was used.

and/or R = Me, R′ = H). No methyl ester formation was detected. When twice the quantity of diazomethane was used, MeTpMeT was the sole product. As a second example, the ribonucleoside derivative ApU (II, R = H) was treated with an approximately 160 molar excess of diazomethane and was quantitatively converted into ApMeU (II, R = Me). Neither esterification nor methylation of the adenine residue was detected. When the ApMeU was incubated with spleen phosphodiesterase, it was completely hydrolyzed to give adenosine 3′-phosphate and 3-methyluridine. It is noteworthy that in their methylating action on ApU, diazomethane and dimethyl sulfate (see above) are complementary in that they are specific toward the uracil and adenine residues, respectively.

This added reassurance to the idea that, under the reaction conditions used, diazomethane did not bring about the esterification of internucleotidic linkages, and hence it seemed unlikely that it would promote the degradation of a polynucleotide. In order to test this hypothesis, a solution of poly-U in 0.05 M aqueous sodium acetate was treated with a large excess of ethereal diazomethane. No dialyzable material could be detected in the products, which were shown by the acid hydrolysis assay to contain 78% of methylated uracil residues. However, when the sedimentation constant of this methylated material (s_{20} 0.8) was found to be much lower than that of the original poly-U (s_{20} 2.0), it seemed likely that some polymer degradation had occurred. This observation is in accord with the findings of Kriek and Emmelot (1963) who studied the action of diazomethane on soluble and messenger RNA (m-RNA) under similar conditions and observed appreciable degradation. The latter workers proposed that this breakdown was promoted by esterification of the internucleotidic linkages. This hypothesis is not in accord with our studies with 5′-mononucleotides and dinucleoside phosphates. We, therefore, wish to propose an alternative mechanism indicated in reaction 1.



(1)

Diazomethane is known to act as a basic catalyst; it has, for example, been observed to promote the methanolysis of *O*-acetyl derivatives of nucleosides (Bredereck, 1947). As indicated in eq 1, diazomethane is acting as the catalyst in the well-established mechanism of basic hydrolysis of polyribonucleotides (Brown and Todd, 1953). The terminal 2′,3′-cyclic phosphate group produced would be expected to be stable under the experimental conditions. In the above methylation of poly-U, however, no degradation products which were small enough to permit the identification of a cyclic phosphate function were isolated.

Conclusions

From the above discussion, it would appear that both diazomethane and dimethyl sulfate are valuable methylating agents for nucleic acids. Furthermore, they are to some extent complementary to each other in their reactivity. Clearly, if it is required to effect extensive methylation of a polyribonucleotide, the use of diazomethane might lead to some breakdown. On the other hand, it appears to be difficult to achieve more than 50% methylation with dimethyl sulfate. This may be influenced by an electrostatic effect: methylation of cytosine and adenine residues leads to strongly basic systems which are largely protonated at pH 7–8. Thus, complete methylation of poly-A or poly-C would lead to a poly zwitterion. It is noteworthy that poly-C (Akinrimisi *et al.*, 1963) and poly-A (Fresco and Doty, 1957) both form stable twin-stranded helical complexes at the points of half-protonation. Nevertheless, it should be possible to effect a considerable amount of methylation of polynucleotides with both reagents without appreciable degradation. As indicated above, this could prove to be a useful tool in the determination of the base sequences of nucleic acids.

Another general observation about methylation is perhaps worth making. Current theories (for principal references, see Broom *et al.*, 1964) suggest that the methylated bases present in nucleic acids are introduced at the polymeric level under the influence of a group of methylase enzymes. The biological source of methyl groups (*S*-adenosylmethionine) chemically resembles dimethyl sulfate rather than diazomethane. It is thus easy to see how the N^1- and N^6-methyladenines, N^7- and even N^1-methylguanine residues are derived. However, it is harder to understand the mechanism of formation of N^6-dimethyladenine and also the N^2-methylated guanine residues in chemical terms, if methylation *always* occurs at the polymeric level. Possibly some of these methyl groups are introduced earlier in the biogenetic scheme.

