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

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ABSTRACT: The reaction between the carbodiimide reagent, *N*-cyclohexyl-*N'*-β-(4-methylmorpholinium)ethylcarbodiimide, and *Escherichia coli* transfer ribonucleic acid has been investigated. The extent of reaction was examined under a variety of conditions of temperature, salt type, and concentration, and reagent and ribonucleic acid concentration. The presence of magnesium ions was found to reduce the extent of reaction considerably. The results have been interpreted in the light of the transfer ribonucleic acid nucleotide sequence data and the physicochemical data that are available. The results are consistent with the existence of three forms of tertiary structure, all based upon the cloverleaf type of secondary structure. The biologically active form is a very compact structure in which most of the limited number of unpaired nucleotides that are exposed are in the anticodon loop. The loss of two of the biological activities of transfer ribonucleic acid following the carbodiimide modification has been examined.

In the absence of magnesium ions, the amino acid acceptor activities are all lost rapidly. In the presence of this divalent cation, the pattern of loss varies very much from one amino acid accepting species to another. The effect of the carbodiimide reaction on the binding of aminoacyl transfer ribonucleic acid to ribosomes has also been studied.

It is clear that a rather detailed knowledge of the structure of tRNA will be necessary for a full understanding of the mechanism of protein synthesis. A number of nucleotide sequences have now been determined (for review, see Madison, 1968). All these sequences can be fitted into the cloverleaf form of secondary structure, and while there is no proof that this model is correct, all the available physical and chemical evidence is compatible with it (Madison, 1968). It has also become apparent that tRNA has a tertiary structure which can exist in more than one form (Fresco *et al.*, 1966).

One approach that has been extensively used to study the structure and function of tRNA has employed reagents that chemically modify the constituent nucleotides (see Miura, 1967, for review). The reaction between *N*-cyclohexyl-*N'*-β-(4-methylmorpholinium)ethylcarbodiimide (hereafter referred to as the carbodiimide), a water-soluble carbodiimide reagent, and the uridine and guanosine 5'-phosphates was first described by Gilham (1962). Augusti-Tocco and Brown (1965), in this laboratory, demonstrated that the extent of the carbodiimide reaction with residues in polynucleotides and nucleic acids was limited by the secondary structure. The presence of potentially reactive nucleotides in a region of double-stranded secondary structure prevented their modification by the reagent. The extent of reaction between the carbodiimide and tRNA was found to be quite limited and this was attributed to the presence of most of the nucleotides in helical regions. Knorre and his coworkers have also described the reaction between the carbodiimide and tRNA (Knorre *et al.*, 1966; Girshovich *et al.*, 1966). Brostoff and Ingram (1967) have reacted purified yeast alanine tRNA with the carbodiimide; this was followed by nuclease digestion to yield modified oligonucleotides which were presumed to have been derived from the exposed, unpaired regions of the molecule. Ho and Gilham (1967) have shown that tRNA, following extensive modification by the carbodiimide, may be digested with pancreatic ribonuclease to oligonucleotides terminating only in cytidine.

In the preceding paper (Metz and Brown, 1969) a study of the reaction between the carbodiimide and a variety of model mono-, oligo-, and polynucleotides was reported. It was found that the reagent reacted very specifically with UMP and GMP of the four main ribonucleotides, under conditions of pH, ionic strength, and temperature in which normal nucleic acid secondary structure would be maintained. The reaction went largely to completion under convenient conditions of reagent concentration and temperature. Nucleotides in single-stranded stacked or unstacked polynucleotides were able to react with the carbodiimide, subject to certain minor limitations. Nucleotides in double-stranded helical polynucleotides were markedly inhibited from reacting. However, such helices were slowly denatured as the equilibrium between the double-stranded and single-stranded components was perturbed by the reaction of the reagents with the latter. The rate of perturbation of a double helix was dependent upon the nature and environment of the polynucleotide in a manner consistent with what is known of the effect of these factors on helix stability in general. It was concluded that when the double-stranded helices are reasonably stable, the carbodiimide may be used to distinguish between single-stranded

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and double-stranded regions of the secondary structure of a polynucleotide.

In the present study the reaction between the carbodiimide and *Escherichia coli* tRNA was investigated. The extent of the modification was determined under a variety of conditions of temperature, salt type, and concentration, and reagent and tRNA concentration. The data have been interpreted to indicate the existence of some three forms of tertiary structure, all based upon the cloverleaf type of secondary structure. The biologically active form, which exists in the presence of magnesium ion, is a very compact structure. The loss of two biological activities of tRNA, its amino acid acceptor activity, and its ability to bind to ribosomes, following the *N*-Cyclohexyl-*N'*-β-(4-methylmorpholinium)ethylcarbodiimide modification, has also been examined.

Materials and Methods

Materials and Methods are as described in the preceeding paper (Metz and Brown, 1969) with the additions described below.

Preparation of tRNA. E. coli 58–161 cell paste was extracted with phenol at room temperature, the phases were allowed to separate by standing, and the RNA in the aqueous phase was precipitated with ethanol. The precipitate was extracted twice with a mixture of one volume of 2-ethoxyethanol and two volumes of 2 M NaCl and the supernatant was precipitated with ethanol. This sRNA preparation was supplied by the Microbial Research Establishment, Porton, Wilts. Analysis of a number of batches of this material indicated that the protein content (Lowry *et al.*, 1951) was less than 1.5%, the DNA content (Burton, 1956) was less than 1.0%, and the polysaccharide content (Littauer and Eisenberg, 1959) less than 2.0%. Polyacrylamide gel electrophoresis (Richards *et al.*, 1965) showed the preparations to be free from degraded RNA (*i.e.*, smaller than 4 S) and to contain little if any rRNA. Apart from the tRNA, the chief minor RNA species seen on the gels were 5S RNA and a component which corresponded in size to a dimer of tRNA.

The sRNA preparations were further purified by a second phenol extraction, stripping of bound amino acids (Sarin and Zamecnik, 1964), and chromatography on a column of Bio-Gel P-100, carried out according to the procedure of Schleich and Goldstein (1966). The main peak from the first passage through the column was recovered and rechromatographed under identical conditions. The tRNA was recovered by ethanol precipitation and was dialyzed first against 1 M NaCl, 0.01 M EDTA, and then against three changes of glass-distilled water. The product was lyophilized. As judged from the column chromatography elution profiles, no degraded RNA material was present in the final product, and the proportion of higher molecular weight species (*i.e.*, larger than 4 S) was less than 1%. The mean molar residue absorptivity, ϵ(P), of the tRNA was determined to be 7230 (phosphorous was estimated according to Ames and Dubin, 1960). The amino acid acceptor activities of the purified tRNA preparations were as follows (millimicromoles per milligram): tyrosine, 1.4; lysine, 3.0; arginine, 2.3; serine, 1.6; valine, 3.6; histidine, 0.6; threonine, 2.0; isoleucine, 7.0; aspartic acid, 1.8; phenylalanine, 1.1; leucine, 4.8; methionine, 1.5; and proline, 1.3. The total acceptor activity for the 13 amino acids tested was 32 mμmoles/mg. The theoretical total acceptor activity, for all amino acids, is 37 mμmoles/mg, assuming a molecular weight of 27,000. This suggests that most of the tRNA molecules in the purified preparation are biologically active.

Amino Acid Acceptor Activity. The aminoacyl-tRNA synthetase preparation was prepared from frozen cells of *E. coli* MRE 600 (a gift from the Microbial Research Establishment). Cells were broken in a Hughes press (Hughes, 1951), starting temperature −20°, extracted with 1.5 volumes of TM buffer (0.01 M Tris-Cl (pH 7.5 at 5°), 0.001 M $MgCl_2$, and 0.005 M 2-mercaptoethanol) and the mixture was centrifuged at 20,000*g* for 30 min, in the cold. The supernatant was centrifuged at 105,000*g* for 2 hr, and the supernatant from this second centrifugation was dialyzed overnight against TM buffer. The final preparation was stored in small aliquots at −20° or in liquid nitrogen. The protein concentration was determined from ultraviolet absorption (Layne, 1957).

The amino acid acceptor activity assay mixtures contained: 0.2 ml of 0.5 M Tris-Cl (pH 7.4), 0.1 M $MgCl_2$, 0.2 ml of 0.05 M ATP (neutralized), 0.1 μCi of [^{14}C]amino acid, *ca.* 100 μg of synthetase protein, and up to 300 μg of tRNA; the total volume was 0.74 ml. The mixtures were incubated at 37° for 30 min after which they were cooled and 3 ml of 7% cold trichloroacetic acid was added. The precipitates were filtered on Millipore filters, washed with three lots of 5 ml of trichloroacetic acid, dried under a heat lamp, and counted, either in a gas-flow counter or in a scintillation counter using a toluene-based scintillation mixture.

Preparation of [^{3}H]*N-Cyclohexyl-N'-β-(4-methylmorpholinium)ethylcarbodiimide-tRNA.* tRNA (8 mg/ml) was dissolved in 0.02 M borate buffer (sometimes containing 0.02 M $MgCl_2$) and the pH was adjusted to 8.0. Aliquots (0.4–0.8 ml) were mixed with equal volumes of [^{3}H]carbodiimide iodide solution (100 mM in water) and the mixtures were incubated at 30°. At the end of the reaction period the mixtures were cooled in ice and the RNA was precipitated by the addition of 0.1 volume of 20% potassium acetate (pH 5.0) and 2.5 volumes of cold 95% ethanol. The RNA was recovered by centrifugation after standing for 1 hr at −20°. The precipitate was dissolved in 1.0 ml of cold 1 M sodium acetate (pH 5.0) and reprecipitated with 2.5 volumes of ethanol. This was repeated twice more. Finally the RNA precipitate was washed twice with cold absolute ethanol and dried *in vacuo*.

