are given in Fig. 2. Peak II was recovered quantitatively (0.142 g.) in its original position (fractions 6–11). Peak III appeared in the expected position (fractions 12–18), but was reduced in amount (0.127 g. recovered). Material corresponding to peak IV (0.052 g.) had also been formed and appeared in the expected position (fractions 21–28).

In another experiment, the combined peaks II and III (0.264 g.) were dissolved in 5 ml. of reagent grade chloroform and the solution was heated at 50° for 1 hour. The solvent was removed and the residue dissolved in a small volume of benzene. Chromatography on silicic acid under standard conditions as described above gave peak II, its amount being constant (0.140 g.). Recovery of peak III was 0.069 g., while the newly formed peak IV was present in an increased amount (0.050 g.).

When peak IV (0.162 g.), collected from several runs, was rechromatographed under standard conditions, both peaks III and IV were obtained in equal amounts (about 0.06 g. of each, Fig. 3).

2′(or 3′)-*O*-(Carbobenzyloxy-DL-phenylalanyl)-adenosine.—To an ice-cold solution of 2′-*O*-carbobenzyloxy-DL-phenylalanyl-5′-*O*-trityladenosine (0.137 g., 0.17 mmole, peak IV) in chloroform (5 ml.) was added a solution of hydrobromic acid in acetic acid (0.11 ml., 0.41 mmole). After 5 minutes at 0°, the solution was concentrated under a high vacuum to remove last traces of acetic acid. The residue was taken up in a small volume of chloroform and the solution applied to a silicic acid column. Standard conditions were used except that elution was carried out using a gradient of 20% methyl alcohol in chloroform. Two peaks were obtained. The first peak was the major one and appeared when the methyl alcohol concentration was 11% (0.077 g., 81%). The second minor peak appeared when the methyl alcohol concentration in chloroform was 14% and was presumably 2′(or 3′)-*O*-phenylalanyladenosine. Analyses were performed on a sample obtained by freeze-drying a solution of the first peak in benzene containing a little methyl alcohol. The sample was homogeneous by paper chromatography in solvents A and B, the R_f's being, respectively, 0.84 and 0.85.

Anal. Calcd. for $C_{27}H_{28}O_7N_6$ (548.54): C, 59.1; H, 5.14; N, 15.34. Found: C, 58.43; H, 5.39; N, 14.31.

2′(or 3′)-*O*-(DL-Phenylalanyl)-adenosine.—A sample (0.02 g.) of 2′(or 3′)-*O*-carbobenzyloxy-DL-phenylalanyladenosine as prepared above was hydrogenated in 80% aqueous acetic acid (3 ml.) at 0° for 2 hr. using 25 mg. of palladium-on-barium sulfate catalyst as described for the preparation of the corresponding uridine ester. After removal of the catalyst by filtration in the cold, the acidic solution of 2′(or 3′)-*O*-DL-phenylalanyladenosine was frozen. Aliquots were removed for different analyses from this stock solution. Lyophilization of the acidic solution to a powder caused some breakdown of the ester linkage. Paper electrophoresis of the solution in 1 *M* acetic acid gave a single ultraviolet-absorbing band which showed positive ninhydrin and hydroxylamine reactions. The analysis performed on material eluted from paper electrophoresis with water gave an adenosine to phenylalanine ratio of 0.90. Paper chromatography in solvents A and B gave single ultraviolet-absorbing and ninhydrin-positive spots: R_f in solvent A, 0.55; (R_f of adenosine, 0.35; that of phenylalanine, 0.41). R_f in solvent B, 0.59 (R_f of adenosine, 0.47; R_f of phenylalanine, 0.59). Paper chromatography in solvent C gave three products, adenosine, phenylalanine and phenylalanine amide. The last product was identified by direct comparison with a sample prepared by the method of Anderson, *et al.*[39]

Hydrolysis of 2′(or 3′)-*O*-DL-Phenylalanyladenosine.—A solution of the compound (0.206 μmole) in 0.1 *M* phosphate buffer (*p*H 7, 3 ml.) was treated at 25° and 34° with potassium periodate (0.42 μmole). The rate of hydrolysis of the ester linkage[40] was followed by the decrease in absorption at 235 mμ using the Cary model 14 spectrophotometer. The hydrolysis of the aminoacyl ester was complete after 538 min. at 25°, the half-life being 48 min. At 34°, the half-life was 22 min. The consumption of periodate at the completion of the reaction was theoretical as calculated for the concentration (determined spectrophotometrically) of the adenosine ester. Subsequent treatment of the reaction products with ammonium carbonate buffer[41] (*p*H 10) at 37°, followed by chromatography in solvent A showed the products to be adenine, phenylalanine and a small amount of a faster traveling unidentified ultraviolet-absorbing material.

(39) G. W. Anderson, J. Blodinger and A. D. Welcher, *J. Am. Chem. Soc.*, **74**, 5310 (1952).

(40) Under identical conditions, the oxidation of the *cis*-glycol system in adenosine was complete within 3 minutes. In another control experiment, DL-phenylalanine was shown to be inert to periodate ions.

(41) P. R Whitfeld, *Biochem. J.*, **58**, 390 (1954).

[CONTRIBUTION FROM THE INSTITUTE FOR ENZYME RESEARCH, UNIVERSITY OF WISCONSIN, MADISON, WIS.]

Studies on Polynucleotides. XXI.[1] Amino Acid Acceptor Ribonucleic Acids (2). The Labeling of Terminal 5′-Phosphomonoester Groups and a Preliminary Investigation of Adjoining Nucleotide Sequences[2]

BY R. K. RALPH, R. J. YOUNG AND H. G. KHORANA

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The conversion of a phosphomonoester group to the coresponding phosphoranilidate was developed as a method for the labeling of those ends of polynucleotide chains which bear such groups. The method involved the reaction of the oligo- or polynucleotide with diisopropylcarbodiimide in the presence of an excess of aniline in a mixture of water, dimethylformamide and *tert*-butyl alcohol at room temperature and a constant pH of about 8. The conversion of the 5′-phosphate groups in oligonucleotides pApApA and pUpUpUpU and amino acid acceptor RNA was 60–80% complete in 24–48 hr. The conditions used did not cause any detectable isomerization (C_3'–C_5' → C_2'–C_5') of the inter-ribonucleotidic linkages. For determination of the nucleotide sequences at the phosphomonoester terminus of acceptor ribonucleic acids (RNA), the labeling was carried out with C^{14}-labeled aniline. The reaction was carried out on RNA itself or on the mixture of oligonucleotides obtained by pancreatic ribonuclease action followed by removal of mono- and dinucleotides by chromatography. Subsequent steps included digestion with spleen phosphodiesterase and anion exchange chromatography. Many radioactively labeled oligonucleotide peaks containing 5′-phosphoranilidate end groups were thus obtained. The major peak corresponded to the anilidate of the dinucleotide pGpCp. The total results obtained by this technique showed: (1) while the majority of the acceptor RNA chains end in pG group, some chains end in pA and one or more may have the pU end group. (2) A considerable proportion of the chains have the terminal dinucleotide sequence pGpC; however, in contrast with the common sequence (pCpCpA) at the amino acid acceptor (right) terminus, the chains become distinctive soon after the pG or pA end groups at the left terminus. Recently proposed models of acceptor RNA structure are discussed in the light of the present findings.

Introduction.—Recent studies on the biosynthesis of polypeptide chains in cell-free systems have led to the recognition of a number of discrete steps.[3] The activation of the α-amino acids occurs by pyrophosphorolysis of adenosine-5′ triphosphate to form aminoacyladenylates. The latter donate the aminoacyl

(1) Paper XX: D. H. Rammler and H. G. Khorana, *J. Am. Chem. Soc.*, **85**, 1997 (1963).

(2) This work has been supported by grants from the National Science Foundation, Washington, D. C., The National Cancer Institute of the National Institutes of Health, Bethesda, Md., and the Life Insurance Medical Research Fund, New York, N. Y.

(3) There are a large number of excellent review articles which summarize the recent developments: (a) P. C. Zamecnik, "Harvey Lectures," Series LIV (1958–1959), p. 256; (b) M. B. Hoagland in "The Nucleic Acids," E. Chargaff and J. N. Davidson, Ed., Vol. III, Academic Press, Inc., New York, N. Y., 1961, p. 349; (c) F. Lipmann, W. C. Hulsmann, G. Hartman, H. G. Boman and G. Acs, *J. Cellular Comp. Physiol.*, **54**, 75 (1959); (d) P. Berg, *Ann. Rev. Biochem.*, **30**, 293 (1961); (e) M. V. Simpson, *ibid.*, **31**, 333 (1962).

groups to the 2′- or 3′-hydroxyl group of terminal adenosine residues of a class of low molecular weight ribonucleic acids, designated variously as soluble, acceptor, carrier, transfer or adapter ribonucleic acids (RNA). There is believed to be at least one, and probably more than one, specific acceptor RNA[4] chain for each amino acid. The acceptor RNA serves as the carrier of the activated amino acids to the site of protein synthesis. Because of the central importance of the acceptor RNA in information transfer in biological systems, the problems of separation and sequential analysis of these molecules are currently under study in a number of laboratories.

The available information on the acceptor RNA relevant to nucleotide sequences may be summarized. All of the amino acid acceptor chains are rather similar in size, containing 70–90 nucleotide residues[3,5] and all the chains terminate in the common sequence pCpCpA,[4] the terminal adenosine carrying the aminoacyl groups.[3,6] At the opposite terminus most of the chains have been concluded to have a pG[4] end group.[7] That the majority of the sequences in the different acceptor RNA chains are statistically different has been forcefully demonstrated by Holley and co-workers[8] by a study of highly purified amino acid acceptor RNA chains. Heterogeneity in nucleotide sequences after the terminal pCpCpA sequence has also been shown by Berg and co-workers[9] and by Herbert and Wilson.[10] Aside from these studies on absolute nucleotide sequences, only statistical data[11] have so far been available on the chemical structures of the total mixtures of acceptor RNA.

In the present paper, we report on the initial phase of our work on the radioactive labeling of the 5′-phosphomonoester end groups in amino acid acceptor ribonucleic acids. This approach may be regarded as complementary to the enzymatic approach developed previously by Berg[9] and by Herbert[10] for the P^{32}-labeling of the pCpCpA terminus. A preliminary investigation has been carried out of the nucleotide sequences adjacent to the 5′-phosphomonoester terminus and this shows that while the majority of the acceptor RNA chains have the terminal pG residue, some end in a pA group and a few per cent of the total number of chains probably have a pU end group. While a considerable proportion (about 40%) of the total chains have the terminal dinucleotide sequence pGpCp, there is extensive heterogeneity with respect to sequences immediately after the terminal nucleotide. A brief report of these findings has been made previously.[12]

Conversion of 5′-Phosphomonoester Groups to 5′-Phosphoromorpholidates in Mono- and Oligoribonucleotides.—Previously[13] an approach to the marking of phosphomonoester groups in oligonucleotides was described which involved the reaction of the terminal groups with dicyclohexylcarbodiimide in the presence of an excess of methyl alcohol to form monomethyl esters. Although in this approach no side reactions could be detected when non-radioactive methyl alcohol was used, experiments at the start of the present work using C^{14}-methyl alcohol revealed side reactions[14,15] which proved serious in application of the method to the acceptor RNA. Thus the total radioactivity incorporated into the acceptor RNA was far in excess of that which would be expected for selective methylation of the phosphomonoester groups. Despite attempts at changing the conditions of the reaction and use of ethyl alcohol, a somewhat less reactive alcohol than methyl alcohol, incorporation of the label selectively at the 5′-phosphate terminus of the acceptor RNA was not realized. The approach was therefore abandoned.