Experimental Section

Paper Chromatography and Electrophoresis. Ascending paper chromatography on Whatman No. 1 paper was carried out in the following solvent systems (see Table I): A, propan-2-ol–ammonia (*d* 0.88)–water (7:1:2); B, saturated aqueous ammonium sulfate–propan-2-ol–0.1 M potassium phosphate buffer pH 7.2 (79:2:19); C, ethanol–1 M aqueous ammonium acetate (5:2); D, propan-2-ol–acetic acid–1% aqueous am-

TABLE I: R_F Values in Systems A–E.[a]

Substance	A	B	C	D	E
Thymidine 5′-methyl hydrogen phosphate	0.50				
3-Methylthymidine 5′-methyl hydrogen phosphate	0.73				
2′-Deoxyguanosine 5′-methyl hydrogen phosphate	0.23	0.28			
2′-Deoxy-7-methylguanosine 5′-methyl hydrogen phosphate	0.32	0.58			
Uridine 5′-methyl hydrogen phosphate	0.22	0.59			
3-Methyluridine 5′-methyl hydrogen phosphate	0.52	0.51			0.38
Thymidylyl-(3′→5′)-thymidine (TpT)	0.47				
3-Methylthymidylyl-(3′→5′)-thymidine (MeTpT)	0.61				
Thymidylyl-(3′→5′)-3-methylthymidine (TpMeT)	0.61				
3-Methylthymidylyl-(3′→5′)-3-methylthymidine (MeTpMeT)	0.76				
Adenylyl-(3′→5′)-uridine (ApU)			0.26		
Adenylyl-(3′→5′)-3-methyluridine (ApMeU)			0.41		
1-Methyladenylyl-(3′→5′)-uridine (MeApU)			0.34		
Adenosine 2′(3′)-phosphates			0.13	0.21	
1-Methyladenosine 2′(3′)-phosphates			0.06	0.25	0.16
N^6-Methyladenosine 2′(3′)-phosphates			0.19	0.38	
Cytidine 2′(3′)-phosphates	0.13				0.28
3-Methylcytidine 2′(3′)-phosphates	0.27				0.34
3-Methylcytidylyl-(3′→5′)-cytidine 3′-phosphate (MeCpCp)					0.17
Uridine 2′(3′)-phosphates			0.26		
3-Methyluridine 2′(3′)-phosphates			0.45		
Uridine			0.65		
3-Methyluridine			0.82		

[a] See Experimental Section for a description of the systems.

monium acetate (45:35:20); E, butan-1-ol–acetic acid–water (5:2:3); F, isobutyric acid–1 N ammonia–0.1 M EDTA (100:60:1.6). Paper electrophoresis on Whatman No. 4 paper was conducted in a carbon tetrachloride cooled apparatus (*ca.* 35 v/cm) with the following buffers: 0.05 M sodium acetate, pH 4.5; 0.05 M phosphate, pH 7.7; 0.05 M triethylammonium bicarbonate, pH 8; 0.067 M phosphate, pH 8; 0.05 M sodium borate, pH 9.8; 0.05 M sodium carbonate, pH 10.5.

Methylation of Aqueous Solutions of Substrates. WITH ETHEREAL DIAZOMETHANE. Ethereal diazomethane was estimated by titration with standardized benzoic acid. Aqueous solutions of substrates and varying excesses of ethereal diazomethane were shaken together at 0° in vessels closed by Bunsen valves. Rapid reactions generally ensued.

WITH DIMETHYL SULFATE. Dimethyl sulfate was added slowly to a rapidly stirred aqueous solution of the substrate at 20°. The pH was maintained at the required value by the automatic addition of alkali. A Radiometer automatic titrator (Model TTT 1a) was used.