Binding of Aminoacyl-tRNA to Ribosomes. Aminoacyl-tRNAs were prepared as follows. Reaction mixtures contained in a total volume of 1.0 ml: 0.1 M Tris-Cl (pH 7.4), 0.016 M magnesium acetate, 0.08 M potassium chloride, 0.0036 M 2-mercaptoethanol, 0.002 M ATP, 0.008 M phosphoenolpyruvate (potassium salt), 2 μl of pyruvate kinase (ammonium sulfate suspension, 10 mg/ml, Boehringer Corp., London), 2 mg of tRNA, 10 μCi of [^{14}C]amino acid, 19 other amino acids (25 mμmoles each), and 0.1 ml of aminoacyl-tRNA synthetase preparation. The [^{14}C]amino acids used were [^{14}C]lysine (180 mCi/mmole) and [^{14}C]phenylalanine (504 mCi/mmole) both obtained from the Radiochemical Centre, Amersham. The tRNA was either unmodified or had been reacted with [^{3}H]carbodiimide iodide. The reaction mixture was incubated at 37° for 30 min. It was then cooled in ice and shaken with one volume of phenol (saturated with 0.2 M potassium acetate, pH 5.0). After centrifugation, the aqueous phase was re-extracted with phenol. The combined phenol phases were extracted with 0.25 volume of 0.2 M potassium acetate.

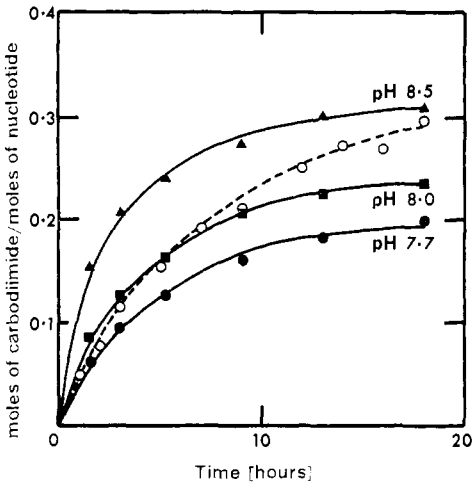

FIGURE 1: The reaction between the carbodiimide and tRNA at different pH values. tRNA was dissolved in 0.02 M borate buffer or in 0.02 M Tris-Cl buffer at a concentration of 6 mg/ml and the pH was adjusted to the specified value. Reaction mixtures contained 20 μl of tRNA solution and 20 μl of [^{14}C]carbodiimide iodide (100 mM in water) and were incubated at 30° for the desired time prior to processing on CM-cellulose columns. (●—●) Borate buffer (pH 7.7), (■—■) borate buffer (pH 8.0), (▲—▲) borate buffer (pH 8.5), and (○- - -○) Tris-Cl buffer (pH 8.0).

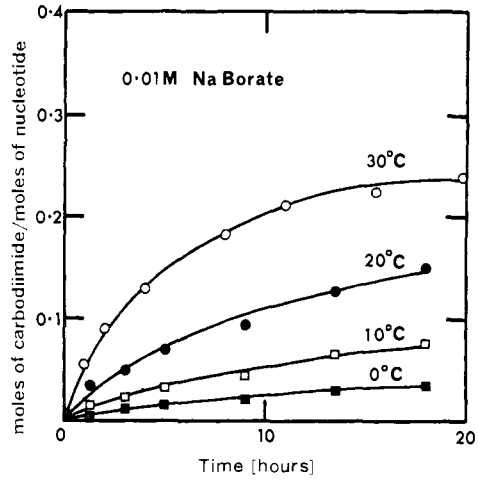

FIGURE 2: The reaction between the carbodiimide and tRNA in 0.01 M borate buffer (pH 8.0) at different temperatures. The reaction conditions are as described in Figure 1. (○—○) 30, (●—●) 20, (□—□) 10, and (■—■) 0°.

The aqueous phases were combined, made 0.2 M in potassium acetate and precipitated with 2.5 volumes of 95% ethanol at −20°. The RNA was dissolved in 1 ml of 0.2 M potassium acetate and was reprecipitated twice more with ethanol. The final precipitate was washed twice with absolute ethanol and dried.

Washed ribosomes were prepared from *E. coli* MRE 600. Frozen cells were broken in a Hughes press prior to extraction with 1–2 volumes of 0.01 M Tris-Cl (pH 7.8), 0.014 M magnesium acetate, 0.06 M potassium chloride, and 0.006 M mercaptoethanol. Following centrifugation (20,000*g*, 30 min) the supernatant was treated with a few micrograms of DNase (Worthington, twice recrystallized) at 4° for 5 min. The preparation was centrifuged at 30,000*g* for 30 min and the supernatant was recentrifuged under the same conditions. The supernatant was incubated at 37° for 45 min without additions (Clark and Marcker, 1966). The mixture was clarified by centrifuging at 30,000*g* for 10 min and then the ribosomes were pelleted at 105,000*g* for 2 hr. The pellet was resuspended in the buffer and centrifuged down again. The pellet was then resuspended in 0.01 M Tris-Cl (pH 7.8), 0.5 M ammonium chloride, 0.06 M potassium chloride, 0.01 M magnesium acetate, and 0.006 M 2-mercaptoethanol, and centrifuged at 105,000*g* for 1.5 hr. The pellet was again resuspended in this buffer and centrifuged down. The ribosomes were suspended in 0.01 M Tris-Cl (pH 7.8), 0.06 M potassium chloride, 0.01 M magnesium acetate, and 0.006 M 2-mercaptoethanol at a concentration of about 20 mg/ml. Following centrifugation at 25,000*g* for 15 min to remove aggregates, the supernatant was stored in small aliquots at −20°.

The aminoacyl-tRNA binding assay was carried out according to the procedure of Nirenberg and Leder (1964). The following were included, where appropriate, in the 50-μl incubation mixtures: 2.45 A_{260} units of ribosomes, 23 mμmoles of base residues of poly U, and 22 mμmoles of base residues of poly A. The magnesium ion concentration was 20 mM in all cases. Incubations were at 24° for 20 min.

rRNA. This was prepared from *E. coli* MRE 600 according to the method of Robinson and Wade (1968) and was a gift from Dr. H. E. Wade. An ϵ(P) value of 7800 for rRNA in 0.01 M sodium acetate (pH 5.0) was used; this was calculated from the $E^{1\%}_{260}$ value of Kurland (1960) and from the base composition data of Spahr and Tissières (1958).

Results

The Extent of the Reaction of the Carbodiimide with tRNA. The time course of the reaction between the carbodiimide and tRNA is shown in Figure 1. The general shape of the curves is typical for this reaction under a variety of conditions. It will be seen that the rate and extent of reaction vary significantly as a function of the pH and buffer type. It is likely that the decreased extent of reaction at pH 7.7 compared with that at pH 8.0 is attributable to a lesser degree of completion of the carbodiimide–nucleotide reaction at the lower pH. The increased extent on going to pH 8.5 is doubtless due to the weakening of the secondary structure which will occur as the alkaline ionization of the bases commences. It is evident that close control of pH is important if reproducible results are to be obtained.

With regard to the choice of buffer, it will be seen that the data for borate and Tris-Cl buffers (both at pH 8.0) are quite similar for times up to about 9 hr. At longer times the extent of reaction in Tris-Cl buffer increases progressively compared with that in borate. It seems plausible to attribute the origin of this quite reproducible effect to the slow drift in pH that was found to occur in Tris-Cl buffer in the presence of the carbodiimide (Metz and Brown, 1969). This upward divergence was linear with time, and it would be expected to exert a greater effect on the carbodiimide tRNA reaction as the time of reaction increases. Thus borate is the buffer of choice for the reaction of the carbodiimide reagent with tRNA. This may be contrasted with the result of the preceding paper (Metz and Brown, 1969) where no difference between the two buffers was found for the carbodiimide–nucleotide reactions

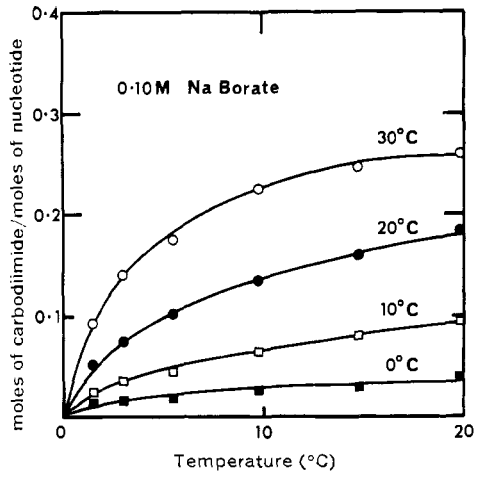

FIGURE 3: The reaction between the carbodiimide and tRNA in 0.10 M borate buffer (pH 8.0) at different temperatures. The reaction conditions are described in Figure 1. (○—○) 30, (●—●) 20, (□—□) 10, and (■—■) 0°.