The approach developed in the present work aimed at the activation of a phosphomonoester group by means of a carbodiimide reagent to form a phosphoramidate.[16] Since it was important that the phosphoramidate linkage have adequate stability during conditions to be used subsequently for chemical or enzymic degradation of the polynucleotide chains, on the basis of previous knowledge,[16] derivatives of aromatic amines appeared suitable. Further, because of its commercial availability in C^{14}-labeled form, aniline was chosen as the amine.[17]

The standard method used in all the previous work[16,18] involved the healed reaction of a phosphomonoester with dicyclohexylcarbodiimide in the presence of an amine. A serious side reaction was the formation of guanidines of the type I, the accumulation of which inhibited the reaction.

$$C_6H_{11}N{=}C(NHR)NHC_6H_{11} \quad \text{I}$$

However, by carrying out reactions at elevated temperatures it proved possible to drive the amidate forma-

(4) (a) Amino acid acceptor ribonucleic acids is designated here simply as "acceptor RNA." The general system of abbreviations is as currently used in *J. Biol. Chem.* and previously defined in earlier papers in this series [see especially, H. G. Khorana and J. P. Vizsolyi, *J. Am. Chem. Soc.*, **83**, 675 (1961)]. Thus the single letters A, U, G and C represent the nucleosides of, respectively, adenine, uracil, guanine and cytosine. The letter "p" to the left of the nucleoside initial indicates a 5′-phosphomonoester group and the same letter to the right indicates a 3′-phosphomonoester group. Alkaline hydrolysis of an inter-ribonucleotide bond would give a mixture of 2′- and 3′-phosphomonoester groups. This mixture is also simply represented by the letter p to the right of the nucleoside initial. In polynucleotides, it follows that in going from left to right, the chain is specified in the $C_3' \rightarrow C_5'$ direction. (b) The common -pCpCpA end of acceptor RNA chains is designated as the right end while the opposite terminus is the left terminus.

(5) (a) See in particular ref. 3d for a full discussion; (b) W. A. Klee and G. L. Cantoni, *Proc. Natl. Acad. Sci. U. S.*, **46**, 322 (1960).

(6) H. G. Zachau, G. Acs and F. Lipmann, *ibid.*, **44**, 885 (1958); J. Preiss, P. Berg, E. J. Ofengand, F. H. Bergmann and M. Dieckmann, *ibid.*, **45**, 319 (1959); R. S. Schweet, F. C. Bound, E. Allen and E. Glassman, *ibid.*, **44**, 173 (1958).

(7) M. F. Singer and G. L. Cantoni, *Biochim. Biophys. Acta*, **39**, 182 (1960); W. Zillig, D. Schactschabel and W. Krone, *Z. physiol. Chem.*, **318**, 100 (1960).

(8) R. W. Holley, J. Apgar, S. H. Merrill and P. L. Zubkoff, *J. Am. Chem. Soc.*, **83**, 4861 (1961).

(9) U. Lagerkuist and P. Berg, *J. Mol. Biol.*, **5**, 139 (1962); P. Berg, U. Lagerkuist and M. Dieckmann, *ibid.*, **5**, 159 (1962).

(10) E. Herbert and C. W. Wilson, *Biochim. Biophys. Acta*, **61**, 750, 762 (1962).

(11) See, *e.g.*, K. S. McCully and G. L. Cantoni, *J. Mol. Biol.*, **5**, 80 (1962); *J. Biol. Chem.*, **237**, 3760 (1962); T. Nihei and G. L. Cantoni, *Biochim. Biophys. Acta*, **61**, 463 (1962); V. M. Ingram and J. G. Pierce, *Biochem.*, 1, 580 (1962).

(12) R. J. Young, R. K. Ralph, P. T. Gilham and H. G. Khorana, *Federation Proc.*, **21**, 372 (1962); R. K. Ralph, R. J. Young and H. G. Khorana, *J. Am. Chem. Soc.*, **84**, 1490 (1962).

(13) H. G. Khorana, *ibid.*, **81**, 4657 (1959).

(14) A side reaction occurring at a level of under 1% per nucleotide group would not be readily detectable by the usual techniques. However, the use of the much more sensitive radioactive technique would readily detect such side reactions. It is also emphasized that for the introduction of a C^{14}-label specifically at the terminus of a long polynucleotide chain, a side reaction occurring even at the level of a fraction of a per cent is undesirable. The seriousness of the interference from the side reaction would increase with increase in the length of the polynucleotide chain.

(15) The side reactions leading to the incorporation of the C^{14}-label in the interior of the chain in the previously described approach perhaps involve the addition of methyl alcohol to dicyclohexylcarbodiimide as the first step. The resulting *O*-methylisourea could cause methylation of the heterocyclic rings.

(16) (a) R. W. Chambers and H. G. Khorana, *J. Am. Chem. Soc.*, **80**, 3749 (1958); (b) J. G. Moffatt and H. G. Khorana, *ibid.*, **83**, 649 (1961).

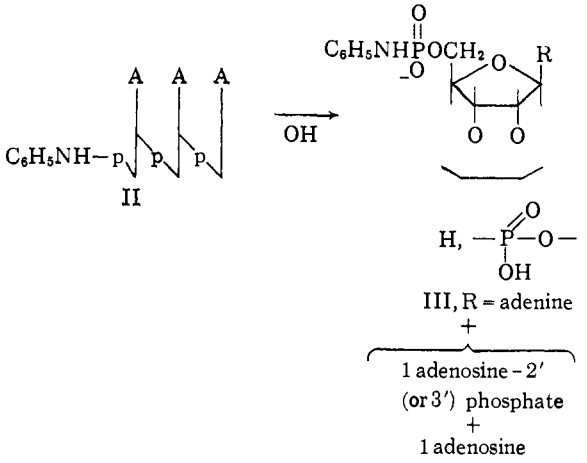
(17) All of the ribonucleoside-5′ phosphoranilidates were prepared essentially by the method previously reported[16b] and their stabilities at 37° and different pH's studied. The results which are reported in the Experimental section showed satisfactory stability at pH 4 and above.

(18) J. G. Moffatt and H. G. Khorana, *J. Am. Chem. Soc.*, **83**, 663 (1961). It should further be noted that under the conditions previously used diesters of phosphoric acid appeared to be inert to further reaction. For example, adenosine-2′(or 3′),5′ diphosphate was converted quantitatively to adenosine-2′,3′-cyclic phosphate, 5′-phosphoromorpholidate.

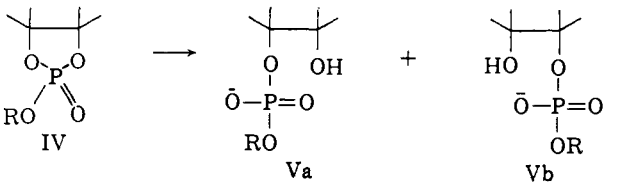
tion to completion. Since these conditions were expected to be rather severe for work with ribopolynucleotides, milder conditions were sought. An important technical problem was that of devising a solvent system which would give homogeneous reaction mixtures with the polynucleotides and the carbodimide reagent, components with contrasting solubility properties. The solvent system found satisfactory was a mixture of water, dimethylformamide and *tert*-butyl alcohol, the carbodiimide used being diisopropylcarbodiimide. To circumvent the retardation of the reaction rate through accumulation of the guanidine (I, R = phenyl), the reactions were carried out at a constant pH around 8 using a pH Stat which automatically added 1 *N* hydrochloric acid to offset the basicity resulting from the formation of the guanidine base. Further, a large excess (750 moles/mole of the phosphomonoester end group) of aniline was used in order to avoid the possibility of amidate formation with the amino groups of the heterocyclic bases. Under these conditions, adenosine-5′ phosphate was converted to adenosine-5′ phosphoranilidate in over 80% yield in about 12 hr., a large amount (300 moles/mole of the nucleotide) of the guanidine (I, R = phenyl) being concomitantly formed. The formation of the latter was indicated by the amount of the acid consumed to keep the pH constant during the reaction. By the same technique, the terminal phosphomonoester group in the trinucleotide, pApApA (1-μmole scale experiment), was converted in 70–80% yield to the corresponding phosphoranilidate (shorthand formulation II).[19] The latter was the only new nucleotidic product detected and it was characterized by the products of alkaline degradation (Chart I). Adenosine-2′(or 3′) phosphate, 5′-phosphoranilidate (III), adenosine-2′(or 3′) phosphate and adenosine were formed in equal amounts.

CHART I

PRODUCTS OF ALKALINE HYDROLYSIS OF THE TRINUCLEOTIDE-PHOSPHORANILIDATE (II)



Two further experiments were then carried out to check if any reaction with the C_3'–C_5' inter-ribonucleotidic linkage occurred. Any activation of the internucleotidic linkage would result in the formation of a trisubstituted ester (partial formula IV) which would hydrolyze to give roughly an equal mixture of C_2'- and C_3'-linked diesters Va and Vb as the main products, in addition to some 2′,3′-cyclic phosphate.[20] A sensitive test for detection of any activation of the inter-ribonucleotidic linkage was therefore considered to be



degradation of the products with a specific phosphodiesterase (for example, pancreatic ribonuclease). Products of the type Vb if formed, would be resistant, the starting C_3'-esters being fully susceptible. In the first experiment uridine-3′ methyl phosphate was subjected to the standard reaction conditions and then re-isolated by paper chromatography. From its suceptibility to pancreatic ribonuclease no isomerization to uridine-2′ methyl phosphate could be detected. The second experiment was carried out with the tetranucleotide pUpUpUpU. Reaction under the standard conditions resulted in conversion of the terminal group to the 5′-phosphoranilidate in about 80% yield and the product appeared to be completely susceptible to the action of pancreatic ribonuclease.[21] The method was then tested with acceptor RNA using C^{14}-aniline.

Labeling Experiments with Acceptor RNA and its Degradation Products.—The first experiment carried out on acceptor RNA under the standard conditions[22] included 0.1 mc. of C^{14}-aniline in a total of 0.75 mmole of this base. Since the first aim was to ascertain if the radioactivity incorporated into the acceptor RNA was not random, the labeled RNA after work-up was hydrolyzed to mononucleotides with alkali. Figure 1

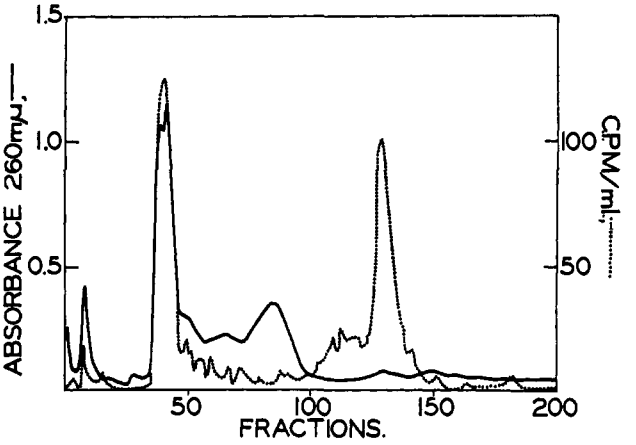


Fig. 1.—Chromatography of alkaline hydrolysate of labeled acceptor RNA on a DEAE–cellulose (carbonate) column (25 cm. × 2 cm. dia.) For conditions of elution see text.

shows the pattern of elution of the ultraviolet-absorbing material and radioactivity on chromatography of the alkaline hydrolysate. Although a considerable proportion of the radioactivity was eluted in the mononucleotide region, analysis showed the radioactivity not to be associated with the nucleotidic material. Thus, paper electrophoresis of an aliquot of the combined peak showed the radioactivity to migrate much faster than the nucleotidic material, the latter being a mixture, mainly, of adenosine-, uridine- and cytidine-2′(3′) phosphates. Treatment of the material

(19) For the system of shorthand formulations see H. G. Khorana, "Some Recent Developments in the Chemistry of Phosphate Esters of Biological Interest," John Wiley and Sons, Inc., New York, N. Y., 1961, Chapter 5.

