The Investigation of Nucleic Acid Secondary Structure by Means of Chemical Modification with a Carbodiimide Reagent. I. The Reaction between *N*-Cyclohexyl-*N'*-β-(4-methylmorpholinium)ethylcarbodiimide and Model Nucleotides*

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ABSTRACT: In order to establish a chemical modification method for the study of nucleic acid secondary structure, the reaction between the carbodiimide reagent, *N*-cyclohexyl-*N'*-β-(4-methylmorpholium)ethylcarbodiimide, and the nucleotides has been investigated. It has been confirmed that the reagent reacts specifically with uridine monophosphate and guanosine monophosphate, of the four main ribonucleotides, under conditions of pH, ionic strength, and temperature in which nucleic acid secondary structure would be maintained. The rate of the carbodiimide–nucleotide reaction is not very dependent upon the ionic strength, buffer type, or the presence of magnesium ions.

The reaction goes largely to completion under convenient conditions of reagent concentration and temperature. The carbodiimide–nucleotide compounds are stable in neutral and acidic solutions but are labile in alkaline. In the reaction with the residues of a polynucleotide, neither chain scission nor cross-linking has been detected. Nucleotides in single-stranded stacked or unstacked configurations may react with the carbodiimide, subject to certain minor limitations. Nucleotides in double-stranded helical configurations are markedly inhibited from reacting. However, such helices are slowly denatured as the equilibrium between the double-stranded and single-stranded components is perturbed by the reaction of the reagent with the latter. The rate of perturbation of a double-stranded helix is dependent upon its nature and environment in a manner that is consistent with what is known of the effect of these factors on helix stability from quite independent methods. When the double-stranded helices are sufficiently stable, the carbodiimide reagent may be used to distinguish between single-stranded and double-stranded regions of the secondary structure of a polynucleotide.

Whereas our understanding of the secondary structure of both natural and synthetic polynucleotides has advanced substantially in recent years (Felsenfeld and Miles, 1967; Michelson *et al.*, 1967), we are still some way from being able to give an adequate account of the conformation of the polynucleotide chains in, for example, tRNA or rRNA. This is because the presently available experimental techniques are not sufficiently powerful, a situation which is unlikely to change radically until the tool of X-ray crystallography can be brought fully to bear. In the meanwhile, existing techniques must be refined and extended.

One approach that has been used involves the chemical modification of the purine and pyrimidine bases of the polynucleotides. If the ability of a base to react with the modifying reagent depends upon the configuration of the nucleotide, then information on the secondary structure may be obtained. Most commonly a base may be sterically hindered from reacting when it is part of a hydrogen-bonded base pair, as in the double helix. While many reagents that react with the purine and pyrimidine bases of polynucleotides are known (for review, see Miura, 1967), and while in a number of cases there is evidence that the conformation of the polymer affects the rate of reaction, there are very few instances where the data are sufficient to permit a quantitative interpretation. The requirements that must be met if a particular reagent is to be validly employed in obtaining information on the secondary structure of polynucleotides are quite extensive. In particular is it necessary that the reaction occurs under reasonably physiological conditions and that the denaturation of the secondary structure during the reaction be minimal.

Existing reagents for the chemical modification of polynucleotides suffer from various disadvantages and improved ones are to be desired. In the present paper, the use of a carbodiimide reagent possessing a number of advantageous properties will be described. *N*-Cyclohexyl-*N'*-β-(4-methylmorpholinium)ethylcarbodiimide (hereafter referred to as the carbodiimide) is a carbodiimide whose structure is shown in Figure 1.[1] It was first synthesized by Sheehan and Hlavka (1956) who required a water-soluble carbodiimide for peptide-bond synthesis. Gilham (1962) reported that this reagent would react with uridine and guanosine 5'-phosphates (but not with the adenosine or cytidine phosphates) at pH 8 to give a 1:1 adduct in which the base was substituted at a ring nitrogen atom (see Figure 1). More recently, Ho and Gilham (1967) have described these reactions in greater detail. Naylor *et al.* (1965) found that pseudouridine would react with two mole-

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[1] The carbodiimide is a base and exists in various salt forms. The iodide, bromide, and *p*-toluenesulfonate salts have been used in the present work.

cules of the carbodiimide to give a disubstituted derivative. Augusti-Tocco and Brown (1965), in this laboratory, first reported that the extent of the carbodiimide–nucleotide reaction was, in polynucleotides, a function of the secondary structure. Using poly U and poly (A + U), and denatured and native DNA, as models, it was shown that the inhibition of the reaction by the double-helical secondary structure was very marked. The extent of reaction with tRNA was quite limited, especially in the presence of magnesium ions. Knorre and his coworkers have also investigated the reaction of carbodiimide with DNA (Drevitch *et al.*, 1966) and with tRNA (Knorre *et al.*, 1966).

The aim of the present work was to extend the previous observations on the reaction between the carbodiimide reagent and the nucleotides and nucleic acids in order to improve our understanding of their secondary structure. In the present paper, a variety of mono-, oligo-, and polynucleotides have been employed as model substances for the purpose of calibrating this chemical modification technique. In the subsequent paper (Metz and Brown, 1969), the method will be applied to the problem of elucidating the secondary and tertiary structure of tRNA.

Materials and Methods

Materials. The carbodiimide *p*-toluenesulfonate (an alternate name is 1-cyclohexyl-3-(2-morpholino-(4)-ethylcarbodiimide metho-*p*-toluenesulfonate) and 1-cyclohexyl-3-(2-morpholino-(4)-ethyl)thiourea were purchased from the Aldrich Chemical Co. Inc., Milwaukee, Wis. Mononucleotides and poly U were purchased from Calbiochem Ltd., dinucleoside phosphates from Sigma London Chemical Co. Ltd., and poly A and poly (A,G) from Miles Laboratories Inc. The scintillator 2-(4′-butylphenyl)-5-(4′′-biphenylyl)-1,3 4-oxidiazole was purchased from CIBA (A.R.L.) Ltd., Duxford, Cambs. [^{14}C]-Methyl iodide, [^{3}H]methyl iodide, and [^{14}C]UMP were obtained from the Radiochemical Centre, Amersham. Calf thymus DNA was a gift from Dr. S. Arnott or was purchased from Sigma. [^{32}P]RNA, from the single-stranded RNA bacteriophage μ2, was a gift from Dr. H. Matthews. The tetranucleotide, GpGpGpCp, was isolated from a pancreatic RNase digest of μ2 RNA and was a gift from Dr. Sheila Lee. The source of the tRNA will be described in the following paper (Metz and Brown, 1969). Poly (A,U) was synthesized using a polynucleotide phosphorylase prepared from *Micrococcus lysodeikticus* (fraction IV of Thanassi and Singer, 1966).

The carbodiimide bromide was prepared from the carbodiimide *p*-toluenesulfonate by means of ion-exchange chromatography. Dowex 1 resin, obtained in the chloride form, was converted into the bromide form. A column (20 × 1.5 cm) was filled with the resin and equilibrated with water. A solution containing 2 g of carbodiimide *p*-toluenesulfonate in 50 ml of water was loaded on the column and elution with water was effected (25 ml/hr). The carbodiimide bromide began to elute after 14 ml and the whole peak came off in the following 55–60 ml. With at least one batch of carbodiimide *p*-toluenesulfonate a small additional peak was seen in the elution profile at the very end of the broad carbodiimide bromide peak, when an ultraviolet monitor was employed. The material in the extra peak had an absorbance maximum at 323 mμ. It was not further characterized and in most cases it was separated from the main carbodiimide bromide product and rejected. The

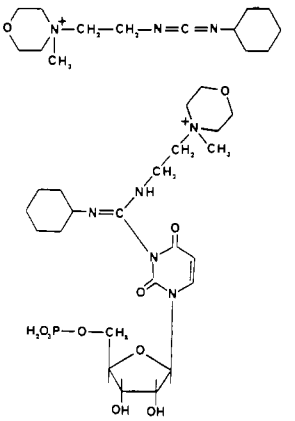

FIGURE 1: The structure of the carbodiimide and its adduct with 5′-UMP. The carbodiimide moiety is N=C=N.

carbodiimide bromide solution was lyophilized. The hygroscopic solid was dissolved in water or buffer and stored at −20°. The concentration of a carbodiimide bromide solution was determined by drying aliquots to constant weight.

The carbodiimide iodide, radioactively labeled in the methyl group, was prepared in two stages. The thiourea precursor was converted into the nonmethylated carbodiimide intermediate essentially according to the procedure of Sheehan and Hlavka (1956), and this was then methylated using isotopically labeled methyl iodide. 1-Cyclohexyl-3(2-morpholino-(4)-ethyl)carbodiimide, the carbodiimide intermediate, was prepared from the corresponding thiourea as follows. A mixture of 8 g of the thiourea and 12 g of yellow mercuric oxide in 100 ml of dry acetone was heated under reflux for 6 hr. The mercuric sulfide formed was removed on a sintered-glass filter and the acetone was removed under reduced pressure. Any residual solid material in the yellow oily residue was removed by further filtration and the oil was extracted twice with a little benzene. Residual benzene was distilled at atmospheric pressure and the carbodiimide intermediate was distilled under reduced pressure (around 150°, 1 mm). The intermediate was stored at −20° until required; if the period of storage exceeded a day or so, it was redistilled immediately before use. I thank Mr. Z. Kosinski for preparing this material. The intermediate was reacted with methyl iodide (either [^{14}C]CH_3I, specific activity 30 mCi/mmole, or [^{3}H]CH_3I, 200 mCi/mmole) as follows. The radioactive methyl iodide (0.5 mCi of [^{14}C]- or 25 mCi of [^{3}H]-CH_3I) was diluted with 1.0 ml of [^{12}C]CH_3I. *Caution:* the boiling point of methyl iodide is 42°; one end of the tube containing the radioactive material should be placed in solid carbon dioxide before opening. The methyl iodide was added to a stoichiometrically equivalent quantity of the carbodiimide intermediate. The mixture was left to react for 3 hr at room temperature in a desiccator ($CaCl_2$ as desiccant) with occasional mixing. The reaction product, a white, sticky solid, was triturated three times with diethyl ether (sodium dried) to remove residual quantities of the reactants. Residual ether was removed under reduced pressure, and the carbodiimide iodide was recrystallized from boiling benzene (the solid material being extracted with aliquots of boiling solvent until no further crystalline material was obtained on cooling). The white crystals were recovered by filtration on a Millipore filter and washed three times with cold benzene prior to drying under

