Dickman, S. R., Aroskar, J. P., and Kropf, R. B. (1956), *Biochem. Biophys. Acta 21*, 538.
Dickman, S. R., Madison, J. T., and Holtzer, R. L. (1962), *Biochemistry 1*, 568 (this issue).
Gomori, G. (1942), *J. Lab. Clin. Med. 27*, 955.
Harris, R. J. C. (1961), Protein Biosynthesis, New York, Academic Press, Inc.
Hirs, C. H. W. (1953), *J. Biol. Chem. 205*, 93.
Hirs, C. H. W., Moore, S., and Stein, W. H. (1953), *J. Biol. Chem. 200*, 493.
Hirs, C. H. W., Stein, W. H., and Moore, S. (1958), Symposium on Protein Structure, Neuberger, A., editor, New York, John Wiley and Sons, p. 211.
Hummel, B. C. W. (1959), *Canad. J. Biochem. Physiol. 37*, 1393.
Keller, P. J., Cohen, E., and Neurath, H. (1958), *J. Biol. Chem. 233*, 344.
Keller, P. J., and Cohen, E. (1961), *J. Biol. Chem. 236*, 1407.
Keller, P. J., Cohen, E., and Neurath, H. (1961), *J. Biol. Chem. 236*, 1404.
Krebs, H. A. (1950), *Biochem. Biophys. Acta 4*, 249.
Littlefield, J. W., Keller, E. B., Gross, J. W., and Zamecnik, P. (1955), *J. Biol. Chem. 217*, 111.
Madison, J. T. (1962), Ph.D. thesis, University of Utah.
McIlwain, H. (1961), *Biochem. J. 78*, 213.
Morris, A. J., and Dickman, S. R. (1960), *J. Biol. Chem. 235*, 1404.
Nakanishi, K. (1959), *J. Biochem. (Japan) 46*, 1263.
Nayyar, S. M., and Glick, D. J. (1954), *J. Histochem. & Cytochem. 2*, 282.
Northrup, J. H., Kunitz, M., and Herriott, R. M. (1948), Crystalline Enzymes, ed. 2, New York, Columbia University Press.
Potter, V. R., and Elvehjem, C. A. (1936), *J. Biol. Chem. 114*, 495.
Rabinovitch, M., and Olson, M. (1959), *J. Biol. Chem. 234*, 2085.
Ryle, A. P., Sanger, F., Smith, L. F., and Kitae, R. (1955), *Biochem. J. 60*, 541.
Sanger, F., and Thompson, E. O. P. (1951), *Biochem. J. 49*, 463, 481.
Sanger, F., and Thompson, E. O. P. (1953), *Biochem. J. 53*, 366.
Schneider, W. C. (1957) *in* Methods in Enzymology, vol. 3, Colowick, S. P., and Kaplan, N. O., editors, New York, Academic Press, Inc., p. 680.
Siekevitz, P., and Palade, G. E. (1958), *J. Biophys. Biochem. Cytol. 4*, 557.
Siekevitz, P., and Palade, G. E. (1960), *J. Biophys. Biochem. Cytol. 7*, 619.
Van Lancker, J., and Holtzer, R. L. (1959), *J. Biol. Chem. 234*, 2359.

Chemical Studies on Amino Acid Acceptor Ribonucleic Acids.* I. Some Properties of Yeast Amino Acid Acceptor Ribonucleic Acid and Mapping of the Oligonucleotides Produced by Ribonuclease Digestion

VERNON M. INGRAM AND JOHN G. PIERCE†

From the Division of Biochemistry, Department of Biology, Massachusetts Institute of Technology, Cambridge, and the Department of Physiological Chemistry, University of California Medical Center, Los Angeles

Received December 29, 1961

In order to investigate the distribution of uracil-5-ribosyl phosphate and of the other "additional" nucleotides in yeast acceptor RNA preparations, digests of the RNA by pancreatic ribonuclease have been fractionated by gel filtration and characterized by fingerprinting. The separation of mixtures of oligonucleotides has been improved by removing terminal phosphate groups. Analysis of a number of oligonucleotides indicates that the "additional" nucleotides of acceptor RNA are not confined to sequences common to all the acceptor RNA molecules. Three dinucleotides containing "additional nucleotides" have also been isolated. Gel filtration and ion-exchange cellulose papers have been used in simplified methods for the determination of the labeling of acceptor RNA with C^{14} amino acids.

Low-molecular-weight acceptor ribonucleic acids have been the subject of recent study both because of their ability to accept activated amino acids in systems carrying out protein synthesis and because of the occurrence in them of nucleotide components other than the four common ribonucleotides (Dunn *et al.*, 1960; Otaka *et al.*, 1959). The presence of these additional nucleotides will be of great value in sequence determination when preparations of a single molecular species of an acceptor RNA[1] are available. Experiments leading toward isolation of

* This investigation was supported by grants (No. A-3409 (C1) and C-2290) from the U. S. Public Health Service and was presented in part at the 140th Meeting of the American Chemical Society, Chicago, September, 1961.

† Permanent address: Department of Physiological Chemistry, University of California Medical Center, Los Angeles.

an acceptor RNA specific for a single amino acid have been reviewed recently by Berg (1961).

The present paper reports some properties of unfractionated yeast acceptor RNA. In particular, the distribution of the additional nucleotides has been studied to determine whether they reside in specific sequences common to each amino acid acceptor RNA molecule, as has been found for the terminal trinucleotide sequence (Hecht *et al.*, 1959; Canellakis and Herbert, 1960), or whether each might occur in a variety of sequences. In these experiments oligonucleotides resulting from digestion of the acceptor RNA by pancreatic ribonuclease were fractionated into groups by chromatography on cross-linked dextran gels (Sephadex); they were then subjected to two-dimensional mapping procedures similar to those described by Rushizky and Knight (1960) but modified to lessen the time involved. The mapping procedures and analysis of nucleotide composition of the various fractions have been facilitated by the use of ion-exchange paper to remove interfering substances.

Experimental

Materials and Methods

Preparation of Acceptor RNA.—Yeast acceptor RNA was prepared by a modification of the method of Monier *et al.* (1960). Fresh pressed baker's yeast, 200 g, was processed according to their method to the stage where the first alcohol precipitate was dissolved in water and the solution was clarified by centrifugation in the cold.

A column of DEAE-cellulose (Serva Lab., Heidelberg, Germany; 2.3 × 20 cm), which had been washed with dilute NaOH and dilute HCl, was equilibrated with 0.01 M phosphate buffer, *p*H 7.2, at 2°. The above solution of crude acceptor RNA was applied and eluted at 44 ml hour with a linear gradient of 300 ml of 0.01 M phosphate, *p*H 7.2, and approximately 285 ml of 0.2 M phosphate, pH 7.2, containing 1.5 M KCl. The potassium salt was used to prevent crystallization of the buffer salts. The effluent was monitored with a recording ultraviolet photometer, after which a sufficient number of fractions were measured to locate those with an $A_{260} > 10$ and a ratio A_{260}/A_{280} between 1.9 and 2.1. For this purpose, aliquots of the fractions were diluted 1:100 with 0.01 M phosphate, *p*H 7.2, and read in a Beckman model DU spectrophotometer.

The RNA located in this manner was dialyzed against deionized water in the cold, in 8/32 Visking tubing previously boiled in deionized water. After lyophilization the yield was usually about 140 mg of RNA.

A final purification step consisted of rechromatography of the RNA (dissolved in 20 ml H_2O, *p*H adjusted to 6.9) under the same conditions. Fractions having ratios of A_{260}/A_{280} between 1.90 and 2.12 were dialyzed and lyophilized. The absorbancy ratio of the combined fractions had been 2.11 but fell to 1.99 after extensive dialysis. The rechromatography of 800 mg of material gave 480 mg of acceptor RNA. All concentrations of RNA were measured spectrophotometrically at 260 mμ in dilute salt solution, with an absorbancy index of 21.4 cm^2mg^{-1} (Stephenson and Zamecnik, 1961).

In some experiments, dialysis before and after rechromatography was replaced by desalting with a Sephadex G-25 column (Pharmacia Fine Chemicals Inc., Rochester, Minn.), 50 × 4.5 cm, in deionized water (Porath, 1960; Gelotte, 1960). A product similar to that obtained with dialysis was obtained except that its digestion with pancreatic ribonuclease was more rapid than usual.

Base Composition.—Analysis was performed with a modification of the technique used by Dunn *et al.* (1960) and by Spahr and Tissieres (1959). Approximately 20 mg of acceptor RNA was hydrolyzed by incubating with 1 ml of 0.3 N KOH at 37° for 16 hours. After dilution with water to 10 ml, the sample was applied directly to a column (0.9 × 20 cm) of Dowex CG-1x8 in the formate form, minus 400 mesh (California Corporation for Biochemical Research). The nucleotides were eluted at room temperature with the following: 0.010 M formic acid (100 ml); 0.020 M formic acid (300 ml); 0.15 M formic acid (600 ml); 0.05 M ammonium formate–0.01 M formic acid (800 ml); 0.10 M ammonium formate–0.10 M formic acid (1800 ml). Finally, a linear gradient from the last buffer to 3 M ammonium formate (approximately 60 ml of each) was applied; this removed pGp[1] as well as traces of some as yet unidentified compounds. The extinction coefficients used were those given by Beaven *et al.* (1955). The mean of results for several preparations of acceptor RNA are given in Table I. Within experimental error, constancy of base composition of different preparations made in this laboratory was obtained. The values are, however, somewhat different from those reported by others (Monier *et al.*, 1960).

Guanosine-2′(3′),5′-diphosphates.—The material

[1] Acceptor RNA is the abbreviation used for ribonucleic acid preparations possessing amino acid acceptor ability. The abbreviations recommended by the *Journal of Biological Chemistry* are used in the text for the mononucleotides and oligonucleotides, *e.g.*, Ap for the 2′ and (or 3′ phosphates of adenosine and ApGp for adenyl-(3′-5′) guanosine phosphate; ψ-uridylic acid (ψ-Up) represents uracil-5-ribosyl phosphate; Tp, thymine ribosyl phosphate; MeAp, N^6-methyladenine ribosyl phosphate; MeGp, the methylated guanine nucleotides (1-methylguanylic acid, N^2-methylguanylic acid or a compound with similar properties [Davis *et al.*, 1959], and the N^2-dimethyl nucleotide); pGp, guanosine-2′(3′),5′-diphosphate. Pu signifies a purine nucleotide and Py a pyrimidine nucleotide. In the figures, the nucleotides are lettered as by Rushizky and Knight (1960) to facilitate direct comparison with their results; *e.g.* U for Up and G diphos for guanosine diphosphate.

