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Polynucleotides Containing 2′-Chloro-2′-deoxyribose†

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ABSTRACT: Polynucleotide phosphorylase from *Micrococcus lysodeikticus* in the presence of Mn^{2+} accepts 2′-chloro-2′-deoxyuridine 5′-diphosphate as well as 2′-chloro-2′-deoxycytidine 5′-diphosphate as substrates. Poly(2′-chloro-2′-deoxyuridylic acid) (poly(Ucl)) and poly(2′-chloro-2′-deoxycytidylic acid) (poly(Ccl)) with *s* values of 7.1 and 10.5 S, respectively, are obtained. Both polymers are stable to alkali and pancreatic ribonuclease. Both are degraded by snake venom phosphodiesterase, spleen phosphodiesterase, DNase I, and micrococcal nuclease at rates considerably slower than for poly(rU) and poly(rC).

The role played by the functional group at the 2′ position of the sugar ring of polynucleotides, in determining structure, function and stability of these compounds, is not well understood (Cross and Crothers, 1971, and references cited therein). Modification of polynucleotides in this position might give some indication as to the factors involved. In addition to polynucleotides containing ribose and deoxyribose, the preparation and properties of homopolynucleotides containing 2′-*O*-methyl-2′-deoxyribose have been described (Bobst *et al.*, 1969; Zmudzka *et al.*, 1969a; Zmudzka and Shugar, 1970). While this manuscript was in preparation the synthesis of two more modified polynucleotides, poly(2′-azido-2′-deoxyuridylic acid) (Torrence *et al.*, 1972) and poly(2′-fluoro-2′-deoxyuridylic acid) (Janik *et al.*, 1972), was reported. The work described here was undertaken to determine the effect of substituting a chlorine atom at the 2′ position. The covalent radius of chloride, r_c^{Cl} = 0.99 Å, should approximate to the spatial bulk of the hydroxyl group (r_c^{O} = 0.74 Å, r_c^{H} = 0.28 Å) and therefore be a good substitute for the hydroxyl group. A recent X-ray study has shown that the crystal structure of 2′-chloro-2′-deoxyuridine (Ucl)[1] is very similar to that of uridine (Suck *et al.*, 1972). A further point of interest concerns the ability of the homopolynucleotide duplex, poly(rI·rC), to induce the formation of interferon and increase resistance to viral attack in cell cultures (Colby, 1971). It has been suggested that this property is a function both of the stability of the duplex (DeClercq and Merigan, 1969), and of its susceptibility to attack by nucleases (DeClercq *et al.*, 1969). A study of the chloro polymers, their stability to enzymic degradation and ability to form stable duplexes, thus provides an opportunity to test this hypothesis.

Materials and Methods

Synthesis of Nucleosides. The synthetic sequence followed for the preparation of Ucl and Ccl was essentially that of Codington *et al.* (1964) and Doerr and Fox (1967). However, 5′-*O*-trityl-O^2,2′-anhydrouridine was conveniently prepared in a single stage from 5′-*O*-trityluridine by treatment with diphenyl carbonate in dimethylformamide at 140° following a procedure by Ruyle and Shen (1967). Ccl was prepared as described below from 2′-chloro-2′-deoxy-4-thiouridine (s^4Ucl).

Synthesis of Nucleotides. Phosphorylation of nucleosides was carried out using $POCl_3$ as described for ribonucleosides by Yoshikawa *et al.* (1967). The method of Hoard and Ott (1965) employing carbonyldiimidazole was used to convert 5′-UclMP to 5′-UclDP; the method of Michelson (1964) employing diphenyl phosphorochloridate was used to prepare 5′-CclDP. The quantity of aUDP and aCDP, respectively, present as impurity was estimated as follows. A sample of the diphosphate (*ca.* 10 A_{260} units) in 100 μl of 0.1 M Tris·HCl (pH 8.9) was digested with alkaline phosphatase (10 μg) for 30 min and the products were applied to a paper chromatogram and developed in system A. The two spots obtained (Ucl, R_F 0.74; aU, R_F 0.65; Ccl, R_F 0.72; aC, R_F 0.67) were

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[1] The following abbreviations are used: Ucl, 2′-chloro-2′-deoxyuridine; Ccl, 2′-chloro-2′-deoxycytidine; s^4Ucl, 2′-chloro-2′-deoxy-4-thiouridine; aU, arabinosyluracil; aC, arabinosylcytosine; poly(Um), poly(2′-*O*-methyluridylic acid); poly(Cm), poly(2′-*O*-methylcytidylic acid); poly(Ucl), poly(2′-chloro-2′-deoxyuridylic acid); poly(Ccl), poly(2′-chloro-2′-deoxycytidylic acid); poly(Uz), poly(2′-azido-2′-deoxyuridylic acid); poly(Uf), poly(2′-fluoro-2′-deoxyuridylic acid); poly(Ua), poly(2′-amino-2′-deoxyuridylic acid).

excised and eluted with methanol–water (1:1, v/v), and the quantities were determined spectrophotometrically.

Arabinosyluracil was prepared from O^2,2'-cyclouridine as described by Brown *et al.* (1956) and arabinosylcytosine 5'-phosphate was obtained from Terra Marine Bioresearch, La Jolla, Calif. $^{32}POCl_3$ was obtained from the Radiochemical Centre, Amersham, England.

Polynucleotide phosphorylase from *M. lysodeikticus* (specific activity 30), bovine pancreatic ribonuclease, spleen phosphodiesterase (1 mg/ml), snake venom phosphodiesterase (1 mg/ml), and alkaline phosphatase (1 mg/ml) were purchased from Boehringer, Mannheim, Germany. Micrococcal nuclease (*Staphylococcus aureus*) was purchased from Sigma Chemical Corp., St. Louis, Mo. DNase I and snake venom phosphodiesterase used for the nucleoside analyzer work from Worthington, Freehold, N. J. [^{14}C]Poly (rU) and [^{3}H]-poly(rC) were purchased from Miles Laboratories, Elkhart, Ind. [^{3}H]Poly(dT) was kindly supplied by Dr. W. McClure, Max-Planck Institut für Biophysikalische Chemie, Göttingen.

Thin-layer chromatography (tlc) was carried out using 0.2-mm layer SiO_2 plates (PF-254) supplied by E. Merck and Co., Darmstadt, Germany, and preparative tlc using similar plates of 2 mm thickness, from the same supplier. Paper chromatography was carried out by the descending method using Schleicher und Schüll 2043b (washed) paper in system A (ethanol–1 M ammonium acetate, 7:3, v/v) or as specified.