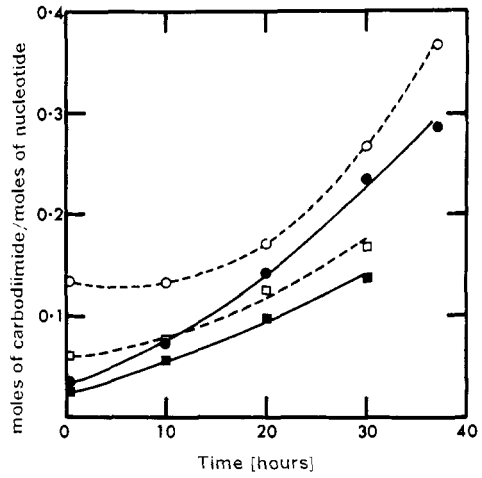

FIGURE 5: The extent of reaction between the carbodiimide and tRNA after 16 hr as a function of temperature. The data in this figure are compiled from the 16-hr values of Figures 2 and 4. The corrected curves are obtained by subjecting these values to correction for the fact that the mononucleotide reactions do not go to completion, as described in the text. (●—●) 0.01 M borate buffer; (○- -○) 0.01 M borate buffer, corrected; (■—■) 0.01 M borate–0.01 M $MgCl_2$; (□- -□) 0.01-borate-0.01 M $MgCl_2$, corrected.

that were studied. This can be attributed to the fact that these previous reactions were completed by about 10 hr. Most of the reactions to be described in the present paper have been carried out in both buffers. Normally, the results for the borate buffer alone will be reported.

The time course of the carbodiimide–tRNA reaction in 0.01 M borate buffer (pH 8.0) at different temperatures is shown in Figure 2. Both the rate and extent of reaction increase with temperature. This is attributable to two effects, namely, the change in the parameters of the mononucleotide–carbodiimide reaction as a function of temperature, and the change in the structure (secondary and tertiary) of tRNA with temperature. The course of the reaction at a higher ionic strength, 0.10 M borate buffer, is shown in Figure 3; this is very similar

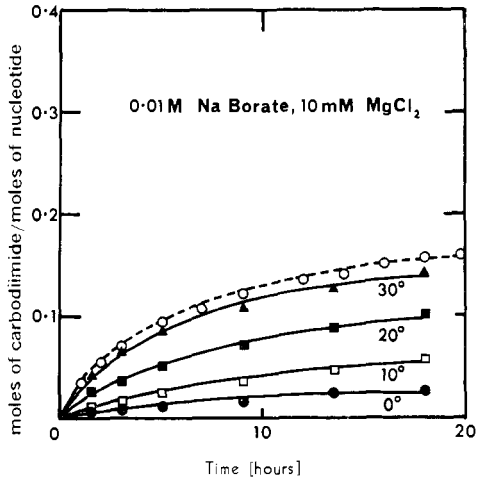

FIGURE 4: The reaction between the carbodiimide and tRNA in 0.01 M borate buffer (pH 8.0)–0.01 M magnesium chloride at different temperatures. The reaction conditions are as described in Figure 1. The tRNA was initially dissolved in 0.02 M borate–0.02 M magnesium chloride. The dashed curve shows the course of the reaction is 0.01 M Tris-Cl (pH 8.0)–0.01 M magnesium chloride at 30°. At the lower temperatures, the curves for Tris-Cl and borate buffers were superimposable. (▲—▲) 30, (■—■) 20, (□—□) 10, (●—●) 0, and (○- - -○) Tris-Cl buffer, 30°.

to the previous case. The reaction in the presence of magnesium ions is depicted in Figure 4, where it will be seen that the extent of reaction at any particular temperature is about half the value that applies in the absence of this ion.

It is possible to attempt a correction of the data of Figures 2–4 to allow for the variation in the extent of reaction between the mononucleotides residues and the carbodiimide as a function of temperature. If this can be done, then the corrected extent of reaction will reflect the proportion of nucleotides that are not prevented from reacting by the secondary (and tertiary) structure of the molecule. In the preceeding paper (Metz and Brown, 1969), data on the extent of reaction of the mononucleotides with the carbodiimide (at 16 hr) as a function of temperature were given. From these results it is possible to calculate factors which will correct the extent of reaction of tRNA (at 16 hr) to the value that would apply had the carbodiimide–mononucleotide gone to completion. The use of such corrections depends upon the assumption that the mononucleotides are good models for the nucleotide residues in tRNA. The data for the extent of reaction of the carbodiimide with tRNA after 16 hr, both the experimental results (taken from the data of Figures 2 and 4) and the corrected values, are shown in Figure 5. In all cases a gradual increase in the extent of reaction with increasing temperature is found. The corrected curves should reflect the changing characteristics of the structure of tRNA with temperature. These include both changes in the actual conformation of the RNA and changes in the stability of the conformation, since the carbodiimide tends to perturb secondary structure as well as reacting with non-base-paired residues.

The experiments described so far have all employed a carbodiimide concentration of 50 mM. The variation in the extent of reaction after 16 hr as a function of the carbodiimide concentration is shown in Figure 6.

It is apparent that no distinct plateau is reached under any of the conditions studied. These curves may be compared with the corresponding data for the mononucleotide (Figure 9 of

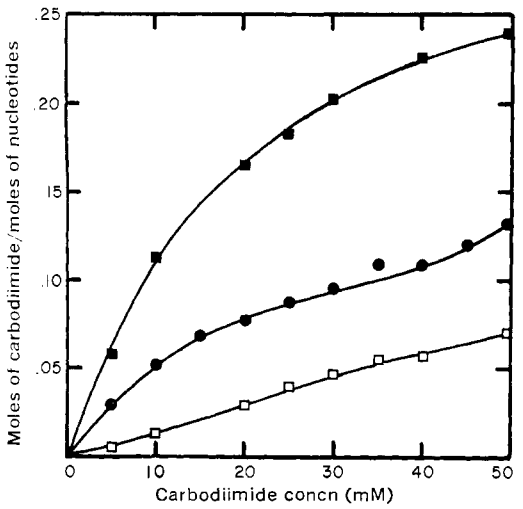

FIGURE 6: The extent of reaction of the carbodiimide with tRNA as a function of the carbodiimide concentration. tRNA was dissolved in buffer (twice the final molarity) at a concentration of about 8 mg/ml and the pH was adjusted to 8.0. Reaction mixtures contained 20 μl of tRNA solution plus an appropriate volume of [^{14}C]carbodiimide (100 mM in water) and water, to give a final volume of 40 μl. The time of incubation was 16 hr. The final buffer molarities are indicated below. (■—■) 0.01 M borate, 30°; (●—●) 0.01 M borate–0.01 M $MgCl_2$, 30°; (□—□) 0.01 M borate–0.01 M $MgCl_2$, 15°.

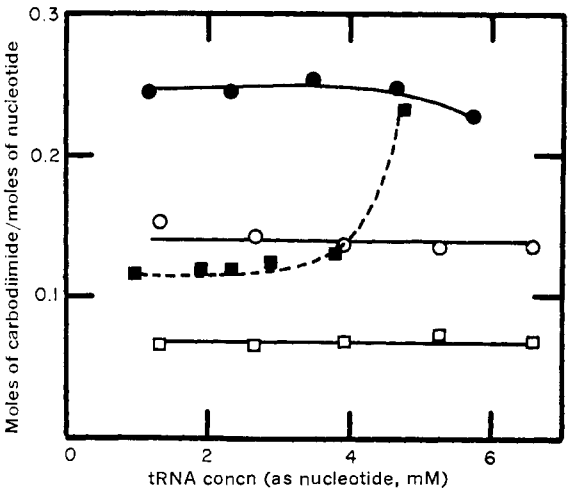

FIGURE 7: The extent of reaction of the carbodiimide with tRNA was a function of the tRNA concentration. Solutions of tRNA (*ca.* 5 mg/ml) in buffer (twice the final concentration) were adjusted to pH 8.0. To 20-μl aliquots of [^{14}C]carbodiimide iodide (100 mM in water) there were added appropriate volumes of the RNA solutions and buffers to give final volumes of 40 μl and the final buffer concentrations were indicated below. After 16-hr incubation at the required temperature, the mixtures were processed on CM-cellulose columns. (●—●) 0.01 M borate buffer, 30°; (○—○) 0.01 M borate buffer–0.01 M $MgCl_2$, 30°; (■—■) 0.01 M borate buffer–0.002 M $MgCl_2$, 30°; (□—□) 0.01 M borate buffer–0.01 M $MgCl_2$, 15°.

the preceding paper) and for the poly (A + U) double helix (Figure 13 of the preceding paper). The magnitude of the proportionate increase in the extent of reaction of tRNA on elevating the carbodiimide concentration from 25 to 50 mM is significantly greater than the corresponding increase for the mononucleotides. This implies that the increase in reagent concentration must perturb the tRNA structure, thereby allowing more residues to react. The curve in Figure 6 corresponding to the reaction in 0.01 M borate at 30° is the one which most closely resembles those for the mononucleotide models. The addition of magnesium ions and the decrease in temperature do not result in an improvement of the relative resistance of the tRNA to reaction as the carbodiimide concentration is increased. These results imply that we are not dealing with a simple polynucleotide system, containing just single- and double-stranded chains, where the addition of a divalent ion or lowering of the temperature would be expected to increase the resistance of double-helical regions to chemical modification without affecting the availability of the residues in single-stranded segments. Another result which points in the same direction is that when the ionic strength of the borate buffer is increased tenfold (see Figures 2 and 3) the extent of reaction is at no point decreased. Increasing the ionic strength in the case of poly (A + U) results in a markedly greater resistance to the reagent (Metz and Brown, 1969).

The results shown in Figure 6 indicate that there is no obvious optimum value for the carbodiimide concentration in experiments with tRNA. In the present investigations, a concentration of 50 mM has normally been used. This will cause the reaction with available nucleotide residues to go to substantial completion, though some perturbation of the secondary (or tertiary) structure will necessarily occur.