Competitive Methylation of Methyl Esters of Thymidine and 2′-Deoxyguanosine 5′-Phosphates. A. A solution of the pyridinium salts of the methyl esters of thymidine and 2′-deoxyguanosine 5′-phosphates (3 mg of each) in water (1 ml) was shaken with ethereal diazomethane (0.05 M; four 1-ml portions, 32 equiv). Paper chromatography (system A) showed the presence of both starting materials (R_F 0.50 and 0.23, respectively) and two fluorescent products, one of which (R_F 0.32) corresponded to 2′-deoxy-7-methylguanosine 5′-methyl hydrogen phosphate (Haines *et al.*, 1962).

B. This differed from A in that twice the quantity of ethereal diazomethane (four 2-ml portions, 64 equiv) was used. In addition to all the above products, a small amount of a material which corresponded to 3-methylthymidine 5′-methyl hydrogen phosphate (R_F 0.73) (Haines *et al.*, 1964) was detected.

Competitive Methylation of Methyl Esters of Uridine and Guanosine 5′-Phosphates. A solution of the pyridinium salts of the methyl esters of uridine 5′-phosphate (3.2 mg) and guanosine 5′-phosphate (3.6 mg) in water (1 ml) was shaken with ethereal diazomethane (0.5 M; 0.4 ml, 30 equiv). Paper chromatography (system B) indicated three new products, two of which corresponded (Haines *et al.*, 1964) to the 5′-methyl hydrogen phosphates of 3-methyluridine (R_F 0.51, 50% yield) and 7-methylguanosine (R_F 0.41, 50% yield). The third product (R_F 0.17) was fluorescent like the latter.

Methylation of Thymidylyl-(3′→5′)-thymidine (TpT). A. A solution of TpT (Li^+ salt, 2.3 mg) (Gilham and Khorana, 1958) in water (1 ml) was shaken with ethereal diazomethane (0.5 M; 2 ml, 245 equiv). Paper chromatography (system A) indicated two products

(R_F 0.61 and 0.76) but no unchanged TpT (R_F 0.47). Paper electrophoresis (pH 10.5) revealed two anionic species with mobilities about one-third and two-thirds that of TpT.

B. Experiment A was repeated with twice the quantity of ethereal diazomethane (4 ml, 490 equiv). Only one product [R_F (system A) 0.76] was obtained; it had about one-third the electrophoretic mobility of TpT at pH 10.5 and almost the same mobility at pH 4.5.

Methylation of Adenylyl-(3′→5′)-uridine (ApU). A. WITH ETHEREAL DIAZOMETHANE. A solution of ApU (Ca^{2+} salt, 1.15 mg) (Griffin and Reese, 1964) in aqueous sodium acetate (0.05 M; 0.5 ml) was shaken with ethereal diazomethane (0.1 M; 5 × 0.5 ml, 165 equiv). Paper chromatography (system C) showed one product (R_F 0.41), uncontaminated with starting material (R_F 0.26). At pH 10.5, the material was electrophoretically homogeneous, having about one-half the mobility of ApU; at pH 8 (bicarbonate buffer) it had about the same mobility as ApU.

The product was lyophilized, dissolved in aqueous ammonium acetate (0.5 M; 0.4 ml), and incubated with spleen phosphodiesterase (0.08 ml) at 37° for 24 hr. Paper chromatography (system C) of the hydrolysate showed the presence of two components only (R_F 0.13 and 0.82) which corresponded with marker spots of adenosine 3′-phosphate and 3-methyluridine, respectively. This was confirmed by paper electrophoresis (pH 9.8).

B. WITH DIMETHYL SULFATE. A solution of ApU (Ca^{2+} salt, 14.9 mg) in water (2.5 ml) was treated with dimethyl sulfate (65 mg initially and a further 65 mg after 2 hr; total, 100 equiv), while the pH was maintained at 7.0 ± 0.1 by the automatic addition of 0.5 M KOH. After 8 hr, the products were extracted with two 5-ml portions of ether. Paper electrophoresis (pH 4.5) revealed a *cationic* species and a material with the same mobility as ApU. The products were added to a Dowex 1×2 (Cl^-, 14 cm × 1 cm^2) anion-exchange column, which was washed with distilled water. The eluate, so obtained, was concentrated and then lyophilized to yield a substance (10 mg) which was homogeneous both paper chromatographically [R_F (system C) 0.34] and electrophoretically (pH 4.5 and 7.7).