(20) D. M. Brown, D. I. Magrath and A. R. Todd, *J. Chem. Soc.*, 4396 (1955).

(21) Very faint bands (ultraviolet-absorbing) were detected on paper chromatograms. Their amounts were too small for identification tests. These could have originated from the enzymic method used for preparation of the tetranucleotide. Trace contaminations by C_2'–C_5' linkages seemed unlikely but could not be excluded in this experiment.

(22) C^{14}-Aniline supplied as the hydrochloride caused the formation of some precipitate in the experiments in which polynucleotides were used as triethylammonium salts. The addition of stoichiometric amounts of 4-morpholine-N,N,-dicyclohexylcarboxamidine[16b] prevented the formation of the precipitate.

in fractions 35–45 with bacterial alkaline phosphatase followed by paper chromatography again showed the radioactivity to be non-coincident with the nucleosides or related compounds.[23] (Experiments described below further confirmed that interference from side reactions was not serious). The small peak of radioactivity eluted in fractions 110–120 contained a nucleotide which was identified as III (R = adenine). The identification was based on ultraviolet absorption spectrum characteristics, identical mobility in solvent B with a sample of III obtained by alkaline hydrolysis of the phosphoranilidate II and by direct comparison of the product, obtained by digestion with bacterial alkaline phosphomonoesterase, with authentic adenosine-5′ phosphoranilidate. The expected anilidate corresponding to guanosine (III, R = guanine) was present in the next main radioactivity peak eluted in fractions 121–139 and it was also characterized by techniques just described. It should be added that by alkaline hydrolysis of a portion of the labeled acceptor RNA and separation by a combination of paper electrophoresis (pH 5) and paper chromatography (solvent B), conversion of the end 5′-phosphate groups to the corresponding anilidates was determined to be in the range of 60–80%.

Further experiments on determination of nucleotide sequences adjoining the labeled terminus were carried out, either after labeling the acceptor RNA itself or by labeling of the mixture of oligonucleotides containing 5′-phosphate end groups obtained by prior degradation of acceptor RNA with pancreatic ribonuclease. Since the results were similar (see Experimental), only the experiments on the rinonuclease digest of acceptor RNA are discussed in detail.

An exhaustive pancreatic ribonuclease digest of acceptor RNA was fractionated by a convex gradient chromatographic technique. The elution pattern of ultraviolet-absorbing material is shown in Fig. 2. Soon after the emergence of the dinucleotides, the total of the higher oligonucleotides was eluted with 1 *M* triethylammonium bicarbonate. This mixture (fractions 110 to end, Fig. 2) was ascertained to contain all of the oligonucleotides containing pA and pG end groups while a little pUp was identified in the dinucleotide fraction (fractions 54–110 of Fig. 2) (see Experimental). The 1 molar eluate, which would consist of oligonucleotides of the types A and, mainly, B (Chart II), was subjected to the standard labeling technique. It should be noted that during the activation with the carbodiimide reagent, while the 5′-phosphate groups of terminal oligonucleotides (type A, Chart II) would be converted to phosphoranilidates, the 3′-phosphate end groups of all of the oligonucleotides would be converted to 2′,3′-cyclic phosphates.[13,18]

The total oligonucleotide mixture following the labeling technique was digested exhaustively with spleen phosphodiesterase.[24a,b] It is recalled that the mode of action of this enzyme on oligonucleotides has been established to be stepwise from the end of the chain bearing a 5′-hydroxyl group[24] and that the presence of a 5′-phosphomonoester group confers resistance toward the enzyme.[24b,25] Digestion of the complex oligonucleotide mixture obtained above was

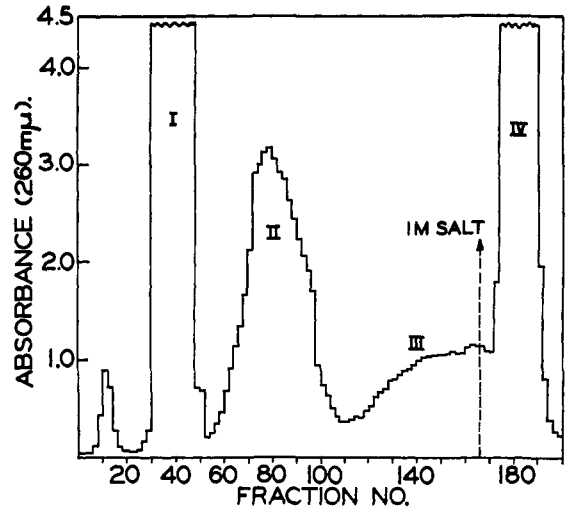


Fig. 2.—Chromatography of pancreatic ribonuclease digest of acceptor RNA on a DEAE–cellulose (carbonate) column using convex gradient technique. For details see text.

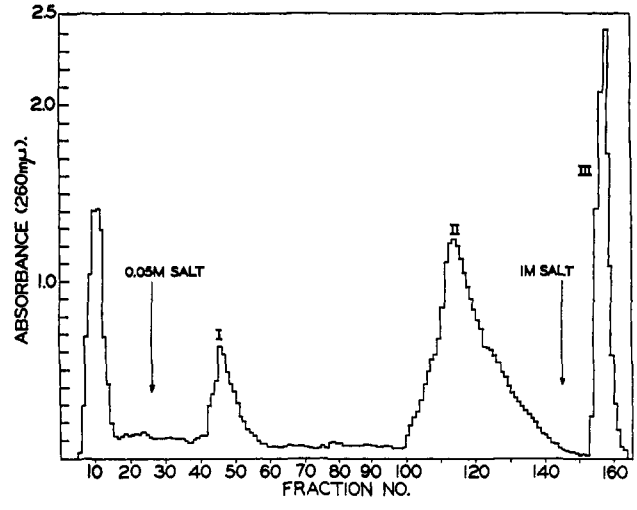
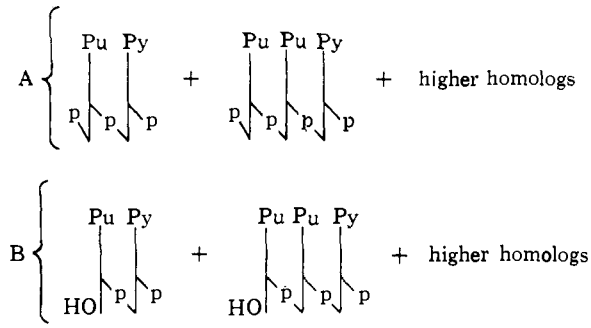


Fig. 3.—Separation of products of spleen phosphodiesterase action from resistant labeled oligonucleotides. For details see text.

therefore performed with a view to degrading to mononucleotides the oligonucleotides of the type B which were derived from the body of the original polynucleotide chains and therefore formed the major portion of the total oligonucleotide mixture. Subsequent chromatography (Fig. 3) showed, as expected, the presence of some cyclic mononucleotides, a major fraction corresponding to ribonucleoside-3′ phosphates and, subsequently, the undegraded oligonucleotide

CHART II

TYPES OF OLIGONUCLEOTIDES IN PANCREATIC RIBONUCLEASE DIGEST OF ACCEPTOR RNA



(23) The conclusion is drawn that the radioactivity in the fractions 35–45 may represent the phosphoranilidate derivative of either inorganic phosphate or polyphosphate which may have contaminated the acceptor RNA preparation used. It should also be noted that the specific radioactivity as a function of ultraviolet absorption was much lower in fractions 35–45 than that in fractions 100–150.

(24) (a) R. J. Hilmoe, *J. Biol. Chem.*, **235**, 2117 (1960); (b) W. E. Razzell and H. G. Khorana, *ibid.*, **236**, 1144 (1961).

(25) L. A. Heppel and R. J. Hilmoe, in "Methods in Enzymology," Vol. II, N. O. Kaplan and S. P. Colowick, Ed., Academic Press, Inc., New York, N. Y., 1955, p. 565.

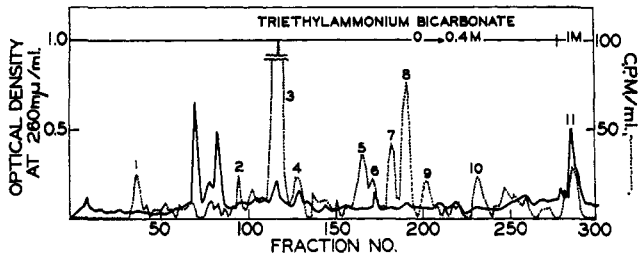


Fig. 4.—Chromatography of labeled oligonucleotides (approximately 270 optical density units at 260 mμ) on a DEAE–cellulose (carbonate) column (1 cm. × 50 cm.); elution was with a linear gradient 0–0.4 *M* triethylammonium bicarbonate (pH 7.5) with 2 l. in each vessel; rate of flow was 0.5 ml./min., ten ml. fractions being collected; 1 ml. of each tube was plated and radioactivity measured in a thin end window gas flow counter; ultraviolet absorption, solid line; radioactivity, broken line.

material was eluted as a sharp peak with a high salt concentration. Although several-fold concentration of the oligonucleotides containing the labeled-end groups was thus effected, it appears that complete removal of all the oligonucleotides except those containing the 5′-phosphoranilidate groups was not realized.[26] It was encouraging that no significant radioactivity was present in the mononucleotide fraction, the major part of the total nucleotidic material, and that essentially all of the original radioactivity was recovered in the resistant 1 molar salt fraction of Fig. 3.

The oligonucleotides present in the 1 molar eluate (Fig. 3) were subjected to the action of pancreatic ribonuclease to convert the terminal 2′,3′-cyclic phosphate groups to 3′-phosphomonoester groups and then separated on a DEAE–cellulose (carbonate) column and gave the elution pattern shown in Fig. 4. The distribution of the ultraviolet-absorbing material and of the radioactivity in the different peaks is given in Table III. The analysis carried out on the pooled radioactive peaks gave the following results (see also Table III).

Phosphomonoester End Groups and Adjoining Sequences in Acceptor RNA.—Peak 3[27] **(Fig. 4)** contained about 40% of the total radioactivity and may therefore be regarded as representing 40% of the total terminal sequence of all the different acceptor RNA species. Chromatography of the pooled peak in solvent B showed the radioactivity to be associated mainly with one nucleotidic band, which was homogeneous also when tested by paper electrophoresis at pH 7.5. After alkaline hydrolysis and separation of the resulting products by paper electrophoresis (pH 5), it gave Anil-pGp[28] and Cp in equal amounts. Further, alkaline hydrolysis after treatment with phosphomonoesterase gave Anil-pGp and cytidine in equal amounts. The original compound was thus concluded to be Anil-pGpCp.

Another minor radioactive nucleotidic product was also present in peak 3. It traveled faster than the above main product in solvent B. Although its identity could not be established, it was shown to give Anil-pGp after alkaline hydrolysis.

(26) About 20% of the total ultraviolet-absorbing material subjected to the action of the spleen phosphodiesterase remained undegraded. This would correspond to about 10% of the total optical density of the ribonuclease digest (some 50% of the total was removed during preliminary fractionation; Fig. 2). The amount to be expected statistically for oligonucleotides containing 5′-phosphate end groups and formed on ribonuclease digestion would be expected to be certainly under 5% of the total ultraviolet absorption present in the ribonuclease digest.

(27) Peaks 1 and 2 and 4 of Fig. 4 remain unidentified.

(28) Abbreviation for guanosine-2′(or 3′) phosphate-5′ phosphoranilidate (III, R = guanine). Similarly, the adenosine analog is abbreviated to Anil-pAp (III, adenine).

