The Reversible Chemical Modification of Uracil, Thymine, and Guanine Nucleotides and the Modification of the Action of Ribonuclease on Ribonucleic Acid*

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ABSTRACT: Uridine, uridine 5′-phosphate, thymidine 5′-phosphate, guanosine 5′-phosphate, and deoxyguanosine 5′-phosphate react with *N*-cyclohexyl-*N*′-β-(4-methylmorpholinium)ethylcarbodiimide *p*-toluenesulfonate in water at pH 8–8.5 and, under these conditions, there is no detectable reaction with adenosine or cytidine derivatives. The products of the reaction are shown to be adducts containing one molecule of the carbodiimide reagent attached to the base of the nucleotide and, in the case of the pyrimidine derivatives, an N_3-substituted structure is proposed. The carbodiimide groups can be readily removed from the derivatives by treatment at pH 10–11. The reversible blocking procedure for uridine compounds has been used to impose greater specificity on the action of pancreatic ribonuclease. Uridylyl-(3′–5′)-uridine and uridylyl-(3′–5′)-cytidine are both resistant to ribonuclease degradation after they have been treated with the above reagent and, on the basis of these results, a procedure has been developed by which ribonucleic acid may be cleaved at the cytidine 3′-phosphoryl positions only. In this procedure ribonucleic acid is first treated with the above reagent and then hydrolyzed with ribonuclease.

The enzyme is subsequently removed or destroyed and the blocking groups are removed. The analysis of the products of this procedure shows that those components which arise from cleavage at uridine 3′-phosphoryl bonds in the ribonucleic acid have been reduced to a low level.

The study of the fine structure of nucleic acids is dependent on the availability of methods for the specific degradation of polynucleotide chains and, although there are known enzymes which have useful specificities in their degradative action on ribonucleic acid, the number of such enzymes and the variety of their specificities remain limited. One approach, by which the range of specificity in degradation might be increased, would involve the chemical modification of the substrate or the enzyme in such a way that the specificity of the enzyme action would be either enhanced or changed. In 1962 we briefly described a method by which uridine and guanosine moieties in ribonucleic acid could be specifically and reversibly modified by reaction with *N*-cyclohexyl-*N*′-β-(4-methylmorpholinium)ethylcarbodiimide *p*-toluenesulfonate (CMC-*p*-toluenesulfonate)[1] and we presented some preliminary results on the modification of ribonuclease action caused by such chemical modification of ribonucleic acid (Gilham, 1962). In the present work these reactions are described in more detail and we report a method by which the action of pancreatic ribonuclease can be made more specific in that only cytidine 3′-phosphoryl bonds in RNA are hydrolyzed.

The reported specificity and the mild conditions of the reaction, together with the observation that the modification of uridine and guanosine takes place within the hydrogen-bonding regions of these moieties, has allowed other applications of the reaction. For example, Augusti-Tocco and Brown (1965) have used the extent of the reaction of the CMC reagent with nucleic acids as a measure of the randomly coiled regions or the nonhydrogen-bonded sections in such molecules. The reaction of the CMC reagent with ribonucleic acid has been used in combination with pancreatic ribonuclease to prepare the nine trinucleotides containing a terminal cytidine (Lee *et al.*, 1965). Naylor *et al.* (1965) have reported the use of CMC-*p*-toluenesulfonate for the selective chemical modification of uridine and pseudouridine in polynucleotides and the effect of these modifications on the specificities of ribonuclease and phosphodiesterases. The rate and the extent of reaction between CMC-*p*-toluenesulfonate and RNA have also been observed, together with the effect of such reaction on the amino acid acceptor activity of tRNA (Knorre *et al.*, 1966; Girshovich *et al.*, 1966).

Uridine, uridine 5′-phosphate, thymidine 5′-phosphate, guanosine 5′-phosphate, and deoxyguanosine 5′-phosphate (at 0.2 M concentration) all react to

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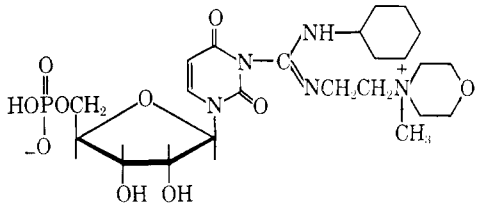
[1] Abbreviations used: CMC, *N*-cyclohexyl-*N*′-β-(4-methylmorpholinium)ethylcarbodiimide; UpU, uridylyl-(3′–5′)-uridine; UpC, uridylyl-(3′–5′)-cytidine; CpU, cytidylyl-(3′–5′)-uridine; UpA, uridylyl-(3′–5′)-adenosine; pU, uridine 5′-phosphate; Up, uridine 3′-phosphate.

completion at room temperature within a few hours in the presence of CMC-*p*-toluenesulfonate (at 1 M concentration) if the reaction mixtures are maintained at pH 8–8.5, although thymidine phosphate reacts about half as fast as the other compounds. All of the above nucleotides have p*K* values in the vicinity of 9 and, since the rates of reaction increase with an increase in the pH of the reaction mixtures, and with a decrease in the p*K* value of the nucleotide, it seems likely that the reacting species is, in each case, the anion of the pyrimidine or purine base. Thus, no reaction could be detected with adenosine and cytidine phosphates under the above conditions; however, it is to be expected that other nucleotides (*e.g.*, inosine phosphate), which also have p*K* values near 9, will react with the carbodiimide reagent in a similar way.