The progress of synthesis of nonradioactive polynucleotide was monitored visually by applying aliquots of the reaction solution to paper chromatograms (2 × 14 cm, Whatman No. 3MM paper) and developing by the descending technique using ethanol–1 M ammonium acetate (1:1, v/v). In experiments involving radioactive nucleotides and polynucleotides, either this technique (for poly(rU)) or that of Bollum (1966) was used to separate the polymeric material. After drying, the origin of the chromatogram was excised and counted. Radioactivity counting was performed in a liquid scintillation counter (Trib-Carb Model 4312) using a toluene-based scintillation solution.

Phosphate determinations were carried out as described by Hurst and Becking (1963).

Circular dichroism curves were obtained on a Roussel-Jouan Dichograph II.

Ultraviolet (uv) absorption–temperature profiles were recorded on a Gilford Model 2000 recorder coupled to a Beckman Model DUR spectrophotometer.

Sedimentation coefficients were determined by analytical ultracentrifugation in a Spinco Model E ultracentrifuge equipped with uv absorption optics in sodium citrate buffer, pH 7.5 (0.05 M Na^+).

Spectrophotometric titrations were performed in a Zeiss PMQ II spectrophotometer using a flow cell and an Orion Research digital pH meter (Model 201).

Nucleoside analyses were carried out using an analyzer (Uziel *et al.*, 1968) incorporating a column (26 × 0.5 cm) with a Beckman M71 ion exchange resin. Nucleosides were eluted with 0.4 M ammonium formate buffer pH 4.75 or 0.4 M ammonium borate pH 7.0 at 50°C at a flow-rate of 0.3 ml per min (Gassen and Leifer, 1970).

Experimental Section

Synthesis of 2'-Chloro-2'-deoxycytidine. 2'-Chloro-2'-deoxy-4-thiouridine (278.5 mg; 1 mmole) was dissolved in water (200 ml) and a 2-ml aliquot of a solution containing 4.725 g of anhydrous sodium sulfite and 1.188 g of sodium hyposulfite in 50 ml of water was added. A brisk stream of air was drawn through the solution *via* a sintered-glass disk. Further 2-ml aliquots were added at hourly intervals up to 4 hr. Examination of the uv spectrum of the reaction solution showed λ_{max} changing from 329 to 318 nm. After 5.5 hr bubbling was stopped, and 1 M ammonium acetate (10 ml) was added to the solution, with sufficient concentrated ammonia to bring the pH to 8.7. The solution was stirred magnetically. After about 1 hr the pH was readjusted to 8.7 with a little more ammonia, and then allowed to stir overnight. A large new maximum at *ca.* 270 nm was seen, the $\epsilon_{270}:\epsilon_{318}$ being *ca.* 50. The aqueous solution was evaporated, traces of water being removed by addition and evaporation of pyridine using an oil pump. The temperature throughout the reaction was not allowed to rise above 25°. The residue was thoroughly triturated with dry pyridine (3 × 15 ml) and the inorganic salts were filtered off. The pyridine was evaporated, and traces of pyridine were removed from the residual gum by addition and evaporation of water. The gum was dissolved in a little methanol and applied to tlc plates (2 mm thickness) which were developed with methanol–chloroform (40:60, v/v). The major band (R_F 0.74) was excised, and the product eluted with methanol. The methanolic solution was evaporated to give a gum which did not crystallize, but gave a single spot on tlc (R_F 0.62) in the above system, and on paper chromatography (R_F 0.72) in system A. The yield was estimated spectrophotometrically as 6.48 × 10^3 A_{269} units in H_2O (81%). Upon thawing a frozen concentrated aqueous solution of the product (*ca.* 31 mg/ml) white crystals were obtained. These showed a melting phase, 109–115°, followed by formation of a new crystalline phase in the range 118–130°. This sintered at 190–220°, decomposing at 230–240°. Doerr and Fox (1967) report sintering at 190–220°, and decomposition at 230–235°.

Radioactive Substrates. The labeled nucleotides [^{32}P]5'-UclMP, [α-^{32}P]5'-UclDP, [^{32}P]CclMP, and [α-^{32}P]CclDP were prepared according to the methods described above, using ^{32}P-labeled phosphorus oxychloride to prepare the monophosphates.

Polymerization of [^{32}P]5'-UclDP. The polymerization mixture contained Tris-HCl (pH 8.5, 100 mM), $MnCl_2$ (5 mM), [α-^{32}P]5'-UclDP (4.6 mM), GpU (0.05 mM), and 45 units of polynucleotide phosphorylase per ml of solution. Incubation temperature was 37°. Aliquots (0.01 ml) were withdrawn at intervals, applied to a strip of Whatman No. 3MM paper, and developed and counted as described in Materials and Methods. After 8 hr the reaction was stopped by freezing in liquid air. Isolation of polymer was carried out by thawing, repeated extraction of protein with chloroform–isoamyl alcohol (5:2, v/v), concentration of the aqueous layer to *ca.* 0.5 ml by evaporation, and successive passages over Sephadex G-25, G-200, and G-25 again. The isolated polymer was stored in aqueous solution, frozen at −20°. The yield was 8.7 A_{260} units (7.3%) of polymer from a 3-ml incubation. Activity on isolation was *ca.* 1.3 × 10^5 cpm/A_{260} unit.

Polymerization of [α-^{32}P]5'-CclDP. Polymerization and work-up were carried out exactly as described for [α-^{32}P]-5'-UclDP, but [α-^{32}P]CclDP (5.1 mM) was used instead. The progress of polymerization was followed by the method of Bollum (1966) and the reaction was allowed to run 24 hr. The yield was 12.7 A_{269} units (H_2O) from a 2-ml incubation (21%). The activity on isolation was 5.1 × 10^6 cpm/A_{269} unit.

Extinction Coefficient of Poly(Ccl) and Poly(Ucl). In a 1-ml cuvet were placed 0.1 ml of Tris·HCl (1 M pH 8.5), 0.8 ml of water, and 0.05 ml of polymer solution, to give an optical

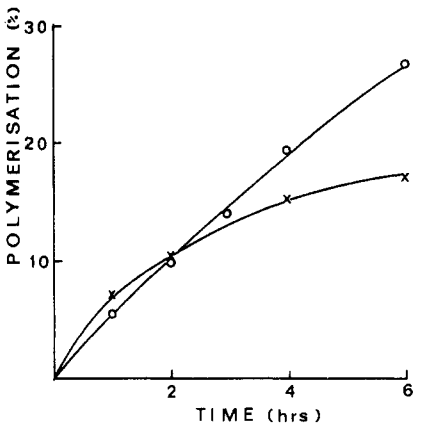

FIGURE 1: Polymerization of UclDP (×) and CclDP (○). Conditions as described in Methods.

density at 269 or 260 nm, respectively, of *ca.* 0.6. The uv spectrum from 250 to 275 nm was recorded using a Zeiss spectrophotometer. Snake venom phosphodiesterase (5 μg) in 0.05 ml of the same buffer was added, and the cuvet was incubated for 1 hr at 37°. The spectrum was then re-recorded over the same range, and after correcting for the dilution the hyperchromicity was calculated.