TABLE I

NUCLEOTIDE COMPOSITION OF ACCEPTOR RNA TOGETHER WITH APPROXIMATE COMPOSITION OF FRACTIONS OBTAINED BY CHROMATOGRAPHY (FIG. 4) ON SEPHADEX OF A RIBONUCLEASE DIGEST OF THE RNA (MOLES/100 MOLES OF NUCLEOTIDES AND NUCLEOSIDES)[a]

Fraction (Fig. 4)	Ap	Cp	Up	ψ-Up	Gp	pGp	Tp	MeAp	MeGp
Acceptor RNA[a]	21.1 (0.4)	26.4 (0.1)	16.1 (0.4)	4.6 (0.2)	29.2 (0.2)	1.15 (0.09)	1.3 (0.1)		
Frac. 1	14.6	12.0	4.7	−[d]	61.0	++[d]	7.6	−	−
2	37.5	11.2	8.6	2.2	33.0	1.7	1.1	1.5	3.4
3	11.8	43.4	22.7	2.2	17.0	−	0.9	trace	1.5
4	18.5	28.0	27.3	7.8	18.9	−	−	−	trace
Total of Frac. 1, 2, 3, 4[b]	22	28	18	3.2	25	0.6	1.1	0.5	1.8
Total[c]	21	25	21	4.0	26	+	1.4	++	1.8

[a] Values are the mean of determinations on five different preparations except for G-diP and TMP, which were determined on two preparations. Standard error is given in parentheses. Adenosine and cytidine were the only nucleosides which could be detected in measurable quantities. The proportions of adenosine to cytidine in these analyses varied between 1:1 and 1.4:1. Prolonged alkaline hydrolysis (up to 64 hours) did not change the nature or the amounts of these nucleoside end-groups. The ratio of 2′(3′)-CMP to 2′(3′)-AMP also remained the same after 64 hours of hydrolysis. 1.3 moles of nucleosides (S.E. 0.06)/100 moles were found in the acceptor RNA. [b] Total calculated by adding the compositions of each of the 4 fractions and including correction for the *percentage* of the total optical density units in each fraction. [c] Determined from a map of a hydrolysate of the entire sample. [d] + Indicates that material was present but not enough could be eluted from the particular map for an accurate determination; − indicates not detectable.

in the eluate resulting from the final ammonium formate gradient was lyophilized and redissolved in 1.0 ml of water; spectrophotometric and phosphate analyses were then carried out. Phosphate was determined by the method of Fiske and SubbaRow (1925) after hydrolysis of a 0.2-ml aliquot with 0.2 ml of 12 N HCl in a sealed tube at 110° for 2 hours. The solution contained 0.85 μmole of phosphate per ml. A second 0.1-ml aliquot was diluted to 3.0 ml with 0.1 N hydrochloric acid, and absorbancies were obtained whose ratios (250/260, 280/260, and 290/260 mμ) agreed well with the mean of those given by Beaven *et al.* (1955) for 2′-GMP, 3′-GMP, and 5′-GMP at *p*H 1. With their ϵ_{260} (11.8 mM^{-1} at *p*H 1) it can be calculated that the solution contained 0.41 μmoles of a guanine-containing nucleotide. Since the extinction coefficient does not change from guanosine to guanylic acid, it seems permissible to use this coefficient for the diphosphorylated compound. The ratio of base to phosphate is therefore 1.0:2.1, in agreement with the formulation of the material as guanosine-2′(3′),5′-diphosphate.

Labeling of Acceptor RNA with C^{14}-Amino Acids.—This was carried out by a method derived from that of Zamecnik *et al.* (1960). Yeast enzyme solution was prepared as follows: pressed baker's yeast was crumbled into a jar of liquid nitrogen. The frozen crumbs were pressed in a 50-ml Hughes press which was at a starting temperature of −20°. The paste was diluted with 1.5 volumes of an ice-cold solution containing 0.14 M KCl, 0.001 M $MgCl_2$, and 0.01 M Tris chloride, *p*H 7.2, in which ribosomes are kept intact. Three small crystals of pancreatic DNase (Worthington Biochemical Corp., Freehold, N. J.) were added to the viscous solution, which was then placed at room temperature with occasional stirring for 10 minutes. Particles were removed by centrifugation in the Spinco model L centrifuge at 30,000 rpm in the number 40 rotor for 2 hours. The clear supernatant was carefully decanted and dialyzed overnight in the cold against 3 liters of 0.01 M phosphate buffer, *p*H 7.2, in Visking 8/32 tubing, previously boiled in deionized water. The final solution contained 2 mg RNA/ml, as determined by the orcinol reaction (Mejbaum, 1939) with AMP as the standard, and also 24.6 mg protein/ml as determined by the procedure of Lowry *et al.* (1951). The enzyme preparation was stored in many small aliquots at −20°, although thawing and refreezing the enzyme solution a *few times* did not appear to alter the ability of the enzymes to label acceptor RNA with valine or tyrosine.

In a typical labeling experiment with valine, the reaction mixture (see Table II) was incubated at 38° for 15 minutes and the reaction terminated by placing the tubes in ice. Either the enzyme or the acceptor RNA were omitted from controls.

The acceptor RNA, now containing bound C^{14}-valine, was then separated from the mononucleotides and from excess C^{14}-valine by chromatography on a Sephadex G-50 column (0.9 × 25 cm, equilibrated with 0.10 M ammonium acetate). After application of the cold incubation mixture, elution was carried out with 0.10 M ammonium acetate at room temperature. The first 5.8 ml of effluent from the time that the sample was first applied was discarded and two fractions of 4.0 ml and 1.0 ml were then collected. The first contained acceptor RNA, labeled or unlabeled, and enzyme proteins, and the second was found to contain virtually no acceptor RNA, radioactivity, amino acids, or small nucleotides. The acceptor RNA content of the first fraction was determined spectrophotometrically. In addition a 0.5-ml aliquot was plated and counted in a Nuclear Chicago model 181A or 186 window counter with no correction for self-absorption. Table II gives the results obtained in a series of experi-

TABLE II
LABELING OF ACCEPTOR-RNA WITH C^{14}-VALINE AND C^{14}-TYROSINE[a]

RNA Sample	Enzyme	RNA	A_{260}	A_{280}	First Fraction cpm/ml	First Fraction cpm/mg	Second Fraction cpm/ml	Second Fraction A_{260}
				C^{14}-Valine				
—	+	−	0.011	—	1	—	0	—
190	−	+	0.192	0.100	0	—	8	—
190	+	+	0.224	0.117	324	1,085[b]	23	—
LS1	−	+	0.180	0.094	2	—	0	—
LS1	+	+	0.185	0.099	266	1,090[b]	19	—
				C^{14}-Tyrosine				
—	+	−	0.013	—	80	—	13	0.011
190	−	+	0.206	0.109	20	—	2	0.020
190	+	+	0.234	0.122	713	2,050[b]	61	0.023
LS1	−	+	0.202	0.106	22	—	2	0.022
LS1	+	+	0.210	0.106	516	1,580[b]	53	0.013

[a] The incubation mixture consisted of 50 μl of a solution of acceptor RNA (28 mg/ml) with 10 μl of 1 M $MgCl_2$, 50 μl of 1 M Tris-HCl buffer (*p*H 7.30), and 50 μl of a solution containing the sodium salts of ATP and CTP (30 and 3 mg respectively per ml of water, brought to *p*H 8) added. This solution of ATP plus CTP was stable for several days when kept frozen. A solution (10 μl) of labeled amino acid was added to the above (1.0 μC in 10 μl) of L-valine-1-C^{14} (Picker, Boston, specific activity 2.90 μC/μM) or of L-tyrosine-C^{14}, uniformly labeled (Nuclear Chicago, 10.0 μC/μM) followed by 20 μl of the yeast enzyme solution together with water to make a final volume of 0.5 ml. [b] These figures correspond to approximately 18 mμM C^{14}-valine/μM RNA (m.w. 25,000 chosen on the basis of end-group analyses) and 9.6 or 7.5 mμM C^{14}-tyrosine/μM RNA, since counting was at an efficiency of 24%. Much higher incorporations can be obtained, if less RNA is used.

ments including those in which valine was replaced by 1.0 μC in 10 μl of L-tyrosine-C^{14} (uniformly labeled, Nuclear Chicago, specific activity 10.0 μC/μmole).

An alternative method of labeling was also developed. DE-20 ion-exchange paper (Reeve Angel & Co., 9 Bridwell Place, Clifton, N. J.; 10 in. wide × 6 in.) was washed with *p*H 1.9 buffer (8.7% acetic acid and 2.5% formic acid) and allowed to dry at room temperature. The incubation mixture (0.1 ml) was as given in Table II, with all amounts reduced to one fifth. The Tris buffer contained 0.15 M $MgCl_2$; no other magnesium salt was added. The crude yeast enzyme had been further purified by passing it through a G-25 Sephadex column in 0.02 M Tris-HCl buffer, *p*H 7.2, containing 0.001 M $MgCl_2$, 0.010 M β-mercaptoethanol.

Fifty μl of the incubation mixture was applied as a compact spot to the DE-20 paper 1 in. below the edge of an elution trough (method similar to that of Sanger and Tuppy, 1951). Elution was carried out for 1 hour with the *p*H 1.9 buffer to remove excess amino acids. The paper was dried *in situ* for several hours. The RNA-containing spot was located under an ultraviolet lamp, cut out, placed in polypropylene vials, and moistened with scintillation fluid NE219 (Nuclear Enterprises, Winnipeg, Canada) prior to counting in a Tri-Carb scintillation counter (Packard Instruments). The results agree with those from the other method, although the degree of incorporation of C^{14}-valine was twice as high, owing probably to the removal of the C^{12}-amino acid contaminants from the enzymes.