The assignment of the N_3-substituted structure, I, to the product from uridine 5′-phosphate was based



I

mainly on elemental analysis, electrophoretic mobility, and ultraviolet spectra. On electrophoresis at pH 7.5 the derivative had a rate of movement, compared to that of its parent nucleotide, of 0.25, a value not unexpected for a molecule which has about twice the molecular weight and half the net charge of the parent compound. The spectrum of compound I had λ_{max} 264 mμ at pH 7 (Figure 1), and initially this spectrum was unchanged at pH 10.3 suggesting that the molecule no longer contained a dissociable hydrogen atom at the N_3 position. The structurally analogous compound, 1,3-dimethyluracil, has λ_{max} 266 mμ at these pH values, whereas the *O*-substituted uracil derivative, 1-methyl-4-ethoxy-2-pyrimidone, has λ_{max} 274.5 at pH 7 (Shugar and Fox, 1952). An analogous structure can be assigned to the product from thymidine 5′-phosphate since its λ_{max} of 270 mμ at pH 7–11 (Figure 2) compares well with the λ_{max} value of 272 mμ (at pH 5–12) given for the structurally similar compound, 1,3-dimethylthymine (Wittenburg, 1966). In the case of the products from the guanine nucleotides, their structures may also consist of CMC groups substituted at the N_1 position, the position equivalent to the N_3 position in the pyrimidines, since it is clear from the spectra (Figure 3) that the products no longer contain the dissociable hydrogen atom at the N_1 position. All of the CMC derivatives can be hydrolyzed back to the parent compounds by treatment at pH 10–10.5 although, here again, the thymidine derivative is slow to react and requires a higher pH than that required by the other derivatives in order to achieve a reasonable rate of hydrolysis. The spectral changes on hydrolysis are shown in Figures 1–3.

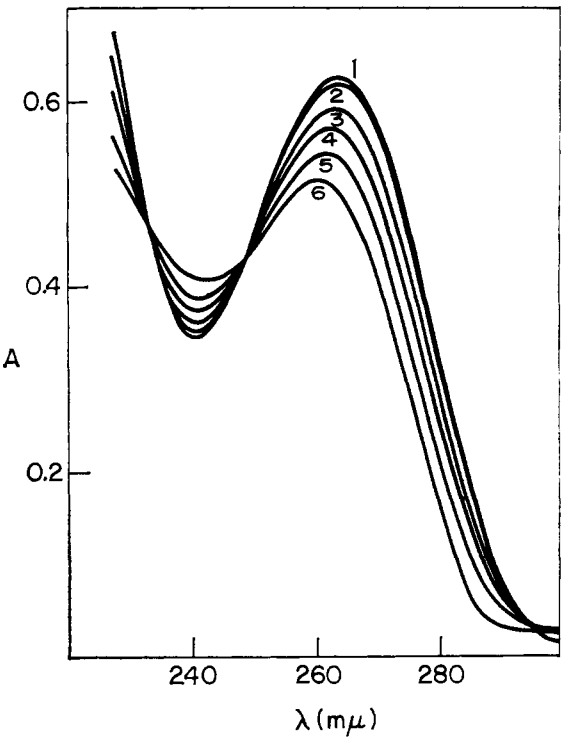


FIGURE 1: Curve 1 represents the spectrum of CMC-uridine 5′-phosphate in 0.02 M sodium phosphate buffer (pH 7) and also its initial spectrum in 0.2 M sodium carbonate buffer (pH 10.3). Curves 2, 3, 4, 5, and 6 are the spectra of this compound after standing at 25° in the sodium carbonate buffer for 1, 30, 60, 120, and 270 min, respectively. After 270 min there was no further change in the spectrum with time.

The specificity of pancreatic ribonuclease is such that, in ribonucleic acid, only uridine 3′-phosphoryl and cytidine 3′-phosphoryl bonds are cleaved, and the discovery of the specific blocking reaction of CMC-*p*-toluenesulfonate with uridine moieties offered a potential method by which this specificity might be restricted to the cleavage of cytidine 3′-phosphoryl bonds only. However, for maximum efficiency in such a procedure, there are three main conditions which should be satisfied. (a) The reagent should be capable of blocking the uridine residues in RNA without interfering with the internucleotide linkages. (b) The 3′-phosphoryl bonds adjacent to the blocked uridine residues should be completely resistant to hydrolysis by the enzyme. (c) The presence of the blocked uridine groups should not interfere with the normal cleavage of the cytidine 3′-phosphoryl bonds.

These requirements were tested in a study of the reactions of three dinucleoside phosphates. UpU, on reaction with the CMC reagent, gave a disubstituted derivative which was completely resistant to the action of ribonuclease. On hydrolysis, however, the recovered UpU was found to be completely degradable by ribonuclease, a result which demonstrated that the 3′–5′-

