

six peaks had been eluted, the total nucleotidic material was eluted with 1 *M* triethylammonium bicarbonate. The 1 *M* eluate (about 30% of material applied to the column) was evaporated to dryness and the process of dissolution in water and evaporation repeated to remove most of the salt. The total mixture was then chromatographed on a 9" wide strip Whatman No. 40 paper using solvent C. Each of the peaks obtained up to fraction 96 was also recovered by evaporation of the appropriate fractions. Characterization of the products recovered from the different peaks and the chromatogram was as follows.

**C<sub>5</sub>'-Pyridinium uridine-3' phosphate (XX)** (4.4% was in fractions 28-31, peak 2), was eluted at a 0.05 *M* triethylammonium bicarbonate concentration; ultraviolet absorption:  $\lambda_{\max}$  260  $\mu$ , shoulder at 267  $\mu$ ;  $\lambda_{\min}$  232  $\mu$  in water;  $\lambda_{\max}$  260  $\mu$  shoulder at 267  $\mu$ ; and  $\lambda_{\min}$  243  $\mu$  at pH 11.5. Paper chromatographic and electrophoretic mobilities are in Tables I and II.

**Uridine-3',5' cyclic phosphate** (7.5%) constituted peak 3, being eluted in fractions 32-34. The paper chromatographic and electrophoretic mobilities are in Tables I and II. In its behavior to acid and alkali and in its resistance to pancreatic ribonuclease, the product was identical with the sample synthesized earlier.<sup>25</sup>

**Uridine-3' phosphate** (9.7%) was present in peak 4 (fractions 42-52) being eluted at 0.079 *M* triethylammonium bicarbonate concentration.

**Uridine cyclic dinucleotide** (5.2%) was present in peak 5 (fractions 58-64) being eluted at 0.082 *M* triethylammonium bicarbonate concentration. The paper chromatographic and electrophoretic mobilities (Tables I and II) were consistent with its structure. Treatment with bacterial phosphomonoesterase under the standard conditions did not alter its electrophoretic mobility at pH 7.1. Incubation with pancreatic ribonuclease caused only partial degradation to uridine-3' phosphate under the conditions which caused complete degradation of the dinucleotide uridylyl-(3'→5')-uridine-3' phosphate to the mononucleotide. Treatment with 0.5 *N* sodium hydroxide at 25° caused only about 20% hydrolysis in 6 hr. and about 65-70% in 54 hr., while uridylyl-(3'→5')-uridine was completely hydrolyzed in 6 hr. under the above conditions. The main product from the cyclic dinucleotide on alkaline hydrolysis was uridine-2'(3') phosphate, there being detected a small amount of an intermediate, presumably linear uridine dinucleotide.

**Uridylyl-(3'→5')-uridine-3' phosphate (XVIII, *n* = 0)** was present in peak 6, being eluted in fractions 84-96 (0.091 *M* triethylammonium bicarbonate concentration). The position of

elution, paper chromatographic and paper electrophoretic properties (Tables I and II) were all consistent with its structure. Treatment with bacterial phosphomonoesterase caused complete conversion to uridylyl-(3'→5')-uridine. The latter was identical in paper electrophoretic mobility with a synthetic sample and gave, after incubation with pancreatic ribonuclease, uridine-3' phosphate and uridine in equal amounts. The dinucleotide itself was completely degraded to uridine-3' phosphate on incubation with pancreatic ribonuclease.

**Uridine Cyclic Trinucleotide (XIX, *n* = 1).**—The material eluted by 1 *M* triethylammonium bicarbonate after elution of the preceding compound, uridylyl-(3'→5')-uridine-3' phosphate, was separated by paper chromatography in solvent C on a sheet of paper (Whatman No. 44). The cyclic trinucleotide constituted a major band<sup>43</sup> (*R<sub>f</sub>* 0.25, solvent C) of the 1 *M* fraction (about 3.5% of total polymeric mixture). Its paper chromatographic and electrophoretic properties are given in Tables I and II. The electrophoretic mobility was almost identical with the previously synthesized thymidine cyclic dinucleotide. It was resistant to the action of the alkaline phosphomonoesterase. Degradation with pancreatic ribonuclease gave a product which was identified as uridine-3' phosphate. (Sufficient material was not available for a comparative rate study with known substrates.)