Chromatography on Cross-Linked Dextrans (Sephadex).—Columns of Sephadex G-25 or G-50 were packed by the procedures described by Porath (1960) and Gelotte (1960) with the gel particles equilibrated and suspended either in water or in 0.5% NaCl. If packed in the presence of salt, the columns were then washed with distilled water and used directly at room temperature for the desalting of acceptor RNA preparations. For most oligonucleotide fractionations the columns were equilibrated with 0.05 M ammonium acetate, which was also used as the eluant. Desalted nucleic acid was recovered by lyophilizing; nucleotide fractions were first concentrated to 2–3 ml in a rotary evaporator; then they were placed in polyethylene vials, covered by a perforated top, frozen, and lyophilized in a dessicator over P_2O_5 and NaOH. Although ammonium acetate could be removed readily by freeze-drying from fractions containing the larger oligonucleotides, *e.g.* fraction 1, Figure 4, further desalting was found necessary for other fractions.

Digestion with Pancreatic Ribonuclease.—After initial desalting on columns (2.1 × 30 cm) of Sephadex G-25, 100–180 mg batches of acceptor RNA preparations were dissolved in 2 ml of deionized water and the solution was brought to *p*H 8.0 with 0.1 N NaOH. Pancreatic ribonuclease (1 mg per 100 mg RNA, Worthington Biochemical Corp., Freehold, N. J.) in 100 μl of water was added and the *p*H was maintained at 8.0 with a *p*H stat (Radiometer Inc., Copenhagen). Digestion was continued for 2 hours with an additional 0.5 mg of enzyme added after the first hour. Digests were kept frozen until elec-

TABLE III
COMPOSITION OF SOME OLIGONUCLEOTIDES ELUTED FROM AREAS SHOWN IN FIGURE 8

Indicated Area in Fig. 8	Nucleotide Sequence Assigned[a]	Major Nucleotides Found After Alkaline Hydrolysis	Additional Nucleotides Found After Alkaline Hydrolysis
1	Cp	Cp[d]	—
2[b]	ApCp	Ap, Cp	—
3[b]	ApApCp	Ap, Cp	—
5[b]	GpCp	Ap, Cp, Gp	MeGp
6	ApUp	Ap, Up	ψ-Up
7[b]	(ApGp)Cp	Ap, Gp, Cp	MeGp
8	(ApApGp)Up	Ap, Gp, Up	ψ-Up, MeAp, MeGp
9	ApGpUp, GpApUp	Ap, Gp, Up	ψ-Up, MeAp, MeGp, Tp
10	GpUp	Gp, Up	ψ-Up, MeGp
11	Up	Up	—
13[c]	GpGpUp	Ap, Gp, Up	Tp (trace)
15		Ap and/or Cp, Gp, Up	—
16		Ap and/or Cp, Gp, Up	—
17	(ApApGp)Up, (ApGpGp)Up	Ap, Gp, Up	ψ-Up, MeAp (trace)

[a] Based on data of Rushizky and Knight (1960). [b] Samples recovered from areas 2, 3, 5, and 7 were subjected after hydrolysis to electrophoresis at *p*H 3.4 on the water-cooled metal plate. Under these conditions A and C are separated as well as 2′-GMP, 3′-GMP, and Up. The spots are not as tight as with the glass plate, which may have made detection of traces of the other nucleotides less sensitive. Insufficient material was obtained from area 14 for identification. [c] Quantities of material recovered from areas 13–17 were less than for areas 6 and 8–10. [d] A trace of Up, presumably from deamination of Cp, was found in hydrolysates of material from areas 1, 2, 3, 5, and 7.

trophoresis or chromatography on Sephadex. The latter was begun about 1 hour after completion of the digestion.

Preparation of Samples for Mapping: Desalting of Oligonucleotide Fractions and Removal of KOH After Hydrolysis to Mononucleotides.—In high-voltage paper electrophoresis of most fractions from the Sephadex columns, it was found that traces of salt caused some double or smeared spots. Greatly improved results were obtained with the following procedure, based on a description by Smellie (1960). The sample (0.4–0.8 mg) was dissolved in 15–20 μl of water and the solution was run on to a 2 × 6 cm strip of cation-exchange paper in the hydrogen form (either paper loaded with Amberlite IRC-50 (WA-2) or carboxymethylcellulose paper; H. Reeve Angel and Co.). The strips had one pointed end, and the elution with water from the paper into 100-μl capillary pipettes was as described by Sanger and Tuppy (1951). After removal of the ammonium ions by the paper, the remaining acetic acid was removed by evaporation *in vacuo* of the eluates over P_2O_5 and NaOH. The samples were taken up in 15–20 μl of water or buffer for loading on to the paper for electrophoresis. Maps of the same sample with or without desalting appeared to be the same except for distortions caused by the salt.

A convenient rapid procedure for the alkaline hydrolysis of small samples of nucleic acid, of fractions removed from Sephadex columns, or of material eluted from areas cut from oligonucleotide maps is as follows: Samples were taken up in thin-walled capillaries with 30 μl of 0.3 N to 1 N KOH; the capillaries were sealed and heated at 80° for 45 minutes (E. Herbert, personal communication, 1962). Although 0.3 N KOH was sufficient for satisfactory hydrolysis of 0.5 mg of nucleic acid, buffer salts in the other fractions sometimes required the use of 0.5–1.0 N KOH. It should be noted (Table III) that only traces of deamination of Cp to Up occurred under these conditions. After hydrolysis, the contents of a capillary were run on to ion-exchange paper, as described above, for removal of K^+ ions. In early experiments, adenylic acid showed a tendency to be retained relative to the other nucleotides; therefore the elution volume from a 2 × 6 cm strip should be at least 100 μl. Carboxymethylcellulose paper was used for most experiments, as less time was required for elution. In fractions rich in guanylic acid, some material remained in suspension when samples were redissolved in 15–20 μl of water or buffer for electrophoresis. The electrophoresis, however, did not seem to be adversely affected.

Mapping (Fingerprinting) Procedures. — The general approach toward mapping follows that of Rushizky and Knight (1960) and the earlier work of Markham and Smith (1952a,b) and of Davidson and Smellie (1952), with some variations to lessen the time required and to simplify the procedure. Two types of electrophoresis apparatus have been used, with voltages ranging from 28–35 v/cm and current of 20–100 ma.

METHOD A.—The apparatus is the arrangement with two polished 0.25-in. (0.64 cm) glass plates which was originally used for the "fingerprinting" of hemoglobins (Ingram, 1958). With an ammonium formate buffer, *p*H 2.7 (Rushizky and Knight, 1960), a run of 1.25 hours at 2000 v could be carried out without excessive heating. Samples were loaded on to moist Whatman 3 MM

papers; more compact spots resulted if buffer was blotted from a small line (0.8 cm) at the origin immediately prior to the placing of the samples on the paper. After electrophoresis, the papers were dried and equilibrated in a chromatography jar for 1 hour over the 1:1 ammonium formate (*p*H 3.8)–*tert.* butyl alcohol system (Rushizky and Knight, 1960) and then chromatographed by ascending chromatography for 16–20 hours. Rectangular chromatography jars, 14 × 12 × 7 in. (36 × 30 × 18 cm) are well suited for papers 12 in. (30 cm) square.[2]

METHOD B.—An alternative method, which spread the constituents over a larger area, employed a cooled metal plate (Stretton and Ingram, 1962), 24 × 14 in. (61 × 36 cm). The paper, 12 × 52 in. (30 × 132 cm), was placed between polyethylene sheets; 7 in. (18 cm) was folded back from each end to make double wicks. Good results were obtained by "dry loading" (Ryle *et al.*, 1955) on a 1.5-cm line and by use of a buffer of 10% acetic acid brought to *p*H 2.7 with concentrated ammonium hydroxide. Runs were 2.5–3 hours at 2500–3000 v. The position of components was observed by ultraviolet light after drying, and the paper was cut in half before chromatography as above.

METHOD C.—The same mapping methods were used for determination of approximate compositions of nucleotides, including the "additional nucleotides," of hydrolysates, except that the chromatographic solvent was the isopropanol-water (70:30) system of Markham and Smith (1952a), which includes ammonia (6.6 ml of concentrated NH_4OH in a beaker) in the gas phase (19 liters). Because of the relatively low R_F values of nucleotides in the system, a double thickness of 3 MM paper was machine-sewn along the top of the chromatogram to permit additional solvent migration.

Each type of electrophoresis apparatus has some advantages. The relatively short total distance of travel with the glass plates and the *p*H 2.7 buffer leads to good separation between Gp and Up, which in turn gives good discrimination between the "additional" components after chromatography. The fact that compact spots result is particularly useful in the analysis of alkaline hydrolysates. Adenylic and cytidylic acids, however, do not separate well under these conditions, and runs at *p*H 3.5, where separation would occur (Davidson and Smellie, 1952), result in excessive heating. However, on the cooled metal plate with ammonium formate buffer, *p*H 3.5, complete separation of Ap and Cp is obtained (with 2′-GMP and 3′-GMP also separated), but the guanylic acids are rather close to uridylic acid. The best visualization of many of the "additional" components was obtained on the metal plate with the *p*H 2.7 ammonium acetate made from 10% acetic acid and concentrated ammonia.

Approximation of the amounts of separated nucleotides was made by elution of the spots from paper with 0.01 N KOH followed by determination of spectra with a Cary spectrophotometer, Model 11 M. A beam-focusing device enabling use of a microcell containing 0.3 ml permitted examination of the spectrum of the "additional" nucleotides obtained from the hydrolysis of samples of 0.4–0.6 mg of starting nucleic acid or of fractions from columns.

Electrodialysis in Starch Gels.—Electrodialysis against a series of membranes with calibrated porosities was carried out as previously described (Pierce and Free, 1961). Azure B (National Aniline Division, Allied Chemical Corp., New York) was used as a stain for acceptor RNA in the same way as naphthalene black is used with proteins (Smithies, 1959).