We have investigated the influence of the RNA concentration on the reaction between the carbodiimide and tRNA. It was thought that at the comparatively high RNA concentrations employed in the present experiments (*ca.* 3 mg/ml) aggregation might occur. The occurrence of aggregation, which is likely to be dependent upon the RNA concentration, could decrease the extent of the carbodiimide reaction. The results shown in Figure 7 show no evidence for the occurrence of aggregation, even in the presence of 10 mM Mg^{2+} at 15° (except possibly at the highest RNA concentration used, in the absence of Mg^{2+} and at 30°). However, the data for the reaction in the buffer containing the intermediate magnesium ion concentration (2 mM) shows a striking and unexpected result. At nucleotide concentrations below 4 mM the extent of reaction is similar to that in 10 mM Mg^{2+}, but between the nucleotide concentrations of 4 and 5 mM there is an abrupt increase in the extent of reaction, to a level corresponding to that which occurs in the absence of the cation. The fact that this effect occurs at a constant Mg^{2+} concentration suggests that it is the stoichiometric ratio of nucleotide to Mg^{2+} that governs the extent of reaction of the tRNA, rather than the Mg^{2+} concentration as such. The upswing of the curve in this experiment occurs at around the point at which the nucleotide concentration (4 mM) is equivalent to the divalent cation concentration (2 mM), and this is also suggestive of a stoichiometric relationship.

The converse experiment to the previous one was performed. In this, the extent of the reaction of the carbodiimide with tRNA was measured at constant tRNA concentration while the magnesium ion concentration was varied. The results are depicted in Figure 8. The same experiment was carried out with rRNA for comparison. As the Mg^{2+} concentration is increased from zero, the extent of reaction increases somewhat, until, between concentrations of 2 and 3 mM, it abruptly falls and soon reaches the value that obtains in 10 mM Mg^{2+}. Essentially the same result was found in 0.01 M Tris-Cl buffer except that the initial rise up to 2 mM Mg^{2+} was less marked. In the

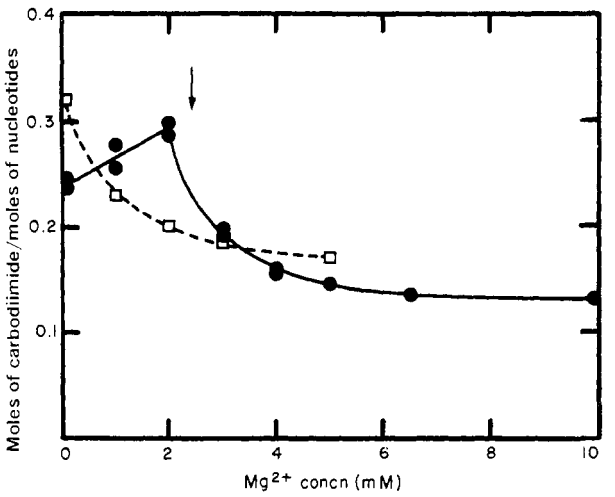

FIGURE 8: The extent of the reaction of the carbodiimide with tRNA and with RNA as a function of the Mg^{2+} concentration. Solutions of RNA were prepared in 0.02 M borate buffer containing twice the final $MgCl_2$ concentration and were adjusted to pH 8.0. To 20-μl aliquots of these solutions was added 20 μl of [^{14}C]carbodiimide iodide solution (100 mM in water). The mixtures were incubated for 16 hr at 30°. Reaction mixtures contained 190 mμmoles of tRNA (expressed as nucleotides) or 270 mμmoles of rRNA. Each point plotted is the mean of duplicate determinations. The arrow indicates the Mg^{2+} concentration at which the amount of the divalent ion in the reaction mixtures is equivalent to the amount of the tRNA nucleotides. (●—●) tRNA and (□- -□) rRNA.

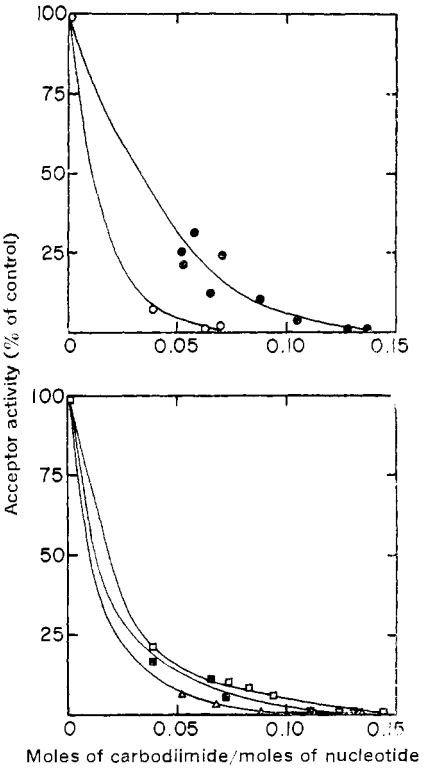

FIGURE 9: Loss of amino acid acceptor activity of tRNA as a function of the extent of the carbodiimide reaction in 0.01 M borate buffer. [^{3}H]Carbodiimide iodide was reacted with tRNA in 0.01 M borate buffer (pH 8.0) at 30° for varying times, as described in Materials and Methods. The acceptor activity of the different preparations was then determined. The acceptor activity is expressed as the per cent relative to the unreacted control tRNA. (●—●) Lysine, (○—○) tyrosine, (□—□) valine, (■—■) arginine, and (△—△) serine.

present experiment, the discontinuity in the tRNA curve again occurs in the vicinity of the point of stoichiometric equivalence between nucleotide and magnesium ion. The result for rRNA is in marked contrast to that for tRNA. In the former case, increasing the magnesium ion concentration effects a gradual and continuous transition in the proportion of nucleotides that react with the reagent.

The results shown in Figures 7 and 8 are essentially complementary and appear to reflect some sort of configurational change that occurs when the number of magnesium ions present is about equal to half the total number of nucleotide residues of the tRNA. One point must be made concerning the procedure employed in the experiment shown in Figure 8. In order to obtain consistent and reproducible results it was necessary to dissolve the tRNA in the buffer solution already containing magnesium ions (at twice the final concentration to allow for dilution by the reagent). In some experiments the tRNA was dissolved in buffer lacking magnesium and a solution of $MgCl_2$ (at ten times the final concentration) was added to give the desired molarity. The extent of reaction of the carbodiimide with tRNA in solutions prepared in this way was very variable and irreproducible. A number of variables were examined in order to gain a better understanding of this effect but no adequate explanation could be inferred. Despite this, reproducible results were obtained, as illustrated, if the experiments utilized tRNA dissolved in buffers containing Mg^{2+} at the outset.

All the experiments that have been described have been carried out with unfractionated tRNA, though this material is substantially homogeneous with respect to molecular weight. In a preliminary experiment, the time course of the reaction of the carbodiimide with a tRNA preparation enriched in the alanine-acceptor species (prepared by a countercurrent distribution method) was examined. The results were quite similar to those for the unfractionated material. This suggests that the behavior of single tRNA species is not likely to be very different from the observed average properties of the unfractionated material.

The Effect of the Carbodiimide Modification on the Biochemical Reactivity of tRNA. tRNA participates in a number of distinct biochemical processes. Amino acids are enzymically attached to the appropriate tRNA species and then transferred into polypeptide chains *via* the ribosome–messenger complex. The transfer function itself involves a sequence of steps, the first and best understood being the nonenzymatic binding of the aminoacyl-tRNA to the ribosome plus messenger codon. Other enzymatic reactions that involve tRNA as a substrate include the removal and renewal of the CpCpA-terminal sequence, and methylation and similar modifications of the putative tRNA precursor species. We have investigated the effect of the carbodiimide modification on the amino acid acceptor activity and on the messenger-stimulated binding by ribosomes of the aminoacyl-tRNA.

When determining the acceptor activity of chemically modified tRNA, one possibility that must be considered is that the reagent will become detached from the RNA during the course of the reaction, resulting in an enhanced activity value. If this occurs, a further possibility is that the liberated reagent will react with some other component of the assay system, *e.g.*,

TABLE I: Loss of Amino Acid Acceptor Activity of tRNA Following Reaction with the Carbodiimide.[a]

Amino Acid	Mg^{2+}	Moles of the Carbodiimide/Moles of Nucleotide	Acceptor Act. (% of control)
Histidine	−	0.12	8
Threonine	−	0.11	0
Leucine	−	0.12	0
Isoleucine	−	0.11	0
Aspartic acid	−	0.11	0
Methionine	−	0.12	3
Proline	−	0.11	0
Histidine	+	0.042	74
Threonine	+	0.043	13
Leucine	+	0.045	70
Isoleucine	+	0.046	69
Aspartic acid	+	0.039	26
Methionine	+	0.046	54
Proline	+	0.049	44

[a] The procedure used was described in Figure 8. The carbodiimide reaction was carried out in 0.01 M borate buffer (pH 8.0) at 30°, in the presence or absence of 0.01 M $MgCl_2$, as indicated.

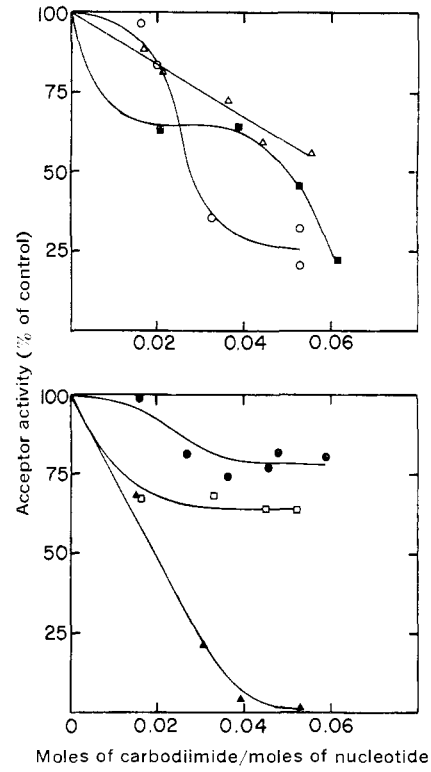

FIGURE 10: Loss of amino acid acceptor activity of tRNA as a function of the extent of the carbodiimide reaction in 0.01 M borate buffer (pH 8.0)–0.01 M $MgCl_2$. The conditions are as described in Figure 9, except that the borate buffer contained 0.01 M $MgCl_2$. (△—△) Serine, (■—■) phenylalanine, (○—○) arginine, (●—●) valine, (□—□) lysine, and (▲—▲) tyrosine.

the enzyme, with deleterious result. Using [^{3}H]carbodiimide-tRNA, we have found that no loss of carbodiimide is detectable in 1 hr at 30° in the following buffers: 0.1 M Tris-Cl (pH 8.0), 0.1 M phosphate (pH 7.0 or 6.0), 0.1 M acetate (pH 5.0), or in unbuffered aqueous solution. Further, no loss of label was detectable in the full reaction mixture for acceptor activity determinations after 30 min at 37°. In another experiment, the carbodiimide was added directly to the assay system in an amount corresponding to the full amount of carbodiimide that was typically bound to tRNA. For the five amino acids whose activities were assayed no significant difference could be detected between assays in the presence and absence of the carbodiimide. Thus it may be concluded that the reagent is not lost from the modified tRNA under the conditions of the acceptor activity assay and even if it were, no inhibition of the assay would be expected.