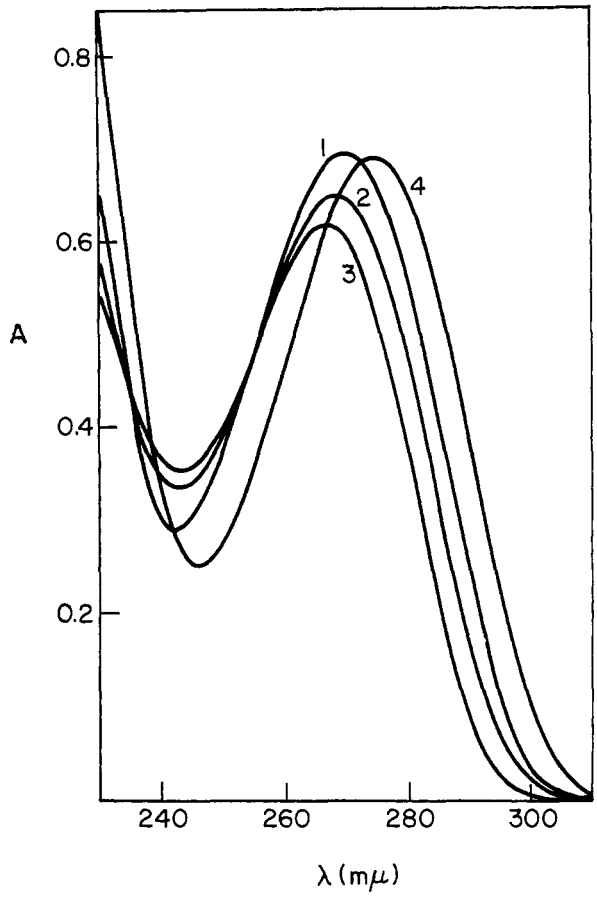


FIGURE 2: Curve 1 represents the spectrum of CMC-thymidine 5′-phosphate in 0.02 M sodium phosphate buffer (pH 7) and also its initial spectrum in 0.2 M sodium carbonate buffer (pH 10.9). Curves 2 and 3 are spectra of the derivative after standing at 25° in the sodium carbonate buffer for 2.5 and 8 hr, respectively. After 8 hr there was no further change in the spectrum with time. Curve 4 is the spectrum of the same concentration of CMC-thymidine 5′-phosphate in dilute hydrochloric acid (pH 1.5).

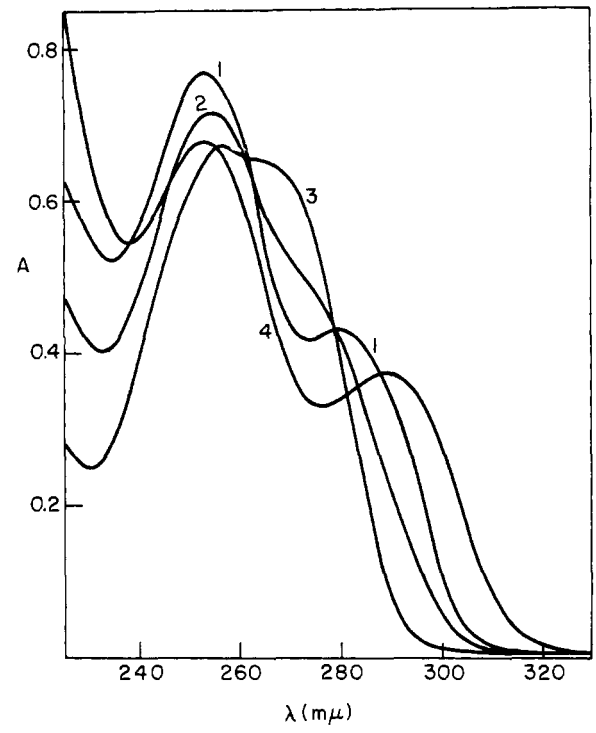


FIGURE 3: Curve 1 represents the spectrum of CMC-guanosine 5′-phosphate in 0.02 M sodium phosphate buffer (pH 7) and also the initial spectrum of the compound in 0.2 M sodium carbonate buffer (pH 10.3). Curves 2 and 3 are spectra of the derivative after standing at 25° in the sodium carbonate buffer for 20 and 120 min, respectively. After 120 min there was no further change in the spectrum with time. Curve 4 is the spectrum of an equal concentration of the compound in dilute hydrochloric acid (pH 1.5). The spectra of CMC-deoxyguanosine 5′-phosphate were identical with those of CMC-guanosine 5′-phosphate.

internucleotide linkage had not been affected during the blocking reaction or during the removal of the CMC groups. UpC gave a mono-CMC derivative and was also resistant to enzymic hydrolysis. Again, after hydrolysis, the reformed UpC could be completely degraded enzymatically to uridine 3′-phosphate and cytidine. On the other hand, the mono-CMC derivative of CpU was hydrolyzed by ribonuclease in the normal way to yield cytidine 3′-phosphate and CMC-uridine, a result which demonstrated that the cleavage of cytidine 3′-phosphoryl bonds is not affected by a neighboring blocked uridine residue.

In the application of this procedure to ribonucleic acid it was, at first, necessary to develop a method for the complete removal of ribonuclease activity from enzyme digests so that, subsequent to the incorporation of the CMC groups and the ribonuclease hydrolysis, the blocking groups could be removed from the uridine moieties without the risk of further enzyme hydrolysis. Although extraction with phenol is an efficient method for removing the enzyme from ribonuclease digests it was not useful in this case since it was found that, in a mixture of phenol and water, oligonucleotides which were carrying CMC groups were more soluble in the phenol-rich phase than in the water-rich phase. Two alternative methods were developed. The first procedure allows for the removal of the CMC groups under conditions where the residual enzyme is inactive. The CMC groups are removed from the oligonucleotides by treatment of the enzyme digest with formamide and diethylamine. The solvents are then evaporated and the enzyme is removed by extraction with phenol in the usual way. The second method is found to be even more efficient and has the added advantage in that there is no loss of nucleotide material (Lee *et al.*, 1965). In this approach the ribonuclease is destroyed by the proteolytic enzyme preparation, Pronase. It is found that, while it is not possible to remove

ribonuclease activity from solution by direct treatment with Pronase, the enzyme activity can be completely destroyed if the ribonuclease is first treated with a reducing agent (mercaptoethanol) in the presence of a denaturing solution (50% dimethylformamide). In each of the above experiments the amount of residual ribonuclease activity can be measured by observing the extent of hydrolysis of uridylyl-(3′–5′)-adenosine to uridine 2′,3′-cyclic phosphate and adenosine.