Peak 5[27] **(Fig. 4)** was first digested with alkaline phosphomonoesterase and the products hydrolyzed with alkali. Chromatography in solvent D showed mainly cytidine and a small amount of uridine as the nucleosides. The nucleotidic products (remaining at origin on chromatography in solvent D) were digested with alkaline phosphomonoesterase and the products were shown by a combination of paper chromatography and paper electrophoresis to be C^{14}-labeled guanosine-5′ phosphoranilidate (Anil-pG), adenosine and guanosine. From these results, peak 5 is concluded to contain either a trinucleotide with Anil-pGp at one end and Cp at the other and/or the dinucleotide Anil-pGpUp. It should be noted that since the radioactive containing material in this peak was not purified, it is also possible that pGpUp (from unlabeled portion of the end group) was the uridine-containing dinucleotide in this peak.

Peak 6 (Fig. 4) contained Anil-pAp[27] as the end group as determined by examination of the products after alkaline hydrolysis by the standard technique described in Experimental section. The peak is therefore concluded to represent a trinucleotide of the structure Anil-pAp(A or G)p(C or U)p.

Peak 7 (Fig. 4) was shown to contain Anil-pGp end group while peak 8 gave after alkaline hydrolysis both Anil-pGp and Anil-pAp. Both these peaks are also concluded to contain trinucleotides with pG and pA end groups.

Peaks 9, 10 and 11 of Fig. 4 also were shown to give after alkaline hydrolysis Anil-pGp (peak 9 could contain Anil-pAp end group). It should also be noted that 1 molar fraction (peak 11) also gave pGp after alkaline hydrolysis, showing that the labeling of end groups was indeed incomplete.

Discussion.—A method for the radioactive marking of the phosphomonoester end groups in polynucleotide chains has been developed. The reaction used causes cyclization of the 2′- or 3′-phosphomonoester groups in ribopolynucleotides and is therefore applicable only to the labeling of 5′-phosphomonoester groups in such polynucleotides. In deoxyribopolynucleotides, however, it would be expected to label both the 3′- and 5′-phosphomonoester end groups.

Although in the application to acceptor RNA not all of the radioactivity appeared to be associated with the end group, it has been demonstrated that interference from any side reactions is not serious. Indeed, from the detailed analysis of Fig. 3 and 4, it was clear that essentially all the radioactivity was associated with phsophate end groups. Furthermore, since the radioactivity released in the mononucleotide region in Fig. 1 was in fact shown not to be a part of any nucleotidic material, the possibility exists that the radioactivity was accepted by a low molecular weight impurity present in the acceptor RNA sample used. From the total of results, the method is concluded to have adequate specificity for labeling and therefore detecting phosphomonoester groups in polynucleotides.

Previously only pG has been recognized as the terminal group in acceptor RNA and from the equivalence of pGp formed on alkaline hydrolysis with the nucleoside released from the opposite terminus it has been previously concluded that all acceptor RNA chains terminate in pG chains. In the present work, the amount of pGp isolated pure was in the range of 60–70% of the total nucleoside formed from the opposite terminus.

On the other hand, in the present work, pA and a very small amount of pU were definitely recognized as end groups in acceptor RNA. The comment has recently been made by Cantoni, *et al.*, that the pA end groups may have their origin in non-acceptor RNA or

from partial enzymic digestion of the acceptor RNA samples used in our work. While these possibilities cannot be rigorously excluded until pure acceptor RNA specific for individual amino acids are examined, it should be pointed out that the samples used in this work had been carefully chromatographed on DEAE-cellulose columns using sodium chloride gradient and the acceptor RNA recovered emerged as a sharp peak under these conditions. Further, the presence of pA end groups in acceptor RNA has been confirmed in more recent work[29] using another method for labeling 5′-phosphate groups.[30]

The present work has shown that in contrast with the common pCpCpA sequence at the right terminus, the sequences at the left terminus become largely heterogeneous immediately after the terminal nucleotide. True, about 40% of the total terminal sequence was identified as pGpC and in this portion there could be longer stretches of common sequence. However, several radioactive peaks in the trinucleotide region were found and it is possible that all of the four possible trinucleotide sequences pGp(A or G)p(C or U)p are present and, in addition, trinucleotides with pA end groups. A certain fraction of higher oligonucleotides was also evidently present (1 molar fraction of Fig. 4). Further detailed analysis of the terminal sequences on the total mixture of acceptor RNA would only be of statistical value and it is therefore proposed to carry out such analysis on purified acceptor RNA activities.

That acceptor RNA has considerable secondary structure has been clear for some time.[3d,31] However very specific models have recently been proposed[32,33] which postulate DNA-like complementary structure for the greater part of the molecules, the one half folding back onto the second half. A specific prediction[34] has been made for the "doubling-up" to begin by Watson–Crick type of hydrogen bonding between the third cytidine of the right terminus with the pG end group of the left terminus. Since considerable progress has been and is likely to be made in the near future in the determination of absolute sequences from the right terminus, complementary progress from the pG terminus should be decisive in considerations of the proposed models. The findings to date do not provide support for the prediction of specific base pairing to begin with the third nucleotide (C) from the right end. The fourth nucleotide from the same end has been shown to be A to the extent of 55–60% in liver and yeast acceptor RNA[10] and to the extent of 65% in *E. coli* RNA,[9] the remainder 15–20% being G. The base-pairing postulate mentioned above would then predict the second nucleotide from the pG end to be 55–60% U and 15–20% C in yeast acceptor RNA. In the present work, approximately 40% pGpC was found, but the pGpU sequence, if present in one of the peaks after peak 3 (Fig. 4), was certainly not a major common sequence. Furthermore, there is the finding that about 15% of total end group in acceptor RNA is pA.

It is clear that the determination of absolute nucleotide sequences from the pG and pA (*left*) terminus would be most revealing for proposals of the secondary structures of acceptor RNA. Further work along these lines will be reported in forthcoming publications.

(29) (a) R. J. Young and H. G. Khorana, *J. Am. Chem. Soc.*, **85**, 244 (1963). (b) Very recently, the presence of pA, pU and a little pC end groups in addition to the pG end group in yeast acceptor RNA has been demonstrated in another laboratory: D. Bell, R. V. Tomlinson and G. M. Tener, *Biochem. Biophys. Research Commun.*, **10**, 304 (1963).

(30) The possibility of some acceptor RNA chains lacking any 5′-phosphate end groups should also be considered in the future work. Such end groups would go undetected in alkaline hydrolysis and the present technique of labeling 5′-phosphate end groups.

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Experimental

General Methods. (1) **Paper chromatography** was performed by the descending technique using mostly Whatman paper No. 40. The solvent system used were: solvent A, isopropyl alcohol–cond. ammonia–water (7:1:2); solvent B, *n*-propyl alcohol–concd. ammonia–water (55:10:35); solvent C, ethyl alcohol–1 *M* ammonium acetate (pH 7.5) (7:2.5, v./v.); solvent D, *n*-butyl alcohol–water (86:14); solvent E, ethyl alcohol–0.5 *M* ammonium acetate (pH 3.8, 7:2.5, v./v.).

(2) **Paper electrophoresis** was performed mostly in an apparatus similar to that described by Markham and Smith.[35] The buffers used were: 0.1 *M* triethylammonium bicarbonate (pH 7.5) and 0.1 *M* triethylammonium acetate (pH 5), both freshly made. For the compounds which carried the phosphoranilidate group the separation was carried out in a 2° room.

The bands or spots, ultraviolet absorbing and/or radioactive, were eluted from paper mostly by descending chromatography using weakly ammoniacal water. For guanosine compounds 1 *M* ammonia was used.

(3) **Counting and Scanning of Chromatograms Containing Radioactive Compounds.**—For counting, appropriate aliquots were plated on aluminum planchets and after drying counted with a thin mica window gas flow counter the efficiency of C^{14} counting being about 15%. The plating was carried out so as to give infinite thinness. For scanning of paper chromatograms a Baird Atomic 4π gas flow windowless paper scanner was used.

(4) **Enzymic Degradations.**—The phosphomonoesterases used were the bacterial alkaline phosphomonoesterase.[36] A highly purified preparation containing about 2 mg. protein/ml. and free from any detectable ribonuclease or deoxyribonuclease activity was furnished by Dr. J. Schwartz of The Rockefeller Institute. The standard conditions used were: 0.2–0.5 μmole of the compound in a total volume of 0.05–0.1 ml. including 0.01 ml. of 0.5 *M* tris-hydroxymethylaminomethane buffer (pH 8) and 0.005 ml. of the enzyme preparation. The incubation was at 37° for 2–4 hr. A prostatic phosphomonoesterase preparation free from any detectable phosphodiesterase activity was generously given by Dr. G. Schmidt of Tufts University Medical School, Boston, Mass. The standard conditions for dephosphorylations of mono- and oligonucleotides with this preparation were: about 1 μmole of adenosine-2′(3′) phosphate, 0.03 ml. of 1 *M* ammonium acetate buffer pH 5.9 and 0.05 ml. of the enzyme preparation were made up to a total volume of 3 ml. The dephosphorylation was complete within a 15-min. period of incubation at 37°. The release of inorganic phosphate was determined by the method of Chen, *et al.*,[37] as modified by Ames and Dubin.[38]

Spleen phosphodiesterase was prepared by the method of Hilmoe.[24a] The preparation thus obtained was then taken through the calcium phosphate gel step devised by Razzell and Khorana.[24b] This last step was necessary to remove some phosphomonoesterase and, in addition, an activity which deaminated adenosine to inosine. All preparations were standardized against thymidine-3′ *p*-nitrophenyl phosphate prior to use with oligonucleotides. The standard conditions used for oligonucleotides were: oligonucleotide mixture (about 200 optical density units at 260 mμ) in a total volume of 0.6 ml.; 1 *M* ammonium acetate buffer, pH 5.9, 0.2 ml.; ethylene diaminetetraacetate, pH 6.5 (0.1 *M*), 0.02 ml.; spleen phosphodiesterase preparation, 0.2 ml. Degradation to mononucleotides was complete in 4 hr. at 37°. The degradation by this enzyme was followed by removing aliquots and further incubation with prostatic phosphomonoesterase. No phosphomonoesterase activity was detected in the preparation of spleen phosphodiesterase. This was shown by a test experiment in which after incubation of the ribonuclease digest of the amino acid acceptor ribonucleic acid with this enzyme, the resistant oligonucleotides were hydrolyzed with 1 *N* potassium hydroxide. The recovery of guanosine diphosphate (pGp) was quantitative.

Degradation by the crystalline pancreatic ribonuclease (a gift of Armour and Co., Chicago, Ill.) was performed either in a pH stat, the pH being maintained constant at 7.0 by the automatic addition of 0.1 *N* sodium hydroxide. Alternatively the following conditions were used: An aqueous solution (0.5 ml.) of the amino acid acceptor ribonucleic acid (6 mg.) in 0.2 *M* tris-acetate buffer pH 7.5 was treated with 0.4 mg. of the crystalline enzyme. In larger scale runs a substrate to enzyme ratio by weight of 15:20 was maintained.[39] Incubation was carried out for about 18 hr. at 37°. Toluene was added after the first 4–5 hr. to avoid bacterial contamination. The preparation of ribonuclease used was

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checked to be completely free from phosphomonoesterase by incubation of guanosine-5′ phosphate with an excess of the enzyme for 4 hr.

(5) **Column chromatography** was performed mostly on a column of DEAE–cellulose in the carbonate form. The commercially procured exchanger was thoroughly stirred in 5-bed volume of 0.1 *N* sodium hydroxide and then filtered from alkali on a Buchner funnel. It was resuspended in fresh 0.1 *N* sodium hydroxide solution and recollected by suction. It was washed thoroughly with water and then suspended in a large excess of 2 *M* ammonium carbonate solution. The exchanger as a suspension in this salt was continuously stirred and packed into the appropriate glass column using a head pressure of about 8 ft. The packed exchanger was washed overnight with 2 *M* ammonium carbonate to ensure the exchanger in the carbonate form and then washed thoroughly with water before use.