For poly(Ccl) ϵ_{270} was 5.55×10^3, assuming ϵ_{270} of 5′-CclDP is 8×10^3 (Doerr and Fox, 1967) at pH 8.5, and the hyperchromicity for poly(Ccl) was 44%. For poly(rC) hyperchromicity at 270 nm upon digestion was 48% (ϵ_{270} 6.0×10^3), with an ϵ_{270} of 5′-CDP = 9×10^3. For poly(Ucl) ϵ_{260} was 8.8×10^3, assuming an ϵ_{260} of 5′-UclDP is 1.03×10^4 (Codington *et al.*, 1964) at pH 8.5, and the hyperchromicity of poly(Ucl) was 17%. For poly(rU) 12% hyperchromicity at 260 nm was found on digestion (ϵ_{260} 8.7×10^3).

Analysis of Polynucleotides. Poly(Ucl). Approximately 1 A_{269} unit of polymer in aqueous solution was lyophilized and dissolved in 20 μl of 0.1 M Tris·HCl (pH 8.75) containing 5 μg of alkaline phosphatase and 50 μg of snake venom phosphodiesterase in a volume of 5 μl each. The mixture was incubated 20 min at 37°, then applied to a nucleoside analyzer. 2′-Chloro-2′-deoxyuridine was heated under the same conditions without enzymes for the same length of time, and also analyzed.

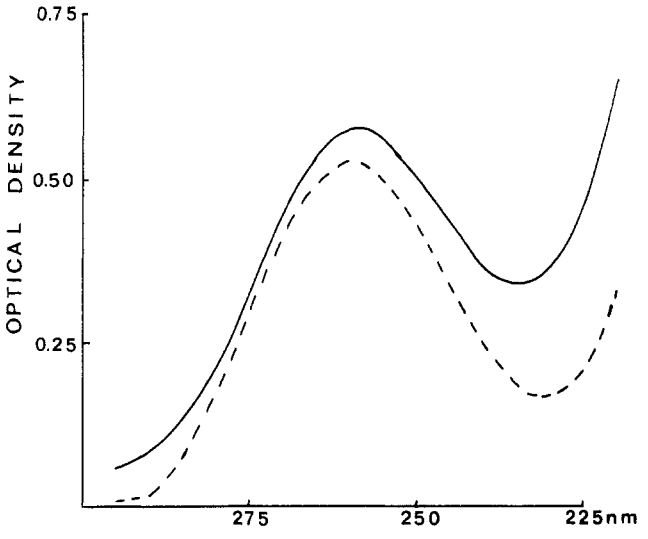

FIGURE 2: Uv spectra of poly(rU) (- - -) and poly(Ucl) (——) in phosphate buffer pH 7.4.

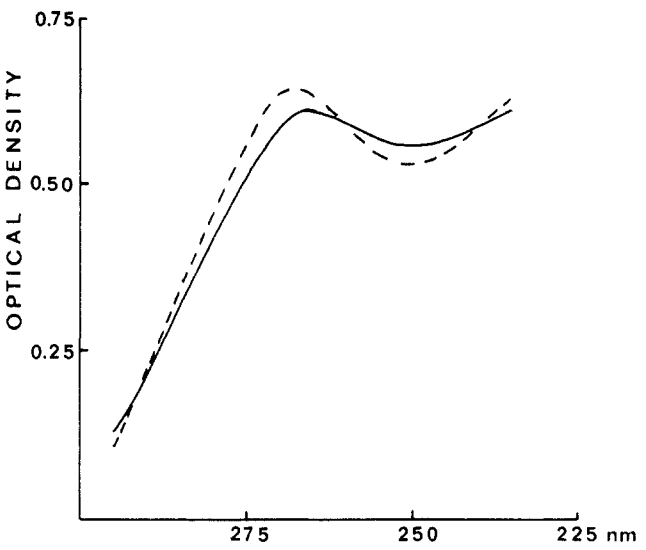

FIGURE 3: Uv spectra of poly(rC) (- - -) and poly(Ccl) (——) in phosphate buffer pH 7.7.

Poly(Ccl). Polymer (1.05 A_{270} units) was dissolved in 0.1 M Tris·HCl (pH 8.75), 12 mM $MgCl_2$ (22 μl) containing 5 μg of alkaline phosphatase, and 50 μg of snake venom phosphodiesterase, incubated at 37° for 15 min and then applied to the nucleoside analyzer. 2′-Chloro-2′-deoxycytidine was heated under the same conditions without enzymes for the same length of time and also analyzed.

pK_a Determination and Thermal Transition of Poly(Ccl). Poly(Ccl) was dissolved in 5 ml of sodium citrate–sodium chloride buffer (0.1 M Na^+) to give an optical density at 267 nm of *ca.* 0.6. The electrode of a digital-reading pH meter was inserted in the solution, which was pumped continuously through a flow-cell in a Zeiss spectrophotometer. The pH was lowered in small steps from 7.7 to 4.0 by the addition of microliter quantities of concentrated HCl. Optical densities at 285 and 252 nm were monitored after each addition.

Degradation Experiments. These were carried out under the conditions indicated in the legends. The incubation mixture, excluding the enzyme, was prepared and preincubated 5 min at 37°. An aliquot was removed for zero-time value, and the enzyme was added immediately. Further aliquots were removed at the times indicated. Counts recorded were corrected for the slight dilution discrepancy between the zero reading and other readings were introduced by this technique. In the experiments involving spleen phosphodiesterase, pretreatment of the polymers with alkaline phosphatase to remove phosphate groups at the 5′ end of the chain was performed, since these inhibit the phosphodiesterase strongly.

In general a best curve was drawn through the available points and figures for percentage degradation are taken from this curve. In those experiments in which breakdown was very small, and obscured by scatter, a least-squares fit was made to the available points and figures for breakdown, if any, are derived from this line.