In the initial experiments on ribonucleic acid the nucleic acid was treated with CMC-*p*-toluenesulfonate and then with ribonuclease, and the enzyme was removed by the diethylamine procedure. The products were separated on a DEAE-cellulose column in the presence of 7 M urea (expt A, Figure 4). For comparison, a similar experiment was carried out except that the products were retreated with ribonuclease before fractionation on the column (expt B, Figure 4). It can be seen that in the first experiment there are less of the smaller and more of the larger oligonucleotides compared to those in the second experiment, a result which is consistent with the expected change in specificity of the enzymic degradation caused by the blocking groups. It will also be noted that the separation of the oligonucleotides of different chain lengths is less precise in the first experiment. This is to be expected also, since, unlike the products from expt B, the type of products in expt A should not have identical purine:pyrimidine ratios for a given chain length. It has previously been shown that, for DEAE-cellulose chromatography, identical purine:pyrimidine ratios for molecules of the same chain length is a requirement for the fractionation of oligonucleotides into peaks of equal chain length (Bartos *et al.*, 1963). The main components present in the mono- and dinucleotide peaks in the two experiments are listed in Table I. Experiment A produced molecules terminating in cytidine 3′-phosphate only, while expt B gave the regular set of ribonuclease products.

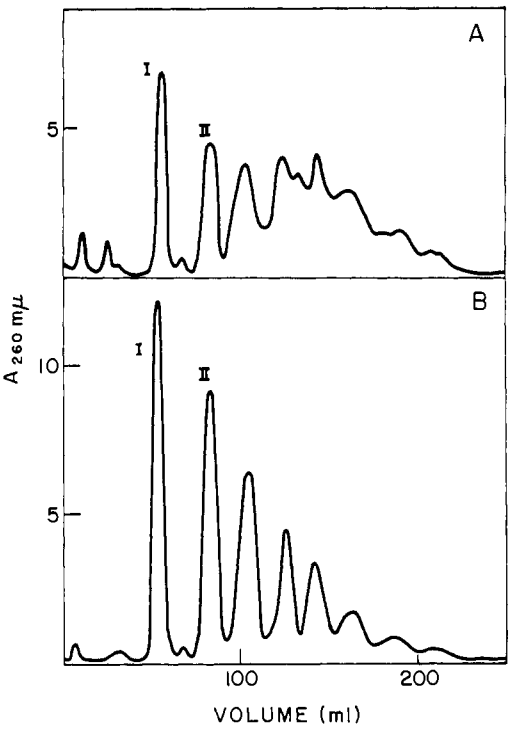


FIGURE 4: Elution pattern. (A) The elution pattern from the chromatography of the ribonuclease digest of CMC-tRNA on a DEAE-cellulose column (0.9 × 21 cm). The solvent was 7 M urea–0.01 M Tris-chloride (pH 7.5, 400 ml) containing a linear gradient of sodium chloride from 0 to 0.5 M. Flow rate, 6 ml/hr. (B) Elution pattern of the chromatography of a ribonuclease digest of the same amount of CMC-tRNA which was retreated with ribonuclease after the CMC groups had been removed. The column and the eluting solvent were identical with those used for A above.

In a more definitive set of experiments RNA was treated with the CMC reagent and hydrolyzed with ribonuclease, the enzyme being subsequently destroyed by the Pronase procedure. The blocking groups were removed and the mono- and dinucleotides separated by paper chromatography and paper electrophoresis (expt I, Table II). Experiment II was carried out in an identical way except that, after the removal of the CMC groups, the products were retreated with ribonuclease. Experiment III represents a control experiment in which the same amount of untreated RNA was hydrolyzed directly with ribonuclease. It can be seen that, as expected, the quantities of uridine 3′-phosphate and the dinucleotides terminating in uridine 3′-phosphate are greatly reduced in expt I while a new dinucleotide, UpCp, is found to be present. The amounts of the products in expt II and III are essentially the same, demonstrating that the CMC reaction and the deblocking reaction do not greatly affect other structural aspects of the molecules. One exception to this is the case of pseudouridine which has previously been shown to form a stable adduct with the carbodiimide reagent (Naylor *et al.*, 1965) and thus, pseudouridine 3′-phosphate does not appear in the products of expt I or II.

The specificity exhibited by the CMC reagent for uracil, thymine, and guanine nucleotides may well be of use in the modification of the actions of other enzymes. It has already been shown that CMC-blocked nucleotides are capable of halting the normal hydrolytic progression of exonucleases along a polynucleotide chain. Also, recent work in this laboratory has demonstrated that the blocking of a guanosine moiety in RNA confers resistance to hydrolysis by the enzyme, ribonuclease T_1, at that position.