RESULTS

Approximate Molecular Size.—Three preparations were subjected to electrodialysis in starch gels made with *p*H 5.0 acetate buffer, 0.012 M. The gel patterns showed all three to run essentially as single bands. The membranes, whose porosity had been altered by treatment with $ZnCl_2$, previously had been calibrated with a group of proteins of known molecular size (see Fig. 2 in Pierce and Free, 1961). The results, which were identical for the three acceptor RNA preparations, indicated a molecular size of approximately 35,000; this value is in reasonable agreement with those calculated from sedimentation, diffusion, and viscosity data (see Berg, 1961) and from equilibrium centrifugation (Goldstein and Craig, 1960).

Mapping of Unfractionated Digests from Yeast Acceptor RNA.—Figure 1 shows a map of a pancreatic ribonuclease digest of acceptor RNA prepared on the cooled metal plate. The spots are labeled as in the maps of digests of tobacco mosaic virus nucleic acid (Rushizky and Knight, 1960; see also Rushizky *et al.*, 1961). A similar pattern, particularly with respect to relative positions, is seen, with the following exceptions: the spot directly below uridylic acid has been identified (by chromatographic behavior and ultraviolet absorption spectra) as ψ-uridylic acid; the spot partially overlapping and just above the dinucleotide GpCp (GC) is an MeGpCp; a spot corresponding to ApApApCp (Rushizky and Knight, 1960) has not been detected in our digests. There also is ultraviolet absorbing material spread between the tri- and tetranucleotide spots which might be due to insufficient resolution of the major spots. However, the spots for the mono- and dinucleotides are clearly separated and thus the smearing may represent in part oligonucleotides containing "additional components." The conditions used for the enzymatic digestion yielded complete or nearly complete

[2] Rectangular jars 14 × 13 × 7 in. were found to be very satisfactory (Shandon Ltd., supplied in the U.S.A. by Consolidated Laboratories Inc., Chicago Heights, Ill.).

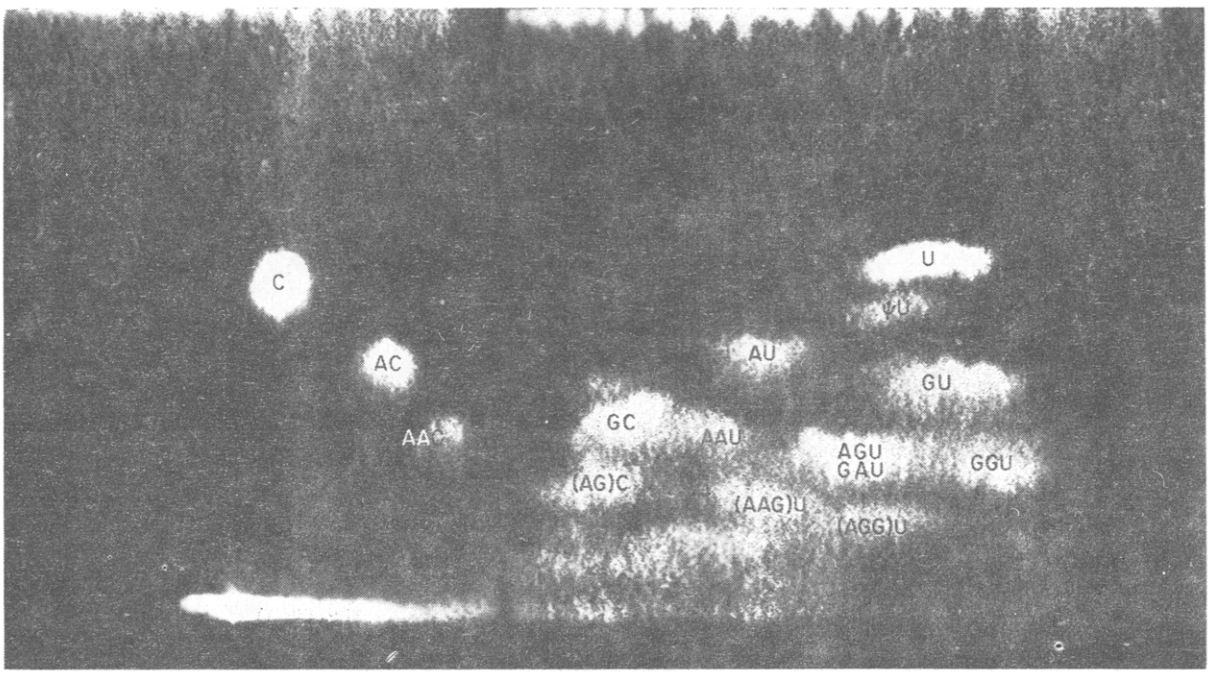

FIG. 1.—Oligonucleotide fingerprint (method B in Methods section) of approximately 0.75 mg of a pancreatic ribonuclease digest of acceptor RNA from yeast. The spots are labeled according to maps of digests of tobacco mosaic virus RNA (Rushizky and Knight, 1960). A = Ap, C = Cp, G = Gp, U = Up, ψU = ψ-Up.

digestion, as no traces of nucleotides containing cyclic phosphates (Rushizky *et al.*, 1961) were seen. In a later experiment where the enzyme-substrate ratio was 1:1000, cyclic compounds were readily detected.

Mapping of Acceptor RNA Digests After Treatment with Alkaline Phosphatase.—In an attempt to increase the resolution (Petersen, 1961) of the mapping procedure, the terminal phosphate groups of the oligonucleotides in the digests were removed by incubation with alkaline phosphatase from *E. coli* (kindly supplied by Prof. C. Levinthal). In the digests originating from pancreatic RNase (Fig. 2) there is considerable increase in resolution, especially among the higher oligonucleotides. In Figure 2, cytidylic acid appears now as a streak of cytidine on the cathode side, whereas uridylic acid and ψ-uridylic acid are now seen as uncharged uridine and ψ-uridine respectively.

In Figure 3, resolution of the oligonucleotides produced by Taka RNase T_1 (Sato and Egami, 1957; Takahashi, 1961) is also increased by removal of the terminal phosphate. In particular, a spot (below and to the left of Gp in Fig. 3a) suspected of containing both CpGp and ApCp is now (Fig. 3b) well resolved into CpG and ApG just underneath the guanosine spot. As a diagnostic tool, this modification of nucleotide mapping holds considerable promise.

The virtual absence of adenosine or guanosine in Figure 2, and of adenosine, cytidine, and uridine in Figure 3b, is additional evidence for the absence of nuclease and phosphodiesterase activity in the *E. coli* alkaline phosphatase.

Distribution of the "Additional Nucleotides" in Pancreatic Ribonuclease Digests.—A group fractionation of ribonuclease digests of the acceptor RNA preparations was carried out on columns of Sephadex G-25 prior to mapping to determine if the sensitivity of the mapping procedures for detection of the "additional" components could be increased. Figure 4 shows a typical result. The elution was carried out in 0.05 M ammonium acetate in order to obviate or reduce the effects of charged groups (Gelotte, 1960); thus the separation should be primarily on the basis of molecular size. The fingerprints (Fig. 5) of the oligonucleotide fractions show this to be the case, as the larger nucleotides are in fractions 1 and 2, whereas fraction 4 contains Cp, Up, and ψ-Up (below but partially overlapping Up, the uppermost right hand spot), together with some di- and trinucleotides. From the relative intensities of the Up and ψ-Up spots in fractions 3 and 4, it can be seen that ψ-Up emerges somewhat later than Up from the columns.

The approximate compositions of the four fractions from Figure 4, after alkaline hydrolysis, are given in Table I, and it was found that the "additional" nucleotides were present in more than one fraction. The data were obtained from spectra of spots eluted from fingerprints of the alkaline hydrolysates. Together they accounted for the composition of the total sample of acceptor RNA, in reasonable agreement with the values determined by column procedures. Fractions 1 and 2 are the most complex in composition; maps of

