The possibility that this inhibitor may have an effect on uridine utilization, mediated through its uptake or conversion into other uridine-containing metabolites, cannot be excluded. Our present studies are directed toward further characterization of this inhibitory factor and to a further study of its mode of action.

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Reaction of Pseudouridine and Inosine with *N*-Cyclohexyl-*N'*-β-(4-methylmorpholinium)ethylcarbodiimide*

Nancy W. Y. Ho and P. T. Gilham†

ABSTRACT: Inosine or its 5'-phosphate reacts with the *p*-toluenesulfonate salt of *N*-cyclohexyl-*N'*-β-(4-methylmorpholinium)-ethylcarbodiimide in aqueous solution at pH 8–8.5. The product of the reaction is a 1:1 adduct in which the nucleoside or its phosphate is attached to the substituted carbodiimide cation (presumably at the N_1 position of the nucleoside base). The reaction of the reagent with pseudouridine is more complicated in that two monosubstituted compounds (N_1 and N_3) are formed initially and these react further to give the N_1,N_3-disubstituted derivative. This product is unstable at the pH of the reaction mixture and partly hydrolyzes to give the stable N_3-substituted compound. The N_3 derivative is stable under the hydrolytic conditions which will remove carbodiimide groups from uridine, guanosine, inosine, and 5-methyluridine, and this property thus constitutes a method for the specific chemical modification of pseudouridine positions in RNA molecules. In addition, the N_3 substitution of pseudouridine in a polynucleotide confers resistance to the action of pancreatic ribonuclease at the phosphodiester linkage attached to the 3' position of the pyrimidine nucleoside. Pseudouridylyl-(3'–5')-adenosine derivatized in this way is not attacked by this ribonuclease whereas derivatized cytidylyl-(3'–5')-pseudouridine is converted by action of the enzyme into cytidine 3'-phosphate and the carbodiimide derivative of pseudouridine.

Those ribonucleosides and deoxyribonucleosides whose bases have p*K* values in the vicinity of 9 have been shown to react with the Cmc[1] cation *N*-cyclohexyl-*N'*-β-(4-methylmorpholinium)ethylcarbodiimide (I) (see Chart I) (Gilham, 1962; Lee *et al.*, 1965; Naylor *et al.*, 1965; Ho and Gilham, 1967; Ho *et al.*, 1969). The reactions proceed rapidly in water at pH 8–9 and under these conditions nucleosides such as adenosine and cytidine and their deoxyribo counterparts do not react. The products are thought to be stable guanidine derivatives in which the carbodiimide group of the Cmc cation is substituted at the N_3 positions in pyrimidines (uridine and thymidine) and at the N_1 positions in the purines (guanosine and deoxyguanosine) (Gilham, 1962; Ho and Gilham, 1967). The phosphate derivatives of these nucleosides undergo a similar reaction as do the guanine, uracil, and thymine moieties in polynucleotides and nucleic acids. In the case of polynucleotides and nucleic acids, the conditions under which these chemical modifications are made and the conditions necessary for the subsequent removal of the Cmc groups are of a sufficiently mild nature that the structural integrity of the poly-

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[1] Abbreviation used is: Cmc, *N*-cyclohexyl-*N'*-β-(4-methylmorpholinium)ethylcarbodiimide.

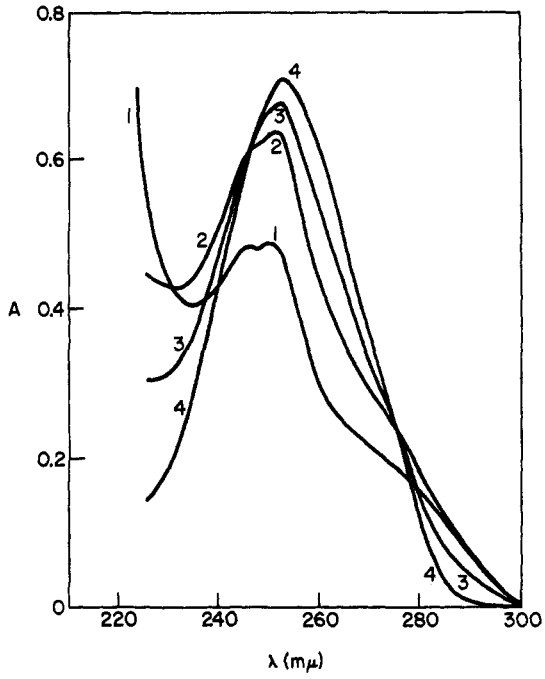
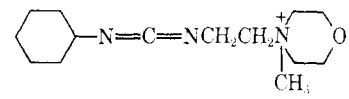
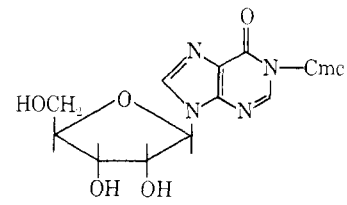


FIGURE 1: Curve 1 is the spectrum of 1-Cmc-inosine 5′-phosphate in 0.02 M sodium phosphate buffer (pH 7) and its initial spectrum in 0.2 M sodium carbonate buffer (pH 10.3). Curves 2, 3, and 4 are spectra of 1-Cmc-inosine 5′-phosphate after standing in 0.2 M sodium carbonate buffer (pH 10.3) at 25° for 1, 5, and 60 min, respectively. No further change in the spectrum was observed after 60 min.