Results

Synthesis of 2′-Chloro-2′deoxycytidine. 2′-Chloro-2′-deoxycytidine was prepared from 2′-chloro-2′-deoxy-4-thio-

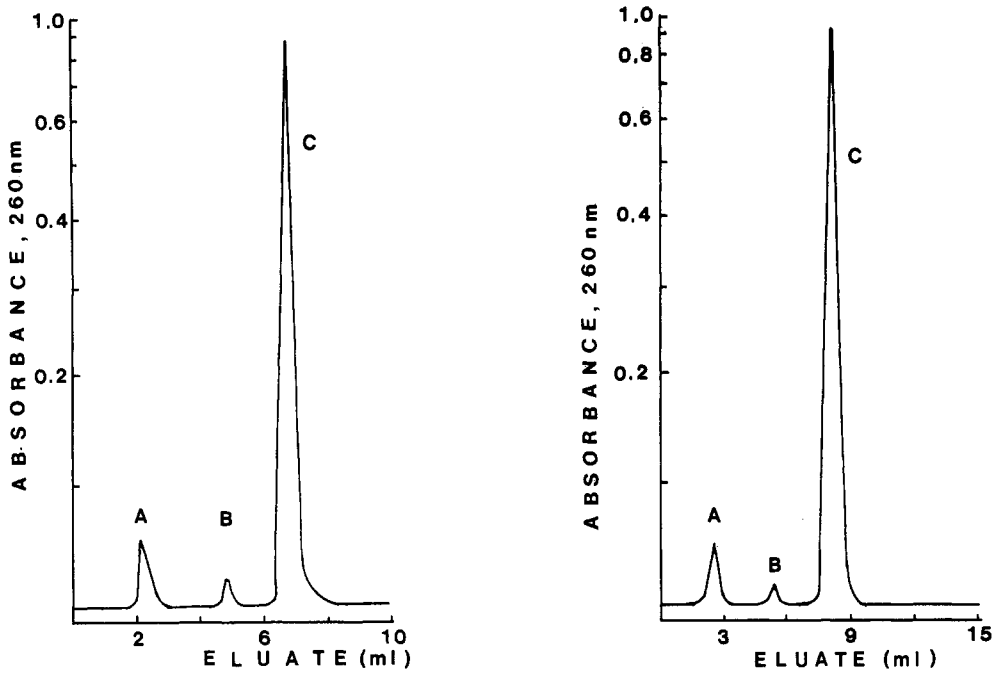

FIGURE 4: Analysis of poly(Ucl) with a nucleoside analyzer. (a, left) Column eluted with 0.4 M formate buffer (pH 4.75). A, breakthrough; B, aU + U; C, Ucl. (b, right) Column eluted with 0.4 M ammonium borate (pH 7.0). A, breakthrough + U; B, aU; C, Ucl.

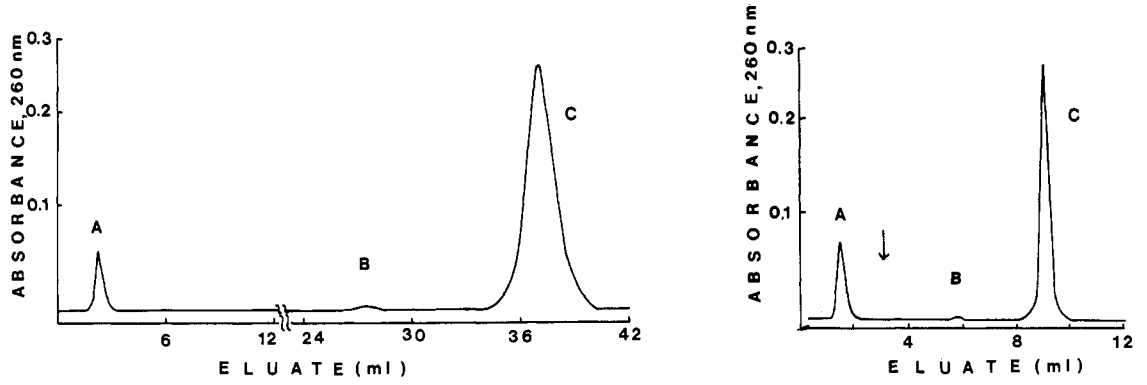

FIGURE 5: Analysis of poly(Ccl) with a nucleoside analyzer, (a, left) Column eluted with 0.4 M formate buffer (pH 7.5). A, breakthrough; B, aC + C; C, Ccl. (b, right) Column eluted with 0.4 M ammonium borate (pH 7.0). A, breakthrough; B, aC; C, Ccl; ———, position for C.

uridine (s^4Ucl) by utilizing the bisulfite–oxygen reaction described by Hayatsu (1969). The 2′-chloro-2′-deoxyuridine-4-sulfonate so formed was treated with ammonia under conditions of controlled pH to afford a good yield of the required nucleoside. Ccl is unstable to heat and decomposes slowly at room temperature even in the solid phase. This necessitated the development of a room-temperature preparation of the nucleoside and also preparation of a fresh batch before each phosphorylation.

Phosphorylation of Nucleosides. Despite care in the preparations, 5′-UclDP was found to contain about 2.5% of the corresponding arabinonucleoside diphosphate. Since the method of analysis involves incubation at pH 8.9 at 37° with alkaline phosphatase to remove the phosphate groups, and the chloronucleoside forms the corresponding aranucleoside under these conditions, this figure is an upper limit to the level of impurity. Incubation of 2′-chloro-2′-deoxyuridine for 30 min resulted in the formation of about 0.8% arabinouracil, for 1 hr of about 4.5% and for 2 hr of about 8%. No contamination by aCDP could be detected in 5′-CclDP.

Polymerization Experiments. Both 5′-UclDP and 5′-CclDP were substrates for polynucleotide phosphorylase from *Micrococcus lysodeikticus*, on incubation at pH 8.5 in the presence of Mn^{2+} ions, and GpU as primer. Figure 1 shows the incorporation of the labeled nucleotides into polymeric material. After 24 hr the acid-precipitable material in the 5′-CclDP incubation contained almost 50% of the total radioactivity. However, a good deal of polymer is lost during protein extraction. Both polymers showed a slight shift in the absorption maximum in the uv spectrum from that of the corresponding diphosphate, poly(Ucl) to 258 nm from 260 nm and poly(Ccl) to 267.5 nm from 270 nm. The uv spectra of poly(rU) and poly(Ucl) (Figure 2) as well as of poly(rC) and poly(Ccl) (Figure 3) differ slightly. Under the conditions used for formation of the chlorinated polymers, no evidence for the polymerization of aUDP or aCDP could be detected by visual inspection. This is consistent with previous observations on these nucleotides using polynucleotide phosphorylase in the presence of Mg^{2+} (Cardeilhac and Cohen, 1964; Michelson *et al.*, 1962).