For chromatography a linear or a convex gradient of triethylammonium bicarbonate (pH 7.5) was used as described in the individual experiments.

Materials.—Ribonucleoside-5′ phosphates were commercially procured samples which were carefully checked for purity by paper chromatography at 1–2 μmoles/1 inch spot level. Uridine-3′ methyl phosphate and uridylyl-(3′→5′)-uridine were synthetic samples prepared by Dr. Rammler.[40] Diisopropylcarbodiimide[41] and C^{14}-aniline[42] were procured commercially.

The tetranucleotide, pUpUpUpU, and the trinucleotide, pApApA, were prepared by degradation of polyadenylic acid and polyuridylic acid, respectively, with the pork liver nuclei enzyme, a preparation which was made in the laboratory of Dr. Leon A. Heppel. The conditions of degradation for the uridine tetranucleotide preparation were as follows:

Uridine Tetranucleotide, pUpUpUpU.—Polyuridylic acid (3055 optical density units at 260 mμ) in 30 ml. of water; tris-acetate buffer pH 7.1, 3.3 ml. of 0.5 *M*; 0.1 *M* magnesium chloride, 1.34 ml.; 0.25 *M* sodium fluoride, 5 ml.; 2% bovine albumin solution, 32 ml.; 0.05 *M* mercaptoethanol, 0.07 ml.; enzyme preparation, 0.5 ml. The mixture was incubated at 37° for 11.5 hr. and was then boiled for 4–5 min. The coagulated protein was removed by centrifugation (chromatography of an aliquot in solvent B separated the digest into six bands). The total digest was diluted to 100 ml. with water to reduce the concentration of anions to below 0.015 *M*, the pH adjusted to 8 with ammonia. The total was then applied to a column (24.5 cm. × 3.5 cm.) of DEAE–cellulose (bicarbonate form). Elution was carried out with a linear gradient of triethylammonium bicarbonate, the mixing vessel contained 4 l. of water and the reservoir an equal volume of 0.25 *M* triethylammonium bicarbonate (pH 7.5). At the end of this gradient, elution was continued with a gradient of 0.25 *M* to 0.45 *M* triethylammonium bicarbonate, there being 4 l. of volume in each vessel. The column was washed finally with 1 *M* triethylammonium bicarbonate. Sixteen well separated peaks were thus obtained. Most of the peaks proved to be mixtures of compounds bearing terminal 5′-phosphate, 3′-phosphate, or both or none. The desired tetranucleotide was present in peak 9. The total optical density units (260 mμ) in this peak were 338. Paper electrophoresis at pH 7.5 in 0.1 *M* triethylammonium bicarbonate buffer showed it to contain four bands. One main component was the tetranucleotide pUpUpUpU. It was purified by paper electrophoresis of the total material in peak 9 on strips of paper. The recovery of the tetranucleotide was 142 optical density units (260 mμ). It was characterized as follows:

A portion was hydrolyzed in 1 *N* potassium hydroxide for 24 hr. at room temp. After neutralization of the alkali with Dowex-50 (pyridinium form), the products were separated in solvent B. Uridine, uridine-2′(3′) phosphate and 5′-*O*-phosphoryl uridine-2′(3′) phosphate[43] were produced in the expected ratio of 1:2:1. Another portion (1 μmole of nucleotide) was incubated with pancreatic ribonuclease under the standard conditions. Complete degradation to uridine, uridine-3′ phosphate and 5′-*O*-phosphoryluridine-3′ phosphate occurred. A third portion (0.5-μmole of nucleotide) was incubated in tris-hydroxymethylaminomethane buffer, pH 8.9, 0.06 ml. of 0.1 *M*, and venom phosphodiesterase[44] (0.05 ml. of a preparation previously standardized with thymidylyl-(3′→5′)-thymidine). The incubation was for 8 hr. at 37°. Complete degradation to uridine-5′ phosphate was observed.

Adenosine Trinucleotide, pApApA.—Degradation of polyadenylic acid was carried out essentially by the above procedure except that the enzyme digest was directly chromatographed in solvent B for 2 days. The major bands corresponding to di-, tri- and tetranucleotides were pure as tested by paper electrophoresis and enzymic degradation. We are grateful to Dr. Heppel for instruction in the use of the pork liver enzyme and in isolation of the adenosine oligonucleotides.

Yeast Amino Acid Acceptor Ribonucleic Acids.—Two preparations were used, one which was prepared in the laboratory and the second which was procured commercially. Most of the work was done with the first one. The preparation started with 1250 g. of yeast cake obtained from Red Star Yeast Co., Milwaukee, Wis. The yeast was washed with iced water several times to remove nutrients and then processed according to Holley's modification[45] of Monier and Zamecnik's[46] procedure. The product was further purified by chromatography at 4° of 100-mg. portions on DEAE–cellulose (chloride form) column (25 cm. × 2.5 cm.). Elution was carried out with a linear salt gradient. Two liters of 0.1 *M* tris-hydrochloride buffer (pH 7.5) was placed in the mixing vessel and 2 l. of 1.0 *M* sodium chloride + 0.1 *M* tris-hydrochloride (pH 7.5) buffer in the reservoir. Fractions of 10 ml. per 10 min. were collected. The single, large ultraviolet absorbing peak eluted between 0.35–0.45 *M* sodium chloride concentration was collected and the product precipitated with 2 volumes of ethyl alcohol. Tailing material after the main peak was discarded. The precipitate was collected by centrifugation, washed with ethyl alcohol–water (3:1) several times, then with ethyl alcohol and dried *in vacuo*. The yield of the ribonucleic acid obtained as a white powder acid was 72 mg.

For labeling experiments, the amino acid acceptor ribonucleic acid was incubated in tris-buffer (0.1 *M*, pH 8.9) at 37° for 1 hr. to cleave the amino acyl linkages at the terminal adenosine and the solution was then dialyzed against water. The solution was then passed through a column of pyridinium Dowex-50 ion exchange resin and the total effluent and washings of the pyridine salt was lyophilized.

Experiments on the Formation of Adenosine-5′ Phosphoranilidate in Dioxane–Formamide Mixture. (a).—Tri-*n*-hexylammonium adenosine-5′ phosphate (0.005 mmole) was dissolved in a mixture of freshly distilled formamide (1 ml.) and dioxane (4 ml.) and to the solution were added more tri-*n*-hexylamine (5 λ, 2 molar equivalent excess over the nucleotide), aniline (0.45 ml., 5 mmoles) and DCC (0.25 g., about 1.25 mmoles) and the clear solution kept at room temp. Aliquots (1 ml.) were removed at intervals, diluted with water and extracted many times with ether. The residual aqueous layer was concentrated *in vacuo* and the concentrate applied to a strip of Whatman No. 44 paper. The chromatogram was developed in solvent A. Three nucleotidic spots were thus detected. The first (R_f 0.08) corresponded to the unreacted nucleotide, the second with R_f 0.18 was apparently adenosine-5′ phosphoramidate[47] and the major spot with R_f 0.41–0.46 corresponded to the desired adenosine-5′ phosphoranilidate. After 40 hr. the relative intensities of the products were: adenosine-5′ phosphate, 11%; adenosine-5′ phosphoramidate, 6%; and adenosine-5′ phosphoranilidate, 83%. After 123 hr. all the starting material had disappeared. In all the chromatograms, there was an intense ultraviolet light-absorbing pot (R_f 0.9) corresponding to the phenyldicyclohexylguanidine.

(b).—Another experiment was set up with the following components. Adenosine-5′ phosphoric acid (0.005 mmole), triethylamine (0.0015 mmole), water (1.7 ml.), dioxane (3.4 ml.), aniline (0.45 ml., 5 mmoles) and diisopropylcarbodiimide (0.4 ml., 2.54 mmoles). The clear solution was kept at room temp. and the products determined at different intervals as described above. The reaction to form the anilidate was complete in 161 hr., the only nucleotidic product being adenosine-5′ phosphoranilidate. There was an intense spot corresponding to the phenylguanidine.

Preparation and Properties of Anilidates of Ribonucleoside-5′ Phosphates.—Samples of uridine-5′, cytidine-5′ and guanosine-5′ phosphoranilidates were prepared by using method b described for adenosine-5′ phosphoranilidate. The desired anilidates which were the major products were purified by preparative paper chromatography in solvent A on Whatman 31 paper. The bands R_f 0.4–0.5 corresponding to the anilidates were cut out, eluted with water and rechromatographed, if necessary, in the same solvent system on Whatman paper 44. The anilidates had the spectral characteristics given below. The molar extinctions given were based on the amount of the parent nucleotide formed after acidic hydrolysis. For this purpose, known amounts (optical density units) of the four anilidates were kept in 0.1 *N* hydrochloric acid for 2 days at room temp. Hydrolysis to the parent nucleotide was complete in all cases under these conditions. Known aliquots were neutralized and applied to paper chromatograms on Whatman paper 44 in solvent A. The single band observed corresponding to the nucleotide was eluted and its concentration determined spectrophotometrically.

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Deoxycytidine-5′ phosphoranilidate: 0.01 *N* hydrochloric acid, λ_{max} 280 mμ, ϵ_{280} 14,800; λ_{max} 226 mμ, ϵ_{226} 16,600; λ_{min} 250 mμ, ε 4,100; in 0.01 *N* sodium hydroxide, λ_{max} 272 mμ, ε 10,000; λ_{max} 232 mμ, ε 19,600; λ_{min} 253 mμ, ε 6,800.

Uridine-5′ phosphoranilidate: λ_{max} 264 mμ, ε 11,100; λ_{max} 232 mμ, ε 14,400; λ_{min} 248 mμ, ε 8,700 in 0.01 *N* hydrochloric acid; λ_{max} 263 mμ, ε 8,870; λ_{max} 230 mμ, ε 17,740; λ_{min} 249 mμ, ε 7,500, in 0.01 *N* sodium hydroxide.

Adenosine-5′ phosphoranilidate: λ_{max} 257 mμ, ε 15,040; λ_{max} 233 mμ, ε 15,900; λ_{min} 245 mμ, ε 12,100, in 0.01 *N* hydrochloric acid; λ_{max} 260 mμ, ε 15,040; λ_{max} 234 mμ, ε 14,700; λ_{min} 245 mμ, ε 11,280, in 0.01 *N* sodium hydroxide.

Guanosine-5′ phosphoranilidate: in 0.01 *N* hydrochloric acid, λ_{max} 256 mμ, ε 13,240; λ_{max} 235 mμ, ε 14,600; λ_{min} 247 mμ, ε 12,200; in 0.01 *N* sodium hydroxide, λ_{max} 257 mμ, ε 13,800; λ_{max} 234 mμ, ε 16,200; λ_{min} 247 mμ, ε 12,600.

Stability of Nucleoside-5′ Phosphoranilidates as a Function of pH.—About 8 μmoles of each one of the nucleoside-5′ phosphoranilidate was dissolved in 1 ml. each of 0.5 *M* acetate buffer (pH 4), 0.5 *M* acetate buffer (pH 5) and 0.1 *M* phosphate buffer (pH 6). The solutions were incubated at 37° and at intervals aliquots were chromatographed in solvent A. The following results were obtained: pH 6: No hydrolysis was detected of any one of the compounds up to at least 72 hr. After 4 days, there was a bare trace of the parent nucleotide formed in the case of adenosine- and guanosine-5′ phosphoranilidates. The pyrimidine analogs showed no decomposition up to 8 days. pH 5: No decomposition for cytidine, guanosine and uridine compounds for at least 2 days, a trace hydrolysis after 24 hr. in the case of adenosine compound. pH 4: There was no hydrolysis detectable in any of the compounds in up to 6 hr. In 12 hr., 0.5–1.6% hydrolysis to the parent compound was found.