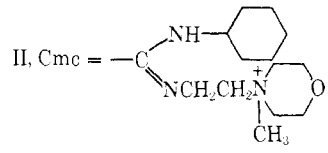
CHART I

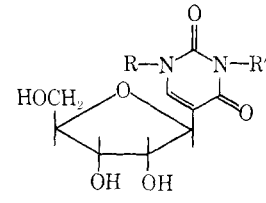


I



II, Cmc =





III, R = R′ = Cmc
IV, R = H; R′ = Cmc
V, R = Cmc; R′ = H

mers can be preserved. Thus, it has been possible to use this type of chemical modification to restrict, in a specific way, the normal activities of ribonucleases and phosphodiesterases (Naylor *et al.*, 1965; Ho and Gilham, 1967; Ho *et al.*, 1969) and these techniques have proved to be of some value in the determination of nucleotide sequences in RNA molecules (Gilham, 1970). The present work extends the study of this type of chemical modification to two of the less common nucleosides, inosine and pseudouridine, which occur in tRNA molecules. Preliminary reports of these studies have been published (Naylor *et al.*, 1965; Ho *et al.*, 1969).

Inosine and inosine 5′-phosphate react with the water-soluble carbodiimide at rates similar to those displayed by uridine and its 5′-phosphate. The reactions are complete in about 2 hr and the product in each case can be shown, by paper electrophoresis, to contain an extra positive charge compared to the starting material. From an analysis of their ultraviolet spectra at neutral and alkaline pH values (Figure 1) it can be seen that the products no longer possess dissociable H atoms at the N_1 positions and thus they are assigned the N_1-substituted structure II, analogous to that proposed for the Cmc derivatives of guanosine and its 5′-phosphate. The spectral characteristics of the inosine derivative are similar to those reported for 1-cyanoethylinosine (Yoshida and Ukita, 1965; Ofengand, 1967). As with the Cmc derivatives of uridine, guanosine, and their 5′-phosphates, Cmc-inosine, and Cmc-inosine 5′-phosphate can be readily reconverted into the nucleoside and nucleotide, respectively, by mild treatment with pH 10 buffer and the spectral changes accompanying this hydrolysis are shown in Figure 1.

It was expected that the reaction with pseudouridine would be more complex because of the two dissociable H atoms present in 5-substituted uracils. An initial indication that an alkali-stable Cmc derivative of pseudouridine can be formed was obtained from a study of the chemical modification of tRNA, and the products of its enzymic hydrolysis (Ho and Gilham, 1967). When tRNA was treated with the Cmc reagent and then hydrolyzed with pancreatic ribonuclease, the amounts of uridine 3′-phosphate and pseudouridine 3′-phosphate produced were reduced to low levels compared to the amounts of these nucleotides obtained from a normal ribonuclease digestion of unmodified tRNA. However, when the tRNA was treated at pH 10 to remove the Cmc groups prior to the enzymic reaction, no pseudouridine 3′-phosphate was formed whereas the amount of uridine 3′-phosphate produced returned to its normal level. The explanation given for this effect was that the chemical modification of tRNA resulted in the formation of stable Cmc-pseudouridine moieties and that the phosphodiester linkages attached to these substituted pyrimidines were resistant to the action of ribonuclease..

This conclusion has now been confirmed by a study of the chemical modification of pseudouridine itself. On reaction with the Cmc reagent at pH 8.5 for 10 hr the nucleoside was completely converted into two products. A comparison of the electrophoretic mobilities of these products showed that one of them possessed two positive charges indicating that it was a disubstituted derivative while the other contained a single positive charge indicating a monosubstituted nucleoside. The monosubstituted derivative is assigned the structure, 3-Cmc-pseudouridine (IV) on the basis of a comparison of its spectral characteristics to those of other substituted uracil derivatives (Table I). In particular, derivative IV shows a strong shift in λ_{max} to a longer wavelength as well as a substantial increase in ϵ_{max} on changing its solution from neutral to alkaline pH (Figure 2). These changes apparently result from the dissociation of the H atom at N_1 and the spectral shifts are also ob-

TABLE I: Spectrophotometric Constants of Substituted Uracils.