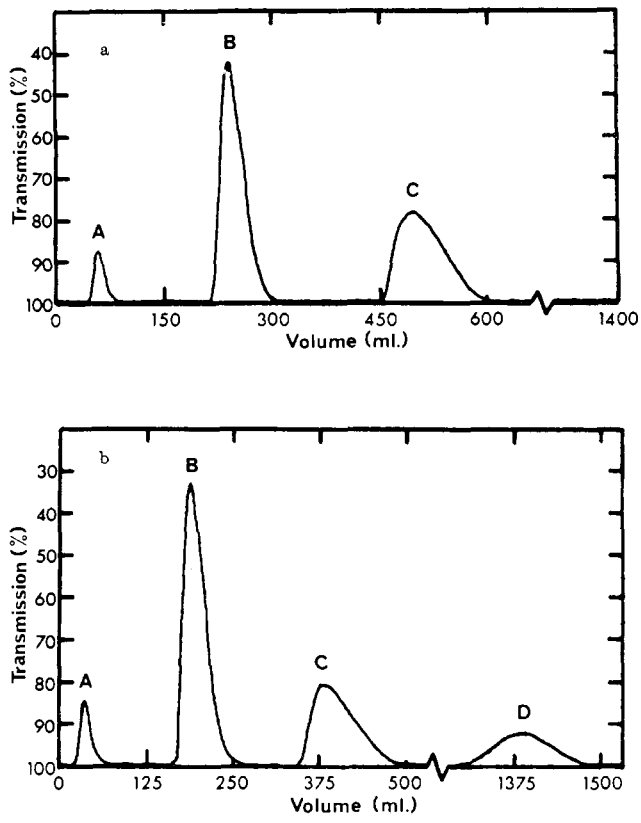

FIGURE 2: Carbodiimide–nucleotide reaction products. (a) The products of the reaction between 5′-UMP and the carbodiimide *p*-toluenesulfonate chromatographed on a DEAE-cellulose column (21 × 2.1 cm), preequilibrated with 0.01 M triethylamine acetate (pH 7.0). The reaction mixture was adjusted to pH 7.0 with acetic acid and diluted to 10 ml prior to application to the column. Elution was effected with a linear gradient of triethylamine acetate (pH 7.0; (0.01–0.15 M, 1400 ml, 100 ml/hr) and was followed by means of an ultraviolet transmittance monitor (at 254 mμ). Peak A is carbodiimide$^+$, peak B is the carbodiimide–UMP adduct, and peak C is *p*-toluenesulfonate. (b) The mixture of 5′-GMP and carbodiimide *p*-toluenesulfonate was chromatographed as in part a but with a gradient of total volume 1500 ml. Peak A is carbodiimide$^+$, peak B is the carbodiimide–GMP adduct, peak C is *p*-toluenesulfonate, and peak D is unreacted GMP.

reduced pressure. The specific activity of the carbodiimide iodide was determined by dissolving a known amount, dried to constant mass, in water, and counting aliquots in scintillation counter using a water-miscible scintillation fluid (see below). The specific activities of the $[^{14}C]CH_3I$ batches used in the present work were 21.0 and 19.0 μCi per mmole and that of the $[^3H]CH^3I$ was 1.68 mCi/mmole.

Properties of the Carbodiimide. The stability of the carbodiimide in aqueous solution was investigated by means of infrared spectroscopy. The Perkin-Elmer Model 237 spectrophotometer was used in conjunction with sample and reference cells fitted with barium fluoride windows. The sample cell had a path length of *ca.* 25 μ and the reference cell had a variable path length in order that precise compensation for the absorbance of the solvent could be effected. The absorbance due to the carbodiimide was measured at 2120 cm^{-1}. At this wavelength, under the present conditions, the transmittance of the aqueous solvent was 30%. In a typical experiment, the carbodiimide *p*-toluenesulfonate was dissolved in the appropriate buffer (40 mg/ml) in a small stoppered tube and incubated at the desired temperature. Aliquots were removed at the outset and then at various subsequent times, their infrared spectra recorded, and the absorbances at 2120 cm^{-1} were determined. The data were fitted to the first-order rate expression

$$\ln \frac{A_0}{A_t} = kt \qquad (1)$$

where A_0 is the absorbance at zero time, A_t is that at time t, and k is the first-order rate constant. Thus for each set of reaction conditions, this rate constant was determined.

The reaction between the carbodiimide and certain buffers was also followed by observing the change in the pH. The pH of a carbodiimide solution was monitored continuously by means of a Jena Micro Dual electrode (from Electronic Instruments Ltd.) and a pH meter, fitted with a pen recorder.

The Reaction between the Carbodiimide and the Nucleotides or Polynucleotides. PREPARATIVE SCALE. Mononucleotide (20 μmoles) was dissolved in 2.0 ml of 0.1 M sodium borate buffer and the pH was adjusted to 8.0 at room temperature. The carbodiimide *p*-toluenesulfonate (200 μmoles) was added as the solid; this resulted in a shift of pH by less than 0.05 pH unit. After incubation at 30° for 6–7 hr, the reaction products were separated on DEAE-cellulose (see Figure 2 for details), and the fractions of interest were recovered by lyophilization.

ANALYTICAL METHOD USING THE ISOTOPICALLY LABELED CARBODIIMIDE. The reaction was carried out in small, stoppered polypropylene tubes. The volume of the reaction mixture was 40 μl. After incubation the mixtures were immediately processed in one of the following ways.

Passage through a CM-cellulose Column (pH 6.0). After pretreatment in 0.5 M NaOH and 0.5 M HCl, CM-cellulose (Whatman) was equilibrated with 0.01 M phosphate buffer (pH 6.0), and the fines were removed by decantation. Small chromatography columns were made from Pasteur pipets fitted with plugs of glass wool at the taper. These were filled with the slurry of CM-cellulose which was packed down under gentle pressure. The effective column size was 6 × 75 mm. The contents of one assay tube were loaded on a column and elution was effected with 0.01 M phosphate buffer (pH 6.0) at a flow rate not exceeding 0.5 ml/min. In preliminary experiments 0.7-ml fractions were collected in scintillation counter vials and 20 ml of water-miscible scintillation fluid was added prior to counting. All nucleotide material eluted within the first four fractions. In most experiments, these four fractions were pooled in a single vial and evaporated to dryness under an infrared lamp. The residue was dissolved in 0.7 ml of water and 20 ml of scintillation fluid was added as before. Under the conditions described, the excess carbodiimide of the reaction mixture remained bound to the CM-cellulose, though if the flow rate exceeded 1 ml/min some carbodiimide tended to be eluted with the nucleotide material. The procedure described above gave complete quantitative recovery of nucleotide material with only a limited number of exceptions, all of which are mentioned below. While UMP was completely recovered from the CM-cellulose column after reaction, in the case of GMP, about 10% of the nucleotide was lost. To allow for this the following modification to the procedure was adopted. The recovery of the nucleotide from the column was determined by measuring the absorbance of the eluted material at the isosbestic wavelength (for the GMP + carbodiimide reac-

tion). The molar extinction coefficient at this wavelength, which is the same for both the reacted and unreacted nucleotide, was determined from the known extinction coefficient of the nucleotide at λ_{max} and the ratio of the absorbances at the isosbestic and maximum wavelengths. After the absorbance determination, the eluted material was counted as before. The extent of reaction of the carbodiimide with the nucleotide was then calculated from the carbodiimide counts bound to the recovered nucleotide.

Passage through a CM-cellulose Column (pH 7.0). The method described in the previous paragraph was satisfactory provided that the net charge on the reacted nucleotide was negative, as is the case for the mononucleotides, mixed polynucleotides of the types poly (A,G) or poly (A + U), and for all RNAs. However, in the cases of ApU, GpC, and poly U, at completion of the reaction with carbodiimide the net change on the molecule is zero. In these cases it was found that the reaction product did not readily elute from the CM-cellulose column at pH 6.0. However, if the column was run at pH 7.0, the elution pattern was exactly as described previously. The recoveries were a little less than complete and so the quantity of the eluted nucleotide material was estimated from the absorbance of the eluate, as described for GMP.

Chromatography on DEAE Paper. Although denatured DNA behaved satisfactorily on the CM-cellulose column (pH 6.0), native DNA gave flow rates too slow to be of use. In this case, the excess carbodiimide was separated from the reacted DNA by chromatography on DEAE paper. Strips of paper (Whatman, Grade DE81, 21 × 3 cm) were pretreated by dipping in 0.01 M acetic acid, then water, and then drying in warm air. The reaction mixture was applied in a streak across the whole width of the strip 3 cm from the bottom and upward elution with 0.5 M sodium acetate (pH 5.0) was effected until the front was *ca.* 15 cm from the line of sample application. After drying in warm air, a 3 × 3 cm square of paper, centered on the line of sample application and containing the DNA was cut out and counted in a toluene-based scintillation mixture.

SPECTROPHOTOMETRIC ANALYSIS. For most of the experiments the Unicam SP 800 double-beam spectrophotometer was employed. It was equipped with a temperature-controlled cell holder and means for continuously recording the absorbance at a single wavelength as a function of time. Stoppered silica cells (10 mm) were used and the wavelength was checked using a Holmium filter. The carbodiimide was always in the bromide salt form. Usually equal concentrations of the carbodiimide bromide were used in both sample and reference cells.

Preparation of Multistranded Polynucleotides. Poly A and poly U were used either directly as purchased or following dialysis, first against 1.0 M NaCl–0.01 M EDTA (pH 8) and then against three changes of water. The homopolymers were each dissolved in water (0.7 mg/ml), aliquots were diluted in 0.2 M NaCl–0.01 M Tris-HCl (pH 7.4), the adsorptions were measured, and thus the concentrations of nucleotides in the homopolymer solutions were determined. Appropriate volumes of these solutions were mixed to give solutions having the desired A:U ratio. These were lyophilized and the resultant polynucleotide mixtures were dissolved in appropriate buffers at concentrations of 6–7 mg/ml. After adjustment of the pH to 8.0, the solutions were kept in tightly stoppered tubes (in the presence of a drop of chloroform) at room temperature for 3–4 days prior to use. The homopolymers were made up and mixed in dilute solution because concentrated solutions of poly A are inhomogeneous. Concentrations of mixed polymer solutions (or of copolymer solutions) were determined by hydrolyzing aliquots in 0.4 M KOH at 37° for 18 hr followed by acidification to pH 1 and measurement of the absorbance at 260 mμ. The concentrations were calculated using molar extinction coefficients that were the appropriately weighted means of the mononucleotide values in 0.1 N HCl. A mixture of poly (A,U) and poly U was prepared in essentially the same way as the poly A + poly U mixtures.

Chain Scission and Cross-Linking. In one experiment 100 μg of ApA was incubated with 2 μmoles of the carbodiimide in 0.01 M borate buffer (pH 8.0; volume 40 μl) at 30° for 24 hr. The reactants and products were then separated by high-voltage paper electrophoresis. In another experiment, 200 μg of tRNA was incubated with 2 μmoles of the bromide carbodiimide in 0.01 M Tris-Cl buffer (pH 8.0; volume 40 μl) at 30° for 44 hr. The reaction mixture was then analyzed by means of polyacrylamide gel electrophoresis (Richards *et al.*, 1965) in the presence of 7 M urea.

Miscellaneous Methods. High-voltage paper electrophoresis was carried out in 0.02 M phosphate buffer (pH 7.2) on Whatman No. 3MM paper, with a potential gradient of 100 V/cm, at 40–50 mA for 30–40 min.

Radioactive counting was done in the Packard scintillation counter. Aqueous solutions were counted using a mixture of 8 g of 2-(4′-butylphenyl)-5(4′′-biphenylyl)-1,3,4-oxidiazole, 600 ml of toluene, 400 ml of 2-methoxyethanol, and 80 g of napthalene. Samples on Millipore filters or on DEAE paper were counted in the presence of a mixture of 6 g of 2-(4′-butylphenyl)-5-(4′′-biphenylyl)-1,3,4-oxidiazole/l. of toluene. Counting efficiencies on Millipore filters were determined using preparations of labeled aminoacyl-tRNA. Aliquots were counted both on the filters, after precipitation by 7% trichloroacetic acid, and as aqueous solutions as described above. The efficiency in the latter instance was determined by internal standardization.

Base compositions of polynucleotides were determined spectrophotometrically according to the procedure of Richards (1968).

Molar extinction coefficients were obtained from the following sources: mononucleotides at pH 7 from the data in the Pabst Circular OR-17 (Pabst Laboratories, Milwaukee, Wis.), mononucleotides at pH 1 from Beaven *et al.* (1955), dinucleoside phosphates from Warshaw and Tinoco (1965), and poly A and poly U from Blake and Fresco (1966).