General Method for the Formation of Nucleoside Phosphoranilidates at Controlled pH.—The following standard composition of the solvent mixture and reaction components was used in experiments with adenosine-5′ phosphate, oligonucleotides as well as the amino acid acceptor ribonucleic acid except for the slight modifications mentioned in the later experiments using C^{14}-labeled aniline. Clear solutions were obtained throughout. Nucleotide or oligonucleotide as the bis-triethylammonium salt (1 μmole), water (0.3 ml.) inclusive of any water introduced from the solution of the nucleotide component, *tert*-butyl alcohol (0.6 ml.), freshly distilled dimethylformamide (0.6 ml.), aniline (0.07 ml., 0.75 mmole), diisopropylcarbodiimide (0.12 ml., about 0.75 mmole): This solution was prepared in the reaction cell of an automatic titrator which was set so as to maintain a constant pH of 8–8.1 by the continual addition of 1 *N* hydrochloric acid. For the pH recording it was necessary to use in all this work a Beckman combination glass-reference electrode No. 39182. The reaction was first carried out with adenosine-5′ phosphate. Aliquots were removed at different intervals and directly examined by paper chromatography in solvent A. The formation of adenosine-5′ phosphoranilidate was about 90% in about 11 hr. at 25–28°, a large excess of the phenylguanidine being concomitantly formed. The amount of the latter was directly registered by the amount of acid added. The total amount of acid added at increasing periods of time was: 1 hr., 22 μmoles; 2 hr., 44 μmoles; 8.75 hr., 210 μmoles; and 11 hr., 260 μmoles.

Conversion of Terminal 5′-Phosphate Group in the Trinucleotide pApApA to Phosphoranilidate.—The reaction was carried out using 36 optical density units at 260 mμ (about 1 μmole) of the trinucleotide. Otherwise the composition of the reaction mixture was exactly as described above. After a total of 16 hr. reaction, 0.2 mmole of 1 *N* hydrochloric acid had been used to maintain the pH at 8.1–8.2. Water (7 ml.) was then added, the total mixture extracted with small (3-ml.) portions of ether and the aqueous layer concentrated under a high vacuum. The total was applied on a strip of Whatman No. 40 paper and the chromatogram developed in solvent A. After developing the chromatogram for 24 hr., the heavy band of the phenylguanidine traveling close to the front was cut out and, after drying, the oligonucleotide was rechromatographed in solvent B for 18 hr. The two major bands detected were those of the unreacted trinucleotide and another stronger band traveling faster which corresponded to the desired anilidate. The extent of reaction as followed by spectrophotometric determination of the concentration was 60%. In another experiment, the reaction had gone to the extent of 75% after a 30-hr. period.

Alkaline Hydrolysis of the Anilidate from pApApA.—The compound was kept in 1 *N* sodium hydroxide at 25° for 24 hr. The solution was treated with an excess of pyridinium Dowex-50 ion exchange resin and the filtered solution after concentration chromatographed in solvent B. Three ultraviolet-absorbing bands corresponding to adenosine (fastest), 2′ or 3′-*O*-phosphoryladenosine-5′ phosphoranilidate (III) (middle band) and adenosine-2′ (or 3′) phosphate (slowest band) were detected. The determination of the optical densities of the band showed the three products to be present in ratio close to 1:1:1. The ultraviolet absorption spectrum of the middle band showed the spectrum characteristic of adenosine-5′ phosphormorpholidate described above.

Stability of Methyluridine-3′ Phosphate under Conditions of Anilidate Formation.—A solution of methyluridine-3′ phosphate (3 μmoles) was lyophilized and the residue dissolved in the required amount (0.3 ml.) of water and the other reaction components added as described above. The reaction was carried out for 22 hr. at pH 8.15–8.2 by which time 0.32 mmole of 1 *N* hydrochloric acid had been consumed. The reaction mixture was then diluted with water and after extraction with ether and concentration, was chromatographed as a band on Whatman paper 40 in solvent A. A single nucleotidic band identical in R_f with marker methyluridine-3′ phosphate was obtained. The band was cut out and eluted with water. The recovery of optical density was about 80%. One-half of the total (1.2 μmoles) was incubated in 0.15 ml. of incubation mixture including 0.02 ml. of pH 7.1 tris-hydroxymethylaminomethane buffer, 0.5 *M*, with pancreatic ribonuclease (0.02 ml. of a 5 mg./ml. solution) for 5 hr. As a control, an equal amount of the sample used as starting material in this experiment was also incubated with the enzyme under identical conditions. Paper chromatography of the incubation mixtures in solvent A showed that degradation to uridine-3′ phosphate was complete in both cases, except for an extremely faint (could be 1% or less in amount) band corresponding in R_f with methyluridine phosphate, which was present in both samples.[48]

Conversion of Uridine Tetranucleotide (pUpUpUpU) to the 5′-Phosphoranilidate and Characterization of the Product.—Triethylammonium uridine tetranucleotide (pUpUpUpU) (40.6 optical density units at 262 mμ, about 1 μmole of the oligonucleotide) was allowed to react under the standard conditions described above. After a 28-hr. reaction period, 0.332 mmole of 1 *N* hydrochloric acid had been consumed. After dilution with water, extraction with ether and concentration of the aqueous solution, the products were chromatographed as a band on Whatman paper 44 in solvent A. After developing for about 18 hr., the paper was dried, the guanidine band cut off and the chromatogram run in solvent B for 24 hr. Two major ultraviolet light-absorbing bands were present and in also three very faint bands, the amount of which was too small for further investigation. The total bands and their amounts in order of increasing R_f's were: (1) a faint band at origin (0.45 optical density unit at 262 mμ, spectrum like that of uridine), (2) the next band corresponded to the starting material pUpUpUpU (4.5 optical density units), the next (strongest) band was that of the anilidate (22.1 optical density units) and the two faster bands were very weak. The optical density recovery was thus only 60%, but as judged by the relative intensities the anilidate formation occurred to the extent of 83%.

The recovered tetranucleotide (pUpUpUpU) was lyophilized and incubated with pancreatic ribonuclease [water, 0.1 ml.; 0.5 *M* tris-buffer (pH 7), 0.025 ml.; and ribonuclease, 0.01 ml. of 5 mg./ml. solution] for 4 hr. at 37°. Subsequent chromatography in solvent B showed products identical in R_f with those of uridine, uridine-2′(3′) phosphate and 2′(3′)-*O*-phosphoryluridine-5′ phosphate. None of the material with R_f of the starting material could be detected.

The total amount of the anilidate of the tetranucleotide was also degraded with pancreatic ribonuclease. The major products after chromatography in solvent B were: uridine (R_f 0.58), uridine-2′(3′) phosphate (0.4) and 2′(3′)-*O*-phosphoryluridine-5′ phosphoranilidate (R_f 0.49). These three products were characterized by comparison with markers, ultraviolet absorption spectra and their electrophoretic mobilities. In addition, a faint slow traveling spot (R_f 0.24) traveling in the region of uridine-2′(3′) 5′diphosphate or the starting material was also detected. The amount of this product (>0.5 optical density unit) was too small for further investigation. It is considered unlikely that it was undegraded starting material.

Nucleoside and Nucleotide Composition of Acceptor RNA.—A sample (about 20 mg.) of the above preparation of acceptor RNA was treated for 24 hr. at 20° with 4 ml. of 2 *N* potassium hydroxide. The hydrolysate was passed through a column of Dowex-50 (pyridinium) resin (12 × 1.3 cm.) and the resin was washed with water (150 ml.). The eluent and washings were evaporated and the residue was dissolved in water at pH 8 and applied to a column (14 cm. × 1.2 cm.) of Dowex-1 × 8% (formate) resin of 200 to 400 mesh. The column was washed with water (50 ml.) and 5-ml. fractions were collected per 10 min. The column was then eluted with a linear gradient of from 0.0 to 2 *M* formic acid (2 × 1000 ml.) and fractions of about 5 ml. were collected per 15 min. The optical density of the eluant fractions at 260 mμ was recorded and individual peaks were combined and the solvent removed *in vacuo* at low temp. The residue was redissolved in water, and the products were identified and estimated by measuring their ultraviolet absorption spectra in 0.01 *M* hydrochloric acid. The identity and amounts of the individual peaks are given in Table I.

(48) A faint trace of methyluridine-2′ phosphate was inherent in the method used for preparation of methyluridine-3′ phosphate; *cf.* D. H. Rammler and H. G. Khorana.[40]

TABLE I

Peak	Compound	Amount
1	Cytidine	0.348 μM
2	Adenosine	.215 μM
3	Uridine	.193 μM
4	Guanosine	.13 μM
5	Cytidylic acid (Cp)	11.6 μM
6	Adenylic acid (Ap)	3.7 μM
7	Adenylic acid (Ap)	4.7 μM
8	Unidentified	7. OD_{260}
9	Unidentified	2.5 OD_{260}
10	Unidentified	7.9 OD_{260}
11	Pseudo-uridylic acid (ψUp)	9.7 OD_{260}
12	Pseudo-uridylic acid (ψUp)	9.1 OD_{260}
13	Guanylic acid (Gp)	6.77 μM
14	Guanylic acid + uridylic acid (Gp + Up)	6.5 μM + 7.87 $M\mu$
15	Guanosine-3',5' diphosphate	0.484 μM
	Guanine-like compound	0.413 μM
	Unidentified λ_{max} 262 mμ	

The ratios of the four main nucleotides are recorded in Table II, together with those reported by Monier, *et al.*[46]

TABLE II

Acid	Present work	Monier, *et al.*[46]
Adenylic	10	10
Uridylic	10.35	10.8
Guanylic	15.7	15.8
Cytidylic	13.8	15.3

Labeling of Amino Acid Acceptor Ribonucleic Acids Followed by Alkaline Hydrolysis.—The reaction mixture was set up in a standard 1.5-ml. vessel by adding the following reactants in the given order: 4-morpholine, N,N'-dicyclohexylcarboxamidine (22.8 mg.); freshly distilled dimethylformamide, 0.6 ml.; *tert*-butyl alcohol, 0.6 ml.; aniline, 0.07 ml.; C^{14}-labeled aniline hydrochloride solution (0.05 ml. containing 0.1 mc. in 0.075 mmole of aniline). A clear solution was obtained at this stage. An aqueous solution (0.25 ml.) of pyridinium amino acid acceptor ribonucleic acid (20 mg.) containing 0.07 ml. of triethylamine was then added followed by diisopropylcarbodiimide (0.12 ml.). The reaction was set up on the pH Stat when 0.318 mmole of 1 *N* hydrochloric acid had been added; a portion (0.5 ml.) was removed and the remainder allowed to react further until a total of 0.38 mmole of hydrochloric acid had been taken up. This major portion of the reaction mixture was diluted with water, extracted thoroughly with ether and dialyzed against water. The material in the bag was then concentrated and hydrolyzed in 1 *N* potassium hydroxide. The total hydrolysate after removal of alkali by exchange with pyridinium Dowex-50 ion exchange resin contained 185 optical density units (260 mμ) and 42,210 c.p.m. in the total solution. The total was applied to the top of a DEAE-cellulose column (25 cm. × 2 cm. diam.) and after a water wash (fractions 1–15 of Fig. 1) elution was carried out with a convex gradient 0–0.2 *M* triethylammonium bicarbonate (total volume of solution 2 l.). The column was subsequently washed with 1 *M* triethylammonium bicarbonate. The elution pattern (optical density and radioactivity) is shown in Fig. 1.