To investigate the effect of the chemical modification on the acceptor activity, tRNA was first reacted with [^{3}H]carbodiimide iodide under various conditions and was recovered by ethanol precipitation. The amino acid acceptor activity of this material was then determined using [^{14}C]amino acids. The aminoacylated tRNA was recovered by acid precipitation as usual, and both isotopes in the washed precipitate were counted. Thus the acceptor activity for a particular extent of modification was determined directly. It should be noted that while the acceptor activity measures the functional integrity of a single type of tRNA species, the amount of carbodiimide bound will reflect the average property of all the species present.

The loss of acceptor activity consequent upon reaction with the carbodiimide at 30° in the absence of Mg^{2+} is shown in Figure 9. In Figure 10, the results of similar experiments, except that the carbodiimide reaction was carried out in the presence of Mg^{2+}, are shown. Progressive carbodiimide modification in the absence of magnesium ions results in a rapid loss of acceptor activity. When the modification is carried out in the presence of magnesium ions the loss of activity varies markedly from one amino acid to another. That the six amino acid specific tRNAs whose losses of activity following modification have been examined in detail are not untypical is suggested by an examination of a further seven acceptor activities at a single extent of modification (Table I).

In the absence of Mg^{2+}, the greater part of the acceptor activity is lost by the time 5% of the nucleotides has reacted with the carbodiimide and almost all is lost at the point when 10% has reacted. It may be recalled that in the absence of Mg^{2+}, at 30°, 10% of the residues has reacted by about 2.5 hr and the reaction proceeds until 25% of the nucleotides has reacted at 15–20 hr (Figure 2). Thus a quite limited extent of reaction suffices for the inactivation of all the acceptor activities tested, in the absence of Mg^{2+}. It seems reasonable to suppose that this limited reaction of the carbodiimide with tRNA is very predominantly with single-stranded segments of the molecule, and not with perturbed double-helical regions.

The variability of the rate of loss of acceptor activity when the carbodiimide modification is effected in the presence of Mg^{2+} is very marked. Altogether, 13 acceptor activities have been examined; after about 3 carbodiimide residues per molecule of 80 nucleotides have reacted, one acceptor activity

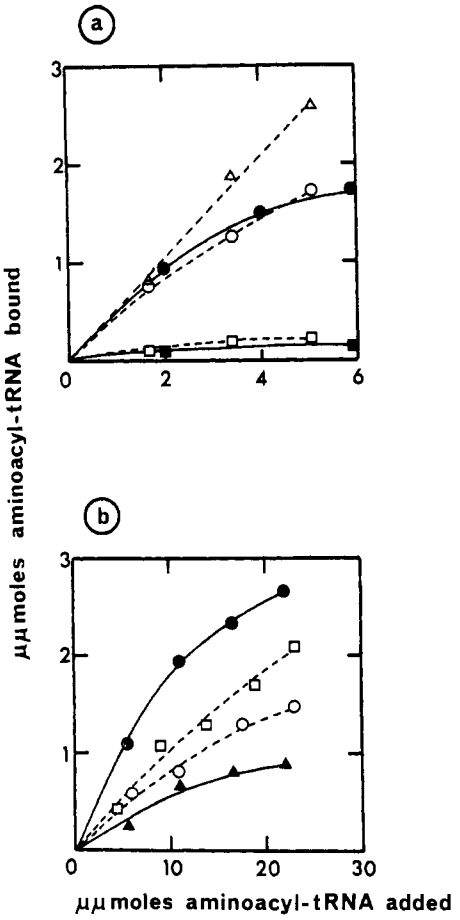

FIGURE 11: Binding of aminoacyl-tRNA to ribosomes. The assay procedure is described in the text. (a) The binding of [^{14}C]Phe-tRNA (17.6 $\mu\mu$moles/A_{260} unit) to ribosomes, in the presence (●—●) and in the absence (■—■) of poly U, is shown. Also shown is the binding of [^{14}C]Phe-[^{3}H]carbodiimide-tRNA (15.4 $\mu\mu$moles of amino acid/A_{260} unit; 1.2 moles of carbodiimide/100 moles of nucleotide) to ribosomes, in the presence (△– –△) and in the absence (□– –□) of poly U. In one experiment, using [^{14}C]Phe-[^{3}H]carbodiimide-tRNA in the presence of poly U, 3.2 A_{260} units of deacylated tRNA was added to each assay tube after the 20-min incubation; after a further 2-min incubation the reaction was stopped in the usual way (○– –○). (b) The binding of [^{14}C]Lys-tRNA (63.3 $\mu\mu$moles/A_{260} unit) to ribosomes, in the presence (●—●) and in the absence (▲—▲) of poly A, is shown. Also shown is the binding of [^{14}C]Lys-[^{3}H]carbodiimide-tRNA (24.9 $\mu\mu$moles of amino acid/A_{260} unit; 1.5 moles of carbodiimide/100 moles of nucleotide) to ribosomes, in the presence (□– –□) and in the absence (○– –○) of poly A.

was retained to the extent of more than 80%, six to between 60 and 80%, three to between 30 and 60%, and three to less than 30%. There is no common feature in the pattern of loss of activity with the extent of modification. It seems plausible to argue here, as before, that the losses of acceptor activity result from chemical modification of single-stranded regions of the tRNA structure.

It was of interest to examine the effect of the carbodiimide reaction on the transfer function of tRNA. The initial step in this process is the binding of an aminoacyl-tRNA molecule to the ribosome in the presence of the specific messenger codon. It seemed logical to investigate the effect of the carbodiimide modification on this binding, rather than on the transfer reaction *in toto*, since the latter could not occur in the absence of the former. The binding assay of Nirenberg and Leder (1964) was used, with poly A and poly U as the synthetic messenger species. For this assay a labeled aminoacyl-tRNA is required. It is apparent that carbodiimide-modified aminoacyl-tRNA cannot be prepared by reacting the carbodiimide with an aminoacyl-tRNA since the ester linkage is labile under the conditions of the carbodiimide reaction (pH 8.0; 1–2 hr). Thus the tRNA must be acylated after modification. It follows that it is possible to react the carbodiimide with tRNA only under conditions where a useful amount of the acceptor activity is retained, namely, is the presence of magnesium ions. It also follows that only the part of a particular amino acid specific tRNA that retains its acceptor activity after modification can be assayed for ribosome-binding activity.

It may be envisaged that the binding of an aminoacyl-tRNA to the ribosome-messenger complex will depend upon two types of interaction: that between the codon and the anticodon, and that between a nonspecific tRNA binding site on the ribosome and some complementary site on the tRNA which is probably common to all species. The former mode of interaction is the more interesting in the present context since the carbodiimide modification will affect this in a way that will depend upon the base composition of the anticodon. Thus, in the present experiments, a very limited extent of the carbodiimide modification has been employed (1.0–1.5 moles of the carbodiimide/100 moles of nucleotides) in the hope of avoiding reaction with nonspecific binding sites on the tRNA. The results of experiments using Phe-tRNA and poly U, and Lys-tRNA and poly A, with and without the carbodiimide modification, are depicted in Figure 11. The binding of phenylalanine-tRNA was not reduced by the carbodiimide modification to the extent of 1.2 mole %. It was, in fact, somewhat enhanced. However, a proportion of this binding was nonspecific since addition of nonacylated tRNA at the end of the incubation decreased the binding to a level equivalent to that of unmodified Phe-tRNA (deacylated tRNA decreased the binding of unmodified Phe-tRNA by a markedly smaller amount). Nirenberg and Leder (1964) have shown that the addition of deacylated tRNA at the end of the incubation has little effect on the specific binding of aminoacyl-tRNA but could reduce the background of nonspecific binding.

The binding of lysine-tRNA to ribosomes in the presence of poly A is also shown in Figure 11. It will be observed that the effect of the carbodiimide modification (1.5 mole %) is to decrease the extent of binding in the presence of poly A and to increase the binding in the absence of the polymer. The net result is a decrease in the binding of lysine-tRNA of about 70%. This is in marked contrast to the result for the phenylalanine tRNA binding.

Discussion

The time course of the reaction of the carbodiimide with tRNA is one in which the rate of reaction decreases continuously as the time increases. This is the case under all conditions of temperature and ionic strength examined, though the actual values of the reaction rates and extents do of course depend upon these variables. The purpose of this discussion is to interpret the present results in terms of a structural model for tRNA.