A solution of the latter methylation product (1 mg) was incubated with spleen phosphodiesterase as described under A above. Paper chromatography (system C) of the hydrolysate indicated two components (R_F 0.65 and 0.06, respectively) corresponding to uridine and 1-methyladenosine 3′-phosphate (Dunn, 1963), and no unhydrolyzed methylation product. Confirmatory evidence was obtained by paper electrophoresis (pH 7.7).

Alkaline Hydrolysis of Dimethyl Sulfate Methylation Product of ApU. A solution of the methylation product (1 mg), obtained in B above, in aqueous 1 N sodium hydroxide (0.1 ml) was kept at 37° for 20 hr. Paper chromatography (system C) indicated two products (R_F 0.65 and 0.19, respectively) corresponding to uridine and N^6-methyladenosine 2′(3′)-phosphates (Littlefield and Dunn, 1958). This was confirmed by paper electrophoresis (pH 7.7).

Methylation of Polyadenylic Acid (Poly-A) with Dimethyl Sulfate. A. To a solution of poly-A[5] (K^+ salt; 10 mg) in water (5 ml) was added dimethyl sulfate (150 mg, 45 equiv[6]) during a period of 1 hr. The pH was maintained at 7.5 by the automatic addition of 0.5 M KOH. After a further period of 5 hr, the products were extracted with three 5-ml portions of ether and then dialyzed against two 250-ml of distilled water at 20° for 48 hr. No nucleotidic material was found in the dialysate. The dialyzed solution was lyophilized to yield methylated poly-A (9 mg), which had s_{20} 6.0 (original poly-A had s_{20} 4.8); extent of methylation (see below for assay method): 45% (acidic hydrolysis), 42% (alkaline hydrolysis).

B. To a solution of poly-A (K^+ salt, 5 mg) in water (2.5 ml) was added dimethyl sulfate (0.51 g, 300 equiv, in six equal portions at hourly intervals), while the pH was automatically maintained at 7.5. After extraction with three 2.5-ml portions of ether, the products were dialyzed as before, but about 4% of the total nucleotidic material was dialyzable. The nondialyzable material was found to be 76% methylated (acidic hydrolysis assay).

Methylation of Polycytidylic Acid (Poly-C) with Dimethyl Sulfate. A. During a period of 3 hr, dimethyl sulfate (100 mg, *ca.* 50 equiv) was added dropwise to a solution of poly-C (K^+ salt, 5 mg) in water (3 ml), while the pH was automatically maintained at 8 by the addition of 0.5 M KOH. After a further 5 hr, the solution was dialyzed against water (2000 ml) at 5° for 48 hr. No nucleotidic material was detected in the dialysate. The solution of nondialyzable material was lyophilized to give methylated poly-C (6 mg). It had R_F 0.00 (systems E and F) and s_{20} 4.0 (original poly-C had s_{20} 3.0); extent of methylation: 46% (acidic hydrolysis assay).

B. A solution of poly-C (K^+ salt, 10.6 mg) in 0.2 M phosphate buffer (pH 7.2; 3 ml) was treated with dimethyl sulfate (24 mg, 6 equiv) and allowed to stand for 2 hr. No nucleotidic material was found in the dialysate. The nondialyzable solution was lyophilized to give methylated poly-C (8 mg) in which 15% (acidic hydrolysis assay) of the cytosine residues were methylated. Both the original poly-C and the methylated material had s_{20} 3.8.

Methylation of Polyuridylic Acid (Poly-U) with Ethereal Diazomethane. A solution of poly-U (K^+ salt, 3 mg) in aqueous sodium acetate (0.04 M; 5 ml) was shaken with ethereal diazomethane (0.25 M; four 1-ml portions, about 150 equiv). The aqueous layer was separated and dialyzed against water (2000 ml). No

[5] All the polyribonucleotides, which were purchased from the Miles Chemical Co., were dialyzed against (a) distilled water, (b) 0.01 M aqueous sodium chloride, and (c) distilled water again.