Uracil Derivative	pH	λ_{max} (mμ)	pH	λ_{max} (mμ)	ϵ_{max} (Alkaline) / ϵ_{max} (Neutral)
3-Cmc-pseudouridine	7	266	10	293	1.40
1-Cmc-pseudouridine	7	263	10	~263	~0.8
1,3-Di-Cmc-pseudouridine	7	265	10	265	1
3-Methylpseudouridine[a]	7	264	12	283	1.27
1-Methylpseudouridine[a]	7	265	12	269	0.78
1,3-Dimethylpseudouridine[a]	7	269	12	270	0.99
3-Methylthymine[b]	5.9	264.5	13	290	1.41
4-*O*-Ethylthymine[b]	5.9	275	13	286	1.26
1-Methylthymine[b]	5.9	273	11.5	271	0.71
1,3-Dimethylthymine[b]	5	272	12	272	1
1-Methyl-4-*O*-ethylthymine[b]	5	280	12	280	1
2,4-Di-*O*-ethylthymine[b]	5	265	12	265	1
Thymidine	7	267	11	267	0.81
1-Cyanoethylpseudouridine[c]	7	267	12	263–265	0.70
1,3-Di-(cyanoethyl)pseudouridine[c]	7	268–265	12	268–270	1

[a] Data from Cohn (1960). [b] Data from Wittenburg (1966). [c] Data from Chambers *et al.* (1963), Ofengand (1965), and Chambers (1965).

served with the structurally analogous compounds: 3-methylpseudouridine and 3-methylthymine. In contrast to this, those derivatives which are structural analogs of 1-substituted pseudouridines (1-methylpseudouridine, 1-methylthymine, thymidine, and 1-cyanoethylpseudouridine) do not display the strong bathochromic shift or the increase in ϵ_{max} on raising the pH of their solutions.

The suggested N_3-substituted structure is consistent with the behavior that polynucleotides containing the modified pseudouridine exhibit in the presence of pancreatic ribonuclease. As discussed below such modification causes the phosphodiester linkage on the 3′ side of the pseudouridine moiety to be completely resistant to the action of the enzyme. The proposed structure, IV, is analogous to that of 3-Cmc-uridine which also protects the adjacent phosphodiester linkage from attack by the ribonuclease when the modified nucleoside is located in a polynucleotide chain.

In contrast to the ready hydrolysis of 3-Cmc-uridine in weakly alkaline solutions 3-Cmc-pseudouridine is relatively stable at high pH. The derivative is not affected by treatment with concentrated ammonia for 2 days at room temperature although the Cmc group can be removed by exposure of the compound to hot 7 M ammonia. This extra stability of the 3-Cmc group in the pseudouridine derivative is probably due to the presence of the negative charge at N_1 caused by dissociation of its proton in alkaline solutions.

Some information on the structure of the di-Cmc derivative has been obtained by studying its behavior in alkaline solution. In cold 7 M ammonia the derivative is converted almost instantaneously into 3-Cmc-pseudouridine. The course of this reaction can be followed spectrophotometrically by using sodium pyrophosphate buffer at pH 8.7 under which conditions the time of hydrolysis is about 3 hr at 20°. Spectra taken during this time are reproduced in Figure 3—the curves have a single isosbestic point and show no indication of the presence of any long-lived intermediate in the conversion. In addition, the product of this reaction, 3-Cmc-pseudouridine can be reconverted into a disubstituted derivative by reaction with carbodiimide at pH 8.5 and this disubstituted product has electrophoretic, chromatographic, and spectral properties indistinguishable from those of the original di-Cmc derivative from which the 3-Cmc nucleoside was obtained. Thus, on the assumption that migration of Cmc groups does not take place during these hydrolytic and synthetic steps the structure of the disubstituted derivative is either the 1,3-di-Cmc-pseudouridine (III) or O^2,3-di-Cmc-pseudouridine. The spectral properties of the compound are not grossly different from those of

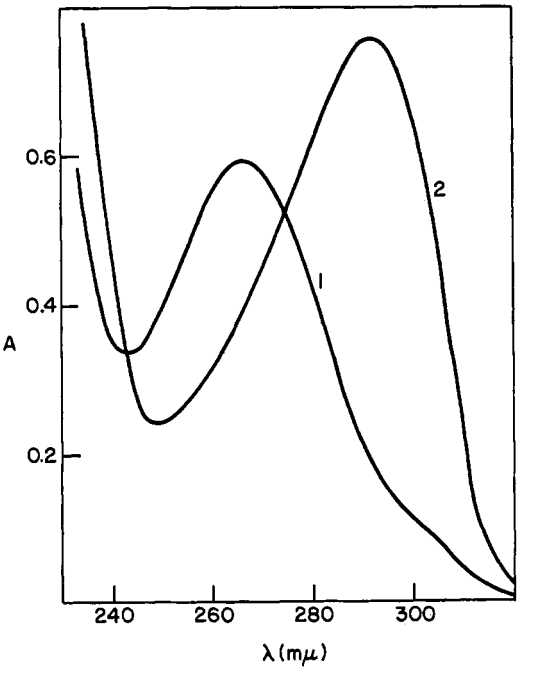


FIGURE 2: Spectra of 3-Cmc-pseudouridine. Curve 1, spectrum taken in 0.02 M sodium phosphate buffer (pH 7). Curve 2, spectrum taken in 0.07 M sodium pyrophosphate buffer (pH 8.7).