First, however, mention must be made of other reports on the reaction between the carbodiimide and tRNA since these

differ in certain respects from our own. Knorre *et al.* (1966) examined the reaction between the reagent and yeast tRNA in Tris-Cl buffer. They observed a step-like characteristic in certain of their time-course curves; an initial rapid reaction was followed by a plateau region which was followed in turn by a further phase of rapid reaction. Knorre *et al.* also reported that the initial rate of reaction of the carbodiimide with tRNA was greater than the initial rate with an equivalent mixture of mononucleotides. A possible origin of the stepwise type of curve may lie in the use of Tris-Cl buffer. It was shown in the preceding paper (Metz and Brown, 1969) that the pH of the reaction mixture slowly increases with time when Tris buffer is used. Since both the initial rate and extent of reaction increase as the pH is increased (Figure 1), it is conceivable that the stepwise curves of Knorre *et al.* may arise from this effect. In our experiments using *E. coli* tRNA in Tris-Cl buffer, however, we have not observed any suggestion of a step-like character in the time-course curves. It is possible, therefore, that there is some difference between tRNAs from *E. coli.* and from yeast (see below).

The initial rapid rate of reaction noted by Knorre *et al.* (1966) and also by the other workers with yeast tRNA (see below) has not been observed in the present experiments. Knorre *et al.* suggested that the greater rate of reaction of the carbodiimide with tRNA, compared with that with the equivalent mixture of nucleotides, might be attributed to an electrostatic type of interaction between the negatively charged macromolecule and the positively charged carbodiimide ion, which would give rise to an increased reagent concentration in the vicinity of the RNA molecules. If such an effect were operative one might expect to observe it in the case of poly U. In fact, the rates of reaction in 8 mM buffer of the carbodiimide with UMP and with poly U are quite similar though the latter reacts more rapidly in 80 mM buffer (1.5 times) (Metz and Brown, 1969). Since the initial rapid phase of the reaction with tRNA was found in buffer concentrations of 10 mM or less, it does not appear that the explanation of Knorre *et al.* is adequate.

Brostoff and Ingram (1967) have examined the time course of the reaction of the carbodiimide with yeast tRNA in borate buffer. They also find an initial very rapid reaction; in fact, at 38°, nearly 2 moles of the carbodiimide attach per molecule of tRNA in the first 60 sec. This is much faster than would be expected if the reaction were occurring with uridine, guanosine, or thymidine residues (Metz and Brown, 1969; Ho and Gilham, 1967). It may well be that there occurs a very rapid reaction between the carbodiimide and one or more minor bases which occur to a considerably greater extent in yeast tRNA than in that from *E. coli*.

Another feature of the results of Brostoff and Ingram (1967) is that after the initial phase of reaction, there follows an extended flat plateau region. This type of curve was also found previously in this laboratory, again when yeast tRNA was reacted with the carbodiimide (Augusti-Tocco and Brown, 1965). In contrast, the present results uniformly display a progressive increase in the extent of reaction with time (Figures 1–4). A likely explanation for this difference is as follows. Both the aforementioned groups used ethanol precipitation to stop the reaction between the reagent and tRNA. It is known that the product of the reaction between the carbodiimide and poly U is soluble in alcohol (Augusti-Tocco and Brown, 1965). Thus it is to be expected that modification by the carbodiimide will tend to increase the solubility of tRNA in ethanol. If this effect is significant at the extents of reaction that occur with tRNA then a tendency for the time-course curves to display a marked flattening as the time increases would not be unexpected. In some preliminary experiments using radioactive-labeled RNA, we have found that reaction with the carbodiimide results in a significant proportion of the counts remaining in solution after ethanol precipitation.

Apart from the differences in the shapes of the time-course curves and in the initial rates of reaction that have already been mentioned, there is some variation in the extents of reaction of the carbodiimide with tRNA among the various reports. These can be attributed to the use of different reagent concentrations, the use of ethanol precipitation, and possibly also to differences in the source of the tRNA. This last point refers not only to differences between yeast and *E. coli* tRNA, but also to the possible presence of contaminant RNA species in the tRNA preparations. The tRNA used in the present experiments was prepared from *E. coli* cells by phenol extraction. The final stages of the preparation involved molecular sieve chromatography to remove higher molecular weight contaminants, followed by extensive dialysis, first against sodium chloride plus EDTA, and then against water. The sodium salt of tRNA thus prepared was substantially free from non-RNA contaminants and from RNA species significantly different in size from the 4S material. It also had good biological activity.

The experiments reported in the preceding paper (Metz and Brown, 1969) on the reaction between the carbodiimide and model polynucleotides could be interpreted essentially on the basis that the reagent reacted rapidly with nucleotides in single-stranded regions of the secondary structure and much more slowly with those in double-helical regions. At first sight, one would be inclined to interpret the results for the carbodiimide–tRNA reaction in the same way. However, there are a number of indications that such an approach would not be adequate. The most compelling are those arising from the examination of the extent of the carbodiimide reaction with tRNA as a function of the Mg^{2+}: nucleotide ratio (Figures 7 and 8). The rather abrupt transition that occurs at the point at which the magnesium ions present are equivalent to the nucleotide phosphate content of the RNA is not an effect that fits easily into the simple model. The behavior of rRNA (Figure 8), in contrast, seems to be quite straightforward; the extent of reaction decreases progressively as the divalent ion concentration is increased. This is consistent with the very reasonable hypothesis that the stability, and possibly the number, of the double-helical segments increases with the Mg^{2+} concentration. The change that occurs in the conformation of tRNA structure on addition of Mg^{2+} is evidently of a different type. Since it occurs at 30° it is unlikely to involve a very substantial reorganization of the secondary structure. We may therefore suppose that a change in the tertiary structure is effected by the addition of a stoichiometrically equivalent quantity of magnesium ions, with the result that the number of single-stranded residues on the outside of the molecule is about halved.

There is other evidence that the pattern of reaction of the carbodiimide with tRNA cannot be interpreted in terms of a simple model involving a consideration of secondary structure alone. As noted earlier, the fact that a tenfold increase in ionic strength does not decrease the extent of the carbodiimide reac-

tion (Figures 2 and 3) is not consistent with the simple model, nor are the data of Figure 6, where the extent of reaction as a function of the carbodiimide concentration in various solutions is indicated.

It will be seen, therefore, that the interpretation of the results on the extent of reaction of the carbodiimide with tRNA is not entirely straightforward. Under any given set of conditions, the extent of reaction will reflect the proportion of uridylate and guanylate residues (and other reactive groups) that are exposed to the solution (*i.e.*, those that are on the outside of the molecule and are not buried in the structure), together with contributions arising from the perturbation by the reagent of regions of secondary and tertiary structure.

The perturbation of polynucleotide secondary structure by the carbodiimide reaction was discussed in the preceding paper (Metz and Brown, 1969). It was there shown that the effect was not great when the secondary structure was reasonably stable. The possibility that the tertiary structure of tRNA may be perturbed by the carbodiimide modification must also be considered. It is conceivable that the conformation of the molecule will be substantially altered by the addition of even a single bulky carbodiimide group. Unfortunately, the absence of a suitable model polynucleotide with a defined tertiary structure makes it impossible to test this hypothesis. There are, however, two considerations which incline us against the idea that chemical modifications need cause a substantial change in the tertiary structure of tRNA. First, many amino acid acceptor activities are largely retained following limited carbodiimide modification (in the presence of magnesium ions). Although we do not know which parts of the molecular structure of tRNA are involved in the acceptor activity reaction, it does seem unlikely that the reaction would occur if the tertiary structure were substantially different from that of the unmodified molecule. Second, the extent of the carbodiimide modification may be quite limited, especially in the presence of magnesium ions. Since it appears improbable that the addition of the first carbodiimide group to the tRNA molecule would change the conformation in such a way as to decrease the extent of reaction, the limited degree of reaction that may be observed argues against a sizable perturbation of the tertiary structure.

For these reasons, we incline to the opinion that the carbodiimide modification of tRNA does not substantially perturb either the secondary or tertiary structure. We recognize, however, that this point has not been rigorously proven. The extent of the two types of perturbation cannot be estimated *a priori*. It is possible, nevertheless, to state that the proportion of single-stranded reactive residues in the unperturbed molecule must be less than, or equal to the proportion that have reacted with the carbodiimide. In other words, the experimental data enable us to place an upper limit to the proportion of nucleotides that are exposed to the environment. The remainder of the discussion should be read with this qualification in mind.

To proceed further, it is necessary to introduce two other types of data into the discussion. These are the results of the primary structure studies on a number of tRNA species and the physicochemical studies that relate the question of the tertiary structure of tRNA.

The complete nucleotide sequences are known for six yeast tRNA species (Holley *et al.*, 1965; Madison *et al.*, 1966; Zachau *et al.*, 1966; RajBhandary *et al.*, 1967; Takemura *et al.*, 1968; Baev *et al.*, 1967), and for two *E. coli* species (Goodman *et al.*, 1968; Dube *et al.*, 1968). All these primary structures can be arranged to give the cloverleaf type of secondary structure (Madison, 1968), which is the most plausible of the secondary structures that have been proposed. It is possible to calculate the proportion of residues of each of the known structures that should react with the carbodiimide, if certain assumptions are made. The procedure adopted will now be stated. The known sequence was put into the cloverleaf conformation with the maximum number of standard pairs. The number of unpaired residues, of the type that react with the carbodiimide, could then be ascertained. In determining the number of unpaired residues that are capable of reacting with the carbodiimide, allowance was made for the fact that certain substituted bases (such as 1-methylguanine) will not be able to react. Pseudouridine residues were counted as two since it is known that two carbodimide groups, can react with one pseudouridine (Naylor *et al.*, 1965). Allowance was also made for the fact the in single-stranded stacked structures composed of nucleotides that can react with the reagent, not all of the residues will be available for reaction due to steric hindrance. It was shown in the previous paper (Metz and Brown, 1969) that in this situation probably two, but not three, adjacent stacked bases are able to react. It is assumed that the results obtained with model polynucleotide are applicable to the looped regions of tRNA. In order to allow for this restriction on the extent of reaction of adjacent stacked bases it is necessary to know the configurations of the single-stranded regions in tRNA. Fuller and Hodgson (1967), on the basis of model-building studies, have proposed a plausible configuration for the anticodon loop. In this, five of the bases are stacked in a continuous sequence while the other two form a separate, weakly stacked pair. This configuration has been used in the calculation of the number of single-stranded residues that react with the carbodiimides. Also following Fuller and Hodgson (1967) we have assumed that the Tψ loop has a similar configuration. In the case of the larger dihydrouridine-containing loop, we have assumed that those bases that normally tend to stack will do so here.