In conclusion, it should be pointed out that CMC-*p*-toluenesulfonate and similar reagents show considerable versatility in that they exhibit a completely different type of specificity when used in solutions of pH values below 7. Under such conditions the bases remain unaffected and the reagents become specific activating agents for terminal phosphate groups in nucleotides and polynucleotides. This activation can be used for the formation of 2′,3′-cyclic phosphates from 2′- or 3′-

TABLE I: Mono- and Dinucleotides from DEAE-cellulose Chromatography.

Peak	RNase Digest of CMC-tRNA (Figure 4A) Total $ODU_{260\ m\mu}$	Components	RNase Digest of CMC-tRNA Redigested with RNase (Figure 4B) Total $ODU_{260\ m\mu}$	Components	Electrophoretic Mobility (pH 3.0) Rel to Up	R_F Value in Solvent D Rel to R_F Value of Cp
I	36		79	Up	1.00	1.00
		Cp		Cp	0.19	1.00
II	46	ApCp	83	ApCp	0.22	0.82
				ApUp	0.62	0.82
		GpCp		GpCp	0.51	0.62
				GpUp	1.03	0.62
		UpCp			0.70	0.82

phosphates and the formation of phosphodiesters or internucleotide linkages from 5′-phosphates (Naylor and Gilham, 1966).

Experimental Section

Materials. *N*-Cyclohexyl-*N*′-β-(4-methylmorpholinium)ethyl carbodiimide *p*-toluenesulfonate was purchased from Aldrich Chemical Co., Milwaukee, Wis. Pancreatic ribonuclease was the product of Worthington Biochemical Corporation, Freehold, N. J., and Pronase was obtained from Calbiochem, Los Angeles, California. Yeast tRNA was purchased from General Biochemicals, Chagrin Falls, Ohio, and was purified by passage through a DEAE-cellulose column in 1 M sodium acetate–0.15 M Tris-acetate solution (pH 7.5). The RNA was recovered from the eluate by precipitation with three volumes of ethanol. Uridylyl-(3′–5′)-adenosine was prepared from the condensation of 2′,5′-diacetyluridine 3′-phosphate with 2′,3′-diacetyladenosine in the presence of dicyclohexylcarbodiimide using the method described by Rammler *et al.* (1963) for the synthesis of UpU. The product was purified by chromatography on Whatman 3MM paper with solvent B. All reactions described below were carried out at 25° unless specified to the contrary.

TABLE II: Quantitative Analysis of Products from the Hydrolysis of CMC-tRNA with Ribonuclease.

Paper Chromatographic Band	Components	Total $ODU_{260\ m\mu}$ Obtained from 5 mg of tRNA I	II	III
A	Up	<0.6	9.1	8.4
	Cp	9.5	13.9	12.9
B	ψp	<0.1	<0.1	2.2
	ApUp	<0.1	4.3	4.3
	UpCp	4.8	<0.1	<0.1
	ApCp	4.5	5.0	4.6
C	GpUp	<0.3	6.8	7.1
	GpCp	6.7	8.2	8.4

Reaction of CMC-p-toluenesulfonate with Nucleotides. A. URIDINE 5′-PHOSPHATE. CMC-*p*-toluenesulfonate (800 mg) was added to a solution of disodium uridine 5′-phosphate (0.4 mmole) in water (2 ml) and the pH of the stirred mixture was kept at 8–8.5 by the periodic addition of *p*-toluenesulfonic acid. For analysis, aliquots of 0.1 ml were withdrawn at various times and added to 0.1 M sodium phosphate buffer (pH 7.0) (0.1 ml). Analysis of these samples by paper chromatography in solvent A showed that the reaction was complete in 3–4 hr. For the isolation of the product, the reaction mixture was finally brought to pH 7 by the addition of *p*-toluenesulfonic acid. The solution was diluted with water and freeze dried, and the product was dissolved in 95% ethanol. This mixture was chromatographed on a column (30 × 2 cm) of cellulose powder (Whatman, Standard Grade) with 95% ethanol as the eluting solvent. The *p*-toluenesulfonates of the carbodiimide and its corresponding urea were eluted first followed by the CMC-uridine 5′-phosphate. The product was concentrated by evaporation and dissolved in methanol. The pH of the solution was adjusted to 5–6 with acetic acid and the product was precipitated by the addition of excess ether. The product was then dissolved in water and freeze dried (105 mg). *Anal.* Calcd for $C_{23}H_{38}N_5O_{10}P$: C, 48.0; H, 6.6; N, 12.2. Found: C, 47.9; H, 6.7; N, 12.2. On paper electrophoresis in 0.05 M sodium phosphate buffer (pH 7.5) the ratio of the mobility of the product to that of uridine 5′-phosphate was 0.25. In 0.02 M sodium phosphate buffer (pH 7) the compound had λ_{max} 264 mμ (ε 9600) and in dilute hydrochloric acid (pH 1.5) the product had λ_{max} 269 mμ. In 0.2 M sodium carbonate buffer, pH 10.3, the spectrum was initially identical with that at pH 7 but slowly changed over

5 hr to a spectrum indistinguishable from that of uridine 5′-phosphate at pH 10.3. The product from this hydrolysis was shown to be uridine 5′-phosphate by paper chromatography in the solvents A and B. Uridine was also found to react under the above conditions to give an analogous product which could be shown to have a net positive charge by virtue of its migration toward the negative electrode on paper electrophoresis at pH 7. This product had λ_{max} 264 mμ (pH 7) and 268 mμ (pH 1.5) and could be converted back to uridine by hydrolysis at pH 10 as described above.