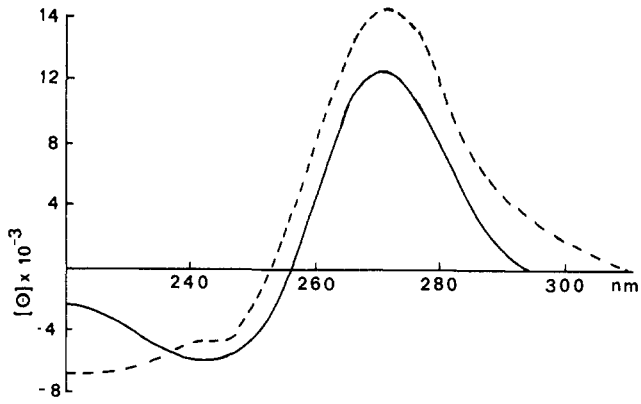

FIGURE 6: CD spectra of poly(rU) (——) and poly(Ucl) (- - -) in 0.1 M phosphate buffer (pH 7.0) at 18°.

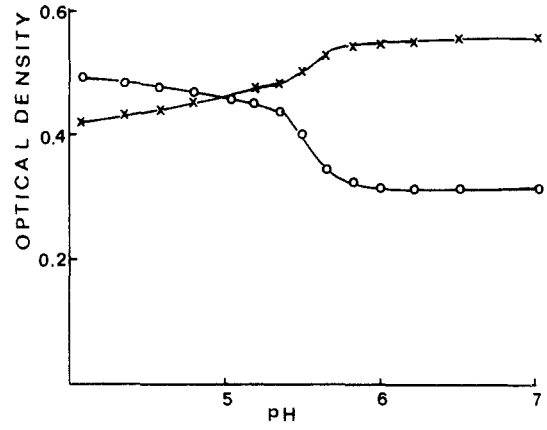

FIGURE 8: Titration curve of poly(Ccl) at 285 nm (×) and 252 nm (○).

Characterization of Polymers. To check the integrity of the polymers, samples of poly(Ucl) and poly(Ccl) were analyzed by degradation with snake venom phosphodiesterase and alkaline phosphatase at 37°, and the mixture of nucleosides obtained was separated on a nucleoside analyzer. Since the ribonucleosides could not be separated from the arabinonucleosides on elution of the column with formate buffer the digestion was repeated and the column eluted with borate buffer. This gave a satisfactory separation of the ribo- from the arabinonucleoside but on the other hand, particularly in the case of poly(Ucl), separation of the ribonucleoside from the breakthrough peak which contains the protein could not be obtained. As can be seen from Figure 4a,b there is a small amount of aU found on digestion of poly(Ucl). The amount is about 0.8%. Heating Ucl for the same length of time under the same conditions also yields an amount of aU of about 0.8%. Increasing the time of incubation for poly(Ucl) and Ucl from 15 to 30 min increased the amount of aU to about 1.5 and about 1.8%, respectively. As noted in the analysis of the diphosphates, incubation of chloronucleoside for 2 hr yielded even a higher proportion of aU, about 8%. This parallel increase of aU formation with incubation time seems to indicate that the amount of aU found upon digestion of poly(Ucl) is to a large extent due to the breakdown of Ucl. The impurity of aC found on digestion of poly(Ccl) is shown in Figure 5a,b. The amount detected is at the limit of the sensitivity of the method employed which is judged to be about 0.02%. The same amount of aC is detected after incubation of Ccl under identical conditions.

Typical values obtained for the sedimentation coefficients of the polymers were $s_{20,w}$ = 7.1 S for poly(Ucl), and $s_{20,w}$ = 10.5 S for poly(Ccl). Generally poly(Ccl) preparations gave higher s values. The circular dichroism spectra of the chlorinated polymers are shown and compared to the ribopolymers in Figures 6 and 7, respectively.

The pK_a determination of poly(Ccl) is shown in Figure 8 and its melting profile in Figure 9.

Degradation of Polymers. The kinetics of enzymatic breakdown of poly(Ucl) and poly(Ccl) in comparison to poly(rU) and poly(rC) are described in Table I–V. The resistance of poly(Ucl) and poly(Ccl) against alkali is represented in Table VI.

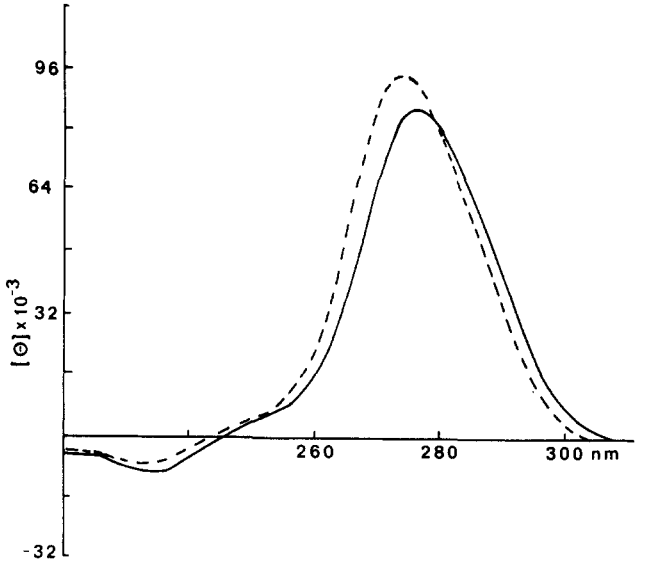

FIGURE 7: CD spectra of poly(rC) (——) and poly(Ccl) (- - -) in 0.1 M phosphate buffer (pH 7.0) at 18°.

Discussion

Polynucleotide phosphorylase accepts a variety of nucleoside 5′-diphosphates with modifications at the 2′-positions as substrates, in the majority of cases in the presence of Mn^{2+}

TABLE I: Degradation Using Pancreatic Ribonuclease.[a]

Time (min)	A		B	
	Poly(rU) (%)	Poly(Ucl) (%)	Poly(rC) (%)	Poly(Ccl) (%)
5	36	100	3	100
10	27	99	2	100
20	23	99	2	100
40	16	98	2	100

[a] The figures represent the per cent of acid-precipitable, undegraded polymer. A: 0.11 mM substrates, 0.08 M Tris·HCl (pH 7.4), and 25 ng of protein, total volume 0.25 ml; B: 0.175 mM substrates, 0.08 M Tris·HCl (pH 7.4), 45 ng of protein, total volume 0.25 ml. Incubation temperature 37°.