[6] This refers to the number of equivalents of methylating agent/atom of phosphorus in the poly-A. This usage of equivalents has also been adopted in the sections dealing with the methylation of poly-C and poly-U (see below).

nucleotidic material was detected in the dialysate. The nondialyzable solution was lyophilized to give methylated poly-U; s_{20} 0.8 (original poly-U had s_{20} 2.0); extent of methylation: 78% (acidic hydrolysis assay).

Assay of Extent of Methylation of Polynucleotides. BY ACIDIC HYDROLYSIS. Methylated polynucleotide (1 mg) was dissolved in 1 N hydrochloric acid (0.1 ml) and the solution was kept under the conditions necessary to effect complete degradation to mononucleotides. The methylated nucleoside 2′(3′)-phosphates were separated from the corresponding unmethylated nucleotides by paper electrophoresis (at a suitable pH) and/or paper chromatography. In each case the resolved fractions were eluted with distilled water and estimated spectrophotometrically at a wavelength near the maximum. The relevant details for the individual polymers are as follows. (i) Methylated poly-A was degraded to a mixture of 1-methyladenosine 2′(3′)-phosphates and adenosine 2′(3′)-phosphates (accompanied by about 10% hydrolysis of the glycosidic linkage of both fractions) after a reaction period of 20 hr at 37°. Paper electrophoresis (pH 7.7) resolved the methylated and unmethylated fractions, which were eluted with distilled water and estimated spectrophotometrically at 260 mμ. Paper chromatography (system D) of the hydrolysate revealed methylated and unmethylated mononucleotides (R_F 0.25 and 0.17, respectively) together with about 10% of a mixture of adenine and 1-methyladenine (both have R_F about 0.6). The latter bases were resolved by paper electrophoresis (pH 7.7). (ii) Methylated poly-C was completely degraded to a mixture of cytidine and 3-methylcytidine 2′(3′)-phosphates after a reaction period of 24 hr at 20°. Paper electrophoresis (phosphate buffer, pH 8) separated the methylated from the unmethylated material. (iii) Methylated poly-U was also completely degraded to a mixture of uridine and 3-methyluridine 2′(3′)-phosphates after a reaction period of 24 hr at 20°. Paper electrophoresis (pH 10.5) separated the methylated from the unmethylated material. The eluted fractions were estimated at 260 mμ.

BY ALKALINE HYDROLYSIS. Methylated poly-A (1 mg) was dissolved in aqueous 1 N sodium hydroxide solution (0.1 ml) and kept at 37° for 20 hr. Paper chromatography (system D) revealed two products corresponding to the 2′(3′)-phosphates of adenosine (R_F 0.21) and N^6-methyladenosine (R_F 0.38).

Pancreatic Ribonuclease Digest of 15% Methylated Poly-C. A solution of 15% methylated poly-C (1 mg) in Tris buffer (0.05 M, pH 7.5, 0.2 ml) was incubated with pancreatic ribonuclease (0.2 mg) for 4 hr at 37°. Paper chromatography (system E) indicated complete degradation of the polymer, and the presence of two products (R_F 0.17 and 0.28). The major component (75%), which had the higher R_F, corresponded to cytidine 3′-phosphate. This was confirmed by paper electrophoresis (phosphate buffer, pH 8); the minor component was less mobile than cytidine 3′-phosphate but slightly more mobile than 3-methylcytidine 2′(3′)-phosphates.

The two components, which had been separated by paper chromatography (system E), were eluted with 0.03 N hydrochloric acid. The eluates were concentrated (about 30 times) and allowed to stand at 20° for 24 hr. The major component was unchanged but the minor component was completely degraded (as shown by paper electrophoresis in phosphate buffer, pH 8) to the 2′(3′)-phosphates of cytidine and 3-methylcytidine. Spectrophotometric assay of the eluted methylated and unmethylated fractions indicated that they were in the proportion of 48:52.

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