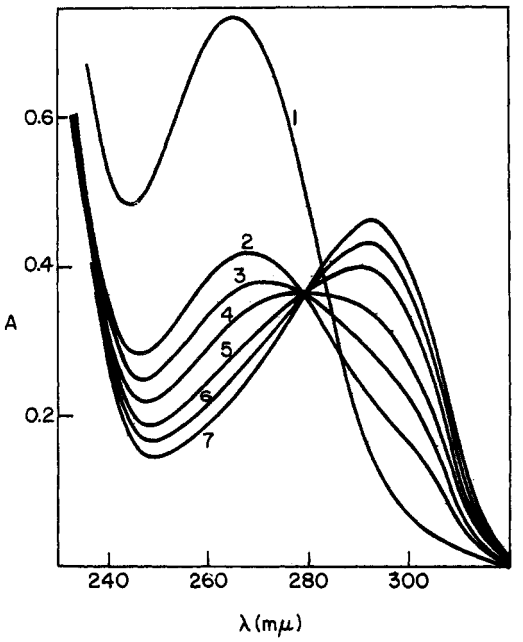


FIGURE 3: Curve 1 is the spectrum of 1,3-di-Cmc-pseudouridine in 0.02 M sodium phosphate buffer (pH 7) and its initial spectrum in 0.07 M sodium pyrophosphate buffer (pH 8.7). Curves 2, 3, 4, 5, 6, and 7 are spectra of the same compound (at a different concentration) after standing in the pH 8.7 buffer at 25° for 1, 8, 18, 38, 68, and 180 min, respectively. After 180 min there was no further change in the spectrum.

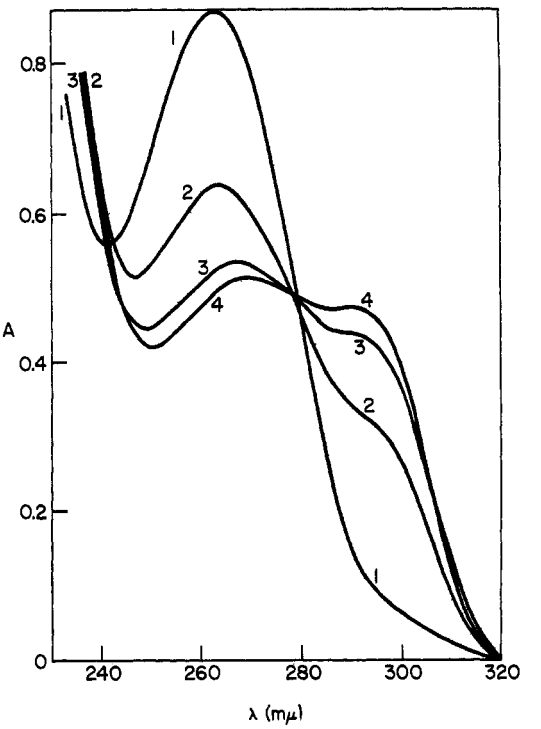


FIGURE 4: Spectra of early-formed mono-Cmc-pseudouridines. Curve 1, spectrum of mixture taken in 0.02 M sodium phosphate buffer (pH 7). Curve 2, spectrum in 0.07 M sodium pyrophosphate buffer (pH 8.7). Curve 3 and 4, spectra taken in dilute sodium hydroxide at pH 9.6 and 13, respectively.

other 1,3,5-substituted uracil derivatives (Table I) and it is thus assigned the structure, III. However, the other structure cannot be completely ignored because model O^2,3,5-substituted uracil derivatives are not available for spectral comparisons.

In order to determine the course of the reaction of pseudouridine with Cmc-*p*-toluenesulfonate experiments were set up such that the products could be analyzed at various times. Only monosubstituted products were present in the reaction mixture up to 15 min and both mono- and disubstituted derivatives were present in all samples taken thereafter. The nucleoside was completely converted into its derivatives in 4–5 hr. By comparison, uridine, under the same conditions, requires 6–7 hr for complete conversion into its Cmc derivative. The physical properties of the disubstituted product isolated from the earlier samples (30 and 45 min) and the later samples (5 and 6 hr) were identical and also indistinguishable from the properties of the di-Cmc derivative isolated from the 10-hr reaction mixture as described above. However, the spectra of the monosubstituted products in the earlier samples differed markedly from those of the products in the later samples. The product obtained after 5 hr was identical to the 3-Cmc-pseudouridine obtained from the 10-hr reaction whereas spectral analysis of the early-formed products indicated the presence of two monosubstituted nucleosides. Both of these products have λ_{max} at pH 7 in the vicinity of 263 mμ and one of them is apparently 3-Cmc-pseudouridine since its λ_{max} changes to *ca.* 290 mμ on raising the pH of the mixture to 13 (Figure 4). The other product is assigned the structure, 1-Cmc-pseudouridine (V) on the basis of its calculated spectral characteristics and a comparison of these to the spectra of compounds of similar structure, 1-methylpseudouridine, 1-methylthymine, thymidine, and 1-cyanoethylpseudouridine (Table I).

The presence of two monosubstituted derivatives in the early stages of the reaction is supported by two other observations. (i) The relative heights of the two peaks in the alkaline spectrum of the mixture of mono-Cmc derivatives changes as the reaction proceeds—the peak at the longer wavelength increases while that at the shorter wavelength decreases. For example, the ratios of ϵ_{263} to ϵ_{290} at pH 10 of samples taken at 15 and 30 min are 1.2 and 0.7, respectively. (ii) The two derivatives have quite different stabilities in alkaline solutions—while the 1-Cmc derivative is stable under the pH conditions of the reaction it can be reconverted into pseudourudine by treatment with 7 M ammonia at room temperature while the 3-Cmc isomer is stable under these conditions. These results tend to support the contention that 1-Cmc-pseudouridine is an early component of the reaction mixture although, because of the lack of model compounds for spectral comparisons, the O^2-Cmc structure cannot be completely excluded.