B. GUANOSINE 5′-PHOSPHATE. CMC-*p*-toluenesulfonate (2 g) was added to a solution of disodium guanosine 5′-phosphate (1 mmole) in water (5 ml) and the pH of the stirred mixture was kept at 8–8.5 by the addition of *p*-toluenesulfonic acid. Analysis of the mixture as described above for uridine 5′-phosphate showed that the reaction was complete in 2–3 hr. In 0.02 M sodium phosphate buffer (pH 7) the CMC-pG had λ_{max} 252 mμ ($\epsilon_{252\ m\mu}$ 13,500) and 279 mμ ($\epsilon_{279\ m\mu}$ 7600). In dilute hydrochloric acid (pH 1.5) the product had λ_{max} 253 mμ ($\epsilon_{253\ m\mu}$ 11,900) and 289 mμ ($\epsilon_{289\ m\mu}$ 6500). In 0.2 M sodium carbonate buffer (pH 10.3) the derivative had an initial spectrum identical with that at pH 7. On standing, the spectrum changed over a period of 2 hr to an alkali-stable curve which was identical with the spectrum of guanosine 5′-phosphate at pH 10.3. Chromatographic analysis of this hydrolysis confirmed that the product was guanosine 5′-phosphate.

C. THYMIDINE 5′-PHOSPHATE. CMC-*p*-toluenesulfonate (0.8 g) was added to a solution of disodium thymidine 5′-phosphate (0.4 mmole) in water (2 ml) and the pH of the stirred mixture was kept at 8–8.5 by the addition of *p*-toluenesulfonic acid. Analysis of the mixture as described above for uridine 5′-phosphate showed that the reaction was complete in 8 hr. In 0.02 M sodium phosphate buffer (pH 7) the CMC-pT had λ_{max} 270 mμ ($\epsilon_{270\ m\mu}$ 8800), and in dilute hydrochloric acid (pH 1.5) the product had λ_{max} 274.5 mμ ($\epsilon_{274.5\ m\mu}$ 8800). In 0.2 M sodium carbonate buffer (pH 10.9) the product had an initial spectrum identical with that at pH 7. On standing over a period of 10 hr the spectrum changed to an alkali-stable curve which was identical with the spectrum of thymidine 5′-phosphate at pH 10.9. The product of this hydrolysis was confirmed as thymidine 5′-phosphate by chromatographic analysis.

D. DEOXYGUANOSINE 5′-PHOSPHATE. CMC-*p*-toluenesulfonate (800 mg) was added to a solution of deoxyguanosine 5′-phosphate (0.4 mmole) in water (2 ml) and the pH of the stirred mixture was kept at 8–8.5 by the addition of *p*-toluenesulfonic acid. Analysis of the mixture as described above for uridine 5′-phosphate showed that the reaction was complete in 5 hr. In 0.02 M sodium phosphate buffer (pH 7) the product had λ_{max} 252 mμ ($\epsilon_{252\ m\mu}$ 13,500) and 279 mμ ($\epsilon_{279\ m\mu}$ 7600), and in dilute hydrochloric acid (pH 1.5) the compound had λ_{max} 253 mμ ($\epsilon_{253\ m\mu}$ 11,900) and 289 mμ ($\epsilon_{289\ m\mu}$ 6500). In 0.2 M sodium carbonate buffer (pH 10.3) the product had an initial spectrum identical with that at pH 7 and, on standing, the spectrum changed over a period of 2 hr to an alkali-stable curve which was identical with the spectrum of deoxyguanosine 5′-phosphate at pH 10.3. The product of this hydrolysis was confirmed to be deoxyguanosine 5′-phosphate by chromatographic analysis.

Reaction of CMC-p-toluenesulfonate with UpU. UpU was prepared using the ribonuclease-catalyzed exchange reaction described by Heppel *et al.* (1955). Ammonium uridine 2′,3′-cyclic phosphate (0.1 mmole) and uridine (98 mg) were dissolved in 0.015 M Tris-chloride buffer (pH 7, 5 ml) and incubated at 37° with pancreatic ribonuclease (0.1 mg) for 75 min. The mixture was then shaken with isoamyl alcohol (0.6 ml) and chloroform (0.15 ml) and streaked on Whatman 3MM paper (60 cm). The mixture was chromatographed with solvent B and the dinucleoside phosphate band was cut out and eluted with water (yield, 63 ODU at 262 mμ). The product was found to be completely degradable to uridine 3′-phosphate and uridine on treatment with excess ribonuclease. UpU (25 ODU at 262 mμ) in water (1.5 ml) was treated with CMC-*p*-toluenesulfonate (60 mg) and the pH of the mixture kept at 8 for 8 hr. The mixture was applied to Whatman 3MM paper, and electrophoresis in 0.05 M sodium phosphate buffer (pH 7.5) gave a single product which moved 4 cm toward the negative electrode while the toluenesulfonate ion moved 6 cm toward the positive electrode. The product remained undegraded by incubation at 37° with ribonuclease (0.2 mg) in water (1 ml) for 5 hr. However, on treatment with dilute ammonia at pH 11 for 7 hr, it was hydrolyzed to a compound which was chromatographically identical with UpU and which could be degraded completely to uridine 3′-phosphate and uridine with pancreatic ribonuclease.