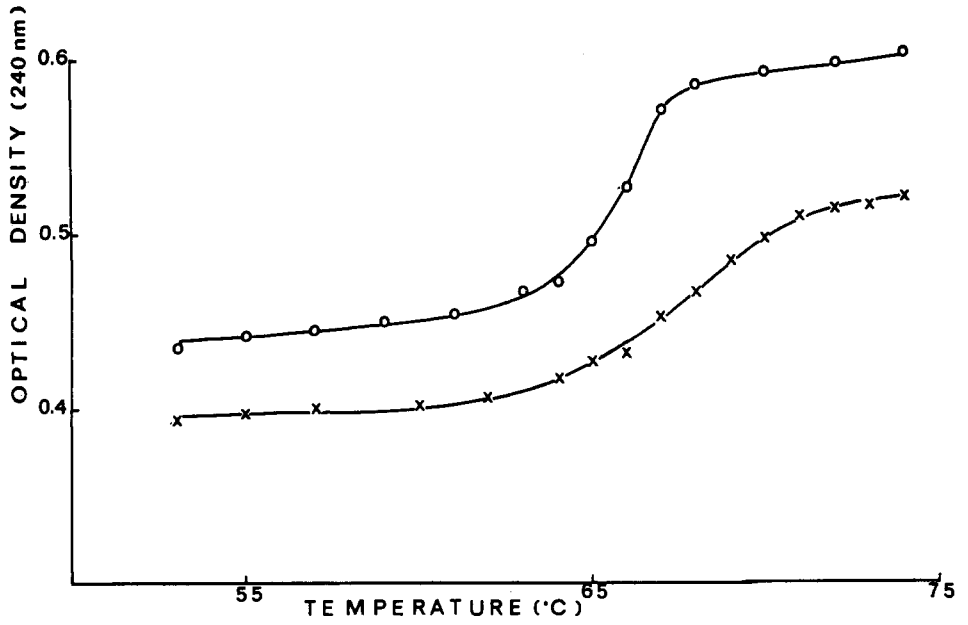

FIGURE 9: Melting curve of poly(rC) (○) and poly(Ccl) (×) at pH 5.0 in sodium citrate (0.1 M Na^+).

(Bobst *et al.*, 1969; Zmudzka and Shugar, 1970; Zmudzka *et al.*, 1969a; Mackey and Gilham, 1971; Janik *et al.*, 1972), but also, as reported for 2′-azido-2′-deoxyuridine 5′-diphosphate recently (Torrence *et al.*, 1972), in the presence of Mg^{2+}. As reported here, 2′-chloro-2′-deoxyuridine- and 2′-deoxycytidine 5′-diphosphate are also substrates. Efficient polymerization could only be observed in the presence of Mn^{2+}. The polymeric products, poly(Ucl) and poly(Ccl), have sedimentation coefficients of 7.1 and 10.5 S, respectively. The 2′-chloro-2′-deoxyuridine 5′-diphosphate seemed to contain traces of the corresponding arabinosyl derivative, whereas none could be detected in 2′-chloro-2′-deoxycytidine diphosphate. Enzymatic degradation of poly(Ccl) and subsequent nucleoside analysis revealed at most 0.02% of aC. The same analysis of poly(Ucl) yielded about 0.8% of aU which could be accounted for by formation from Ucl during incubation as discussed in Results. These results indicate that the contaminating arabinonucleoside 5′-diphosphate in the substrate was not polymerized. This is in accordance with results by Cardeilhac and Cohen (1964) who found arabinonucleoside 5′-diphosphates not to be substrates for polynucleotide phosphorylase.

Poly(Ucl) appears, from its CD spectrum and its hyperchromicity on enzymic degradation, to exhibit markedly more stacking of the uracil rings in solution at pH 7.5 than poly(rU). This finding is in contrast with that for poly(dU) (Zmudzka *et al.*, 1969b), and also poly(Ua) (Hobbs *et al.*, 1972), which show very little evidence of ordered structure in solution and is consistent with what is expected of poly (Um) since the base stacking in UmpU is more pronounced than in UpU (Zmudzka and Shugar, 1970). However, poly (Um) and poly(rU) (Zmudzka and Shugar, 1970) as well as poly(Uz) (Torrence *et al.*, 1972) form helical complexes in

TABLE II: Degradation Using Snake Venom Phosphodiesterase.[a]

	A		B		C
Time (min)	Poly-(rU) (%)	Poly-(Ucl) (%)	Poly-(rC) (%)	Poly-(Ccl) (%)	Poly-(Ccl) (%)
5	57	93	29	97	82
10	37	85	7	94	61
20	12	71	4	89	32
40	1	42	4	78	11

[a] The figures represent the per cent of acid-precipitable, undegraded polymer. A: 0.085 mM substrates, 0.08 M Tris·HCl (pH 8.5), and 0.5 μg of protein, total volume 0.3 ml; B: 0.15 mM substrates, 0.08 M Tris·HCl (pH 8.5), and 0.5 μg of protein, total volume 0.3 ml; C: 0.165 mM substrates, 0.08 M Tris·HCl (pH 8.5), and 2 μg of protein, total volume 0.25 ml. Incubation temperature 37°.

TABLE III: Degradation Using Micrococcal Nuclease.[a]

	A		B		C	
Time (min)	Poly-(rU) (%)	Poly-(Ucl) (%)	Poly-(Ucl) (%)	Poly-(dT) (%)	Poly-(rC) (%)	Poly-(Ccl) (%)
5	64	63	77	3	47	100
10	62	47	54	2	36	100
20	58	26	31	2	36	100
40	56	20	15	2	36	100

[a] The figures represent the per cent of acid-precipitable, undegraded polymer. A: 0.25 mM substrates, 0.3 mM $CaCl_2$, 0.08 M Tris·HCl (pH 8.5), 25 ng of protein, total volume 0.28 ml; B: 0.2 mM poly(Ucl), 0.17 mM poly(dT), other components as A; C: 0.17 mM substrates, 0.3 mM $CaCl_2$, 0.08 M Tris·HCl (pH 8.5), 100 ng of protein, total volume 0.27 ml. Incubation temperature 37°.

TABLE IV: Degradation Using DNase I.[a]

Time (hr)	Poly(dT) (%)	Poly(Ucl) (%)	Poly(Ccl) (%)
0.5	15	100	100
1	5	100	99
2	3	100	99
4	2	100	97

[a] The figures represent the per cent of acid-precipitable, undegraded polymer; 0.12 mM poly(dT), 0.135 mM poly(Ucl), 0.21 mM poly(Ccl), each with 2 mM $CaCl_2$, 8 mM $MgCl_2$, 0.1 M Tris·HCl (pH 7.4), 20 μg of protein, total volume 0.26 ml. Incubation temperature 37°.