With these observations the pattern of the reaction of pseudouridine with the Cmc reagent can be proposed. (i) Both monosubstituted derivatives are formed in the early stages of the reaction (the 1-Cmc compound being produced at a somewhat faster rate than the 3-Cmc isomer); (ii) both of these derivatives react further to form a single disubstituted compound, 1,3-di-Cmc-pseudouridine; (iii) under the pH conditions of the reaction this di-Cmc nucleoside breaks down to the 3-Cmc-pseudouridine only; (iv) the proportions of the 3-Cmc and 1,3-di-Cmc derivatives present in the final product are controlled by the rate at which the di-Cmc compound is hydrolyzed and the rate at which the 3-Cmc derivative is reconverted into the di-Cmc product with the excess Cmc reagent.

With this reaction mechanism it is possible to convert the

pseudouridine moiety of a nucleotide or polynucleotide into its 3-Cmc derivative in essentially 100% yield. This is carried out by simply treating the nucleotide or polynucleotide with excess reagent followed by treatment with dilute ammonia. The effect of this type of modification on the action of pancreatic ribonuclease has been investigated. As mentioned above, the substitution of the 3 position of uridine moieties in polynucleotides confers resistance to the action of this enzyme at the positions occupied by these nucleosides and, in preliminary studies on tRNA, there were indications that the same situation is true also for pseudouridine. To study this effect two dinucleoside phosphates containing pseudouridine were prepared. Pseudouridylyl-(3′–5′)-adenosine was synthesized by the condensation of 2′,5′-diacetylpseudouridine 3′-phosphate with 2′,3′-diacetyladenosine using a method similar to that described by Rammler *et al.* (1963), and cytidylyl-(3′–5′)-pseudouridine was prepared from cytidine 2′,3′-cyclic phosphate and pseudouridine using the reversed ribonuclease-catalyzed reaction first described by Heppel *et al.* (1955). Both of these dinucleoside phosphates were converted into their 3-Cmc derivatives by reaction with the Cmc reagent followed by treatment with ammonia. The Cmc-pseudouridylyl-(3′–5′)-adenosine was then shown to be completely resistant to the action of the enzyme while pseudouridylyl-(3′–5′)-adenosine itself could be degraded to pseudouridine 3′-phosphate and adenosine. On the other hand, the Cmc-cytidylyl-(3′–5′)-pseudouridine could be completely degraded by the enzyme to cytidine 3′-phosphate and 3-Cmc-pseudouridine. These results are analogous to those obtained with the enzyme hydrolysis of oligonucleotides containing modified uridine moieties in that, for a particular phosphodiester linkage, a Cmc-modified pyrimidine on the 3′ side protects against ribonuclease attack while a modified pyrimidine or purine on the 5′ side does not.

A knowledge of the pattern of the Cmc derivatization of inosine and pseudouridine moieties in polynucleotides should be of some value in studies on the primary and secondary structure of tRNA molecules. For example, the reaction of tRNA with the reagent followed by treatment with dilute ammonia should produce molecules in which only the pseudouridine bases have been modified or, at least those which, by virtue of their spatial arrangement in the secondary structure of the molecules, are available for reaction. Also, the Cmc modification of uridine, guanosine, 5-methyluridine, inosine, and pseudouridine in polynucleotides followed by digestion with a particular nuclease or phosphodiesterase provide useful cleavage mechanisms for nucleotide sequence studies (Naylor *et al.*, 1965; Ho and Gilham, 1967; Ho *et al.*, 1969).

Experimental Section

Materials. *N*-Ccylohexyl-*N*′-β-(4-methylmorpholinium)-ethylcarbodiimide-*p*-toluenesulfonate was purchased from Aldrich Chemical Co., Milwaukee, Wis. Inosine, inosine 5′-phosphate, and pseudouridine (A grade) were obtained from Calbiochem, Los Angeles, Calif., and pancreatic ribonuclease was the product of Worthington Biochemical Corp., Freehold, N. J. Pseudouridine 3′-phosphate was isolated from yeast RNA by the method described by Cohn (1960).