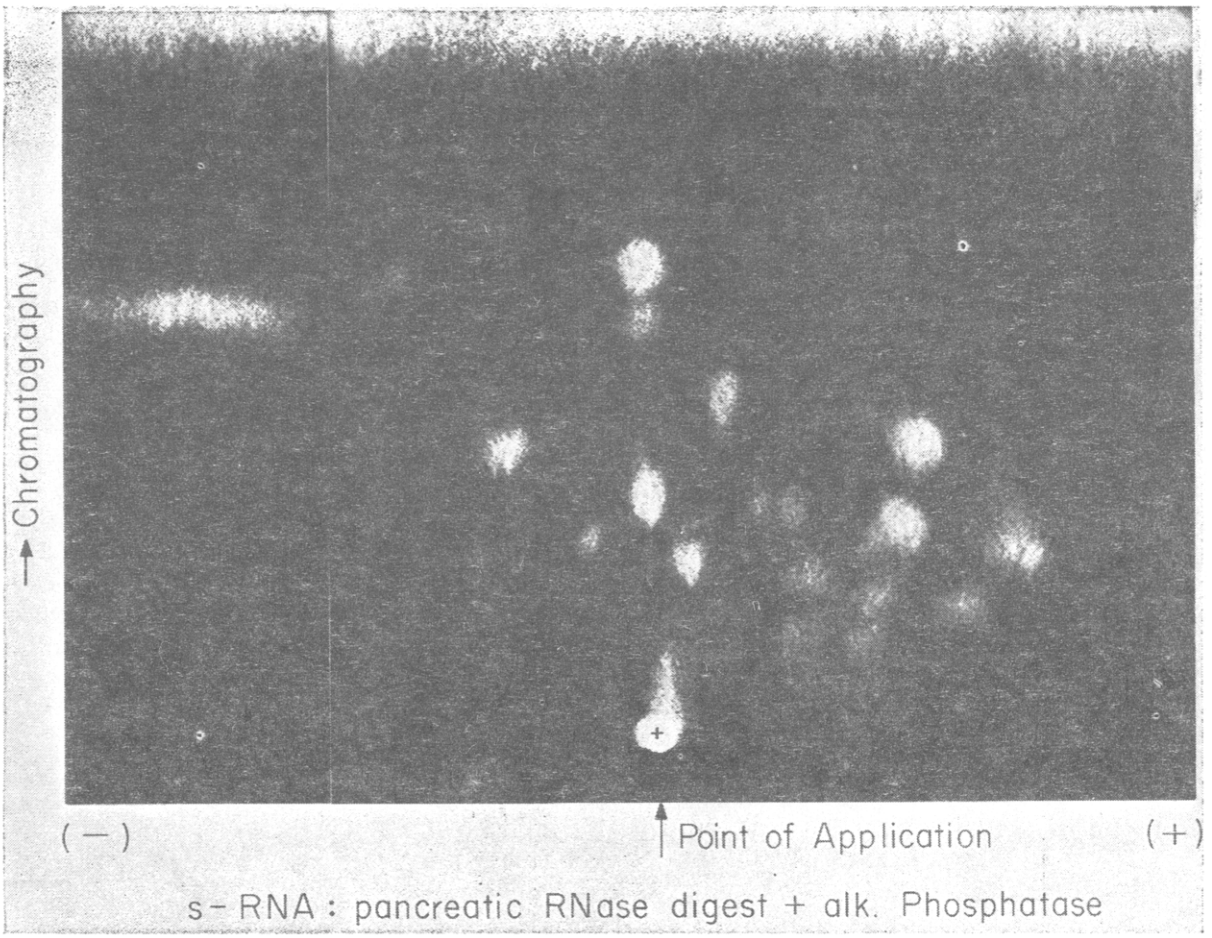

FIG. 2.—Fingerprint of pancreatic RNase digests of *s*-RNA after treatment with alkaline phosphatase (method B; see Methods). Loading: 0.75 mg RNA in 75 μl water had been digested with pancreatic RNase in the usual proportion of 1:100 at *p*H 8.0. Such a sample was treated with 20 μl of 1 M ammonium bicarbonate together with 1 μl of 0.1 M $MgCl_2$ and 10 μl of alkaline phosphatase from *E. coli* (15 μg). The whole was sealed into a glass capillary and incubated at 37° for 30 mins. After evaporation, the residue was applied to the paper in 20 μl of water.

their alkaline hydrolysates are shown in Figure 6. Two unidentified spots, labeled X and Y, are seen in addition to the expected nucleotides. From their electrophoretic behavior, these spots may represent nucleoside 2′(3′),5′-diphosphates other than the guanosine diphosphate end-groups (Singer and Cantoni, 1960; Zillig *et al.*, 1960). The spectrum of the thymine ribotide eluted from spot "T" in Figure 6 clearly showed its identity (Littlefield and Dunn, 1958). Material from the spots attributed to "MeG" from hydrolysates of fraction 2 (Fig. 6) and from hydrolysates of intact acceptor RNA show variation in spectra, indicating that the spots represent a mixture of methylated guanylic acids. The spectrum of the spot labeled "MeA" (Fig. 6) showed that most of the material was N^6-methyl adenylic acid (at *p*H 12: max. 266 mμ, min. 236 mμ; at *p*H 2: max. 265 mμ, min. 238 mμ; see Davis *et al.*, 1959, and Littlefield and Dunn, 1958).

Further Fractionation of Oligonucleotides

EXPERIMENT 1.—Experiments were carried out to study further the distribution of the "additional nucleotides" in various oligonucleotides. In the first, 20 mg of a fraction corresponding to fraction 3 in Figure 4, but containing a somewhat higher proportion of oligonucleotides, was rechromatographed on Sephadex G-50. The sample had been taken to dryness three times to remove as much ammonium acetate as possible. Distilled water was used for elution to see if a fractionation dependent on differences in composition might occur. The elution pattern is shown in Figure 7. While mapping of the three fractions indicated in Figure 7 showed a considerable overlap of nucleotides in the column separation, the first material to be eluted (fraction 1 in Fig. 7) contained no free ψ-Up and little free Cp and Up, and was markedly enriched in tri- and tetranucleotides. A map of the material eluted in the second peak of Figure 7 closely resembled that of fraction 4 in Figure 4, with equivalent resolution. As

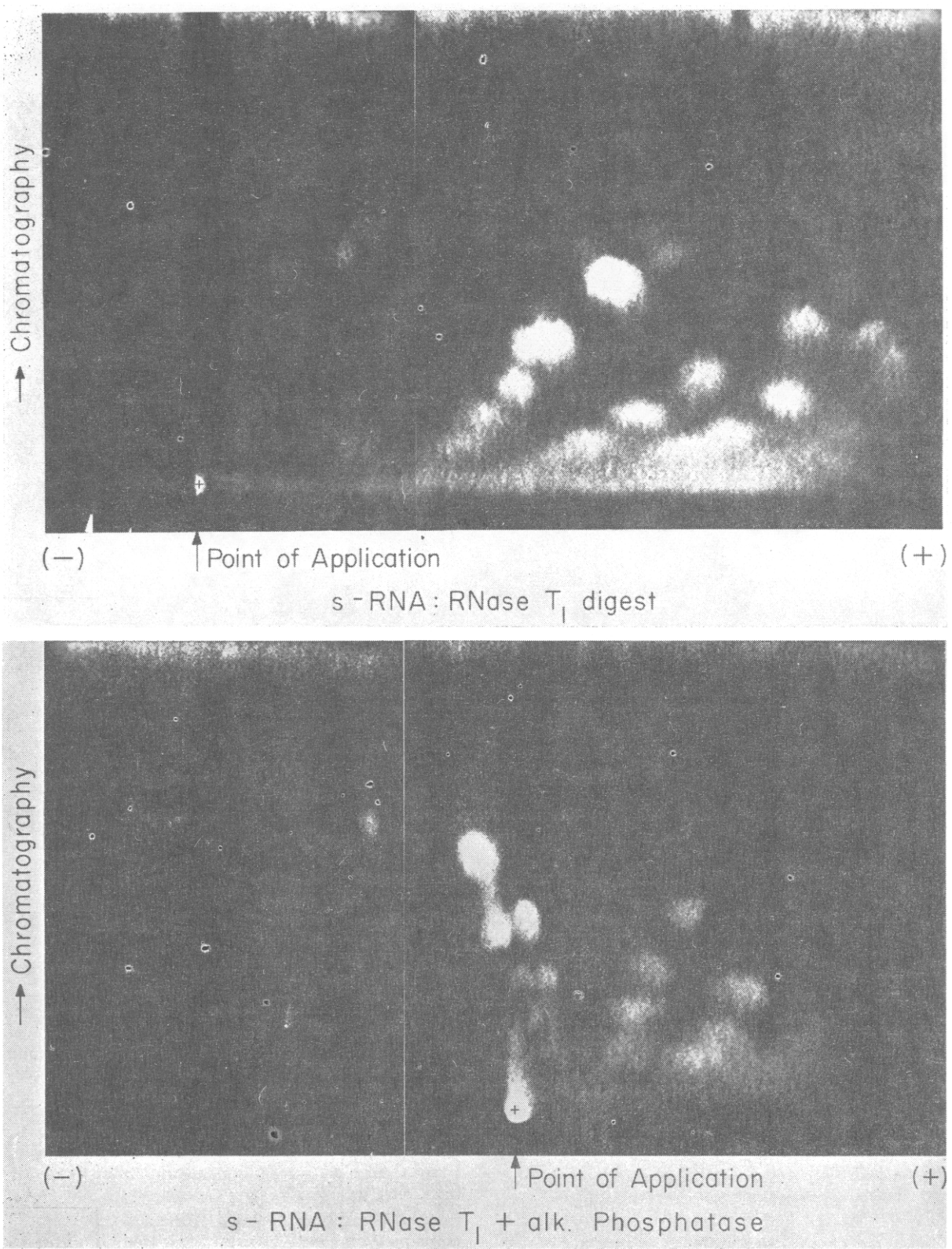

FIG. 3.—Mapping of acceptor RNA digested with Taka RNase-T_1 without (*top*) and with (*bottom*) subsequent treatment with alkaline phosphatase. (Method B; see Methods). Loading: 0.75 mg RNA had been treated as in Figure 2, except that Taka RNase T_1 replaced pancreatic RNase. The very strong spot highest in chromatography in (*top*) is Gp, and in *bottom* is guanosine.

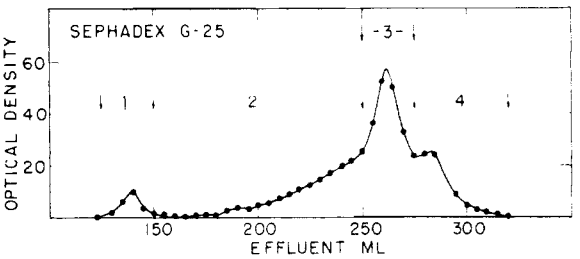

FIG. 4.—Chromatography on Sephadex G-25 (medium grade) of a pancreatic ribonuclease digest of 110 mg of yeast acceptor RNA. Column dimensions 2.1 × 95 cm; fraction size 5 ml; flow rate 30 ml per hour.

fraction 1 in Figure 7 was found to be relatively rich in ψ-Up, Tp, MeGp, and MeAp after alkaline hydrolysis, it was further studied. Its map is shown in Figure 8, together with the tentative identification of the nucleotides in each area on the basis of data from maps of tobacco mosaic virus RNA (Rushizky and Knight, 1960). It should be noted that the spots of Cp, ApCp, ApApCp, and Up (areas 1, 2, 3, and 11) are compact spots well separated from their neighbors. For examination of the "additional" nucleotide content of each area, the numbered areas from five replicate fingerprints were eluted, hydrolyzed with KOH, and mapped again. Table III shows the components observed from each numbered area where sufficient material was recovered. Agreement is seen in all cases with the expected major nucleotide composition except for area 13, which contained some Ap, presumably due to overlap with area 17.