When the calculations are performed as described above, we find that the proportion of residues available for reaction with the carbodiimide lies between 18 and 27%. This range includes the uncertainties in the estimation. The mean value is 22%. It may be noted that the magnitude of this figure is determined very largely by the form of the cloverleaf structure. The restriction upon the reactivity of the single-stranded stacked residues has only a very slight effect.

The above value for the expected extent of reaction of the carbodiimide with tRNA has been calculated on the assumption that the secondary structure alone limits the reaction. From Figure 5 is is apparent that in the absence of magnesium ions, not until a temperature of 25° is reached is an extent of reaction of 22% (at 16 hr) attained. It may therefore be concluded that the simple open cloverleaf structure, lacking a definite tertiary structure, cannot occur either in the presence of magnesium ions at moderate temperatures, or in the absence of the divalent ion at temperatures below 20°. The existence of more than one distinct tertiary structure appears to be an inescapable postulate, unless some entirely novel form of nucleotide–nucleotide interaction is proposed.

The above interpretation has employed the cloverleaf model. It should be noted that this model is constructed on the basis that base pairing is maximized. Thus no alternative simple

base-paired model structure will give a value for the proportion of nucleotides available for reaction with the carbodiimide that is in better agreement with the experimental data.

The type of calculation that has been used to calculate the proportion of the nucleotides in the structures of known sequence that could react with the carbodiimide in the absence of any tertiary structure constraints can be extended somewhat. It is apparent that the contribution to the proportion of reactive residues made by any one region of the tRNA molecule can be assessed. For example, the average contribution made by the anticodon loop is 5%. Thus we might attempt to correlate the experimental values for the extents of the carbodiimide–tRNA reaction, under a variety of conditions of temperature and ionic composition, with the calculated contributions made by the exposure of the different segments of the cloverleaf to the environment. Before persuing this, however, it is convenient to introduce a further class of result, namely, the physiocochemical studies that point to the existence of different tertiary structures.

Henley *et al.* (1966) examined the variation of a number of hydrodynamic parameters of yeast tRNA with temperature. They concluded that the thermal denaturation of tRNA (in the absence of Mg^{2+}) occurred in two steps; the first, between 20 and 40°, seemed to involve a change of shape accompanied by only a relatively small loss of secondary structure. The second step which occurred above 50° involved the denaturation of the secondary structure. Henley *et al.* considered the first step to be indicative of the loss of some type of tertiary structure. In contrast, in the presence of Mg^{2+}, the change in the hydrodynamic parameters was largely suppressed below 60°, at which temperature denaturation commenced (Fresco *et al.*, 1966). The absence of change in the experimental variables below 60° does not preclude the existence of tertiary structure since the observed changes above 60° may reflect the simultaneous destruction of secondary and tertiary structures, both stabilized by Mg^{2+}.

The data on the carbodiimide reaction with tRNA may now be considered in conjunction with the physicochemical data of Fresco *et al.* The rapid increase in the extent of the carbodiimide reaction in the absence of Mg^{2+} above 20° (Figure 5, corrected data) is likely to correspond to the tertiary structure transition that was deduced from the variation in the hydrodynamic parameters. The extent of the carbodiimide reaction corresponds to that expected for the simple open cloverleaf by the time a temperature of 25° is attained. However, this extent of reaction will include some part that must be attributed to perturbation of the secondary structure by the reagent, and therefore the fully opened-cloverleaf configuration will not occur until a somewhat higher temperature is reached. It seems reasonable to suppose that the transition observed by Henley *et al.* (1966) between 20 and 40° involves the opening of a closed-cloverleaf structure into an open cloverleaf, in which all the nonbase-paired residues are exposed but the secondary structure is maintained intact. The extent of the carbodiimide reaction below 20°, in the absence of Mg^{2+}, suggest that the unpaired nucleotides in one, or possibly two, of the loops (or the central part plus the small extra arm) of the cloverleaf are not exposed. Alternatively, some of the residues in each loop may be exposed while others are protected against reaction by the tertiary structure.

In the presence of magnesium ion the extent of reaction of the carbodiimide with tRNA increases gradually, without discontinuity, as the temperature increases (Figure 5). The results of Fresco *et al.* (1966) indicate that no change in the secondary or tertiary structure occurs below 60°. We may therefore suppose that the gradual rise in the extent of the carbodiimide reaction in this case is due to an increasing amount of perturbation of the structure with increasing temperature. The extent of reaction below 10° is about 7% (after correction, Figure 5). This is consistent with a model in which only one, or possibly two, of the loops (or the central part) of the cloverleaf are exposed and therefore available for reaction with the reagent.

The above considerations have led us to a model in which there are three distinct tertiary structural conformations for tRNA in aqueous solution at moderate ionic structure and neutral pH. In the absence of Mg^{2+} and at temperatures above 40°, the simple open-cloverleaf structure occurs, with the unpaired nucleotides in all the loops exposed. On reducing the temperature to below 20°, a tertiary structure transition occurs leading to a configuration in which a smaller proportion of the unpaired bases are exposed, the decrease being equivalent to the number of reactive residues in one, or possibly two, loops. On the addition of at least a stoichiometric equivalent of magnesium ions a second marked conformational transition is apparent. The proportion of nucleotides on the outside of the molecule is now decreased to an extent consistent with there being only one (or possibly two) loops exposed.

A discussion of which of the unpaired residues are likely to be exposed and which protected is complicated by the fact that the carbodiimide reaction only measure the gross number of residues that react. We do not know whether or not all the potentially reactive unpaired nucleotides in a single loop will behave similarly under a given set of conditions. Thus we should not restrict our consideration to models in which whole loops are either exposed or protected but should include those in which only parts of loops are exposed. Clearly, many types of model could fit the present data when fewer than all the unpaired reactive nucleotides react. Other information is required in order to identify the nucleotides that do react.

There is evidence to suggest that, at low temperatures, in the presence of Mg^{2+}, the anticodon loop is exposed. Ribonuclease T1 will specifically cleave certain tRNA species in the anticodon region at 0° in the presence of Mg^{2+} (Penswick and Holley, 1965; Madison *et al.*, 1966; Baev *et al.*, 1967). Under these conditions, scission of other phosphodiester linkages may be avoided. Nirenberg and Leder (1964) have shown that aminoacyl-tRNA binds specifically to the ribosome–messenger complex at 0° in the presence of Mg^{2+}, suggesting that the anticodon loop is exposed under these conditions. Taking the above evidence together with the present results, it seems that a plausible model for the tertiary structure of tRNA in the presence of Mg^{2+} and at moderate temperatures is one in which substantially the only non-base-paired residues that are exposed to the environment are those in the anticodon loop. Most, if not all, of the unpaired nucleotides in the rest of the molecule are not available for interaction with other molecular species. (The nucleotides of the CpCpA-terminal sequence might well be exposed; these residues would not react with the carbodiimide.) Since all the biological activities of tRNA require the presence of Mg^{2+}, the above, very compact structure must be the biological active conformation.

The role of magnesium ions in relation to polynucleotide secondary structure in general and to the structure of tRNA

in particular is still by no means clear (for review, see Felsenfeld and Miles, 1967). It is known that divalent cations are more tightly bound to polynucleotides than are monovalent cations and it would therefore be expected that the former would be more efficient at neutralizing change repulsions between the phosphate groups, thereby stabilizing the secondary structure. The tightness of binding of Mg^{2+} has been evoked to explain such effects as the sharpening of the melting curve and the increase in melting temperature (Monier and Grunberg-Manago, 1962), the increased resistance to nuclease attack (Nishimura and Novelli, 1963, 1964), and the inhibition of the reaction with formaldehyde (Penniston and Doty, 1963a), all of which occur on the addition of magnesium ions to tRNA. The present work has shown that magnesium ions play an important role in stabilizing the tertiary structure of tRNA. In this respect, tRNA behaves quite differently from rRNA. A significant point to emerge is that about 1 equiv of magnesium ions must be bound per phosphate group before the tertiary structure transition occurs. At Mg^{2+} :phosphate ratios that are less than one, no decrease in the extent of the carbodiimide reaction, compared with that which occurs in the absence of Mg^{2+}, occurs. This stoichiometry suggests that the formation of the compact conformation requires more efficient change neutralization of the phosphate groups than can be provided by monovalent cations at normal concentrations. At least some of the magnesium ions might also be required for a more specific structural role, for example, the formation of bridges between phosphate groups in different helical segments.

We now discuss the effect of the carbodiimide modification on the biological activities of tRNA. When tRNA is reacted with the reagent at 30°, in the absence of Mg^{2+}, substantially all the amino acid acceptor activity is lost by the time about four residues have been modified on the average. The rates of loss of activity do not vary much from one amino acid specific species to another. Two types of explanation for this result are possible. It may be that a single-stranded region of tRNA that interacts with the synthetase enzyme in the acylation reaction is always exposed under the reaction conditions used. Alternatively, it may be that a quite limited degree of modification prevents the molecule from folding into the compact, biologically active configuration which must occur in the acceptor activity assay, when Mg^{2+} is of course present. Although both hypotheses are plausible, the second does follow more directly from the previous arguments on tertiary structure. It may be noted that the carbodiimide does not react with the CpCpA-terminal sequence of tRNA, whose integrity is required in the aminoacylation reaction.