Results

The Stability of the Carbodiimide. Any reagent used to modify nucleic acids must itself be substantially stable under the conditions of the modification reaction. We have studied the stability of the carbodiimide under a variety of conditions by means of infrared spectroscopy. Carbodiimides have a characteristic absorbance peak at about 2100 cm^{-1} due to the N=C=N stretching mode (Khorana, 1953). The carbodiimide has such a peak at 2120 cm^{-1} and its decrease has been employed to follow the destruction of the carbodiimide moiety. The transmittance of water at this wavelength is sufficient to permit the use of aqueous solutions. The data fit a first-order kinetic scheme satisfactorily. The first-order rate constant and the corresponding half-life values for the decompo-

TABLE I: Decomposition of the Carbodiimide Followed by Infrared Absorbance.[a]

Carbodiimide	Buffer (M)	pH	Temp (°C)	$k \times 10^2$ (hr^{-1})	$t_{1/2}$ (hr)
Carbodiimide *p*-toluenesulfonate	H_2O		30	0.29	240
Carbodiimide *p*-toluenesulfonate	Sodium borate (0.1)	8.0	30	0.63	110
Carbodiimide *p*-toluenesulfonate	Tris-Cl (0.1)	7.6	30	2.1	32
Carbodiimide *p*-toluenesulfonate	Sodium phosphate (0.1)	7.0	30	75	0.92
Carbodiimide *p*-toluenesulfonate	Sodium cacodylate (0.1)	7.0	30	20	3.4
Carbodiimide *p*-toluenesulfonate	Sodium cacodylate (0.1)	6.0	30	66	1.0
Carbodiimide *p*-toluenesulfonate	Sodium acetate (0.1)	5.0	30	360	0.19
Carbodiimide *p*-toluenesulfonate	Sodium cacodylate (0.01)	7.0	30	4.6	15
Carbodiimide *p*-toluenesulfonate	Sodium phosphate (0.01)	7.0	30	15.6	4.4
Carbodiimide *p*-toluenesulfonate	Sodium phosphate (0.1)	1.3	30		<0.01
Carbodiimide bromide	Sodium borate (0.1)	8.0	30	0.97	72
Carbodiimide iodide	Sodium borate (0.1)	8.0	30	0.66	104
Carbodiimide bromide	Sodium borate (0.1)	8.0	50	8.9	7.8
Carbodiimide iodide	Sodium borate (0.1)	8.0	50	4.3	16.3

[a] pH measurements were made at *ca.* 22°. k is the first-order rate constant of eq 1. $t_{1/2}$ is the half-life of the carbodiimide.

sition of the carbodiimide in a number of buffers are shown in Table I. It is evident that the stability of the carbodiimide varies over a very wide range. The pH is the most important determinant; in general, the lower the pH, the greater the rate of decomposition. However, the data of Table I indicate that the type of buffer anion is also important.

The carbodiimide is seen to be very stable in water and in borate buffer (pH 8.0) at 30°. The three different salt forms of the carbodiimide behave quite similarly (the carbodiimide *p*-toluenesulfonate was used for most of these experiments because of its ready availability). All the reactions involving the carbodiimide that are described in this paper employ either sodium borate or Tris-Cl buffers at pH 8.0 and at temperatures below 37°, and normally at least a tenfold excess of reagent over reacting nucleotide is used.

TABLE II: Reaction between the Carbodiimide and Buffers Followed by the Change in pH.[a]

Buffer (M)	Carbodiimide Concn (mM)	Time (hr)	ΔpH
Sodium borate (0.01)	50	22	0.06
Sodium borate (0.01)–$MgCl_2$ (0.005)	50	29	0.02
Tris-Cl (0.01)	50	22	0.30
Tris-Cl (0.005)	25	22	0.17

[a] Reactions were initiated by dissolving solid carbodiimide *p*-toluenesulfonate in the buffer, or by mixing equal volumes of double-strength carbodiimide *p*-toluenesulfonate solution (in water) with double-strength buffer. All mixtures had initial values of 8.0. The reactions were carried out at 30° and the pH values were monitored continuously. pH changes were found to be linear with time and so only the net changes are given above (the ΔpH values); all changes were increases.

The possibility of reaction between the carbodiimide and the ion-exchange materials, DEAE-cellulose and Dowex 1, was investigated using the infrared absorbance method. No decomposition of the carbodiimide could be detected after 2 hr at room temperature. As a further check on the stability of the carbodiimide, the pH changes occurring on incubation in borate and in Tris-Cl buffers were determined. The results of several such experiments are shown in Table II. There are only slight changes in pH in the case of borate buffer but rather greater pH drifts occur with Tris-Cl buffer. Presumably there is a limited amount of reaction between the carbodiimide and the Tris ion.

The Products of the Reaction between the Carbodiimide and the Mononucleotides. The reaction between 5′-UMP or 5′-GMP and the carbodiimide may be written as follows (showing the charges effective at pH 7, and omitting nonreacting ions): NMP^{2-} + carbodiimide$^+$ ⇄ carbodiimide–NMP^-. On the basis of elemental analysis, electrophoretic mobility, and ultraviolet spectra, Ho and Gilham (1967) proposed that the reaction product, in the UMP case, was the N-substituted derivative shown in Figure 1. The reaction product should be readily separable from the reactants by means of ion-exchange chromatography at neutral pH. The results of the chromatography of such reaction mixtures on DEAE-cellulose columns are shown in Figure 2. The eluted peaks were identified on the basis of their ultraviolet absorption spectra and electrophoretic mobilities. Peak A in Figure 2a is carbodiimide$^+$, peak B is the reaction product carbodiimide–UMP^-, and peak C is the *p*-toluenesulfonate ion (recognized by its characteristic multicomponent spectrum at around 260 mμ). Unreacted UMP would have eluted at the end of the gradient; under the present conditions no unreacted nucleotide was detectable. In the case of the 5′-GMP reaction (Figure 2b) some nucleotide was left unreacted and this eluted as peak D. In both cases no significant amount of material having an absorption maximum at around 260 mμ was detectable in peak A, and no minor peaks were to be seen. The fact that only the expected components were observed indicates that the reac-

tion is specific, in agreement with the original report of Gilham (1962).

Other mononucleotides were tested for their reaction with the carbodiimide using ion-exchange chromatography to analyze the reaction mixture. With 5′-AMP and 5′-CMP no peak corresponding to the modified nucleotide was found. 5′-TMP and 5′-IMP reacted to give products analogous to those of the UMP and GMP reactions. Dihydrouridine monophosphate was also investigated, but no evidence for the existence of a reaction product in which the carbodiimide was bound to the nucleotide could be obtained.

The reaction between the carbodiimide and 2′(3′)-UMP was studied. In this case an important side reaction occurs leading to the formation of the cyclic phosphate derivative of the nucleotide and other products (D. H. Metz, unpublished experiments). Dekker and Khorana (1954) first reported this type of reaction and Naylor and Gilham (1966) have shown it to be the dominant reaction at pH 6. However, undegraded RNA is not expected to contain 2′(3′)-phosphate groups.

Some properties of the carbodiimide–5′-nucleotide compounds were determined. Their electrophoretic mobilities at pH 7.2, relative to the corresponding mononucleotide, were found to be 0.29 for carbodiimide–UMP and 0.37 for carbodiimide–GMP. The ultraviolet spectra of these compounds were measured in 0.10 M borate buffer (pH 8.0); for carbodiimide–UMP, λ_{max} 266 mμ, λ_{min} 241 mμ; for carbodiimide–GMP, λ_{max} 253 and 280 mμ, λ_{min} 234 and 274 mμ.

The stabilities of the two carbodiimide–nucleotide compounds were investigated as a function of pH (see Figure 3). Decomposition occurs readily at pH 10.5 but the compounds are stable over long periods at pH 5. Ho and Gilham (1967) have previously demonstrated the hydrolysis at pH 10.5 and have shown that the parent compounds are regenerated. We have also found the two compounds to be completely stable in 0.1 N HCl over 40 hr. The loss of the carbodiimide moiety from carbodiimide-reacted tRNA at pH 10.5 is also shown in Figure 3. The fact that some of the reagent remains bound at this pH after 6 hr may be attributed, at least in part, to the presence of ψMP residues in the tRNA. Naylor *et al.* (1965) showed that one of the two carbodiimide residues that could react with this minor nucleotide was stable in dilute alkali.

The Course of the Reaction between the Carbodiimide and the Nucleotides. Two methods have been employed for following the carbodiimide reaction. One is based upon the change in the ultraviolet absorption spectrum of the nucleotide when it reacts with the reagent. The other utilizes the binding of isotopically labeled carbodiimide to the nucleotide.

Since the spectra of the modified nucleotides, described in the preceding section, differ from those of the parent nucleotides, it is apparent that the reactions can be followed by means of the absorbance changes. However, the carbodiimide, which has to be present in fair excess, has a significant absorbance in the ultraviolet. The nature of its spectrum depends both upon the cation (carbodiimide$^+$) and anion. The *p*-toluenesulfonate salt form had to be excluded since this anion absorbs at around 260 mμ. The iodide form had an absorbance maximum of 227 mμ (due to the iodide ion). Since at high reagent concentrations a 227-mμ peak of appreciable width would interfere with the nucleotide spectrum, other carbodiimide salts were investigated. They were prepared from the carbodiimide *p*-toluenesulfonate by ion-exchange chromatography on a Dowex 1 resin. A number of salts of the carbodiimide were prepared but all were found to be quite hygroscopic, in marked contrast to both the iodide and *p*-toluenesulfonate derivatives. The bromide form was the one used in the spectroscopic experiments; it was used as a concentrated stock solution, the concentration of which was determined by drying and weighing aliquots. The spectrum of carbodiimide bromide is shown in Figure 4.

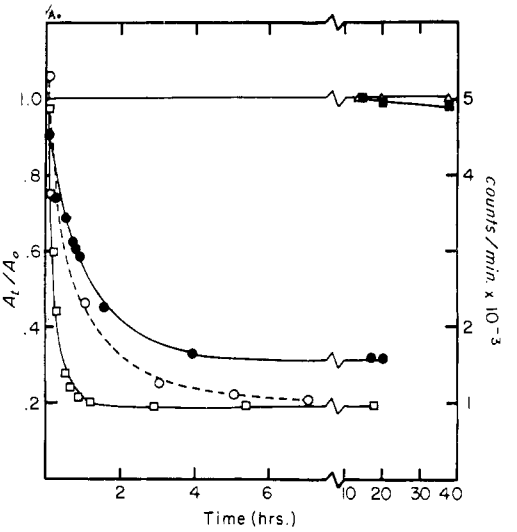

FIGURE 3: The decomposition of the carbodiimide–nucleotide compounds was followed spectroscopically. Stock solutions of carbodiimide–UMP or carbodiimide–GMP in water were added to the appropriate buffer to give suitable absorbances at 284 and 292 mμ, respectively. The absorptions of these solutions, in stoppered spectrophotometer cells, at these wavelengths, were followed as a function of time, at 22°. (△—△) Carbodiimide–UMP in 0.1 M acetate buffer (pH 5.0); (■—■) carbodiimide–GMP in 0.1 M acetate buffer pH 5.0); (●—●) carbodiimide–UMP in 0.1 M triethylamine acetate buffer (pH 10.5); (□—□) carbodiimide–GMP in 0.1 M triethylamine acetate buffer (pH 10.5). In the case of the carbodiimide-reacted tRNA, 50-μl aliquots of a solution of ^{3}[H]carbodiimide-tRNA were added to 0.5-ml aliquots of 0.1 M glycine–NaOH buffer (pH 10.5). The reactions were stopped by addition of 0.1-ml carrier tRNA (5 mg/ml) and 3 ml of 5% trichloroacetic acid. The precipitates were filtered on Millipore filters, washed three times with 5% trichloroacetic acid, dried, and counted. (O- - -O) [^{3}H]Carbodiimide-tRNA.

The changes in the absorption spectra during the carbodiimide bromide–nucleotide reactions are shown in Figure 4. Because of the nature of the carbodiimide bromide spectrum (the absorbance at 260 mμ is about 1.0 at the concentration used in the present experiments), little reliance can be placed upon nucleotide absorption data at wavelengths below about 260 mμ. Nevertheless, there is a clear isosbestic point at 271 mμ in the UMP case, while in the GMP case there are two, one at 280.5 mμ and the other at about 263 mμ.