EXPERIMENT 2.—In the second set of experiments, 100 mg of the RNA was digested with pancreatic RNase as described but with an enzyme-substrate ratio of 1:1000. The group separation on Sephadex G-25 was essentially as given in Figure 4. In mapping, spots indicating the presence of 2′-3′ cyclic phosphates were seen which were not present after redigestion of each chromatographic fraction with an enzyme-substrate ratio of 1:50. After redigestion, five replicate fingerprints of material corresponding to fraction 3 in Figure 4 were made by method B, above, with resolution equal to that shown in Figure 1. A pattern of mono-, di-, and some trinucleotides similar to those of Figure 1 was seen with the following additions: a clearly defined spot above area GC (the same as area 4 in Figure 8) was identified as MeGpCp by elution of the area from the five maps, alkaline hydrolysis, and mapping with chromatography in the isopropanol-ammonia-water system. The ultraviolet spectrum of the MeGp from this area indicated it to be N^2-methyl or N^2-dimethylguanylic acid or closely related to them rather than 1-methylguanylic acid. A spot with almost the same chromatographic mobility as area GU (corresponding to 10 in Fig. 8) but slightly slower in electrophoresis was seen adjacent to area GU. It appeared to have some fluorescence after irradiation with ultraviolet light (see next section) and was found on alkaline hydrolysis to contain only MeGp and ψ-Up. It was probably MeGp ψ-Up. A third spot, as yet unidentified, was seen near the U area, which may correspond to area 12 (Fig. 8).

Examination by alkaline hydrolysis of the other areas of the maps showed, in most areas, the additional nucleotides expected from the data in Figure 8 and Table III, although some spots in this second experiment were considerably less intense. MeGp was the most widespread "additional" nucleotide, occurring in the areas of GpGpUp, (ApGp)Cp, in an area below ApApUp, (traces) in (ApGp)Up, in the MeGpCp, and in the MeGpψ-Up spots. MeAp was found only in (ApGp)Up in this (second) experiment, while Tp was found in the area of GpGpUp and possibly in GpUp. ψ-Up was in MeGpψ-Up and traces in (ApGp)Up; none, however, was detected in the ApUp in this fraction (contrast experiment 1; see 3 in Table III). In this experiment most of the ApUp was found in fraction 4. When fraction 4 was chromatographed twice in the isopropanol-ammonia-water system after electrophoresis, the ApUp area clearly separated into two spots. The lower of these yielded Ap and ψ-Up after alkaline hydrolysis, thus demonstrating the presence of the dinucleotide Ap ψ-Up. The upper spot was ApUp.

To demonstrate that the presence of ψ-Up, MeAp, and Tp in fractions 2 and 3 of Figure 4 (Table I) was not the result of poor resolution on the column, the material corresponding to fraction 2 was redigested with pancreatic RNase (enzyme-substrate ratio 1:50) and rechromatographed on a column of Sephadex G-25. The sample was completely eluted with the expected volume of effluent (*i.e.*, 160–250 ml; Fig. 4) and was divided into three subfractions. The oligonucleotides eluted from 160–205 ml and 205–225 ml were found on hydrolysis to contain relatively high amounts of ψ-Up, Tp, MeAp, and pGp, and the last subfraction to emerge (225–250 ml) was markedly enriched in MeGp relative to the other "additional" nucleotides. Another component, clearly seen in the hydrolysate of the first subfraction (160–205 ml), had the electrophoretic mobility of Ap but less mobility in the isopropanol-ammonia-water system. This component, as yet unidentified, was also found in the analyses by hydrolysates of the ApCp area from maps of fraction 3. It can also be seen as a very faint spot in Figure 6, fraction 2, below area A (Ap).

Fluorescence of ψ-Uridylic Acid on Paper.—During the course of the mapping experiments it was noted that, in addition to the fluorescence in ultraviolet light of some areas containing guanine, a characteristic intensely blue-white fluorescence was found in the spot of free ψ-uridylic acid, particularly when the paper was still slightly

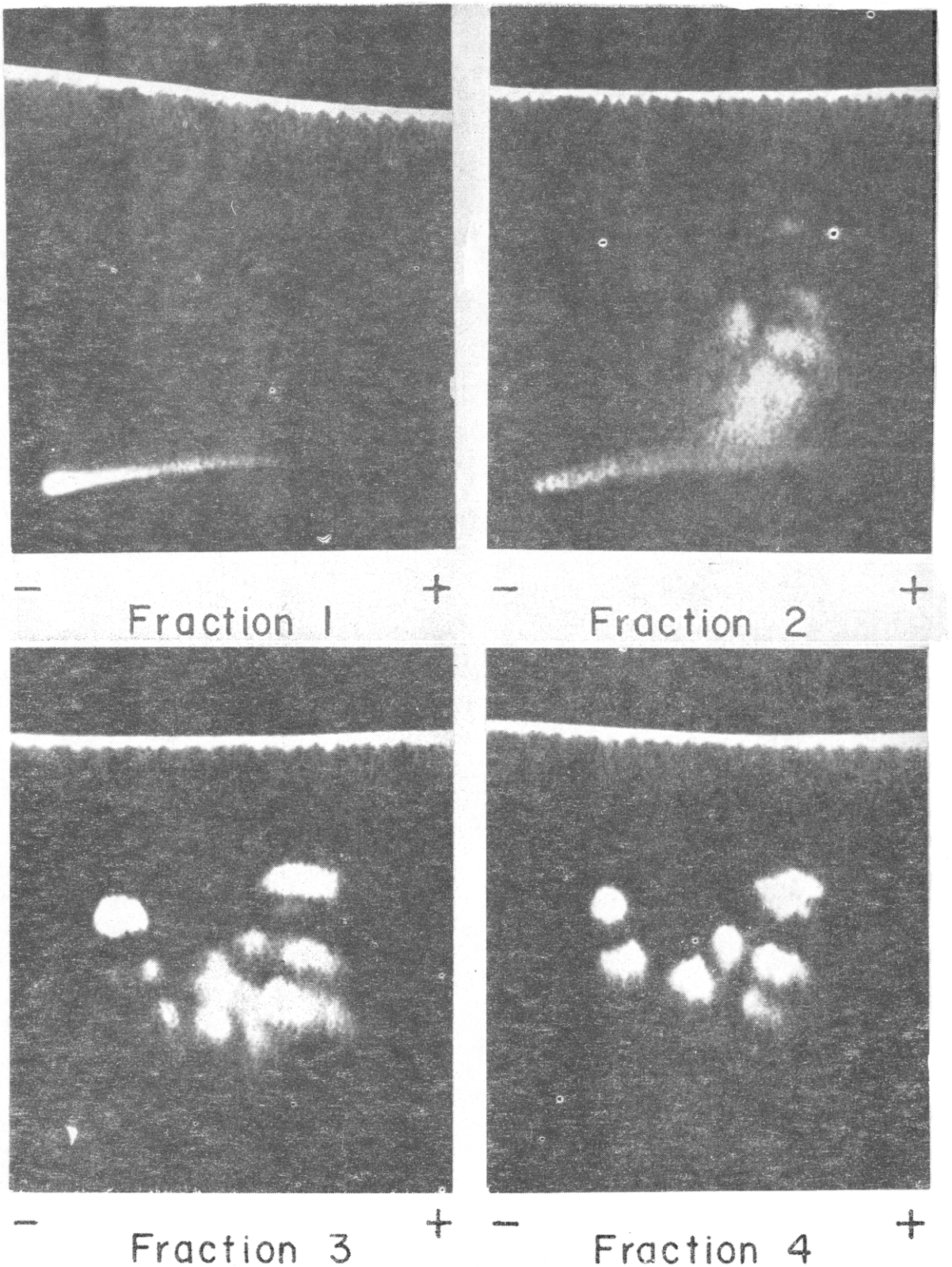

FIG. 5.--Fingerprints by method A (see Methods) of fractions 1 to 4 shown in Figure 4. Fractions 2, 3, and 4 were desalted by elution from the cation exchange paper WA-2. Sample size approximately 0.5 mg for fraction 1, 0.8 mg for fractions 2-4. In the fingerprints of fractions 3 and 4, the two uppermost spots (left to right) are Cp and Up; ψ-Up is directly below Up and partly overlaps it in fraction 4 (see Figure 1 for identification of other spots).