Reaction of Cmc-p-toluenesulfonate with Inosine 5′-Phosphate and Inosine. Cmc-*p*-toluenesulfonate (800 mg) was added to a solution of disodium inosine 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. Aliquots of 0.1 ml were withdrawn at various times and added to 0.1-ml portions of 0.1 M sodium phosphate (pH 7) and the analysis of these samples by paper chromatography in solvent system A showed that the reaction was complete in 2 hr. In this chromatographic system inosine 5′-phosphate and its Cmc derivative have R_F values of 0.16 and 0.55, respectively, on Whatman No. 3MM paper. On paper electrophoresis the product migrates toward the positive electrode in 0.05 M sodium phosphate buffer (pH 7.0) and the ratio of its mobility to that of inosine 5′-phosphate is 0.25. In 0.02 M sodium phosphate buffer (pH 7) the product had λ_{max} 246 mμ (ε 11,000) and 251 mμ (ε 11,000). 1-Cyanoethylinosine has λ_{max} 249–251 mμ at pH 7–12 (Yoshida and Ukita, 1965; Ofengand, 1967). In 0.2 M sodium carbonate buffer (pH 10.3) the spectrum of the product was initially identical with that taken in pH 7 buffer but then gradually changed over a period of 1 hr to a spectrum indistinguishable from that of inosine 5′-phosphate in pH 10.3 buffer (Figure 1). The product from this hydrolysis was shown to be inosine 5′-phosphate by paper chromatography with solvent system A.

Inosine reacted with the Cmc reagent at about the same rate as its 5′-phosphate. In 0.05 M sodium phosphate buffer (pH 6.5) the product had an electrophoretic mobility equal to that of Cmc-uridine. The derivative had similar spectral characteristics to those of the Cmc nucleotide and could be reconverted into inosine under the hydrolytic conditions used for Cmc-inosine 5′-phosphate. In solvent system A on Whatman No. 3MM paper, Cmc-inosine had R_F, 0.86 while inosine itself had R_F 0.67.

Reaction of Cmc-p-toluenesulfonate with Pseudouridine. Cmc-*p*-toluenesulfonate (100 mg) was added to a solution of pseudouridine (16 mg) in 0.05 M sodium borate buffer (pH 8.5; 4 ml). Aliquots of 0.4 ml were withdrawn at 15, 30, 45, 60, 120, 180, 240, 300, and 360 min and each sample was immediately adjusted to pH 6 with dilute hydrochloric acid to stop the reaction. Analysis of the early samples (15 and 30 min) by electrophoresis in 0.05 M sodium phosphate buffer (pH 7) showed that a small portion of the pseudouridine had been converted mainly into a mono-Cmc product which had a mobility toward the negative electrode identical with that of Cmc-uridine. Electrophoretic analysis at pH 7 of later samples (taken after 60 min) showed that the pseudouridine had been largely converted into at least two different products: a mono-Cmc derivative with a mobility identical with that of Cmc-uridine and a di-Cmc derivative with a mobility toward the negative electrode 1.7 times that of Cmc-uridine. The uv spectrum of the di-Cmc product remained constant while the spectrum of the mono-Cmc derivative changed during the course of the reaction. The analysis of these spectra, as described below, indicated that, in the early stages of the reaction the predominant monosubstituted product was the 1-Cmc-pseudouridine. The proportion of this material present in the monosubstituted electrophoretic band decreased with time and the 3-Cmc-pseudouridine ultimately became the sole mono-substituted product. No unreacted pseudouridine could be detected in the reaction mixture after 3–4 hr. For comparison, when uridine was treated with the reagent under identical conditions, 6–7 hr were required for its complete reaction.

3-Cmc-pseudouridine. Cmc-*p*-toluenesulfonate (100 mg) was added to a solution of pseudouridine (4 mg) in water (1 ml). The stirred mixture was kept at pH 8.5 for 10 hr and was then adjusted to pH 6 by the addition of *p*-toluenesulfonic acid. The monosubstituted derivative was separated by paper electrophoresis in 0.05 M sodium phosphate buffer (pH 7), under which conditions it had a mobility identical with

that of Cmc-uridine. The yield of the derivative was about 50% with the rest of pseudouridine having been converted to the di-Cmc derivative. Uv spectra were taken at pH 7.0 in 0.02 M sodium phosphate buffer, pH 8.7 in 0.07 M sodium pyrophosphate buffer, and pH 10.0 in 0.25 M sodium carbonate buffer (Table I, Figure 2). After standing in these buffer solutions for periods of up to 2 hr the derivative showed no change in its initial spectra. On paper chromatography in solvent systems A and B the derivative had R_F values: 0.81 and 0.59, respectively, compared to R_F values for pseudouridine itself: 0.61 and 0.40, respectively.

1,3-Di-Cmc-pseudouridine. The disubstituted derivative was prepared and isolated by paper electrophoresis in sodium phosphate buffer (pH 7) as described above for the preparation of the 3-Cmc derivative. At pH 7, the product had an electrophoretic mobility 1.7 times that of 3-Cmc-uridine and had an R_F value of 0.87 in solvent system A. Spectra were determined in 0.02 M sodium phosphate (pH 7), 0.07 M sodium pyrophosphate (pH 8.7), and 0.2 M sodium carbonate (pH 10) (Table I and Figure 3). In the pH 8.7 buffer the compound had an initial spectrum identical with that at pH 7 and, on standing, the spectrum changed over a period of 180 min to a curve which was indistinguishable from the spectrum of 3-Cmc-pseudouridine at pH 8.7 (Figure 3). At pH 10 the disubstituted derivative was converted almost instantaneously into a product which was spectrally, chromatographically and electrophoretically indistinguishable from 3-Cmc-pseudouridine.