Difference spectra between the reacted and unreacted mononucleotides indicate that the wavelengths where the greatest absorbance changes may be observed in the course of the reactions are 284 mμ for the UMP case and 292 mμ for GMP. It should be mentioned that when the carbodiimide bromide was incubated alone in borate buffer at 30°, a small progressive decrease in absorbance occurred at wavelengths greater than 250 mμ. This absorbance change was small compared with that which occurs during the carbodiimide–nucleotide reaction (less than 10%). Its origin is obscure but in all experiments the effect was allowed for, usually by having the carbo-

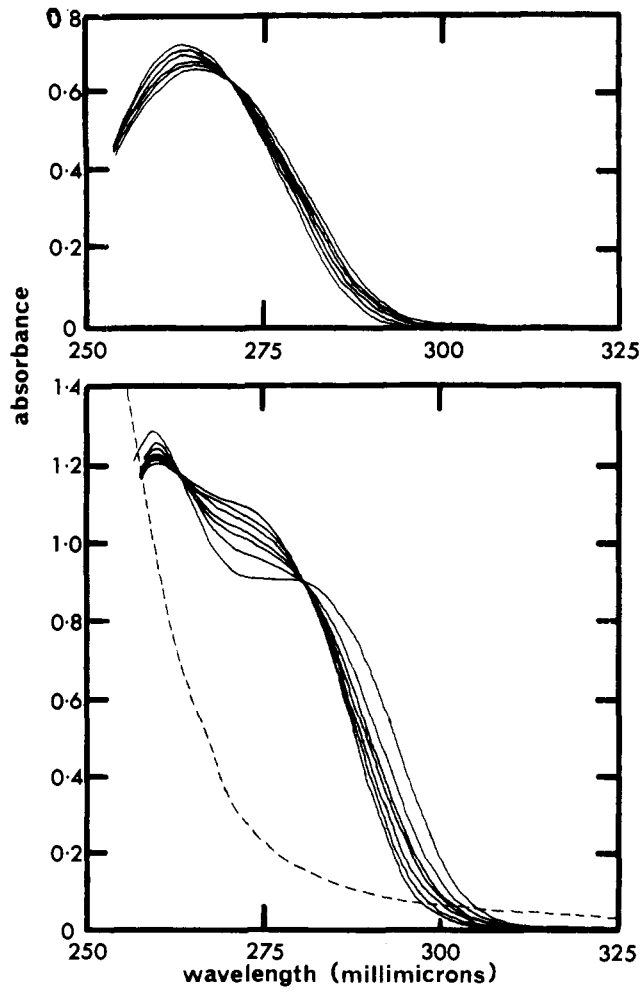

FIGURE 4: The spectral changes accompanying the reaction between the carbodiimide bromide and 5′-UMP and 5′-GMP. Spectra were recorded on the Unicam SP800 spectrophotometer with 10-mm silica cells. The reference cells contained carbodiimide bromide (50 mM) in 0.09 M borate buffer (pH 8.0), thus compensating for the absorbance of carbodiimide bromide in the reaction mixture. The absorbance of the reference cell against a buffer blank is shown in the lower figure (dashed line). (a) The sample cell contained 5′-UMP (7×10^{-5} M) and carbodiimide bromide (5×10^{-2} M) in 0.09 M borate buffer (pH 8.0), at 30°. Spectra were recorded at 0, 1.5, 3, 4.5, 6.5, and 17 hr. The absorbance increased with time at wavelengths greater than 271 mμ. (b) The sample cell contained 5′-GMP (9×10^{-5} M) and carbodiimide bromide (5×10^{-2} M) in 0.09 M borate buffer (pH 8.0), at 30°. Spectra were recorded at 0, .5, 1, 2, 3, 5.5, and 20 hr, the absorbance increasing with time at wavelengths above 280 mμ.

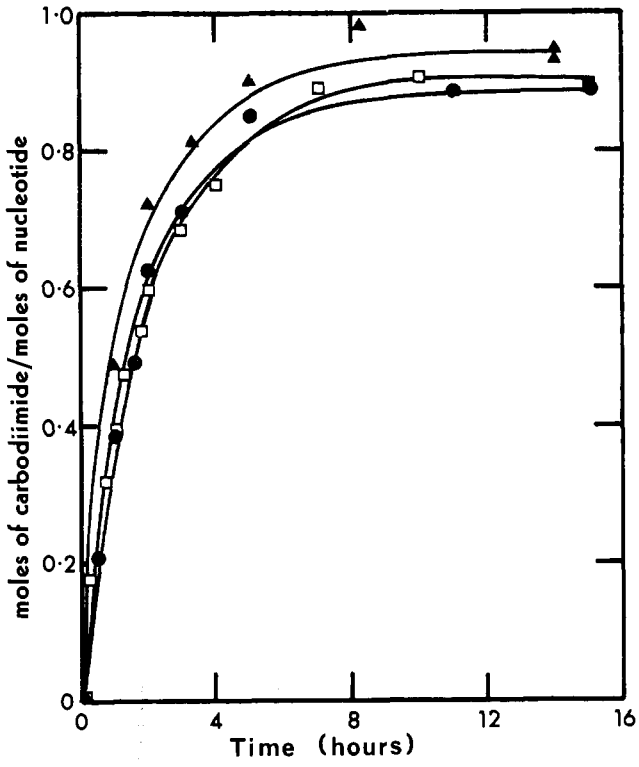

FIGURE 5: The reaction between [^{14}C]carbodiimide and 5′-UMP, 5′-GMP, and poly U. The concentration of [^{14}C]carbodiimide iodide was 50 mM, the buffer was 0.10 M borate (pH 8.0), the temperature was 30°, and the volume of the reaction mixture was 40 μl, in all cases. The UMP concentration was 4.5 mM, that of GMP was 4.0 mM, and that of poly U was 4.0 mM. The reaction mixtures were processed on CM-cellulose columns after incubation. (□—□) UMP, (●—●) GMP, and (▲—▲) poly U.

diimide in both the sample and reference cells. The validity of this correction depends upon the assumption that the absorbance change of the carbodiimide bromide is independent of the presence of the nucleotide.

The isotopically labeled carbodiimide may be prepared without difficulty from [^{14}C]- or [^{3}H]methyl iodide and the nonmethylated carbodiimide precursor. The product is the iodide salt of the carbodiimide base. The major problem that arises with the use of the labeled reagent is the need to separate efficiently the large excess of the carbodiimide from the reacted nucleotide or polynucleotide. Fortunately, the reagent is bound quite stably at acidic pH values and the separation procedure is best carried out under such conditions.

A number of methods have been reported for removing excess carbodiimide, including ethanol precipitation of the nucleic acid (Augusti-Tocco and Brown, 1965), perchloric acid precipitation (Drevitch *et al.*, 1966), Sephadex chromatography (Knorre *et al.*, 1966), and DEAE-cellulose chromatography (Augusti-Tocco and Brown, 1965). The procedures that depend upon the size of the nucleotide reactant were not suitable for the present work since it was desired to study the reaction with mononucleotides as well as with polynucleotides. Ion-exchange chromatography seemed likely to be the most useful type of technique and a number of exchangers and conditions were investigated. While most methods gave adequate separation of the nucleotide and the oppositely charged reagent, the CM-cellulose column technique was the most satisfactory in terms of recovery of nucleotide material and efficiency of removal of unreacted reagent. Full details are given in the Material and Methods section.

Recovery of nucleotide material from the analytical scale CM-cellulose columns was checked in a number of cases. When [^{14}C]UMP was reacted with unlabeled carbodiimide iodide, 97% of the radioactivity put on the column was recovered. [^{32}P]RNA prepared from the bacteriophage μ2 was reacted with the reagent and in this instance 99% of the activity was recovered after elution. The recoveries of other mono-, oligo-, and polynucleotides from the column were determined by absorbance measurements. In certain cases, recoveries of significantly less than 100% were obtained; in these instances

the calculation of the extent of the carbodiimide reaction was based upon the actual amount of nucleotide recovered. This is described fully in Materials and Methods.

Both the spectroscopic and isotopic methods have advantages and disadvantages. Essentially, the former is the more convenient for kinetic studies of the mononucleotides but the latter is far more satisfactory for measuring extents of reaction. The spectroscopic method cannot be used with polynucleotides possessing secondary structure since perturbation of this structure may give rise to a hyperchromic effect which will interfere with observations of the absorbance changes due to the reaction.

The time course of the reactions between the carbodiimide and 5′-UMP, 5′-GMP, and poly U is shown in Figure 5. In all three cases a plateau is reached after 8 hr, at which time 85–95% of the nucleotide has reacted. To analyze and compare these and similar data, it is necessary to consider a scheme of reaction within which the experimental results may be interpreted.

A kinetic expression adequate for present purposes may be derived by commencing with the expression

$$N + C \underset{k_{-1}}{\overset{k_{+1}}{\rightleftharpoons}} X \qquad (2)$$

This represents the reversible reaction between the nucleotide, N, and the carbodiimide, C, to give the product, X. k_{+1} and k_{-1} are the rate constants of the forward and reverse reactions, respectively. The adequacy of the scheme in eq 2 will be considered presently.

Let the concentration of N at times $t = 0$, t, and ∞ be n_0, n, and n_e, respectively, and likewise for the concentration of C (c_0, c, and c_e), and of X (x and x_e). Let it be assumed that $c_0 \gg n_0$ (this will be the case experimentally), so that the concentration of C is effectively constant. We may now write the rate equation for the second-order reversible reaction shown in eq 2:

$$\frac{dx}{dt} = k_{+1}(n_0 - x)c_0 - k_{-1}x \qquad (3)$$

We employ the equilibrium condition that $dx/dt = 0$, eliminate k_{-1}, and integrate, to give the expression

$$-\ln\left(\frac{x_e - x}{x_e}\right) = k't \qquad (4)$$

where

$$k' = \frac{k_{+1}c_0n_0}{x_e} = k_{+1}c_0 + k_{-1} \qquad (5)$$

Equation 4 may be used directly for the analysis of the data obtained from the reaction of the labeled carbodiimide with nucleotides. For spectroscopic data, it may readily be demonstrated that eq 4 transforms to

$$-\ln\left(\frac{A_\infty - A_t}{A_\infty - A_0}\right) = k't \qquad (6)$$

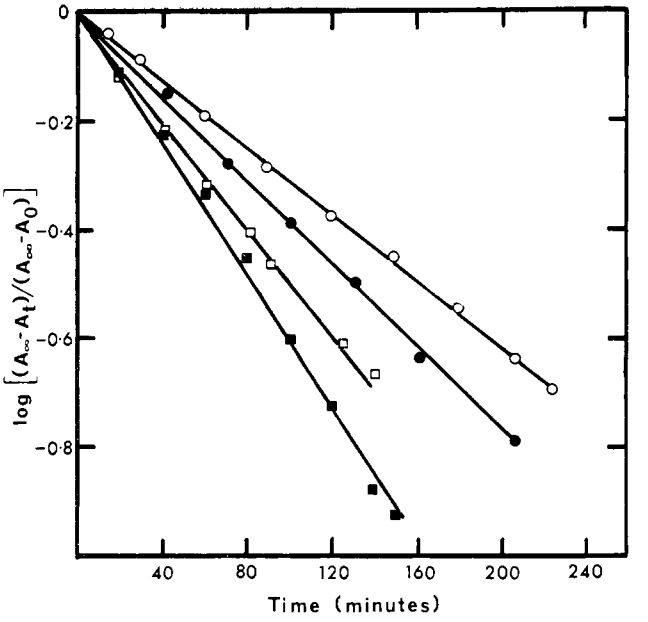

FIGURE 6: The kinetics of the reaction between the carbodiimide and uridine, 5′-UMP, poly U, and ApU. The reactions were followed spectrophotometrically. The reaction conditions are given in Table III. (■—■) Uridine (in 85 mM borate buffer), (○—○) 5′-UMP (in 100 mM borate), (□—□) poly U (in 78 mM borate), and (●—●) ApU.

where A_0, A_t, and A_∞ are the absorbance of the reaction mixture at the times indicated by the subscript.