Reaction of CMC-p-toluenesulfonate with UpC. UpC (84 ODU at 264 mμ) was prepared from uridine 2′,3′-cyclic phosphate (0.1 mmole), cytidine (100 mg), and ribonuclease (0.1 mg) in 0.015 M Tris-chloride buffer (pH 7.5, 5 ml) as described above for UpU. UpC (40 ODU) (264 mμ) in water (1.5 ml) was treated with CMC-*p*-toluenesulfonate (60 mg) and the pH of the mixture was kept at 8 for 5 hr. Paper electrophoresis in 0.05 M sodium phosphate buffer (pH 7.1) showed that the UpC had been completely converted to a product which moved 1.5 cm toward the negative electrode while, on the same paper, a sample of the starting material moved 6.5 cm toward the positive electrode. This derivative was unchanged by incubation at 37° with pancreatic ribonuclease (0.2 mg) in water (1.5 ml) for 5 hr. However, on treatment with 0.03 M sodium carbonate buffer (pH 10.5) for 10 hr the CMC derivative was hydrolyzed to a compound chromatographically and electrophoretically identical with UpC, and this product, on treatment with pancreatic ribonuclease (0.1 mg) for 5 hr at 37° in 0.02 M Tris-chloride buffer (pH 7.5, 0.5 ml), gave a mixture of uridine 3′-phosphate and cytidine only, the products of the digestion being identified by paper chromatography in solvent B.

Reaction of CMC-p-toluenesulfonate with CpU. CpU (63 ODU at 265 mμ) was prepared from cytidine

2′,3′-cyclic phosphate (0.13 mmole), uridine (98 mg), and ribonuclease (0.1 mg) in 0.015 M Tris-chloride buffer (pH 7.5, 5 ml) as described above for UpU. CpU (60 ODU) in water (1 ml) was treated with CMC-*p*-toluenesulfonate (40 mg) and the pH of the mixture was kept at 8 for 5 hr. Paper electrophoresis in 0.05 M sodium phosphate buffer (pH 7.5) showed that the CpU had been completely converted to a product which moved 1.5 cm toward the negative electrode while, on the same paper, a sample of the starting material moved 6.5 cm toward the positive electrode. On incubation at 37° for 5 hr in water (1.5 ml) at pH 7 with pancreatic ribonuclease (0.2 mg) the CMC-CpU was completely degraded to two compounds which were identified by paper chromatography and by ultraviolet spectra as Cp and CMC-U, the latter being compared with the sample of CMC-U prepared as described above.

Preparation of CMC-tRNA and Its Hydrolysis with Pancreatic Ribonuclease. tRNA (100 mg) and CMC-*p*-toluenesulfonate (1 g) were dissolved in water (7 ml). The mixture (together with a few drops of toluene to prevent microbial contamination) was kept at pH 8.5 for 18 hr by the periodic addition of dilute hydrochloric acid. The pH of the solution was then brought to 6 by the addition of dilute acid and the mixture was dialyzed at 4° against 0.01 M sodium chloride solution (6 l., pH 5–6) for 10 hr and then against distilled water (two 6-l. portions, 6 hr each). The product was concentrated to about 8 ml and pancreatic ribonuclease (5 mg) was added together with a few drops of toluene. The mixture was kept at pH 6–7 for 20 hr and then frozen.

Removal of Ribonuclease Activity. A. PHENOL TREATMENT. The pancreatic ribonuclease digest of CMC-tRNA (from 50 mg of tRNA) in water (4 ml) was treated with phenol (3 g) and shaken. The aqueous layer was removed and the phenol layer was washed with 0.05 M sodium chloride solution (three 3-ml portions). The aqueous extracts were combined and washed with ether to remove phenol. Spectrophotometric analysis showed that only 25% of the RNA had been recovered in the combined extracts. Incubation of one-fifth of this extract with UpA (9 ODU, 260 mμ) for 5 hr followed by paper chromatography in solvent B showed partial hydrolysis of the substrate to adenosine and uridine 2′,3′-cyclic phosphate, demonstrating the presence of some residual ribonuclease in the extract.

B. PRONASE TREATMENT. Ribonuclease (1 mg) in water (1 ml) was treated with Pronase (2.5 mg) at pH 7.0 for 50 hr. A portion (0.2 ml) of this mixture was then incubated with UpA (9 ODU at 260 mμ) for 5 hr. Paper chromatography in solvent B showed *ca.* 50% hydrolysis of the UpA.

C. PHENOL TREATMENT AFTER REMOVAL OF CMC GROUPS. The solution of the pancreatic ribonuclease digest of CMC-tRNA (from 21 mg of tRNA) was evaporated to dryness *in vacuo* and taken up in formamide (2 ml). Diethylamine (0.2 ml) was added and the solution was allowed to stand for 18 hr and then concentrated to dryness *in vacuo*. Liquid phenol (2 ml) was added to the residue and the resulting mixture was extracted with 0.1 M ammonium bicarbonate (pH 8.5). The aqueous layer was removed and washed again with phenol (2 ml). The aqueous layer was removed and the two phenol layers were each washed twice with 2-ml portions of the ammonium bicarbonate buffer. The aqueous layers were combined and washed with ether to remove phenol. The recovery of nucleotide material was 70–90%. The extract was tested with UpA as described above and no hydrolysis of the substrate could be detected.