1-Cmc-pseudouridine. Pseudouridine (8 mg) in 0.05 M sodium borate buffer (pH 8.5; 1 ml) was mixed with Cmc-*p*-toluenesulfonate (16 mg) and allowed to stand for 15 min. The pH of the mixture was then adjusted to 6.0 with dilute hydrochloric acid and the products were separated by paper electrophoresis in 0.05 M sodium phosphate (pH 7). The band of monosubstituted derivatives was cut out and eluted and spectra were taken at pH 7 in 0.02 M sodium phosphate and at pH 8.7 in 0.07 M sodium pyrophosphate (Figure 4). The spectrum at pH 9.6 was taken on a solution prepared by the addition of 1 M sodium hydroxide (25 μl) to the sodium pyrophosphate solution (1.5 ml) of the compound and the spectrum at pH 13 was taken on a mixture of this pH 9.6 solution (1.525 ml) and 3 M sodium hydroxide (25 μl). Assuming that the 3-Cmc derivative is responsible for the peak at about 290 mμ which is formed in media of high pH, it is possible to make an approximate estimate of the contribution made by this derivative to the neutral and alkaline spectra of the mixture. Thus, the other monosubstituted derivative (the presumed 1-Cmc-pseudouridine) must have a λ_{max} of about 263 mμ at pH 7 with little or no shift to longer wavelengths at higher pH values, and, in addition, the ratio of its ϵ_{max} in alkaline medium to its ϵ_{max} in neutral medium must be less than one.

Hydrolysis of Cmc-pseudouridine Derivatives with Ammonia. The disubstituted derivative (3 ODU), isolated as described above, was dissolved in 7 M ammonium hydroxide (4 ml). A spectrum taken immediately showed that the compound had been converted into the 3-Cmc derivative and this was confirmed by electrophoretic and chromatographic comparison of the product to the 3-Cmc-pseudouridine prepared as described above.

Samples (3 ODU each) of the mixture of monosubstituted derivatives formed in the 15-min reaction were treated with 4-ml quantities of 7 M ammonium hydroxide at room temperature. Spectra of the samples taken at different times showed that the presumed 1-Cmc-pseudouridine was converted into pseudouridine over a period of 40 hr while the 3-Cmc-pseudouridine remained unchanged. The identities of the products of this hydrolysis were confirmed by paper chromatography in solvent system A.

Samples of 3-Cmc-pseudouridine (5 ODU each) were dissolved in 7 M ammonium hydroxide (7 ml each) and their spectra were taken after standing at room temperature for 24, 48, and 72 hr. The derivative showed no spectral changes over these periods and, on recovery of the compound by evaporation, it was shown, by paper chromatography in solvent system A, that the derivative was essentially unhydrolyzed. A larger sample (20 ODU) of the derivative was dissolved in 7 M ammonium hydroxide (2 ml) and heated at 100°. Aliquots of 0.2 ml were withdrawn at 2, 4, 6, 8, and 10 min and subjected to paper electrophoresis in 0.05 M sodium phosphate buffer (pH 7). Complete conversion of the derivative to pseudouridine was indicated in the 8-min sample.

Reaction of Cmc-p-toluenesulfonate with 3-Cmc-pseudouridine. 3-Cmc-pseudouridine (80 ODU) which had been purified by paper chromatography in solvent system B was dissolved in 0.05 M sodium borate (pH 8.5, 0.3 ml) and mixed with Cmc-*p*-toluenesulfonate (15 mg) and allowed to stand for 20 hr. The mixture was then adjusted to pH 6 with dilute hydrochloric acid. Paper electrophoresis in 0.05 M sodium phosphate buffer (pH 7) showed that over half of the 3-Cmc-pseudouridine had been converted to a disubstituted product which had electrophoretic and spectral properties identical with those of the 1,3-di-Cmc-pseudouridine described above. For comparison, pseudouridine (80 ODU) was treated with the reagent under the same conditions and paper electrophoresis showed the presence of a mixture with essentially the same proportions of mono- and diderivatives as was obtained from the reaction of 3-Cmc-pseudouridine.

Preparation of Cmc-pseudouridylyl-(3′–5′)-adenosine and Its Resistance to the Action of Pancreatic Ribonuclease. Pseudouridylyl-(3′–5′)-adenosine was prepared by the condensation of 2′,5′-diacetylpseudouridine 3′-phosphate (0.01 mmole) with 2′,3′-diacetyladenosine (0.2 mmole) in anhydrous pyridine (1 ml) in the presence of dicyclohexylcarbodiimide (0.5 mmole) using the methods described by Rammler *et al.* (1963). The dinucleoside phosphate (100 ODU) was dissolved in water (1 ml) and treated with Cmc-*p*-toluenesulfonate (100 mg). The solution was kept at pH 8–8.5 for 70 hr at room temperature and was then treated with an equal volume of concentrated ammonia and allowed to stand for 2 hr in order to decompose any di-Cmc-pseudouridine derivative to the stable mono-Cmc derivative. The ammonia was then removed by evaporation *in vacuo* and the solution of the product was passed through a short column of Dowex 50-X8 (NH_4^+) ion-exchange resin. The product was then concentrated and subjected to paper electrophoresis in 0.05 M ammonium formate (pH 3.0). The Cmc derivative which was the only material to migrate toward the negative electrode was obtained in a yield of 85%. The product was desalted by chromatography in solvent system B and a portion (6 ODU) was dissolved in 0.05 M sodium phosphate buffer (pH 7; 0.2 ml) and treated with pancreatic ribonuclease (2.5 μg) at 37° for 4 hr. Paper chromatography in solvent systems A and B showed that the Cmc derivative (R_F 0.64 and 0.34, respectively) had not been attacked by the enzyme. Under the same conditions pseudouridylyl-(3′–5′)-adenosine (R_F 0.30 and 0.14 in solvent systems A and B) was hydrolyzed to pseudouridine 3′-phosphate and adenosine. In another experiment the Cmc derivative was treated with 40 times the amount of ribonuclease (100 μg) and, again, the dinucleoside phosphate remained unhydrolyzed.