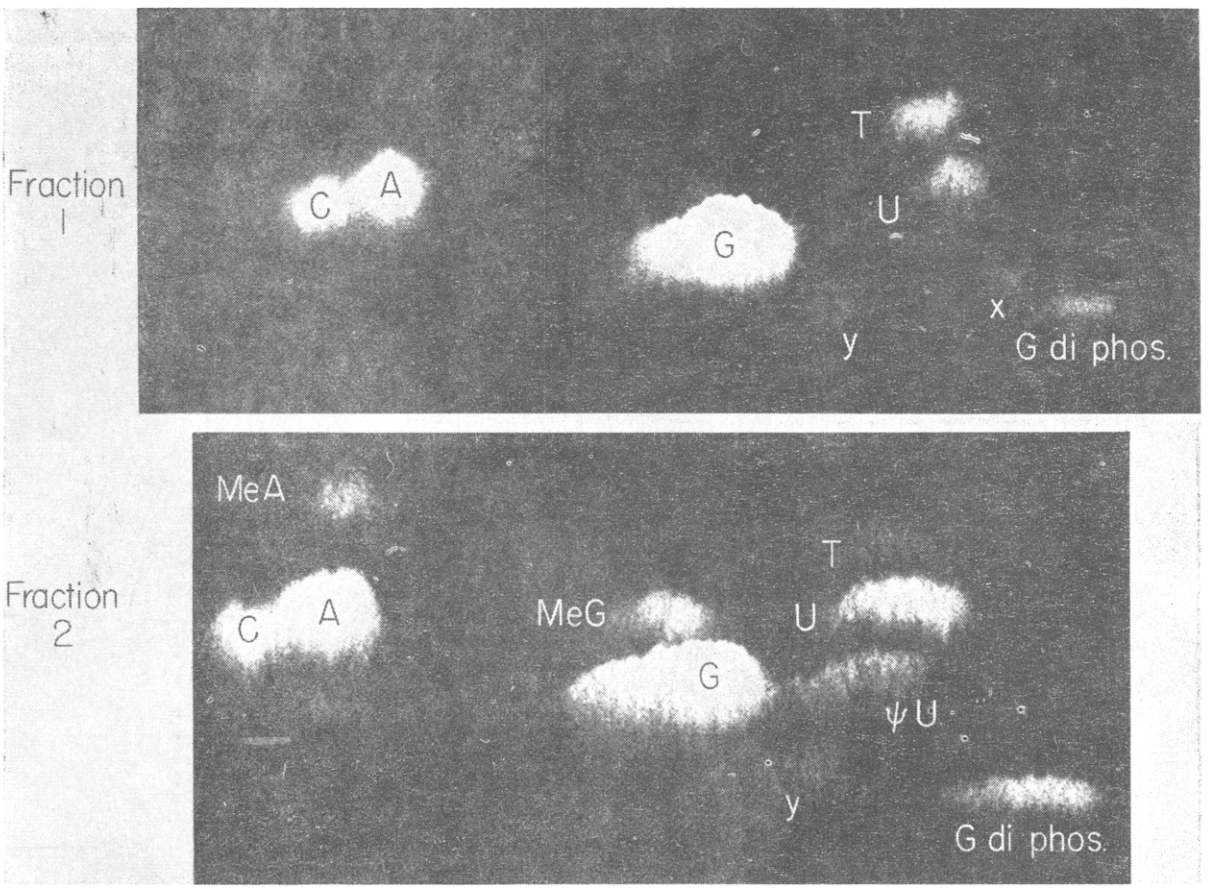

FIG. 6.—Maps prepared by method C (see Methods), showing the constituents of fractions 1 and 2 of Figure 4 after alkaline hydrolysis. The desalted hydrolysate of fraction 1 was "wet-loaded" on a 1.2-cm line perpendicular to the direction of migration; fraction 2 was "dry-loaded" on a 1.5-cm line parallel to the direction of migration and concentrated into a spot by allowing buffer to flow into the line from both sides. Sample size approximately 0.4 mg.

damp with *p*H 3.5 or 2.7 buffer (Smith and Markham, 1950). The latter fluorescence did not appear in well-dried maps unless they were subjected to 2–5 minutes of ultraviolet irradiation (Mineralight Model No. R51, Ultraviolet Products Inc., San Gabriel, Calif.). This behavior allowed easy identification of free ψ-uridylic acid on the maps. The fluorescence was observed after development of the maps with either the *tert.* butyl alcohol–ammonium formate or the isopropanol-ammonia-water systems. Upon rechromatography in the isopropanol system of a map in which the fluorescence had been developed in the ψ-uridylic acid spot, the fluorescent material migrated with an increased R_F, leaving behind a non-fluorescent spot of ψ-uridylic acid in which, however, fluorescence could be again developed. The spot remaining after rechromatography showed the characteristic spectra of ψ-uridylic acid in both acid and alkaline solution. Such fluorescence of ψ-uridylic acid does not appear to have been described before; it is probably distinct from the photolysis of ψ-uridylic acid in solution (Lis and Allen, 1961).

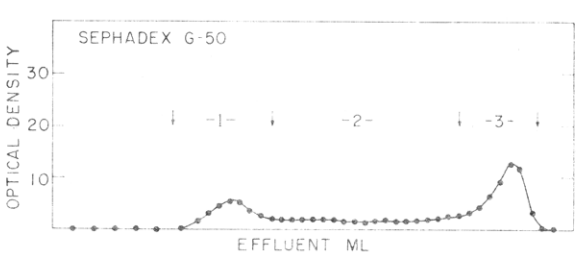

FIG. 7.—Chromatography on Sephadex G-50 (coarse) of approximately 20 mg of a fraction similar to fraction 3, Figure 4. Column dimensions 2.1 × 78 cm. Eluting solution distilled water. Fraction size 5.3 ml; flow rate 32 ml per hour.

DISCUSSION

The additional components that have been reported in yeast acceptor RNA are ψ-Up, Tp, and N^6-methyladenylic acid (Monier *et al.*, 1960; Osawa, 1960). Davis *et al.* (1959) have also described the presence of these additional components as well as 1-methyl guanylic acid and a

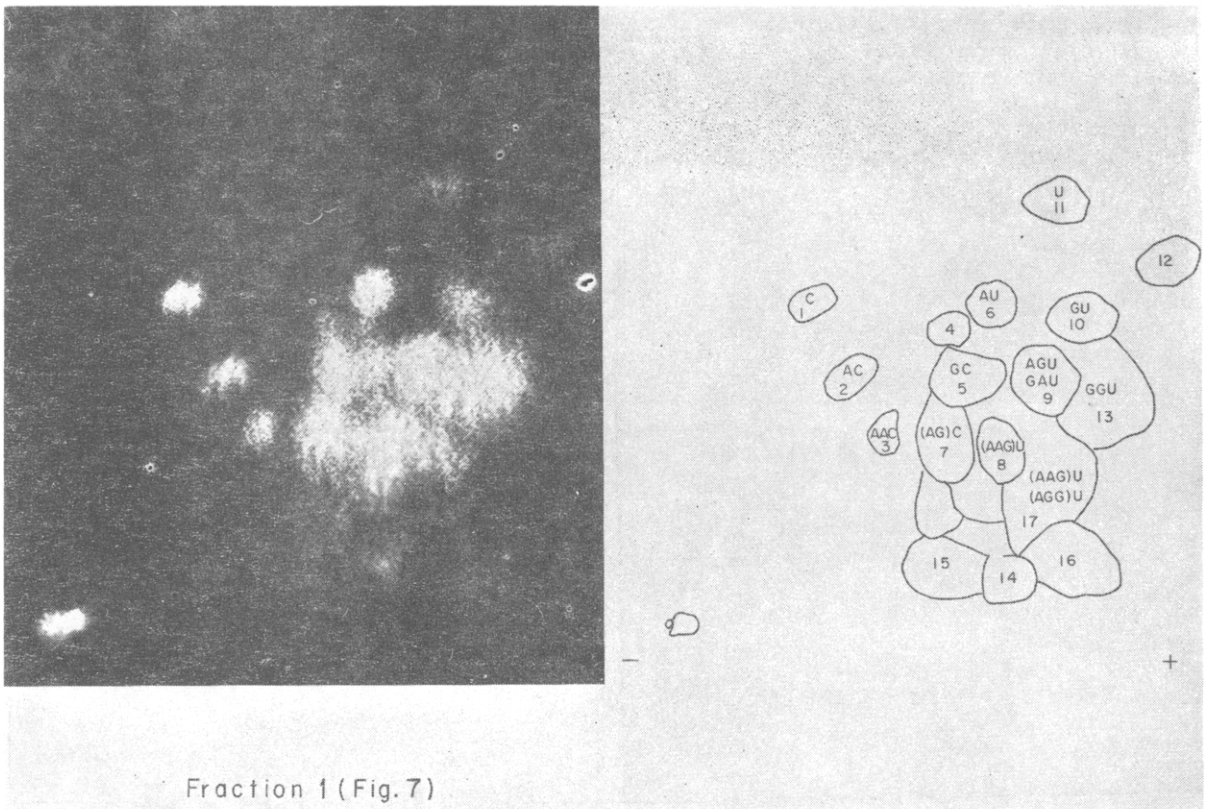

FIG. 8.—Oligonucleotide map of fraction 1, Figure 7 (0.75 mg). Desalting was not necessary. Electrophoresis (wet loading) and chromatography as in Figure 6.

substance similar to N^2-methylguanylic acid in fractions of yeast RNA soluble in 1 M sodium chloride. Many of the original observations concerning the presence of additional components were made with commercial yeast RNA (*e.g* Volkin and Cohn, 1953; Littlefield and Dunn, 1958). With some variations a similar situation holds for acceptor RNA from other species (see Berg, 1961). Whether the thymine ribonucleotide originates during the alkaline hydrolysis of 5-methylcytosine in the case of yeast acceptor RNA is not completely settled, but is unlikely (Dunn, 1960). The N^6-methyladenylic acid arises from the 1-methyl compound during alkaline hydrolysis (Dunn, 1961).

In the present experiments, the distribution of the "additional" components in the fractions obtained by chromatography on Sephadex suggests that, while these additional nucleotides are not randomly replacing their major counterparts, they are probably not restricted to a few sequences common to all acceptor RNA molecules. Thus if one assumes that the RNA chains are 100 nucleotides long (which for the composition of our preparations would give a molecular weight of approximately 30,000) it follows that each molecule might contain a maximum of four or five ψ-Up residues, one or possibly two Tp residues, and two MeGp residues (which represent two or three different guanylic acids); however, not all molecules can contain a MeAp residue. The data shown in Table III together with the analyses of the subfractions of material corresponding to fraction 2 (Fig. 4) and of the maps of material corresponding to fraction 3 (Fig. 4) show MeAp to the present in at least two sequences, one in the larger oligonucleotides of fraction 2 and in the area chiefly occupied by (ApGp)Up. MeGp was found to be widely distributed, occurring in at least five well-separated areas [those corresponding to the major nucleotides (ApGp)Cp, (ApApGp)Up, and (ApGp)Up as well as in the two dinucleotides MeGpψ-Up and MeGpCp]. Tp was found in at least three well-separated fractions or areas from the maps (Table III and text). If one excludes overlapping areas, the data show ψ-Up to be in the areas of (ApApGp)Up and probably in (ApGp)Up, as well as in the dinucleotides MeGpψ-Up and Apψ-Up and in the larger oligonucleotide portion of fraction 2 material. On the other hand, ψ-Up is absent from areas 13, 15, and 16. A small possibility remains that some of the ψ-Up could be in some sequences common to most of the species of RNA in the preparation, since we cannot assign positions to the ψ-Up liberated by pancreatic ribonuclease. However, the rather wide distribution of a great deal of the ψ-Up amongst various nucleotides inclines us

against this last possibility. It is interesting to note, incidentally, that Osawa (1960) had indicated a relation between the ψ-Up content of an RNA preparation and its ability to accept leucine.

It is also of interest that the guanosine diphosphate, presumed to be terminal, has not been detected in any of the smaller nucleotides but is markedly enriched in fractions consisting of the larger oligonucleotides (fraction 1 in Figure 4 and the first material of fraction 2 to emerge from the Sephadex columns). This observation indicates either that a proportion of the non-amino acid acceptor ends of the transfer RNA molecules do not have a pyrimidine residue close to the terminal pGp or that small nucleotides with an extra phosphate group are accelerated in their travel down a Sephadex column.