D. TREATMENT WITH MERCAPTOETHANOL AND PRONASE. Ribonuclease (1 mg) dissolved in water (1 ml) was treated with dimethylformamide (1 ml) and mercaptoethanol (0.1 ml) and kept under nitrogen for 24 hr. The solution was then evaporated to dryness *in vacuo* and the residue was dissolved in degassed water (1 ml). The product was again concentrated to dryness, dissolved in water (1 ml), and treated with Pronase (1 mg) and mercaptoethanol (0.01 ml) under nitrogen. Aliquots of 0.1 ml were removed at various times. To each aliquot was added a solution of UpA (36 $ODU_{260\ m\mu}$ in 0.1 ml of water); the resulting mixture was allowed to stand for 20 hr and then chromatographed on paper in solvent B. There was essentially no hydrolysis of the UpA after 48-hr incubation with Pronase. In a similar experiment except that no mercaptoethanol was added to the Pronase digestion mixture, there was still about 10% hydrolysis of UpA after 48-hr incubation with Pronase.

Fractionation of Ribonuclease Digest of CMC-tRNA on DEAE-cellulose. tRNA (100 mg) was modified with CMC-*p*-toluenesulfonate and hydrolyzed with ribonuclease as described above. The CMC groups and the enzyme were then removed by the diethylamine–phenol procedure (method C) and the product was concentrated to 7 ml. Sufficient urea was added to 2 ml of this product to make the final urea concentration 7 M and the solution was then added to a column (0.9 × 21 cm) of DEAE-cellulose (0.83 mequiv/g) which had been packed with 7 M urea–0.01 M Tris-chloride solution (pH 7.5). Elution was carried out with the 7 M urea–0.01 M Tris-chloride solution (pH 7.5, 400 ml) containing a linear gradient of sodium chloride from 0 to 0.5 M at a flow rate of 6 ml/hr. The elution profile is shown in Figure 4A. The material in the mono- and dinucleotide peaks (I and II, the first two major peaks) were obtained free of urea and salt by dilution of the peak fractions and readsorption of the components to a DEAE-cellulose column of the same size. The urea and salt were removed by washing with 0.05 M triethylamine bicarbonate solution (pH 7.5). The nucleotide material was recovered by elution of the column with 0.5 M triethylamine bicarbonate. The main components of these fractions were determined by paper electrophoresis in 0.05 M ammonium formate buffer (pH 3.0) and by comparison of their spectra and R_F values with those of authentic compounds (Table I). In addition, the identity of UpCp was confirmed by its hydrolysis with ribonuclease to Up and Cp.

TABLE III: R_f Values.

Compound	R_F Values		
	Solvent A	Solvent B	Solvent C
U	0.73	0.44	0.39
Up		0.10	
pU	0.29		0.16
CMC-U	0.81		0.66
CMC-pU	0.64		0.35
pT	0.41		0.26
CMC-pT	0.72		0.42
pG	0.16		0.12
CMC-pG	0.53		0.30
d-pG	0.20		0.18
CMC-d-pG	0.57		0.36
C		0.43	
Cp	0.23		0.09
UpC	0.39	0.20	
CMC-UpC	0.70		0.20
UpU		0.20	
CpU	0.42	0.18	
CMC-CpU	0.73		0.20
UpA	0.40	0.22	0.10
U>p		0.3	
A		0.59	

Another 2 ml of the diethylamine–phenol-treated material was fractionated in the same way except that, prior to the addition of the urea and the DEAE-cellulose chromatography, the sample was again treated with ribonuclease (1 mg) at pH 7 for 24 hr. The elution pattern of the DEAE-cellulose chromatography is shown in Figure 4B and the main components in the first two major peaks are given in Table I.

Quantitative Analysis of Mono- and Dinucleotides from Ribonuclease Digestion of CMC-tRNA. In expt I the ribonuclease digest of CMC-tRNA (obtained from 5 mg of tRNA) dissolved in water (0.3 ml) was treated with dimethylformamide (0.3 ml) and mercaptoethanol (0.03 ml), and the enzyme was destroyed by the mercaptoethanol–Pronase method as described above. After the 48-hr Pronase digestion the product was concentrated to remove mercaptoethanol, dissolved in water (0.5 ml), and mixed with concentrated ammonia solution (0.5 ml). The mixture was allowed to stand for 1 hr to remove the CMC groups and then concentrated to a small volume and applied as a band to Whatman 3MM chromatographic paper (23 cm). Chromatographic elution with solvent D gave a number of bands, the first three of which, A, B, and C, had R_F values (relative to the R_F value of cytidine 3′-phosphate) of 1.00, 0.82, and 0.62, respectively. Each band was eluted and fractionated by paper electrophoresis in 0.05 M ammonium formate buffer (pH 3.0) and the products were identified as described above. The identity of the components and their quantities are given in Table II. A second experiment (expt II, Table II) was carried out in an identical way with the same amount of RNA except that, prior to the chromatographic separation, the product was re-treated with ribonuclease (0.5 mg) for 20 hr at pH 7. A third experiment (expt III) involved the direct hydrolysis of RNA (5.0 mg) in 0.03 M sodium phosphate buffer (pH 7) with ribonuclease (0.5 mg) for 20 hr. The products were analyzed as above and the quantities obtained are listed in Table II.

Chromatography. Paper chromatography was carried out by the descending technique on Whatman No. 1 paper with the following solvent systems: (A) ethyl alcohol (70 ml)–1 M ammonium acetate (pH 7.5, 30 ml), (B) isopropyl alcohol (70 ml)–concentrated ammonia (10 ml)–water (20 ml), (C) *n*-butyl alcohol (50 ml)–acetic acid (20 ml)–water (30 ml), and (D) *n*-propyl alcohol (55 ml)–concentrated ammonia (10 ml)–water (35 ml) (see Table III).

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