Preparation of Cmc-cytidylyl-(3′–5′)-pseudouridine and Its Hydrolysis with Pancreatic Ribonuclease. Cytidylyl-(3′–5′)-

pseudouridine was prepared using the ribonuclease-catalyzed reaction described by Heppel *et al.* (1955). Cytidine 2',3'-cyclic phosphate (0.1 mmole) and pseudouridine (100 mg) were dissolved in 0.015 M Tris-chloride buffer (pH 7; 5 ml) and treated with pancreatic ribonuclease (0.1 mg) at 37° for 75 min. The mixture was then shaken with isoamyl alcohol (0.6 ml) and chloroform (0.15 ml) and streaked on Whatman No. 3MM chromatographic paper (66 cm). The components of the mixture were separated with solvent system B and the dinucleoside phosphate band was cut out and eluted (yield, 30 ODU). This product was found to be completely degradable by ribonuclease to cytidine 3'-phosphate and pseudouridine. The dinucleoside phosphate (20 ODU) was dissolved in 0.05 M sodium borate buffer (pH 8.5; 0.3 ml) and treated with Cmc-*p*-toluenesulfonate (40 mg). The mixture was allowed to stand for 20 hr. At this time paper chromatography in solvent systems A and B showed that about 70% of the material had been converted to Cmc derivatives. The dinucleoside phosphate and its mono-Cmc derivative had R_F values 0.41 and 0.61 (solvent system A); 0.18 and 0.26 (solvent system B), respectively. The mixture was treated with concentrated ammonia for 2 hr and then applied to Whatman No. 3MM chromatographic paper and then separated in solvent system B. The Cmc-dinucleoside phosphate (6 ODU) was dissolved in 0.02 M sodium phosphate buffer (pH 7.0; 0.2 ml) and treated with pancreatic ribonuclease (2.5 μg) at 37° for 4 hr. Paper chromatography in solvent system A showed that about 70% of the Cmc derivative had been converted into a mixture of cytidine 3'-phosphate and Cmc-pseudouridine. With twice the amount of enzyme under the same conditions it was possible to hydrolyze all the dinucleoside phosphate to its components.

Chromatography. Paper chromatography was carried out by the descending technique on Whatman No. 1- or 3MM paper with the solvent systems: (A) ethyl alcohol (70 ml)–1 M ammonium acetate (pH 7; 30 ml) and (B) isopropyl alcohol (70 ml)–concentrated ammonia (10 ml)–water (20 ml).

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A Novel Prostaglandin Derivative Formed from Arachidonic Acid by Rat Stomach Homogenates*

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ABSTRACT: A novel derivative of prostanoic acid was isolated during the biosynthetic conversion of arachiodonic acid into prostaglandins by rat stomach homogenates. The structure proposed is 6(9)-oxy-11,15-dihydroxyprosta-7,13-dienoic acid (I). The elucidation of this structure was based on infrared and nuclear magnetic resonance spectroscopy, mass spectrometry of several derivatives, and products obtained from oxidative ozonolysis. Evidence for the occurrence in minor amounts of an isomer of I, *i.e.*, 6(9)-oxy-11,15-dihydroxyprosta-5,13-dienoic acid (II) was obtained from mass spectrometry of products of oxidative ozonolysis. Prostaglandins E_2 and $F_{2\alpha}$ were also isolated in smaller amounts and identified by mass spectrometry. Two pathways for the formation of I are proposed.

Prostaglandins are oxygenated derivatives of prostanoic acid, a cyclopentane trans substituted in the 1,2 position by a C-7 carboxylic acid and a C-8 alkane. Their structures were originally determined on material isolated from sheep seminal vesicles and human seminal fluid (Bergström and Sjövall, 1960; Bergström *et al.*, 1963; Samuelsson, 1963). They were shown to be derived in sheep seminal vesicles from certain essential fatty acids (Bergström *et al.*, 1964; Nugteren *et al.*, 1966; Hamberg and Samuelsson, 1967). Certain fatty acids that are not precursors to prostaglandins have been shown to inhibit their biosynthesis (Pace-Asciak and Wolfe, 1968; Nugteren, 1970). Prostaglandins are widespread in occurrence in mammalian tissues and possess diverse physiological activ-

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