The expressions 4 and 6 describe the course of a pseudo-first-order reversible reaction, where the rate constant, k', is a function of the concentration of the reactant present in excess.

The reaction in eq 2 is somewhat simplified. Experimentally it is found that the reaction does not go entirely to completion. One possible reason for this is that the simple reverse reaction (rate constant k_{-1}) is significant. An alternative, or additional, explanation is that the following reaction occurs

$$X \xrightarrow{k_2} N + C' \qquad (7)$$

This represents the hydrolysis of X to give the nucleotide and C′, a hydrolyzed form of the carbodiimide which is likely to be a disubstituted urea (NHCONH). It seems reasonable to suppose that the hydrolysis of the reacted nucleotide at pH 10.5 is a base-catalyzed reaction of this type. A kinetic treatment of the complete reaction scheme (eq 2 and 7) is mathematically complicated and is hardly necessary for the purpose of the present discussion. It may be noted that in eq 7 N is regenerated and this can participate again in eq 2. Thus eq 2 and 7 together effect the net conversion of C into C′. Provided that k_2 is small compared with k_1 and that C is present in excess, the eq 7 may be regarded as being included in the eq 2, that is, k_2 can be effectively included in k_{-1}.

The kinetic data from the carbodiimide–nucleotide reactions have been treated in accord with the kinetic expressions just derived. Some typical results using the spectrophotometric method are shown in Figure 6. It is apparent that the data fit the kinetic scheme very well. Data obtained using the isotopically labeled carbodiimide also fit the expected kinetics well. The rate constants are determined from the slopes of the above-

TABLE III: Rate Constants for the Reaction between the Carbodiimide and the Nucleotides.[a]

Nucleotide	Nucleotide Concn (mM)	Buffer (mM)	Method	$k' \times 10^3$ (min^{-1})
5′-UMP	0.18	Borate (100)	sp	7.5
5′-UMP	4.5	Borate (100)	is	6.2
5′-UMP	0.16	Borate (8.3)	sp	12.0
5′-UMP	0.16	Tris-Cl (8.3)	sp	12.0
5′-UMP	5.2	Borate (10) $MgCl_2$ (10)	is	7.2
Poly U	0.17	Borate (78)	sp	11.3
Poly U	0.17	Borate (7.8)	sp	12.1
Poly U	0.17	Tris-Cl (7.8)	sp	11.2
Poly U	4.0	Borate (10) $MgCl_2$ (10)	si	7.2
Uridine	0.17	Borate (85)	sp	14.2
Uridine	0.17	Borate (8.5)	sp	17.5
Uridine	0.17	Tris-Cl (8.5)	sp	17.8
ApU	0.15	Borate (87)	sp	8.8
5′-GMP	0.20	Borate (100)	sp	5.6
5′-GMP	4.0	Borate (100)	is	6.4
GpC	0.22	Borate (87)	sp	6.8
$(Gp)_3Cp$	0.2	Borate (87)	sp	5.9

[a] In all cases the pH was 8.0, the temperature was 30°, and the carbodiimide concentration was 50 mM. In the spectrophotometric experiments (denoted by 'sp') carbodiimide bromide was used, while in the isotope experiments (denoted by 'is'), [^{14}C]carbodiimide iodide was employed. The wavelengths used in the spectrophotometric experiments were 280 mμ for the uracil-containing nucleotides and 292 mμ for the guanine-containing nucleotides. The rate constants, k', were calculated using the eq 4 or 6 as appropriate.

mentioned plots. In Table III there are summarized the data from a number of such experiments.

It will be seen that, where comparable, the data from the spectrophotometric and isotopic experiments are in good agreement. This means that there is no significant difference between the bromide and iodide forms of the carbodiimide in the reaction with the nucleotides. It is also apparent that there is no significant difference between the rates of reaction in borate and in Tris-Cl buffers, at least under the conditions used here.

It is satisfactory that the range of values of the rate constants shown in Table III is relatively small, despite the variations in buffer type and concentration, and the variety of mono-, oligo-, and polynucleotides and nucleosides employed. There is only a threefold difference between the greatest and smallest values. It follows that the times required for 90% completion of the reaction will range between about 3 and 7 hr for the nucleotides

The Position of the Equilibrium in the Reaction between the Carbodiimide and the Nucleotides. Since the carbodiimide is to be used to measure the proportion of single-stranded residues in RNA, it is obviously necessary to know the extent of reaction between the mononucleotides and the reagent under the various conditions used. This is equivalent to a knowledge of the equilibrium constant for the reaction. Two independent methods have been employed to determine the extent of reaction; in the *direct* method, the amount of labeled carbodiimide bound to the nucleotide has been measured. In the *indirect* method, the equilibrium constant for the reaction is determined from the rates of the forward and reverse reactions.

We consider first the indirect method. The equilibrium constant, K, for the eq 2 is given by

$$K = \frac{k_{+1}}{k_{-1}} \tag{8}$$

Since the rate constants k_{+1} and k_{-1} are proportional to the initial rates of the forward and reverse reactions, respectively, K can be deduced from the ratio of these rates, as follows.

For eq 2, the initial rate of the forward reaction is given by

$$\left(\frac{dx}{dt}\right)_0 = k_{+1} n_0 c_0 \tag{9}$$

This converts into an expression involving absorbances, namely

$$\left(\frac{dA}{dt}\right)_0 = \Delta\epsilon k_{+1} n_0 c_0 \tag{10}$$

where the term on the left-hand side represents the initial rate of change of absorbance for the forward reaction, and $\Delta\epsilon$ is the difference between the extinction coefficients of the nucleotide and the reacted nucleotide at the particular wavelength employed for following the reaction. Knowing n_0 and c_0 and the rate of change of absorbance at zero time, $\Delta\epsilon k_{+1}$ may be determined.

The reverse reaction of eq 2 may be followed directly if the nucleotide–carbodiimide compound is isolated. We have

$$\left(\frac{dA'}{dt}\right)_0 = -\Delta\epsilon k_{-1} x_0 \tag{11}$$

where x_0 is the zero-time concentration of X, and the left-hand side of the expression is again the initial rate of change of absorbance. Again, $\Delta\epsilon k_{-1}$ can be determined. Thus, knowing both $\Delta\epsilon k_{+1}$ and $\Delta\epsilon k_{-1}$, K can be calculated.

The rates of change of absorbance at zero time for both the forward and reverse reactions of both the UMP and the GMP cases were determined spectroscopically. The equilibrium constants in 0.10 M borate buffer (pH 8.0) at 30° were deduced (see Table IV). The values of the equilibrium are seen to be relatively large, a satisfactory situation since this means that the reactions go substantially to completion. We may determine the extent of reaction from the equilibrium constant as follows. If α is the proportion of the nucleotide, N, that is converted into X, then from eq 2 it follows that

$$K = \frac{\alpha}{(1 - \alpha)c_0} \tag{12}$$

TABLE IV: Equilibrium Constants for the Reactions between the Carbodiimide and the Mononucleotides.[a]

Nucleotide	$\Delta\epsilon k_{+1}$ (l.^{2} mole^{-2} min^{-1})	$\Delta\epsilon k_{-1}$ (l. mole^{-1} min^{-1})	K (l. mole^{-1})
UMP	$(1.50 \pm 0.09) \times 10^2$	0.136 ± 0.006	1100 ± 85
GMP	$(4.55 \pm 0.29) \times 10^2$	1.02 ± 0.06	450 ± 40

[a] The initial rates of change of absorbance for the forward and reverse reactions in eq 2 were determined for both 5′-UMP and 5′-GMP. The buffer was 0.10 M borate (pH 8.0), at 30°. The same wavelength was used for both forward and reverse reactions; for UMP it was 284 mμ and for GMP it was 292 mμ. The rates of change of absorbance with time were linear for the first 15–20 min for the forward reactions and for at least 6 hr for the reverse reactions. n_0 and x_0 values were determined from the absorbances at the maximum and the isosbestic wavelengths, respectively. Each $\Delta\epsilon k$ value is the mean of four independent determinations using varying reactant concentrations; the errors quoted are standard deviations of the mean.

This rearranges to

$$\alpha = \frac{Kc_0}{1 + Kc_0} \tag{13}$$

Thus for a given value of K, α may be plotted against the reagent concentration, c_0. This has been done in Figure 7. When the carbodiimide concentration is 50 mM, the UMP reaction has gone to 98% completion, while the GMP reaction has reached 96%. At 25 mM, the corresponding values are 96 and 91%. Thus, between these two reagent concentrations, the extent of reaction has been calculated to be greater than 90% and it is relatively insensitive to small changes in reagent concentration.

The extent of the carbodiimide–nucleotide reaction can be determined directly using labeled carbodiimide. Figure 5 shows the time course of the reaction. The proportion of each nucleotide that is reacted is shown in Table V. The value for UMP is close to that determined by the indirect method but the GMP figure is some 10% lower. The reason for this discrepancy is unclear. In all cases, however, the extent of reaction is greater than 85%.

The variation in the extent of reaction as a function of the carbodiimide concentration, determined directly, is shown in Figure 8. This may be compared with Figure 7, where the extent of reaction was determined indirectly. GMP reacts to a rather lesser extent as mentioned above. Also, the extent of reaction at the lower carbodiimide concentrations are less in Figure 8. This is because in the one case the carbodiimide concentration indicated is that present at the start of the reaction while in the other, it is the value of the concentration when equilibrium is reached.

The extent of reaction as a function of temperature was investigated. Some results are indicated in Figure 9. It should be stressed that in these experiments the incubation was for a uniform 16 hr. Although equilibrium is reached in this time at 30°, this may not be the case at lower temperatures. Thus the reduced extents of reaction found at the lower temperatures are not necessarily due to a shift in the position of equilibrium but may be due to failure to reach equilibrium in 16 hr. Whatever the actual situation may be, it is nevertheless apparent that above 20°, usually at least 80% of the nucleotide will have reacted under the conditions examined.

The Reaction of the Carbodiimide with Double-Stranded Polynucleotides. Having considered the reaction between the carbodiimide reagent and the mononucleotides, we may now extend the discussion to the reaction with polynucleotides. It

TABLE V: Extent of Reaction between the Carbodiimide and Various Nucleotides.[a]

Nucleotide	Extent of Reaction (%)
5′-UMP	96
5′-GMP	86
Poly U	93
5′-IMP	90
ApU	97
GpC	85

[a] About 0.2 μmole of the nucleotide was incubated with 2 μmoles of [^{14}C]carbodiimide iodide in 0.10 M borate buffer (pH 8.0) in a volume of 40 μl at 30°. After 13–16 hr the reaction mixtures were processed on CM-cellulose columns. The results are expressed as the proportion of the nucleotide that reacts under the present conditions. Each result is the mean of at least two determinations.

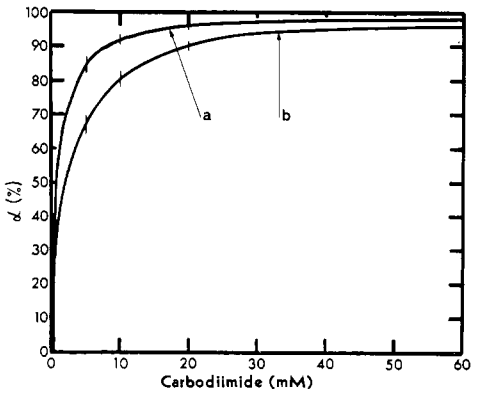

FIGURE 7: The extent of the reaction between the carbodiimide and the mononucleotides, determined indirectly. α is the proportion of the nucleotide that has reacted with the reagent at equilibrium. Curve a is for the UMP reaction, and curve b for the GMP case. The errors bars correspond to the uncertainty in the values of the equilibrium constant, K, which are used to calculate α.