TABLE VI: Degradation Using Alkali.[a]

	A		B
Time (min)	Poly(rU) (%)	Poly(Ucl) (%)	Poly(Ccl) (%)
5	80	100	100
10	74	100	99
20	64	100	99
40	45	100	98

[a] The figures represent the per cent of acid-precipitable, undegraded polymer. A: 0.28 mM substrate in 0.25 ml of 0.1 M potassium hydroxide; B: 0.42 mM substrates in 0.25 ml of 0.1 M potassium hydroxide. Incubation temperature 37°.

the presence of Mg^{2+} at neutral pH where poly(Ucl) (Hobbs *et al.*, 1971), poly(dU) (Zmudzka *et al.*, 1969b), poly(Ua) (Hobbs *et al.*, 1972), and poly(Uf) (Janik *et al.*, 1972) do not. The thermal stability of poly(Um) and poly(Uz) even surpasses that of poly(rU). It is thus clearly seen that the factors controlling base stacking in solution do not correlate simply with the ability to form helical complexes in the presence of metal ions.

In contrast to the results with poly(rU) and poly(Ucl) we find little difference in hyperchromicity between poly(rC) and poly(Ccl) on digestion. The CD spectra reveal little difference in stacking. These results are in agreement with those of Alderfer *et al.* (1972) and Janion *et al.* (1970) in which the stacking of poly(rC) was found to be similar to that of poly (Cm). Thus, it seems that a substituent in the 2′ position can exert different effects on stacking depending on the polymer.

The pK_a of cytidylic acid polymers is markedly affected by the substituent at the 2′ position. Thus, poly(dC) possesses $pK_a = 7.4$ (Inman, 1964), where poly(rC) has a $pK_a = 5.7$. This very ready uptake of protons by poly(dC) and the formation of secondary structure at comparatively low pH suggest that the chain is capable of assuming a conformation suitable for helix formation which is denied to polycytidylic acids which carry a bulkier 2′ substituent. Poly(Ccl) has $pK_a = 5.5$, and poly(Cm) $pK_a = 4.6$ (Zmudzka *et al.*, 1969a). The pK_a of the nucleoside Ccl was measured as 4.14, virtually identical with that of cytidine under the same conditions. In 0.1 M Na^+ at pH 5.0, poly(Ccl) and poly(rC) form secondary structures of very similar stability with T_m 67 and 66°, respectively, although the melting profile of poly(Ccl) is less steep, and the hyperchromicity less than that of poly(rC). That the stability of the secondary structures differs with pH is seen in the observations that, where poly(rC) has a T_m of 79° at pH 4.0, 0.1 M Na^+, poly(Ccl) does not melt below 92°C.

TABLE V: Degradation Using Spleen Phosphodiesterase.[a]

	A		B	C	
Time (min)	Poly-(rU) (%)	Poly-(Ucl) (%)	Poly(dT) (%)	Poly-(rC) (%)	Poly-(Ccl) (%)
5	13	97	69	51	99
10	2	94	54	34	99
20	1	88	38	18	98
30	1	82	33	12	97

[a] The figures represent the per cent of acid-precipitable, undegraded polymer. A: 0.23 mM substrates, 0.1 M NaCl, 0.08 mM EDTA, 0.1 M Tris·HCl (pH 8.9), and 20 μg of alkaline phosphatase, total volume 0.25 ml; B: 0.12 mM substrates, otherwise as A; C: 0.10 mM substrates, 0.11 M NaCl, 0.09 mM EDTA, 0.1 M Tris·HCl (pH 8.9), and 20 μg of alkaline phosphatase, total volume 0.22 ml. After 10 min at 37°, acetic acid (0.01 ml) was added to bring pH to 5.5 in each case, zero readings were taken, and spleen phosphodiesterase (2 units/ml) was added: in A, 0.025 ml, in B, 0.015 ml, in C, 0.01 ml. Incubation temperature 37°.

The alteration of the susceptibility of a polynucleotide chain to digestion by a nuclease upon chemical modification in the sugar ring is likely to be a complex function involving several different factors. The simplest case of induced resistance of a nuclease will occur when the modification alters a functional group which is actively concerned in the hydrolytic mechanism, and is here exemplified by the total resistance of poly(Ucl) and poly(Ccl) to breakdown by pancreatic ribonuclease. Both chlorinated polymers are also resistant to the hydrolytic effect of 0.1 M KOH at 37°, conditions under which poly(rU) is broken down. This is again a direct consequence of the absence of the 2′-hydroxyl group.

Other alterations in the susceptibility of a polynucleotide chain to nucleases will occur as a result of hydrogen bonding and steric, conformational, and other changes induced by the modification. Exact interpretations and comparisons are unlikely to be obtainable without intimate knowledge of the properties and conformation in solution of the polynucleotide and more detailed kinetic data of the enzymatic reaction. Failing these data interpretations must be speculative.

Upon attack by micrococcal nuclease, poly(dT) was digested very rapidly, in accord with previous findings (Anfinsen *et al.*, 1971). However, whereas poly(rU) and poly(Ucl) were degraded initially at similar rates, digestion of poly(rU) had nearly ceased after 5 min, whereas digestion of poly(Ucl) proceeded further, becoming very slow after 20 min. This may be a consequence of different substrate susceptibilities of oligonucleotide fragments produced in the reaction. Alternatively, since the digestion is carried out in 3 mM Ca^{2+}, it is possible that the higher tendency of poly(rU) to form secondary structure in the presence of a divalent metal ion accounts

for the observed difference. Micrococcal nuclease is known to be sensitive to secondary structure in the substrate. Poly(rC) was hydrolyzed upon using rather more enzyme than was required for poly(rU), but poly(Ccl) was not appreciably degraded, even when poly(rC) was totally broken down.

Snake venom phosphodiesterase hydrolyzed poly(Ucl) more slowly than poly(rU) and with the cytidine polymers the difference was even more pronounced. However, with a suitably high concentration of enzyme, even poly(Ccl) could be degraded completely. These observations are consistent with previous findings that the enzyme will hydrolyze dinucleoside monophosphates containing either a 2′-*O*-Me group or the sugar arabinose (Laskowski, 1971, and references therein).

Since poly(Ucl) and poly(Ccl) were not significantly degraded by DNase I in the presence of Ca^{2+} and Mg^{2+} under conditions in which poly(dT) is extensively degraded, it seems that from the point of view of the enzyme, the presence of chlorine at the 2′ position does not give the substrate the appearance of a polydeoxyribonucleotide. However, poly (dU) and poly(dC), which would have afforded better comparisons as model compounds, were unfortunately not available.