Fraction 1 (Fig. 4) is similar to the "core" or "limit polynucleotide" material described by Volkin and Cohn (1953) and by Smith and Markham (1950). The extent of recovery of optical density units in fraction 1 in five preparations was 5.6, 5.5, 4.6, 2.4, and 5.3%, indicating variation in the preparations (conditions of digestion were essentially the same). The nucleotide analyses of three other samples of fraction 1 gave results similar to those of Table I, and redigestion, rechromatography, and remapping of fraction 1 did not show any change in composition. Because of its high content of Gp in relation to Ap the fraction resembles the acid-insoluble fraction of whole yeast and pig liver RNA (Volkin and Cohn, 1953) rather than similar material from digests of tobacco mosaic virus nucleic acid (Reddi and Knight, 1956). Of particular interest is the relatively high proportion of thymine ribonucleotide in fraction 1. If all the oligonucleotides containing Tp were of the type $(Pu)_nTp$, consistent with the others as $(Pu)_mCp$, $(Pu)_rUp$, an over-all purine-pyrimidine ratio of 3:1 would be obtained; the fraction would contain tetranucleotides, which is inconsistent with its chromatographic behavior on both the maps and the Sephadex columns. From the figure of 3500–4500 given by the manufacturer for the molecular size of neutral polysaccharide which will not be retarded by Sephadex G-25, the average molecular size of fraction 1 might be approximately that of an octa- to a decanucleotide. The Pu:Py ratio excluding Tp indicates penta- to hexanucleotides, and it is likely that some exclusion effect (Gelotte, 1960) by virtue of the negatively charged phosphate groups is still present even in 0.05 M ammonium acetate. Thus the intriguing possibility arises that some of the Tp is not terminal in these "core" oligonucleotides, which would then be on the average six residues long; further investigation is required to see if ribonuclease-resistant linkages containing Tp are present and whether Tp bonds are resistant to pancreatic RNase. It should be noted that the only free Tp seen when fractions resulting from pancreatic ribonuclease digestion and containing free pyrimidine nucleotides have been mapped with the propanol-ammonia-water system for chromatography has been *traces* in material similar to fraction 4 in Figure 4.

Acknowledgments

The authors wish to acknowledge the capable assistance of Miss Lee Sullivan, Miss Ann McFarlin, and Miss Dorothy McNall during portions of the investigation.

References

Beaven, G. H., Holiday, E. R., and Johnson, E. A. (1955), *in* The Nucleic Acids, Vol. I, Chargaff, E., and Davidson, J. N., editors, New York, Academic Press, Inc.

Berg, P. (1961), *Ann. Rev. Biochem. 30*, 293.

Canellakis, E. S., and Herbert, E. (1960), *Proc. Nat. Acad. Sci. U. S. 46*, 170.

Davidson, J. N., and Smellie, R. M. S. (1952), *Biochem. J. 52*, 599.

Davis, F. F., Carlucci, A. F., and Roubein, I. F. (1959), *J. Biol. Chem. 234*, 1525.

Dunn, D. B. (1960), *Biochim. et Biophys. Acta 38*, 176.

Dunn, D. B (1961), *Biochim. et Biophys. Acta 46*, 198.

Dunn, D. B., Smith, J. D., and Spahr, P. F. (1960), *J. Mol. Biol. 2*, 113.

Fiske, C. H., and SubbaRow, Y. (1925), *J. Biol. Chem. 66*, 375.

Gelotte, B. (1960), *J. Chromatog. 3*, 330.

Goldstein, J., and Craig, L. C. (1960), *J. Am. Chem. Soc. 82*, 1833.

Hecht, L. I., Stephenson, M. L., and Zamecnik, P. C. (1959), *Proc. Nat. Acad. Sci. U. S. 45*, 505.

Ingram, V. M. (1958), *Biochim. et Biophys. Acta 28*, 539.

Littlefield, J. W., and Dunn, D. B. (1958), *Biochem J. 70*, 642.

Lis, A. W., and Allen, F. W. (1961), *Biochim. et Biophys. Acta 49*, 190.

Lowry, O. H., Rosebrough, N. J., Farr, A. L., and Randall, R. J. (1951), *J. Biol. Chem. 193*, 265.

Markham, R., and Smith, J. D. (1952a), *Biochem. J. 52*, 552.

Markham, R., and Smith, J. D. (1952b), *Biochem. J. 52*, 558.

Mejbaum, W. (1939), *Z. physiol. Chem. 258*, 117.

Monier, R., Stephenson, M. L., and Zamecnik, P. C. (1960), *Biochim. et Biophys. Acta 43*, 1.

Osawa, S. (1960), *Biochim. et Biophys. Acta 42*, 244.

Otaka, E., Hotta, Y., and Osawa, S. (1959), *Biochim. et Biophys. Acta 35*, 266.

Petersen, G. B. (1961), *Biochim. et Biophys. Acta 51*, 212.

Pierce, J. G., and Free, C. A. (1961), *Biochim. et Biophys. Acta 48*, 436.

Porath, J. (1960), *Biochim. et Biophys. Acta 39*, 193.

Reddi, K. K., and Knight, C. A. (1956), *J. Biol. Chem. 221*, 629.

Rushizky, G. W., and Knight, C. A. (1960), *Virology 11*, 236.

Rushizky, G. W., Knight, C. A., and Sober, H. A. (1961), *J. Biol. Chem. 236*, 2732.

Ryle, A. P., Sanger, F., Smith, L. F., and Kitai, R. (1955), *Biochem. J. 60*, 541.

Sanger, F., and Tuppy, H. (1951), *Biochem. J. 49*, 463.
Sato, K., and Egami, F. (1957), *J. Biochem. (Japan) 44*, 753.
Singer, M. F., and Cantoni, G. L. (1960), *Biochim. et Biophys. Acta 39*, 182.
Smellie, R. M. (1960), *J. Chromatog. 4*, 494.
Smith, J. D., and Dunn, D. B. (1959), *Biochem. J. 72*, 294.
Smith, J. D., and Markham, R. (1950), *Biochem. J. 46*, 509.
Smithies, O. (1959), *Biochem. J. 61*, 629.
Spahr, P. F., and Tissieres, A. (1959), *J. Mol. Biol. 1*, 237.
Stephenson, M. L., and Zamecnik, P. C. (1961), *Proc. Nat. Acad. Sci. U. S. 47*, 1627.
Stretton, A. O. W., and Ingram, V. M. (1962), *Biochem. et Biophys. Acta*, in press.
Takahashi, K. (1961), *J. Biochem. (Japan) 49*, 1.
Volkin, E., and Cohn, W. E. (1953), *J. Biol. Chem. 205*, 767.
Zamecnik, P. C., Stephenson, M. L., and Scott, J. F. (1960), *Proc. Nat. Acad. Sci. U. S. 46*, 811.
Zillig, W., Schachtschabel, D., and Krone, W. (1960), *Z. physiol. Chem. 318*, 100.

Pyridoxal-Catalyzed Decarboxylation of Amino Acids*

G. D. KALYANKAR† AND ESMOND E. SNELL

From the Department of Biochemistry, University of California, Berkeley, California

Received March 12, 1962

When α-amino acids (*e.g.*, α-aminoisobutyric acid, α-methylserine, α-phenylglycine) are heated with pyridoxal in dilute aqueous solutions in the absence of metal ions, two closely related but independent reactions occur as follows:

$$RR'CNH_2COOH \xrightarrow{\text{Pyridoxal}} RR'CHNH_2 + CO_2 \quad (1)$$

$$RR'CNH_2COOH + \text{Pyridoxal} \longrightarrow RR'C{=}O + CO_2 + \text{Pyridoxamine} \quad (2)$$

Reaction (1) is analogous to decarboxylation of amino acids by pyridoxal phosphate enzymes. Reaction (2) is a decarboxylation-dependent transamination reaction for which no enzymatic analogy is known. Reactions (1) and (2) are both partially inhibited by those metal ions that catalyze previously studied reactions between pyridoxal and amino acids. These observations are explained in terms of the general mechanism for pyridoxal-catalyzed reactions presented previously (Metzler *et al.*, 1954; Snell, 1958).

Most reactions of amino acids that are catalyzed by pyridoxal phosphate enzymes are also catalyzed at slower rates by pyridoxal in dilute aqueous solution (Metzler *et al.*, 1954; Snell, 1958). Study of such nonenzymatic reactions has provided a sound experimental basis for current concepts of the catalytic role of the coenzyme in pyridoxal phosphate–dependent enzymes. Nonenzymatic decarboxylation of amino acids is known to occur in such systems from the study of Werle and Koch (1949), who used pharmacologic techniques to detect histamine formation from histidine in the presence of pyridoxal. However, no detailed study of this reaction has appeared. Results presented herein show that such nonenzymatic decarboxylation reactions are readily observable. They are of a special interest since, unlike other model reactions catalyzed by pyridoxal, they are independent of or actually inhibited by metal ions. A novel decarboxylation-dependent transmination reaction can be observed under these conditions when amino acids lacking an α-hydrogen atom are employed.

* Supported in part by a grant (A-1448) from the U. S. Public Health Service. These observations were completed approximately 5 years ago and are published now despite their incomplete nature since it is unlikely that the study will be extended further.

† Present address: All India Institute of Medical Sciences, New Delhi, India.

Experimental Procedures

To minimize metal ion contamination, all stock solutions were prepared with distilled water that was passed through a mixed bed deionizing resin, and buffer salts were recrystallized from dilute solutions of disodium ethylenediaminetetraacetate. Procedures were similar to those described by Metzler and Snell (1952a,b). Reaction mixtures (1–10 ml) were heated for the desired time in sealed soft-glass tubes or in flasks equipped with reflux condensers. The contents were cooled and the products formed were identified and analyzed by the following procedures.

Pyridoxal was estimated by the ethanolamine procedure, and *pyridoxal plus pyridoxamine* by the absorbancy at 323.5 mμ and *p*H 6.7 (Metzler and Snell, 1952a). *Pyridoxamine* was separated from pyridoxal by chromatography on paper