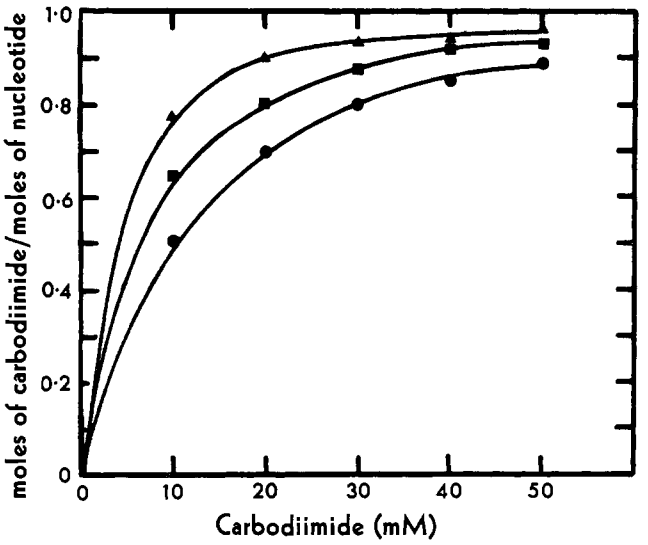

FIGURE 8: The extent of the reaction between the carbodiimide and the nucleotides determined directly. The procedure used was as determined in Table V except that the amount of [^{14}C]carbodiimide iodide was varied as indicated. The ordinate is the proportion of the nucleotide that has reacted after 16.5-hr incubation. (▲—▲) 5′-UMP, (●—●) 5′-GMP, and (■—■) poly U.

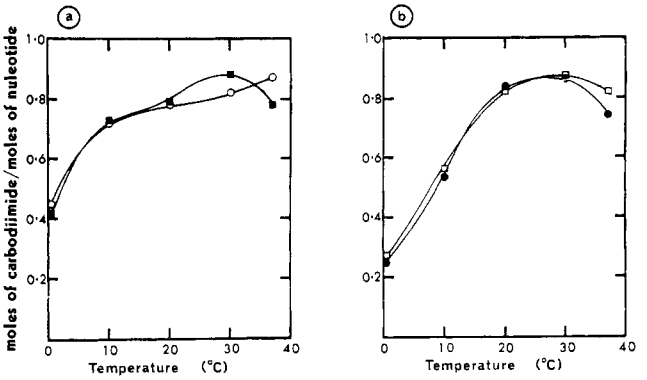

FIGURE 9: The extent of the reaction between the carbodiimide and the nucleotides as a function of temperature. The procedure was as described in Table V. The time of incubation was 16 hr (a) (■—■) 5′-UMP in 0.10 M borate buffer and (○—○) 5′-UMP in 0.01 M borate–10 mM $MgCl_2$; (b) (□—□) 5′-UMP in 0.01 M borate and (●—●) 5′-GMP in 0.01 M borate.

has already been seen that the rate and extent of reaction of the reagent with poly U very closely parallels the UMP case. It may therefore be concluded that the amorphous secondary structure of this polynucleotide does not influence the process of chemical modification. On the other hand, the ordered, stacked secondary structure conformations are expected to affect the reaction and it is to a consideration of these that we now turn.

First, however, it is convenient to dispose of two points that arise when the chemical modification of polynucleotides is considered. These are the possibilities that the reaction may cause either scission or cross-linking of the polynucleotide chain, quite apart from the reaction with the base. The possibilities were tested in two experiments. In one, the dinucleoside phosphate, ApA, which does not react with the carbodiimide, at least in the normal mode, was incubated with the reagent for 24 hr. The mixture was then subject to high-voltage paper electrophoresis under conditions where adenosine, AMP, and ApA could be separated. No AMP was found (conversion of 1% of the ApA into this would have been detected), nor was any adenosine though the presence of the latter would have been somewhat obscured by the electrophoretic behavior of the excess of the carbodiimide. The last difficulty also makes it unlikely that polymerized products would have been detectable. In the other experiment, tRNA was incubated with the carbodiimide for 44 hr. The mixture was then analyzed by polyacrylamide gel electrophoresis in the presence of 7 M urea (used to reveal any hidden breaks in the polynucleotide chain) (Richards and Gratzer, 1968). No degradation products nor higher molecular weight material, over and above that which was present in the unreacted tRNA, was detected. Thus there is no evidence that either chain scission or cross-linking occurs under the conditions of carbodiimide reaction used here.

Returning to the main theme, the two types of double-helical polynucleotide that it seemed appropriate to use in the present study were native DNA and the poly (A + U) duplex. This extends the earlier work of Augusti-Tocco and Brown (1965).

The participation of the thymine and guanine residues of DNA in the established base-paired structure would be expected to sterically hinder the reaction of these bases with the carbodiimide reagent. The marked contrast between the reactivities of native and denatured DNA is seen in Figure 10a. We have found that the extent of the reaction with denatured DNA varies somewhat depending upon the source of the DNA and, in all likelihood, on the precise conditions of the heat denaturation. Between 25 and 35% of the nucleotides in denatured DNA react. Since only two of the four bases of DNA can react with the carbodiimide, this result implies that 50–70% of the nucleotide residues is in the nonhelical conformation. The remaining nucleotides may still be in a double-helical configuration or may be in some other arrangement which sterically inhibits the carbodiimide reaction.

The reaction of the carbodiimide with native calf thymus DNA is shown in greater detail in Figure 10b. The extent of reaction depends upon the source of the DNA and upon the ionic conditions. Sample A was a preparation of DNA that gave a good X-ray diffraction pattern (S. Arnott, personal communication), while sample B was from a commercial source and was used without further purification. This latter sample seems to contain about 5% of the residues in nonhelical segments since about 2.5% of the nucleotides reacts fairly rapidly with the carbodiimide. The subsequent slow reaction is considered to be due to a gradual opening of the double helix (see below).

The reaction of poly (A + U) with the carbodiimide has been studied. Conditions that lead to the formation of the two-stranded complex, as opposed to the three-stranded poly (A + 2U) were employed (Blake and Fresco, 1966). Some typical results are shown in Figure 11. The curves consist of two portions; an initial rapid reaction is followed by a slower linear one. It seems most reasonable to interpret these results on the basis that the initial fast reaction is with what are normally nonhydrogen-bonded UMP residues, while the slower reaction reflects the opening of the double helix. By reacting with transiently unpaired residues (normally these

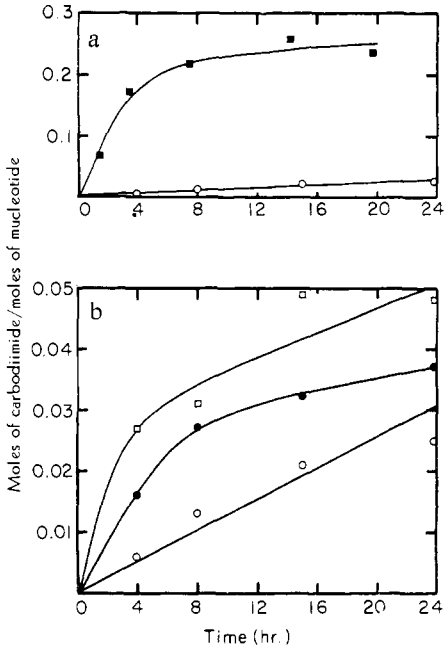

FIGURE 10: The reaction of the carbodiimide with DNA. (a) DNA (70 μg) (sample A) was incubated with 2 μmoles of [^{14}C]carbodiimide iodide in 40 μl of 0.1 M borate buffer (pH 8.0) at 30° for the times indicated. The mixtures were then processed as described in Materials and Methods. Denatured DNA was prepared by heating a solution of native DNA (3.5 mg/ml in 0.2 M borate buffer, (pH 8.0) at 100° for a few minutes followed by cooling in ice. (○—○) Native DNA; (■—■) denatured DNA. (b) The reaction mixtures were as indicated above except as noted. (○—○) Native DNA, sample A, in 0.1 M borate buffer (pH 8.0); (●—●) native DNA, sample B, in 0.1 M borate buffer (pH 8.0); (□—□) native DNA, sample B, in 0.01 M borate buffer (pH 8.0). It may be noted that the zero time control was equivalent to about 0.03 mole of carbodiimide/mole of nucleotide and this value was subtracted from all experimental values. The magnitude of the control accounts for the scatter of some of that data.

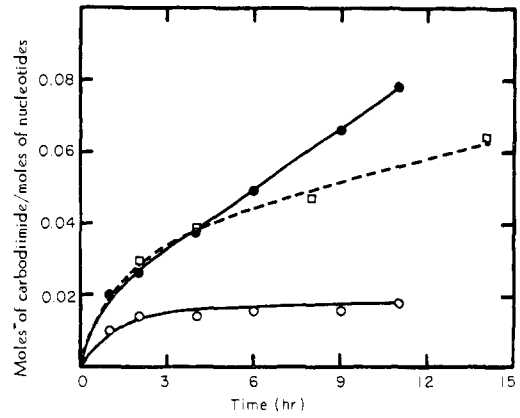

FIGURE 11: The reaction of poly (A + U) with the carbodiimide. Poly (A + U) was prepared as described in Materials and Methods, in 0.1 M borate buffer or 0.1 M Tris-Cl buffer (both pH 8.0). The A:U ratio was 1:1, except that in one case (where borate buffer was used) a 5% excess of poly A was employed. Poly (A + U) (20 μl), containing 150–200 mμmoles of nucleotide, was mixed with 20 μl of [^{14}C]-carbodiimide iodide solution, 100 mM in the appropriate buffer. The mixtures were incubated at the temperature indicated prior to processing on CM-cellulose columns. (●—●) 0.1 M borate buffer, 30°; (○—○) 0.1 M borate buffer, 15°; (□- - -□) 0.1 M Tris-Cl buffer, 30°.

are in the helix), the reagent will tend to shift the helix–coil equilibrium in the direction of the single-stranded amorphous coil. The rate of this perturbation of the double helix by the reagent may be expected to be a function of the stability of the helix, and, so long as the extent of perturbation is limited, it should proceed linearly with time. If the linear portion of the curve is extrapolated back to cut the ordinate, the intercept will be a measure of the proportion of the nucleotides initially present in nonhelical segments. The slope of this part of the curve should be inversely related to the stability of the helical regions.

While the same general shape was obtained in all the experiments with the poly (A + U) system, the actual values of the slopes of the linear portions of the curves and of the intercepts with the ordinate varied depending upon the conditions and on the polymer preparations used. The variation with the preparation may be illustrated by means of the values of the ordinate intercepts for three different polymer mixtures, all in 0.1 M Tris-Cl (pH 8.0) at 30° and with the other conditions as in Figure 11. These were 0.012, 0.028, and 0.017 moles of carbodiimide/mole of nucleotide. There was a similar range of variation in the values of the slopes of the linear portions of the curves though no correlation was noted between the values of the slopes and the intercepts for the different preparations of poly (A + U). For a given preparation, the curves of the type shown in Figure 11 were entirely reproducible. However, preparations of poly (A + U) made on different occasions from the same lots of homopolymers did not give identical results. Prior dialysis of the homopolymers to remove any contaminant oligonucleotides did not reduce the values of the slope or the intercept, nor did the presence of any excess of adenylate residues of between 1 and 5%. It is considered, therefore, that this variability from batch to batch must reflect the precise conditions used during the preparation of the two-stranded complex. It has been established that the two-stranded complex is the equilibrium form for an equimolar mixture of poly A and U at neutral pH (Blake and Fresco, 1966). The nucleotide concentrations used in the present experiments, however, are of the order of 200 times those used in the spectrophotometric experiments which established the nature of the interactions between the homopolymers. It is possible that at the higher concentrations thermodynamic equilibrium is not reached, even after a 3- or 4-day equilibration period.