The major peaks containing radioactivity were investigated as follows:

Fractions 35–45.—The total contained 70 optical density units (260 mμ) with specific activity of 106 c.p.m./optical density unit. It contained cytidylic-2'(3'), adenylic and uridylic acid. A portion was incubated with the bacterial phosphomonoesterase and the products chromatographed in solvent A. Several ultraviolet-absorbing bands were present ranging in R_f from 0.093 and 0.74. Scanning of the paper chromatogram for radioactivity showed that the radioactivity was present in the region between two ultraviolet-absorbing bands with R_f's 0.47 and 0.62. No ultraviolet absorption was associated with the radioactive peak.

Another aliquot of the nucleotide peak was subjected to electrophoresis on Whatman 3 MM paper strip. Subsequent scanning of the strip (very high self absorption) showed that the radioactivity moved ahead of the fastest nucleotide (uridylic acid) band.

Fractions 101–120.—Paper chromatography in solvent A and scanning of the chromatogram showed the radioactivity to be present mainly in the ultraviolet-absorbing spot with R_f corresponding to that of pAp (or pUp)-anilidate (R_f 0.124) and, in addition, a shoulder with R_f corresponding to that of pGp-anilidate (R_f 0.05–0.06). The latter was, however, not conclusively identified. The major radioactive band was identified as pAp-anilidate by its absorption spectrum and the formation of adenosine-5' phosphoranilidate after incubation with the alkaline phosphomonoesterase.

Fractions 121-139.—The major portion of radioactivity was shown to be associated with pGp-anilidate by the standard analytical techniques.

Fractions 251–270 (not shown in Fig. 1).—On continued elution with 1 *M* triethylammonium bicarbonate a little ultraviolet-absorbing material and some radioactivity was eluted. The nature of this fraction was not investigated.

Degradation of C^{14}-Labeled Amino Acid Acceptor Ribonucleic Acids to Oligonucleotides Containing C^{14}-Labeled 5'-Phosphoranilidates. (a) Labeling.—To 4-morpholine-N,N'-dicyclohexylcarboxamidine[16b] (34.6 mg., 0.118 mmole) were added in this order, freshly distilled dimethylformamide (0.6 ml.), *tert*-butyl alcohol (0.6 ml.), aniline (0.07 ml.) and an aqueous solution (0.05 ml.) of C^{14}-aniline hydrochloride (0.1 mc. containing about 0.075 mmole of aniline). A clear solution resulted at this stage. An aqueous solution of lyophilized pyridinium amino acid acceptor ribonucleic acid (30.4 mg. in 0.25 ml. of water) was next added and to the resulting turbid solution was finally added 0.12 ml. of diisopropylcarbodiimide. The clear solution which was obtained was sealed and set up on the pH Stat which added hydrochloric acid to maintain the pH at 8.2. The reaction was carried out for 2 days (temp. about 28–30°) at the end of which 0.464 mmole of 1 *N* hydrochloric acid had been added. Water was added and after several extractions with ether the solution was dialyzed against 0.05 *M* sodium chloride (several changes) and finally against water. The specific activity of the solution remaining inside the bag was 109 c.p.m./optical density unit at 260 mμ.

(b) **Digestion with Pancreatic Ribonuclease and Gross Fractionation of Products.**—The digestion was performed in batches of 400 optical density units. The lyophilized labeled product was adjusted to pH 7 with sodium hydroxide in a total volume of 1.5 ml. of water. Pancreatic ribonuclease (1 mg.) was added and the pH was maintained at 7 by the continual addition of alkali on the pH Stat. After 19 hr., the uptake of alkali was 9.5 μmole and the reaction had come to a halt.

A portion of the total ribonuclease digest (505 optical density units at 259 mμ) was diluted to 10 ml., the pH was adjusted to 8 and the solution applied to a column (21 cm. × 2.4 cm. diam.) of DEAE-cellulose (bicarbonate). The column was washed with one bed volume of water and then eluted with a convex gradient[49] of triethylammonium bicarbonate (0–0.2 *M*). The reservoir contained 2 l. of 0.2025 *M* triethylammonium bicarbonate (pH 7.5) while the mixing vessel contained 400 ml. of water. A flow rate of 1 ml./min. was maintained, 10-ml. fractions being collected. When 1260 ml. of eluent had passed through the column, by which time the dinucleotide fraction had come off, the column was eluted with 1 *M* triethylammonium bicarbonate (pH 7.5). The distribution of the ultraviolet-absorbing material in the different combined fractions from the column was: nucleoside region, 12.6 optical density units at 260 mμ; mononucleotide region, 105 optical density units at 261 mμ (the total radioactivity present in this region was 4312 c.p.m.).[50] The region between mononucleotide and 1 *M* eluate contained a total of 136 optical density units at 262 mμ (the total radioactivity in this region was 10,700 c.p.m.)[51]; 1 *M* eluate, 283 optical density units (260 mμ), contained a total radioactivity of 40,700 c.p.m. This fraction was investigated further for oligonucleotide sequences exactly as described below for the oligonucleotides labeled after their isolation from RNase digest of acceptor RNA.

Isolation of Oligonucleotide Mixture (Including pG and pA End Groups) after Ribonuclease Digestion of Amino Acid Acceptor RNA.—Yeast acceptor RNA (200 mg.) was digested with pancreatic ribonuclease (3.2 mg.) at pH 7 using a pH Stat. After about 20 hr. when alkali uptake had ceased, the reaction mixture was applied to the top of a DEAE-cellulose column (25 cm. × 2.4 cm.). The elution was carried out with the convex gradient of triethylammonium bicarbonate as described above. The oligonucleotide mixture consisting of members larger than dinucleotides (A(G)pU(C)p) (containing more than three net negative charges) was eluted with 1 *M* triethylammonium bicarbonate. The elution pattern is shown in Fig. 2. The distribution of the optical density in different combined fractions was: fractions 1–20, 52.5 optical density units (260 mμ) (mainly cytidine and adenosine); fractions 20–54, 924 optical density units (cytidylic acid and uridylic acid); fractions 54–110, 897 optical density units (mainly dinucleotides (A or G)p(U or C)p); 1 *M* fraction, fractions 110–end, 2155 optical density units (pGpCp and all higher oligonucleotides). In later experiments, the 1 *M* fraction

(49) R. M. Bock and Nan-Sing Ling, *Anal. Chem.*, **26**, 1543 (1954).

(50) This was not investigated further since the earlier experiment on alkaline hydrolysis of labeled ribonucleic acid had shown that these counts are not associated with the mononucleotides.

(51) 2'(3')-*O*-Phosphoryluridine-5' phosphoranilidate (IfI, R = uracil) derived from the uridine-5' phosphate end group (see above) would be expected to be present in this region. This region was, however, not investigated further.

included all ultraviolet-absorbing material eluted after the dinucleotide peak (peak 3, fractions 54–110).

The 1 *M* fraction contained all the oligonucleotides bearing 5′-phosphorylpurine nucleoside (pG and pA) end groups. This was shown by alkaline hydrolysis of an aliquot of this fraction and separation of pGp by paper electrophoresis and estimation by ultraviolet absorption measurement. These measurements gave a value of 2.32 μmoles of pGp in 200 mg. (4,000 optical density units of acceptor RNA). This 1 *M* fraction was used in the subsequent experiment on formation of C^{14}-anilidates.

Identification of 5′-O-Phosphoryluridine-3′ Phosphate in Ribonuclease Digest of Acceptor RNA.—In the preceding experiment, the fractions 54–110 representing the dinucleotide fractions were concentrated to remove the salt. The total was then taken up in 8 ml. of water. One-half of this solution was treated with 4 ml. of 2 *N* potassium hydroxide for 24 hr. at room temp. The hydrolysate was passed through a column of Dowex-50 (pyridinium) resin and the total eluate and washings were evaporated. The residue in 0.5 ml. of water was electrophoresed at pH 7.5 in 0.1 *M* triethylammonium bicarbonate buffer. The heavy mononucleotide band was eluted and estimated to contain 217 optical density units at 260 mμ. Two overlapping bands, observed ahead of the major band, were eluted and reelectrophoresed at pH 3.5 in 0.05 *M* formate buffer. Two spots were again observed. These were eluted separately and chromatographed on paper in solvent B. The faster (in solvent B) spot proved to be non-nucleotidic, whereas the slower traveling spot had R_f identical with that of uridine-2′(3′),5′ diphosphate. The ultraviolet absorption spectrum was also characteristic of uridine. The compound was present in the total amount of 0.86 optical density unit (260 mμ) in the total fraction (897 optical density units).

C^{14}-Anilidate Preparation from Oligonucleotide Mixture Containing 5′-Phosphate End Groups.—The oligonucleotide mixture for the reaction was the fraction eluted with 1 *M* triethylammonium bicarbonate (Fig. 2). Several batches of the oligonucleotide mixture was prepared. The triethylammonium bicarbonate was removed by repeated evaporation from aqueous pyridine and the residue passed through a column of Dowex-50 (pyridinium) ion exchange resin. The total effluent and washings were lyophilized. It was reacted in portions with C^{14}-aniline and diisopropyl carbodiimide.

In one experiment 4-morpholine-N,N′-dicyclohexylcarboxamidine (34.8 mg.) was added to the lyophilized oligonucleotide mixture (590 optical density units at 260 mμ, before conversion to the pyridine salt) and the solvents and other reactants were added in the standard order. The 0.05 ml. of C^{14}-aniline hydrochloride (0.1 mc.) was added just prior to diisopropylcarbodiimide which was added last, after the pH of the reaction mixture was ascertained to be 8.1 on the pH-Stat. The reaction was allowed to proceed for 92 hr. at the controlled pH of 8–8.1. After addition of water (5 ml.) and ether extraction, the aqueous solution was applied to a DEAE–cellulose (carbonate) column (12 cm. × 2.5 cm.). The column was washed with water until optical density (260 mμ) in the effluent was nil. It was then washed with 0.05 *M* triethylammonium bicarbonate until no further chloride ions were eluted. The oligonucleotide mixture was then eluted with 1 *M* triethylammonium bicarbonate. The appropriate fractions were pooled and evaporated. A total of 570 optical density units (recovery over 96%) was recovered and the total radioactivity in this mixture was 53,700 c.p.m.[52] This product was stored in 0.1 *M* ammonium acetate buffer (pH 5.9) at −10° for the next step. In a repeat of this experiment, when the recovery of the optical density was 600 units, the total radioactivity present was 63,000 c.p.m.

In two experiments carried out prior to the above, the extent of reaction was determined by hydrolysis of the product with alkali, neutralization, paper electrophoresis at pH 5 and finally paper chromatography (solvent B) of the band traveling ahead of the mononucleotides on paper electrophoresis. Conversion of the end group pGp to Anil-pGp was 73–80%. In the above experiment with C^{14}-aniline, an aliquot (about 11 optical density units) was hydrolyzed with alkali. The total product was separated by paper electrophoresis. The Anil-pGp band recovered had an optical density of 0.37[53] at 260 mμ and contained a total of 468 c.p.m. The total mononucleotide recovered (10.1 optical density units) contained 140 c.p.m.