Spleen phosphodiesterase degraded poly(Ucl), but at a much slower rate than poly(rU) and poly(dT). Although Bernardi and Bernardi (1968) found that poly(rC) was not digested at pH 5.0, it was broken down with reasonable rapidity at pH 5.5. Poly(Ccl) however, was highly resistant. Since the difference between the pK_a values of poly(rC) and poly(Ccl) is less than 0.2 pH unit, it is unlikely that the difference in substrate susceptibility is due simply to the difference in the degree of secondary structure at this pH, although this may be critical at pH 5.0. Probably other factors are important as in the case of the uridine polymers, and a conformational change in the geometry of the sugar–phosphate backbone may be the controlling factor.

Dunlap *et al.* (1971) have noted that 2′-O-methylation of polynucleotides also renders these polymers resistant to a number of nucleases. Thus, it might be a general phenomenon that modifications at the 2′ position increase the stability of polynucleotides toward nucleases.

It has previously been shown (Hobbs *et al.*, 1971) that poly (Ucl) and poly(Ccl) form stable complexes with their complementary polynucleotides poly(rA) and poly(rI), respectively. For both pairs the T_m-values were slightly higher than those of the ribopolymer pairs, poly(rA·rU) and poly(rI·rC), respectively. The T_m values of the corresponding mixed polymers poly(rA·dU) and poly(rI·dC), however, are reported to be lower. Thus it seems that at least the effect of the chlorine atom on thermal stability of the polynucleotides investigated resembles more that of a hydroxyl group than a hydrogen atom. A comparison to other modified polymers reveals that the T_m value of poly(rA·Um) is considerably higher (Zmudzka and Shugar, 1970), that of poly(rA·Uz) approximately the same (Torrence *et al.*, 1972), and that of poly (rI·Cm) (Zmudzka *et al.*, 1969a) lower than those of the corresponding ribopolymer pairs.

Clearly, detailed structural investigations such as nuclear magnetic resonance studies are needed to explain the influence of the substituent in the 2′ position on polymer structure and stability. In addition to conformational changes of the sugar, the effect of a substituent on steric hindrance to base stacking (Cross and Crothers, 1971), changes in the dipole, and solvation will have to be considered. It has been suggested by Cross and Crothers (1971) that the base overlap in oligonucleotides is affected sterically by the hydroxyl group. Oligonucleotides with a chlorine atom in the 2′ position might be well suited to test this model.

Poly(Ucl) and poly(rU) fulfill the criteria suggested by DeClercq and Merigan (1969) and DeClercq *et al.* (1969) to be important in determining the ability of a polymer duplex to induce interferon. However, both poly(rA·Ucl) and poly (rI·Ccl) are inactive in this respect, though the chloropolymers were found highly resistant to serum nucleases (Black *et al.*, 1972). The presence of a 2′-hydroxyl group thus appears to be a necessary condition for interferon induction, though whether this group is intimately concerned in the interferon triggering mechanism *per se*, or whether the substitution at the 2′ position influences the conformation of the duplex and prevents triggering at a recognition site, or whether the ability of the duplex to be readily degraded is a necessary condition for triggering, remain matters for discussion.

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Purification of Cysteine Transfer Ribonucleic Acid of Bakers' Yeast†

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ABSTRACT: A procedure for the purification of cysteine transfer ribonucleic acid ($tRNA^{Cys}$) of Bakers' yeast has been developed which is based upon the reaction of the sulfhydryl moiety of cysteinyl-$tRNA^{Cys}$ with an organomercurial–polysaccharide. tRNA charged with L-[^{35}S]cysteine was prepared by the reaction of the amino acid with heterogeneous tRNA of yeast in the presence of yeast L-cysteinyl-tRNA synthetase. Preliminary purification of the RNA was necessary to remove protein and unreacted [^{35}S]cysteine from the mixture prior to reaction of the cysteinyl-$tRNA^{Cys}$ with the organomercurial–polysaccharide column material. The $tRNA^{Cys}$ was recovered from the column by elution with 0.02 M Tris-HCl (pH 7.2). A 43-fold purification of the $tRNA^{Cys}$ was accomplished by this procedure. Attempts to charge the purified RNA with other amino acids gave negative results.

The complex function of a specific tRNA would be difficult to study without first having at hand the means of obtaining the tRNA in relatively pure form. Purification of a particular tRNA is complicated by the fact that all species of tRNA are relatively similar in nature and have molecular weights of approximately 25,000 (Litt and Ingram, 1964). This report describes a method for the purification from yeast of a tRNA specific for the amino acid cysteine. The method is unique in that it relies upon the chemical nature of the amino acid rather than upon the nature of the specific tRNA. After reaction of cysteine with unfractionated tRNA in the presence of the L-cysteinyl-tRNA synthetase, the cysteinyl-tRNA is selectively bound to an organomercurial–polysaccharide material through the sulfhydryl group of the tRNA-bound amino acid. tRNAs specific for other amino acids are removed by decantation and $tRNA^{Cys}$ of high purity is eluted. A preliminary report of certain areas of this work has been presented (James *et al.*, 1967).

Materials and Methods

Preparation of Enzyme Fraction from Baker's Yeast. A crude soluble fraction of Bakers' yeast was prepared by a modification of the method of James and Bucovaz (1966). Approximately 1400 g of Bakers' yeast (Federal Yeast Corp.) was frozen in an ether–CO_2 mixture for 8 hr to break the cells. After removing the ether by aspiration, the resulting homogenate was thawed and extracted with 15 g of KCl. Following the KCl extraction, the homogenate was centrifuged at 105,000 · *g* for 1 hr in a Spinco Model L centrifuge with an analytical rotor No. 40. The supernatant liquid obtained was the crude enzyme fraction.

Purification of the Crude Enzyme Fraction. Approximately 350 ml of the crude enzyme fraction was adjusted to 10% saturation with $(NH_4)_2SO_4$ (65 mg of $(NH_4)_2SO_4$/ml of solution) and the resulting precipitate, which contained no major enzyme activity, was discarded. The supernatant liquid was increased to 75% saturation with $(NH_4)_2SO_4$. After centrifugation the supernatant liquid was discarded and the precipitate was dissolved in 100 ml of 0.01 M Tris-HCl (pH 7.5). The redissolved precipitate was subdivided into three fractions (0–35% precipitate, 35–60% precipitate, and 60% supernatant fraction) by additions of $(NH_4)_2SO_4$. The resulting precipitates were dissolved in 0.01 M Tris-HCl (pH 7.5) and all three fractions were dialyzed against 0.01 M Tris-HCl (pH 7.5) for 15 hr to remove $(NH_4)_2SO_4$ and other small molecular

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