Because of the degree of variation in the reaction between the carbodiimide and poly (A + U) described in the preceding paragraph, comparison of the rate and extent as a function of the reaction conditions must be made on single preparations of poly (A + U). Thus the difference between the course of the reaction in borate and in Tris-Cl buffer depicted in Figure 11 is not meaningful since different batches of polymer were used. On the other hand, the variation with temperature shown in the figure is significant since here a single preparation was used. The difference between the 15 and 30° curve is as expected if the slopes of the linear portions of the curves are inversely proportional to helix stability. It is satisfactory that the linear portions both extrapolate back to the same intercept value. The dependence of the reaction upon ionic strength was also explored. Three different preparations of poly (A + U) were each reacted with the carbodiimide in both 0.1 and 0.01 M Tris-Cl buffers (pH 8.0). The slopes of the

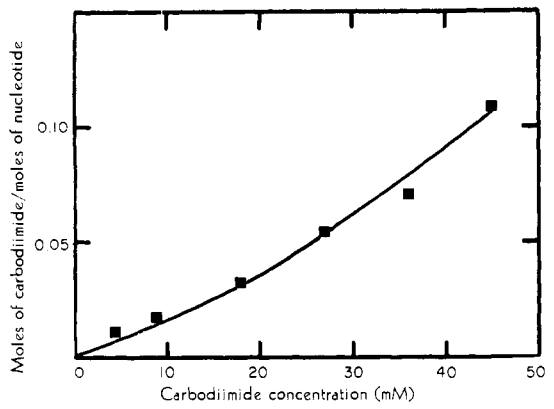

FIGURE 12: The reaction of poly (A + U) and the carbodiimide as a function of the reagent concentration. The experimental conditions were similar to those given in Figure 12. The buffer was 0.1 M borate (pH 8.0), the temperature was 30°, and the time of incubation was 17 hr.

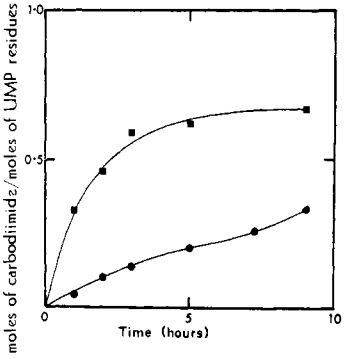

FIGURE 13: The reaction of poly (A,U + U) with the carbodiimide. The poly (A,U) copolymer contained 22% uridylate residues. The copolymer–homopolymer complex was prepared as described in Materials and Methods. The frozen-dried polymer mixture was dissolved in 0.1 M borate buffer (11 mg/ml) and the pH was adjusted to 8.0. After 3-days equilibration at room temperature, 20-μl aliquots of the mixture were mixed with equal aliquots of the [^{14}C]carbodiimide iodide solution (100 mM in 0.1 M borate, pH 8.0) and incubated at 15 or 30°. After the desired times of incubations, the mixtures were passed through CM-cellulose columns. (■—■) 30°; (●—●) 15°.

linear parts of the time-course plots were in all cases greater when the salt concentration was lower. This is also consistent with an inverse relationship between the slope of the plot and helix stability.

The variation in the extent of reaction of the carbodiimide with poly (A + U) as a function of the reagent concentration is shown in Figure 12. It will be seen that the extent is roughly proportional to the carbodiimide concentration.

The reaction between the carbodiimide and the triple-stranded complex, formed by equilibrating a mixture of poly U and poly A in the molar ratio 2:1, was investigated. The course of the reaction was closely similar to that of the double-stranded complex under the conditions employed, *i.e.*, at 30°. Thus it is inferred that under these conditions the second poly U strand in poly (A + 2U) is bound as firmly as the first.

We have examined the reaction between the carbodiimide and the complex formed between the copolymer, poly (A,U) and poly U. Fresco and Alberts (1960) studied this system by observing the hypochromic change on mixing copolymer and homopolymer. They concluded that maximum complex formation occurred at the point where the moles of nucleotide residues in the homopolymer equaled the moles of the complementary residue in the copolymer, under conditions where the two-stranded helix was formed. This result implies that the non-complementary residues of the copolymer (the uridylate residues in the present case) do not remain within the helix but loop out of it. It might be expected that such looped-out residues would react with the carbodiimide reagent while those that are in the helix should be sterically hindered from reacting.

An (A,U) copolymer containing 22% uridylate residues was mixed with an amount of poly U such that the moles of uridylate residues in the homopolymer was equal to the moles of adenylate residues in the copolymer. The complex was reacted with the carbodiimide, as shown in Figure 13. It will be seen that at 30° two-thirds of all the uridylate residues has reacted after 9 hr. This suggests that, at this temperature, the stability of many of the short helical segments between the putative loops is insufficient to prevent reaction with the carbodiimide. This result is not unexpected since the stability of a helical segment is known to depend upon the length of the segment. Thus, while the extended helices of the poly (A + U) system are markedly resistant to attack by the reagent, the shorter ones in the poly (A,U + U) complex are less so. The striking difference between the rates and extents of reaction at 15 and 30° in the case of the copolymer–homopolymer system (Figure 13) is consistent with the existence of many short helices of comparatively low stability. The slopes of the 15° curve are quite shallow, although no plateau is apparent. After 5 hr, 20% of the uridylate residues has reacted. The Fresco–Alberts model would predict that 22% of these residues should be looped out of the helix. Thus the experimental results are not in disagreement with the model. This, however, is the most that can be said since it is not possible to specify, for any given extent of reaction, what proportion of the reacted residues derive from disrupted helical regions and what from single-stranded regions.

The Reaction of the Carbodiimide with Single-Stranded Stacked Polynucleotides. We now consider the effect of the single-stranded stacked polynucleotide structure on the reaction of carbodiimide with the constituent nucleotides. The first class of model compound to be considered is that of the dinucleoside phosphates (XpY, where X and Y are nucleosides). Warshaw and Tinoco (1965) have investigated the conformation of certain of these dimers using the magnitudes of the hyperchromism and optical rotation, compared with the constituent nucleoside and nucleotide, as a measure of the base–base interaction. They divided the dimers into two categories, those in which the bases were predominantly stacked and those in which they were unstacked. GpC was in the former category and ApU in the latter. More recently Simpkins and Richards (1967a) have determined the proportion of stacking in certain dinucleoside phosphates from spectrophotometric titration data and deduced that for ApU this value was 0.29.

The rates of reaction of carbodiimide with ApU and GpC are shown in Table III. The extents of reaction are given in Table V. It will be seen that the uridylate and guanylate residues of the dimers react at rates that are close to those for the

corresponding mononucleotides. It seems, therefore, that the stacking of GpC has no effect on the rate of reaction with the carbodiimide. The possibility is then suggested that the reaction perturbs the stacking. This was resolved by examining the change in the absorbance spectrum of GpC during the reaction. The changes were very similar to those observed in the GMP case (Figure 4b). In particular, distinct isosbestic points were found at 278 mμ and at about 263 mμ. The existence of the former isosbestic point demonstrates that unstacking of the bases does not occur during the carbodiimide reaction since such an effect would be accompanied by a hypochromic shift at this wavelength (Warshaw and Tinoco, 1965).

It may be argued, however, that a dinucleotide is not an adequate model for a single-stranded stacked conformation since only one side of each base interacts with another. Of the polynucleotides that react with the carbodiimide, poly U does not form a stacked structure under normal conditions (Richards *et al.*, 1963; Simpkins and Richards, 1967b), poly G has a pronounced tendency to form aggregates, and poly I forms a triple-stranded structure involving hydrogen-bonded bases. A small quantity of the tetranucleotide, GpGpGpCp, was available and its reaction with the carbodiimide was followed spectrophotometrically. It is to be expected that such an oligomer will exist in a stacked conformation. The work of Lipsett (1964) and others has shown that guanine oligoribonucleotides tend to form aggregates in solution. It was clearly desirable to eliminate aggregation in order that the effect of stacking on the reaction be apparent. The oligonucleotide was dissolved in 0.1 M borate buffer (pH 8.0) at a nucleotide concentration of about 0.2 mM and its absorbance spectrum was recorded. After heating at 75° for 10 min and cooling, a significant hypochromic effect was observed. No further change in the spectrum occurred after incubation for 12 hr at 30° or after a further treatment at 75°. It seems reasonable to infer that the initial heating disrupted any aggregates that were present and this process was not reversed during incubation at 30°. This conclusion is consistent with the results of Lipsett (1964) who found that the rates of aggregation of guanine oligonucleotides were extremely low.

The disaggregated GpGpGpCp was reacted with the carbodiimide and the absorbance spectra were recorded at various intervals. Again, the changes were very similar to those observed during the reaction with GMP (Figure 4b), clear isosbestic points occurring at 277 mμ and at about 265 mμ. The rate of reaction was determined and it was found that there was no significant difference compared with that of the mononucleotide (Table III). Since two out of the three reactive residues of the oligonucleotide are at internal positions, it would seem that the conclusion reached in the case of GpC is confirmed, namely that the existence of stacking neither hinders, nor is itself perturbed by, the reaction with carbodiimide. There is evidence, however, that not all of the guanylate residues in GpGpGpCp react with the carbodiimide. A rough measure of the extent of reaction is provided by the increase in absorbance as a proportion of the initial absorbance at a given wavelength. The values of the ratio $(A_\infty - A_0)/A_0$ at 292 mμ are as follows: for GMP, 0.99; for GpC, 0.52; and for GpGpGpCp, 0.50. These values provide only a rough measure of the relative extents of reaction because the extinction coefficients of the guanylate and cytidylate residues differ at this wavelength. Nevertheless, the value for the tetramer, compared with those for the monomer and dimer, suggests

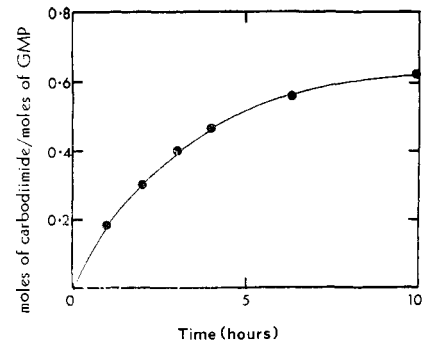

FIGURE 14: The reaction of poly(A,G) and the carbodiimide. The copolymer (A:G base ratio 47:53) was dissolved in 0.02 M borate buffer (pH 8.0; 4 mg/ml), heated to 80° for a few minutes, and then cooled in ice. Aliquots (20 μl) were incubated at 30° with 20 μl of [^{14}C]carbodiimide iodide (100 mM in water). After the desired time, the reaction mixtures were applied to CM-cellulose columns.

that only about two of the three guanylate bases react, on average. Too little of the oligomer was available to enable the extent of reaction to be determined more directly, using labeled carbodiimide.

The question of the effect of stacking on the carbodiimide modification was further pursued using a poly (A,G) copolymer having an A:G ratio of 47:53. The reaction with the carbodiimide was largely completed by 10 hr, at which time some 63% of the guanylate residues has reacted (Figure 14). Since GMP reacts to only 86% completion under similar conditions, it may be deduced that about 74% of the guanylate residues in the sample of poly (A,G) is available for reaction. This result also suggests that not all the residues in a single-stranded stacked conformation, as poly (A,G) may be supposed to be, are available for reaction with the carbodiimide.

It seems reasonable to assume that this limitation on the extent of reaction of residues in a single-stranded stacked helix arises from simple steric hindrance. The reaction of one molecule of the carbodiimide at one particular nucleotide may prevent the reaction of another molecule at an adjacent nucleotide simply because of the bulk of the reagent molecule. Various detailed models are possible; in one, no two adjacent stacked residues may react while in a second, two adjacent residues may react but three may not. More elaborate models are also possible.