Enrichment of Oligonucleotides Containing C^{14}-Labeled 5′-Phosphoranilidate Groups by Digestion with Spleen Phosphodiesterase.—The total 1 *M* triethylammonium bicarbonate eluate (from two experiments) of C^{14}-labeled oligonucleotides (1170 optical density units) was dissolved in 1 ml. of 0.1 *M* ammonium acetate (pH 5). It was incubated with spleen phosphodiesterase in 4 equal portions. (The standard conditions are given above.) The conversion to mononucleotides was followed by removing aliquots, heating at 90° for 2 min., then incubating with prostatic phosphomonoesterase and estimating inorganic phosphate. The action of the spleen phosphodiesterase came to a halt when about 70–80%[54] of the total nucleotidic material had been converted to mononucleotides. The digests were combined and chromatographed successively in two equal portions on a DEAE–cellulose (bicarbonate) column (14 cm. × 2.5 cm.). The column was washed with water, then with 0.05 *M* triethylammonium bicarbonate to remove ribonucleoside-2′,3′ cyclic phosphates andribonucleoside-3′ phosphates and finally with 1 *M* triethylammonium bicarbonate to elute all the oligonucleotides as one sharp peak. A typical elution pattern at this stage is shown in Fig. 3. The combined yield of optical density in the 1 *M* triethylammonium bicarbonate fractions was 269 units (about 20% of the total optical density units applied to the columns). The total radioactivity present in this fraction was 54,200 c.p.m., about 50% of the total radioactivity present in the oligonucleotide mixture after the reaction with C^{14}-labeled aniline. The cyclic phosphate and mononucleotide regions contained only small fractions of the total radioactivity.

Ribonuclease Digestion and Final Chromatography of the C^{14}-Labeled Oligonucleotides.—The total oligonucleotide mixture obtained above was digested with pancreatic ribonuclease under the standard conditions[55] and the resulting products applied on the top of a DEAE–cellulose (carbonate form) column (50 cm. × 1 cm.). After a water wash, elution was carried out with a linear gradient (0–0.4 *M*) of triethylammonium bicarbonate (pH 7.5) with 2 l. of liquid in each of the two vessels. A flow rate of 0.5 ml./min. was maintained, 10-ml. fractions being collected. The ultraviolet absorption and the radioactivity of different fractions is shown in Fig. 4 and Table III. The recovery of the optical density at 260 mμ was 226.8 units (84%) and the recovery of the radioactivity was 49,293 c.p.m. (91%).

TABLE III

ULTRAVIOLET ABSORPTION AND RADIOACTIVITY DISTRIBUTION IN OLIGONUCLEOTIDES CONTAINING 5′-PHOSPHORANILIDATE END GROUPS

Fractions pooled	Radioactive peak numbering	Total optical density (260 mμ)	Total c.p.m. in peak (cor. for background)	Total radioact. recovd. from col.	Identification end group, etc.
0–15		20.0	174		
15–60	1	12.0	3,135	6.4	Unidentified
60–73		32.7	913		
74–80		12.2	60		
80–90		22.0	671		
90–100	2	6.8	1,340	2.7	Unidentified
100–110		11.3	1,320	2.7	
110–124	3	19.0	19,700	40	Mainly Anil-pGpCp + Anil-pG end group
125–132	4	12.2	1,890	3.9	Anil-pG end group
132–158		20.0	2,580		
158–169	5	8.6	2,800	5.7	Trinucleotides with Anil-pG end group and/or Anil-pGpUp
169–175	6	6.2	1,010	2.1	Anil-pA end group (trinucleotide)
178–185	7	5.0	2,640	5.4	Anil-pG end group
187–198	8	10.3	6,650	13.5	Anil-pG + Anil-pA end groups
198–210	9	~8.0	1,100	2.3	Anil-pG or Anil-pA end group
228–238	10	~8.0	1,920	3.9	Anil-pG end group
280–290	11	12.5	1,390[a]	2.8	Anil-pG + free pG end group

[a] This figure is in doubt.

Standard Procedure for Isolation and Identification of End Groups.—The different peaks were pooled as shown in Table III. Triethylammonium bicarbonate was removed by repeated evaporation. Appropriate aliquots were removed for counting and for ultraviolet absorption measurement. For further purification the total or a portion of the total material was streaked as a band on Whatman paper 40 and chromatography was performed in

(52) The efficiency of the Geiger counter tube used is assumed to be about 15%. Accordingly, 1 μmole of C^{14}-aniline would have around 40,000 c.p.m. under the conditions used.

(53) This figure for optical density must be too high (extraneous absorption from paper). Theoretically in about 10 optical density units of mononucleotide, one would expect about 0.1–0.12 optical density of pGp. The c.p.m. noted are consistent with the amount to be expected. The figure of 468 c.p.m. would indicate 0.012 μmole of the labeled end group assuming a specific activity of 40,000 c.p.m./μmole as calculated above.

(54) This estimate is very approximate and uncertainty arises further from the fact that the terminal 3′-phosphoryl groups in all the oligonucleotides would be present, after the reaction with diisopropylcarbodiimide, as 2′,3′-cyclic phosphate groups.

(55) This step was given to hydrolyze the 2′,3′-cyclic phosphate end groups of oligonucleotides to the 3′-phosphate groups.

solvent B. A 1-in. wide strip of the chromatogram was used for the paper chromatogram scanner for locating the radioactive bands. For identification of end groups, the material in the original peak or the purified band was kept in 1 *N* potassium hydroxide for 24 hr. at room temp. and then the total passed through a short column of pyridinium Dowex-50 ion exchange resin column and the column washed with 10% aqueous pyridine. The total effluent was evaporated and the products separated by paper electrophoresis at pH 5 in freshly made 0.1 *M* ammonium acetate buffer. The Anil-pGp or Anil-pAp band traveled ahead of the major nucleotide bands. The bands were eluted and the material freed from salt and further examined by paper chromatography in solvent B. Standard markers of nucleotides and the anilidates were applied alongside. In addition to identification by radioactive scanning, the spots were eluted and characterized by ultraviolet absorption spectra and specific radioactivity.

COMMUNICATIONS TO THE EDITOR

The Atom Connectivity Matrix (ACM) and its Characteristic Polynomial (ACMCP): A New Computer-Oriented Chemical Nomenclature

Sir:

The Atom Connectivity Matrix, or ACM, here described, is a new unambiguous universal symbolism which, with only two simple rules, allows ready transformation, even by non-professionals, of the usual two dimensional pictorial molecular representation (or even more sophisticated models) into a unique mathematical expression readily useful for hand or computer manipulation.

Rule I.—Sketch the molecule with all the atoms (or radicals or nuclides) and interatomic connectivities (bonds, bond orders, force constants, ionic character, dipole moments, or vectorial components) desired to be incorporated into the ACM. Atoms and connectivities should be indicated in the pictorial representation. Anything from skeletal stick formulas to more complicated structures may be written.

Rule II.—Construct a matrix array, the ACM, wherein the a_{ii} elements (those on the matrix diagonal from upper left to lower right) represent the constituent atoms (defined above) and the off-diagonal a_{ij} elements represent the connectivity between the ith and jth atoms. The order of writing the diagonal elements is unimportant.

The characteristic polynomial, ACMCP, obtained by evaluating the ACM as a determinant, is invariant with respect to direction of viewing the molecule and order of numbering the atoms, and has a unique one-to-one correspondence with its pictorial progenitor.

The ACM (and its ACMCP) is versatile and universal in that any order of approximation may be selected, depending upon the molecular details to be represented. A few arbitrarily designated types of approximations and representative examples are:

A. Zero-Order Approximation.—Only the basic linked backbone atoms are listed (hydrogen may be omitted in organic molecules, or fluorine in fluorocarbons, or repeating groups in ionic structures) and the connectivities between pairs of atoms are assigned integral bond order values of 0, 1, 2 and 3 for no, single, double and triple bonds, respectively.

1. Hydrogen chloride: H–Cl

$$\begin{vmatrix} H & 1 \\ 1 & Cl \end{vmatrix} = HCl-1$$

2. Hydrogen cyanide: H—C≡N

$$\begin{vmatrix} H & 1 & 0 \\ 1 & C & 3 \\ 0 & 3 & N \end{vmatrix} = \begin{vmatrix} C & 1 & 3 \\ 1 & H & 0 \\ 3 & 0 & N \end{vmatrix} = HCN-9H-N$$

3. Carbon dioxide: O=C=O

$$\begin{vmatrix} C & 2 & 2 \\ 2 & O & 0 \\ 2 & 0 & O \end{vmatrix} = \begin{vmatrix} O & 2 & 0 \\ 2 & C & 2 \\ 0 & 2 & O \end{vmatrix} = CO^2-8O$$

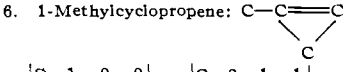
4. Allene: C=C=C

$$\begin{vmatrix} C & 2 & 0 \\ 2 & C & 2 \\ 0 & 2 & C \end{vmatrix} = C^3-8C$$

5. Methylacetylene: C≡C—C

$$\begin{vmatrix} C & 3 & 0 \\ 3 & C & 1 \\ 0 & 1 & C \end{vmatrix} = C^3-10C$$

6. 1-Methylcyclopropene:



$$\begin{vmatrix} C & 1 & 0 & 0 \\ 1 & C & 2 & 1 \\ 0 & 2 & C & 1 \\ 0 & 1 & 1 & C \end{vmatrix} = \begin{vmatrix} C & 2 & 1 & 1 \\ 2 & C & 1 & 0 \\ 1 & 1 & C & 0 \\ 1 & 0 & 0 & C \end{vmatrix} = C^4-7C^2 + 4C + 1$$

7. 2-Methylcyclopropene: (ACMCP identical with 6 above)

8. 3-Methylcyclopropene:

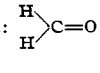
$$\begin{vmatrix} C & 1 & 0 & 0 \\ 1 & C & 1 & 1 \\ 0 & 1 & C & 2 \\ 0 & 1 & 2 & C \end{vmatrix} = C^4-7C^2 + 4C + 4$$

9. Alkyl bromide: R–Br

$$\begin{vmatrix} R & 1 \\ 1 & Br \end{vmatrix} = RBr-1$$

B. First-Order Approximation.—This is the same as the zero-order A above, but includes all atoms.

1. Formaldehyde:

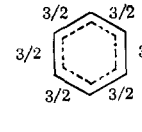


$$\begin{vmatrix} H & 0 & 1 & 0 \\ 0 & H & 1 & 0 \\ 1 & 1 & C & 2 \\ 0 & 0 & 2 & O \end{vmatrix} = H^2CO-4H^2-2HO$$

C. Higher-order approximations may include fractional bond orders (which may be desirable for delocalized aromatic and sandwich-type molecules), directed or vectorial connectivities, etc. In most cases, these would be less commonly used than the simpler types above.

As examples of other atoms or connectivity types used to develop appropriate ACM's, the following are shown:

Delocalized benzene, where the C–C bond order is 3/2.



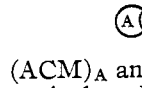
$$= \begin{vmatrix} C & 3/2 & 0 & 0 & 0 & 3/2 \\ 3/2 & C & 3/2 & 0 & 0 & 0 \\ 0 & 3/2 & C & 3/2 & 0 & 0 \\ 0 & 0 & 3/2 & C & 3/2 & 0 \\ 0 & 0 & 0 & 3/2 & C & 3/2 \\ 3/2 & 0 & 0 & 0 & 3/2 & C \end{vmatrix}$$

Note: The above ACM differs from that for the Kekulé or that for the Dewar forms since the ACM is always the exact analog of the pictograph selected.

Localized free radicals, such as ethyl radical, require an added symbol, ϵ, for the electron

$$\cdot C-C = \begin{vmatrix} C & 1 & 0 \\ 1 & C & 1 \\ 0 & 1 & \epsilon \end{vmatrix}$$

Linked rings, A and B, where no direct bonding occurs but integrity of each ring is required for existence of the molecule are exemplified by



$$= \begin{vmatrix} (ACM)_A & 0 \\ 0 & (ACM)_B \end{vmatrix}$$

where $(ACM)_A$ and $(ACM)_B$ are the ACM's for A and B respectively. Note: This principle of zero-order delocalized bonds is appropriate for clathrates, inclu-