When the modification of tRNA is carried out in the presence of Mg^{2+}, the pattern of loss of acceptor activity varies considerably from one amino acid to another. In many cases there is evidence of a stepwise loss of activity; a partial loss after two to four nucleotides have reacted (on the average) may be followed by no further loss of activity during the reaction of a further two or more residues. Penniston and Doty (1963b) found curves of this type when they inactivated tRNA with formaldehyde. They suggested that this shape could be attributed to the known heterogeneity of tRNA, the different molecular species for a particular acceptor activity losing activity at different rates. Such an explanation would fit the present results. There is another possible type of explanation that should not be neglected. The assay for amino acid acceptor activity involves a two-stage enzymic reaction. The enzyme preparation employed was a fairly crude one, and the optimum conditions for each particular acceptor activity were not individually ascertained. It is conceivable that a limited degree of modification of a particular tRNA species could result in a shift in the equilibria of the corresponding assay reactions thereby changing the measured acceptor activity.

A number of workers have attempted to correlate the loss of acceptor activity following various types of chemical modification with the composition of the putative anticodon. We have not been able to find such a correlation in the case of the carbodiimide so no direct deduction can be made concerning the involvement of the anticodon in the acceptor reaction. As discussed earlier, there is good evidence that most of the unpaired residues of tRNA that are exposed in the presence of Mg^{2+} are those in the anticodon loop. Thus the fact that a number of acceptor activities are significantly decreased after two or three nucleotides have reacted with the reagent (on the average) would not be inconsistent with the involvement of the anticodon in the acceptor function of tRNA. Conversely, however, the fact that most acceptor activities are at least partially retained after a few nucleotides have been modified suggests that the anticodon is not involved. On the question of a role for the anticodon in the acceptor function, then, the evidence from the carbodiimide modification is equivocal.

The ribosome-binding experiments were designed to examine the involvement of the anticodon in the transfer reaction of tRNA. Very limited extents of modification (1–2%) were employed with the intent of avoiding loss of the binding activity due to reaction at some nonspecific ribosome-binding site. The fact that phenylalanyl-tRNA completely retained its activity after modification suggests that this type of inactivation was avoided. The known genetic code together with the Wobble hypothesis (Crick, 1966) enable us to predict that the anticodon for phenylalanine tRNA should be GAA, corresponding to the codons UUU and UUC, while that for lysine tRNA should be UUU, corresponding to the AAA and AAG codons. Thus every base in the anticodon of lysine tRNA is capable of reacting with the carbodiimide, while only one of the three Phe species might react. It would be expected, therefore, that lysyl-tRNA would more rapidly lose its ability to be bound to the messenger–ribosome complex following carbodiimide modification than would Phe-tRNA.

The experimental results are consistent with the foregoing expectation. No loss of phenylalanyl-tRNA binding was detectable while lysyl-tRNA binding was markedly reduced. However, caution must be exercised in interpreting these results. Phenylalanyl-tRNA binds much more efficiently to ribosomes in the presence of poly U than does lysyl-tRNA in the presence of poly A. There are a number of possible reasons for this, and for similar differences amongst the binding efficiences of the different codon triplets (Nirenberg *et al.*, 1966). It is possible that the carbodiimide modification of the lysine tRNA occurred at a position other than the anticodon, with the consequence that some other factor that influences the binding was altered.

If the binding experiments are interpreted in the simplest manner, however, then it follows that the lysine tRNA species has been modified at its anticodon without losing its amino acid acceptor activity (because only the binding of those species that have been aminoacylated after modification can be measured). This would imply that the aminoacyl-tRNA

synthetase does not recognize the anticodon region of tRNA. The simplest interpretation of the binding experiment is also consistent with the idea that the anticodon loop of tRNA is exposed in the presence of Mg^{2+}.

The fact that the biologically active form of tRNA possesses a defined and very compact tertiary structure may have a number of consequences. For example, it has generally been assumed that the very specific interaction between a tRNA species and the corresponding aminoacyl-tRNA synthetase would require the recognition by the latter of single-stranded nucleotide sequences; recognition of the exterior of a double helix would not be expected to be sufficiently specific. The present results have suggested that few single-stranded nucleotides apart from those in the anticodon loop are likely to be exposed in the biologically active conformation. One possibility then is that the synthetase interacts with the anticodon. However, the experimental evidence on this point is equivocal (for discussion, see Miura, 1967; Madison, 1968). A second possibility is that the interaction between the enzyme and the tRNA involves a tertiary structure transition of the latter with the result that a single-stranded region of the structure is made available for specific interaction with enzyme. A third possibility is that the synthetase recognizes features of the tertiary structure of tRNA rather than segments of primary structure. While the general form of the tertiary structure for different molecular species of tRNA is unlikely to vary (it being based on the common cloverleaf form of secondary structure), it may be conceived that sufficient distinguishable differences could occur between the different amino acid accepting species so as to give the necessary degree of specificity.

Another possible consequence of the existence of a tertiary structure for tRNA is that changes in this structure may occur during the functioning of the molecule. The existence of conformational differences between tRNAs and their aminoacylated derivatives, or between tRNAs on the two ribosome binding sites, are hypotheses that may be useful in the elucidation of the mechanism of protein synthesis. There is some evidence for the existence of the first-mentioned type of conformational difference (Sarin and Zamecnik, 1965; Kaji and Tanaka, 1967).

In conclusion, it may be observed that the carbodiimide reagent has proved its utility in this study of the secondary and tertiary structures of tRNA. It should be possible to combine the present approach with primary sequence determinations of the reactive segments of the molecule (Brostoff and Ingram, 1967) to yield a more complete account of the different conformations that the tRNA molecule can adopt, and to identify the regions that interact with the ribosome and with the aminoacyl-tRNA synthetase and the other enzymes.

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Chromomycin A_3 Studies in Aqueous Solutions. Spectrophotometric Evidence for Aggregation and Interaction with Herring Sperm Deoxyribonucleic Acid*

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ABSTRACT: Chromomycin A_3 is an antibiotic with antitumor activity and is known to exhibit an inhibitory effect on the deoxyribonucleic acid dependent ribonucleic acid and deoxyribonucleic acid synthesis. The effects of environmental factors on the absorption spectrum and optical rotatory dispersion of the antibiotic were examined, and chromomycin A_3 molecules were found to form aggregates of various sizes. Concentration and magnesium ion were found to cause characteristic changes in the absorption spectrum due to the shifts of the equilibrium positions of various molecular forms present in aqueous solutions. The aglycone, chromomycinone, was studied in the same way and found not to exhibit any aggregative properties on spectrophotometric observations. The mode of interaction of chromomycin A_3 with herring sperm deoxyribonucleic acid in the presence of magnesium ion was studied with the aid of spectrophotometric techniques. The data suggest that in agreement with the aggregative tendency of chromomycin A_3 found in this study, the antibiotic molecules bind deoxyribonucleic acid to form an aggregative state through the bridging with magnesium ion. In this state chromomycin A_3 molecules are believed to be preferentially bound to a site adjacent to those already occupied in an antibiotic–deoxyribonucleic acid complex. The comparative study of the binding with deoxyribonucleic acid was made by the use of a series of the antibiotic analogs and the interacting tendency was shown to be completely parallel with their biological activity. The direct involvement of the side-chain sugar moieties in the binding to deoxyribonucleic acid was indicated. The interaction study was also extended to transfer ribonucleic acid, mononucleotides, amino acids, and bovine serum albumin, and the cooperative binding suggested for deoxyribonucleic acid was found not to occur with transfer ribonucleic acid.

Chromomycin A_3 is one of the antibiotics produced by *Streptomyces griseus* No. 7 (ATCC 13273) and is known to have antitumor activity. Chromomycin A_3 has been used as a clinic medicine under the name of "Toyomycin." Miyamoto *et al.* (1967) elucidated the chemical structure of chromomycin A_3 and a number of biochemical studies have been reported in the literature (Kajiro and Kamiyama, 1965, 1967; Hartmann *et al.*, 1964; Ward *et al.*, 1965; Kamiyama and Kajiro, 1966; Koschel *et al.*, 1966; Kersten *et al.*, 1967). They showed that this antibiotic substance inhibits DNA-dependent RNA and DNA polymerase reactions when Mg^{2+} is present in the system. Studies on the mode of interaction of chromomycin A_3 with DNA have also been carried out by means of biochemical and physicochemical techniques. It has been found that addition of native or heat-denatured DNA from various sources to a solution containing chromomycin A_3 and Mg^{2+} induces changes in the electronic absorption spectrum (Behr and Hartmann, 1965; Ward *et al.*, 1965). Other physicochemical data based on hydrodynamic measurements have indicated that the binding mode of this antibiotic to DNA is quite similar to that observed for actinomycins C and D and different from those for anthracyclines (daunomycin, nogalamycin, etc.) or acridine dyes (Ward *et al.*, 1965; Kersten *et al.*, 1966). Concerning the base specificity in the interaction of chromomycin A_3 with DNA, Ward *et al.* (1965) and Kaziro and Kamiyama (1967) have demonstrated that the antibiotic requires the presence of the guanine base. From the comparative studies on the inhibition of RNA polymerase reaction in cell-free system with chromomycin A_3 and the related materials, it was shown that the presence of sugar moieties is essential for the inhibitory effect on RNA synthesis. Mithramycin (Rao *et al.*, 1962) and olivomycin (Gause *et al.*, 1964; Berlin *et al.*, 1966) are chromomycin-like antibiotics, and all of these are closely related to

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