For a given (A,G) copolymer, it is possible to calculate the extent of reaction of the guanylate residues with the carbodiimide for the various models, assuming a random distribution of nucleotides. For the case in which no two adjacent GMP residues can react, the fraction of all GMP residues that are expected to react is calculated to be 0 60 for the particular copolymer for which we have experimental data. For the model in which two, but not three adjacent residues can react, the corresponding figure is 0.80, while for models in which greater numbers of adjacent stacked nucleotides can react the fraction will be greater than 0.80. The experimentally determined value is 0.74; this would seem to exclude the first model and to be in most reasonable agreement with the second. It should be noted, however that the above calculations assume that the nucleotide sequence in the copolymer is essentially random.

Thus the experimental data for the poly (A,G) system is reasonably consistent with the proposition that two, but not three, adjacent guanylate residues can react with the carbodiimide. The possibility, however, cannot be wholly excluded that the results with GpGpGpCp and with poly (A,G) are due to aggregation of the GMP-rich polynucleotides, the occurrence of which may be expected to decrease the extent of reaction.

An elementary model-building exercise was attempted in order to shed more light on this matter. While there are no proper stereochemical data available for the structure of a single-stranded stacked polynucleotide, it does not seem unreasonable to assume that the configuration will be closely similar to that of one of the strands of the double-stranded RNA structure (Fuller and Hodgson, 1967). Thus we take it that the single-stranded helix will have a helix pitch of 29 Å and 10 or 11 nucleotides per turn of the helix (Arnott *et al.*, 1966). A model of the carbodiimide ion was built from Corey–Pauling–Koltun atomic models (Koltun, 1965). The cyclohexane ring was put in the chair conformation, as was the methylmorpholinium group, more or less. These two groups are not rigidly connected for there can be rotation about the various single covalent bonds through which they are joined. Thus the configuration of the molecule cannot be precisely defined in the model. However, when the different parts are rotated relative to one another, the over-all dimensions of the possible unstrained configurations do not vary greatly. These are about 16 × 7 × 5 Å. The important point is that the minimum thickness of the molecule cannot be less than about 5 Å because this is the thickness of the saturated, puckered ring systems. It is therefore apparent that not every nucleotide in a single-stranded helix can react with the carbodiimide, since the bases are stacked about 3 Å apart. The carbodiimide residues could not avoid being positioned one on top of the other since they would take the place of the bases in the second strand of the notional double helix. In the absence of good stereochemical data on the structures of the polynucleotides and the carbodiimide, it would be unwise to take this model-building exploration further. As far as it goes, it does confirm the experimental evidence that not all the residues in a single-stranded stacked polynucleotide are available for reaction with the carbodiimide. Nevertheless, most stacked residues can react and, at least in the instances examined here, the stacking of the polynucleotide is not perturbed.

Discussion

In the present study we have attempted to establish the utility of the carbodiimide reagent in the investigation of nucleic acid and polynucleotide secondary structure. The important features of the reagent will now be discussed.

The readily soluble carbodiimide is very stable in water and in borate buffer (pH 8.0) and only a little less so in Tris-Cl buffer (pH 7.6). Solutions of the carbodiimide become progressively less stable as the pH is lowered. The decomposition is also promoted by certain buffer anions. It may be noted here that the different salt forms of the carbodiimide that have been used have essentially identical stabilities and rates of reaction with the nucleotides, at least under the variety of conditions that have been studied here. Consequently, they may be regarded as equivalent in their reactions with the nucleotides, although each has particular virtues in particular experimental situations. The carbodiimide causes only a very slight shift in the pH of borate buffer but that for Tris-Cl buffer is more substantial. This fact, together with the superior stability of the carbodiimide in borate buffer, would seem to indicate the exclusive use of this buffer. However, it is well known that borate can complex with *cis*-glycol groups such as occur in the 5′-mononucleotides. Also, we have found that the double-stranded poly (A + U) complex was not formed in 0.01 M borate buffer, as judged by the hypochromic effect on mixing the homopolymers, though it was formed in Tris-Cl buffer of the same molarity. In the circumstance, it seemed most reasonable to study the carbodiimide–nucleotide reaction in both buffers. By doing so, it was hoped to detect any artifacts arising from interaction between buffer ions and either of the reactants. In fact, no significant differences were found between the two buffers when used for the carbodiimide–nucleotide reactions reported in the present paper. This is not the case, however, with tRNA, as will be shown in the following paper (Metz and Brown, 1969).

The carbodiimide reacts specifically with 5′-UMP and 5′-GMP, just two of the four main 5′-monoribonucleotides. That is, no detectable side reactions occur in the case of the reactive nucleotides and the other pair do not react at all. This is in agreement with the earlier reports of Gilham (1962) and of Ho and Gilham (1967). 5′-IMP and 5′-TMP also react though 5′-dihydrouridine monophosphate does not. Naylor *et al.* (1965) have reported that two molecules of the carbodiimide will react with pseudouridine. In general, it appears that those nucleotides that have a keto group at the 4 position of the pyrimidine ring, or at the equivalent 6 position of the purine ring, will react with the carbodiimide to give the N-substituted 3 or 1 adduct, respectively. An equivalent generalization is that the carbodiimide reacts with the nucleotides whose bases have pK values in the vicinity of 9 (Ho and Gilham, 1967). The carbodiimide addition reaction seems to be dependent upon the ionization of the base (Knorre *et al.*, 1966; Ho and Gilham, 1967). Where two N positions are available adjacent to the keto group, as in the case of pseudouridine, both may react. If the base is not fully unsaturated, reaction may not occur, as is the case for dihydrouridine monophosphate. If the 2′(3′)-mononucleotides are reacted with the carbodiimide, cyclization of the phosphate group will occur.

The rate of reaction of the carbodiimide with the nucleotides and related compounds has been examined under a variety of conditions. The most significant finding to emerge was that the range of variation of the rate constants was quite limited. The fact that the reaction rate does not depend markedly upon the buffer type, ionic strength, or the presence of Mg^{2+} ions suggests that the position of equilibrium of the reaction will be similarly independent of these variables. Clearly, this is a desirable property for a reagent that is to be used to study the secondary structure of nucleic acids and polynucleotides. The above-mentioned parameters can be varied thereby changing the secondary structure without directly affecting the rate and extent of the modification reaction. It is also an advantage that the rates of reaction of UMP and GMP are similar since this simplifies the interpretation of data for the reaction with a polynucleotide containing both nucleotides.

We have not investigated the variation in the reaction rate of the mononucleotide as a function of pH. A difficulty in this

connection is the instability of the reagent at pH values below 7. Knorre *et al.* (1966) have considered this variable and concluded that the rate constant was inversely proportional to the hydrogen ion concentration. These workers also investigated the variation of the reaction rate with temperature; the rate constants were found to increase with temperature in the normal way.

The equilibrium constants for the reactions between the carbodiimide and UMP and GMP have values, respectively, of 1100 and 450 l. mole^{-1} at 30°. These are usefully large figures and contrast with the values of around 10 that have been determined for the reaction of formaldehyde with the mononucleotides (Grossman *et al.*, 1961). The consequence of the magnitudes of equilibrium constants is that the reactions go substantially to completion. In no case is the extent of reaction less than 80% with the mononucleotides or the polynucleotides that lack secondary structure when the usual carbodiimide concentration of 50 mM is employed and the temperature is between 20 and 30°. Further, small variations in the reagent concentration (above, say, 25 mM) will not materially affect the extent of reaction of the nucleotide.

The carbodiimide–nucleotide adducts are entirely stable at acidic pH values. They may be dissociated readily at pH 10.5 yielding the original nucleotide (except that only one of the two carbodiimide groups that react with pseudouridine is labile under these conditions). The stabilities of the substituted nucleotides as a function of pH are the reverse of the stability of the carbodiimide itself. This is convenient since, for example, carbodiimide-reacted RNA may be precipitated by trichloroacetic acid and under these conditions the bound carbodiimide will be quite stable but the free form will be very rapidly hydrolyzed. It will be shown in the following paper (Metz and Brown, 1969) that the loss of bound carbodiimide from tRNA is negligible under the conditions that the latter may encounter in *in vitro* biochemical reactions.

When carbodiimide reacts with polynucleotides there is no evidence that the chain is either cross-linked or cleaved. In the absence of ordered secondary structure, the extent of reaction is the same for polynucleotides as for the mononucleotides. The double-stranded secondary structure markedly inhibits the reaction with the carbodiimide, though this inhibition is not complete. Any reagent that reacts specifically with nonbase-paired residues must inevitably perturb a double-helical structure. The rate of reaction with the base-paired residues is expected to be a function of the stability of the helix. It has been shown that this rate of reaction increases with increasing temperature and with decreasing ionic strength. Helix stability, as determined by the melting temperature, for example, is known also to depend upon the base composition and length of the double-stranded region. It would be expected that the rate of the carbodiimide reaction would depend upon these factors also. Some evidence in support of the dependence upon helix length has been deduced from the reaction with the poly (A,U + U) system. There is no evidence that the carbodiimide tends to denature helical polynucleotides in any way except by reacting with the bases. This point cannot, however, be demonstrated rigorously, since there is no double-stranded polynucleotide containing no reactive bases.

It will be seen, therefore, that the single-stranded regions of a polynucleotide will be most clearly distinguished from the double-stranded helical regions when the latter are long and have a high GC content, and when the ionic conditions and temperature are such as to promote maximal helix stability. Conversely, short helices, such as those which are believed to occur in most ribonucleic acids, will be less easily differentiated from single-stranded regions.

It has been demonstrated that the bases in single-stranded stacked polynucleotides can react with the carbodiimide, with certain limitations. Not every nucleotide in, for example, poly G could react because the carbodiimide residues would mutually obstruct. The weight of the evidence is that two, though not three, adjacent bases can react. The effect of this limitation on the extent of reaction of the carbodiimide with an RNA species of unknown nucleotide sequence obviously cannot be computed. It is, however, unlikely that this will lead to an important reduction in the extent of the carbodiimide reaction since only two of the four major nucleotides can react and only one of these has a strong tendency to form stacked structures. In all the single-stranded model systems that have been examined, the carbodiimide reaction has not perturbed the stacking. Conceivably, less strongly stacked structures could be so perturbed; the gain in energy resulting from the reaction of a base that would otherwise have been obstructed could be greater than the loss of stacking energy.

One other very useful property of the carbodiimide reagent may be mentioned here. The enzymic hydrolysis of the internucleotide phosphodiester linkage may be inhibited if one of the nucleotides is modified by the carbodiimide (Gilham, 1962; Naylor *et al.*, 1965; Ho and Gilham, 1967; Brownlee *et al.*, 1968). It is possible in this way to isolate and identify oligonucleotides from the nonhelical regions of a ribonucleic acid (Brostoff and Ingram, 1967). The reagent may also be used to modify the specificity of pancreatic ribonuclease (Ho and Gilham, 1967) and thus to prepare oligonucleotides containing terminal cytidine (Lee *et al.*, 1965).

Altogether, it may be judged that the properties of the carbodiimide are satisfactory for the investigation of nucleic acid secondary structure by means of the chemical modification technique. Its main weakness lies in the fact that it slowly reacts with bases in double-stranded helices, but this is an inevitable property of any reagent of this type and it is unlikely that the extent of this effect will vary substantially from one reagent to another. In its general properties, the carbodiimide is probably superior to any of the chemical modification reagents for nucleotides whose use has been investigated to date.

Acknowledgments

We are indebted to Sir John Randall for provision of facilities. We thank Professor M. H. F. Wilkins for his encouragement, and Drs. Sheila Lee, E. G. Richards, and W. B. Gratzer for useful discussion.

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