**Uridylyl-(3'→5')-uridylyl-(3'→5')-uridine-3' phosphate (XVIII, *n* = 1)** was present (about 2% of total nucleotidic material) in a band traveling slower (*R<sub>f</sub>* 0.15, solvent C) than the cyclic trinucleotide. Its chromatographic properties are in Tables I and II. On incubation with the alkaline phosphomonoesterase it was completely converted to a product (*R<sub>f</sub>* 0.25 in solvent C) which was identified as uridylyl-(3'→5')-uridylyl-(3'→5')-uridine. Degradation of the latter with pancreatic ribonuclease gave uridine-3' phosphate and uridine in a ratio close to 2. The trinucleotide itself on incubation with pancreatic ribonuclease gave only uridine-3' phosphate.

**Higher Oligonucleotides.**—Some 3% of the total polymeric mixture did not leave the origin on paper chromatography in solvent C as used above for separation of the di- and trinucleotides. This material evidently consisted of several components as shown by paper electrophoresis and included the oligonucleotides higher than those characterized above.

(43) This product was further purified by paper electrophoresis at pH 7.1. In this way two minor impurities were removed.

CONTRIBUTION FROM THE INSTITUTE FOR ENZYME RESEARCH, UNIVERSITY OF WISCONSIN, MADISON, WIS., AND THE CHEMISTRY DIVISION OF BRITISH COLUMBIA RESEARCH COUNCIL, VANCOUVER, CAN.]

## Studies on Polynucleotides. XX.<sup>1</sup> Amino Acid Acceptor Ribonucleic Acids (1). The Synthesis and Properties of 2'(or 3')-O-(DL-Phenylalanyl)-adenosine, 2'(or 3')-O-(DL-Phenylalanyl)-uridine and Related Compounds<sup>2</sup>

BY D. H. RAMMLER<sup>3</sup> AND H. G. KHORANA

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The reaction of carbobenzyloxy-DL-phenylalanine with dicyclohexylcarbodiimide gave an excellent yield of the corresponding symmetrical anhydride III, which was isolated in a crystalline form and characterized. The pyridine-catalyzed reaction of III with 5'-O-tri-*p*-methoxytrityluridine followed by an acidic treatment gave mono-*O*-(carbobenzyloxy-DL-phenylalanyl)-uridine, which was, presumably, a mixture of the 2'- and 3'-isomers. Palladium-catalyzed hydrogenolysis of the latter afforded 2'(or 3')-O-(DL-phenylalanyl)-uridine. Analogous reaction of III with 5'-O-trityl-adenosine gave both 3'-O-(carbobenzyloxy-DL-phenylalanyl)-5'-O-trityl-adenosine and the corresponding 2'-isomer which were separated by chromatography on a silicic acid column. The isomers were found to undergo interconversion under mildly acidic or basic catalysis. It proved possible, however, to determine the orientation of the protected aminoacyl residue in the products by phosphorylation and identification of the resulting adenosine-2' or -3' phosphate. Removal of the protecting groups from the above derivatives gave DL-phenylalanyl-adenosine which was, presumably, a mixture of the 2'- and 3'-isomers. The rate of hydrolysis of the aminoacyl nucleoside was determined in pH 7 phosphate buffer. The half-life at 25° was 48 min., while that at 34° was 22 min. The possible causes for the great lability of the aminoacyl linkage in the adenosine ester are discussed. Furthermore, the present work suggests the rapid migration of the amino acyl groups between the 2'- and 3'-hydroxyl groups of terminal adenosine in amino acid acceptor ribonucleic acids.

The first steps in the enzymatic synthesis of a polypeptide chain are the activation of  $\alpha$ -amino acids, by

(1) Studies on Polynucleotides. XIX: D. H. Rammler, Y. Lapidot and H. G. Khorana, *J. Am. Chem. Soc.*, **85**, 1989 (1963).

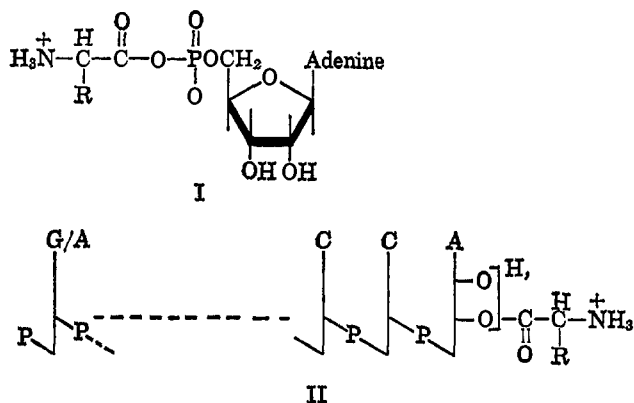
(2) This work has been supported by grants from the National Cancer Institutes of the National Institutes of Health, the National Science Foundation, Washington, D. C., and the Life Insurance Medical Research Fund, New York, N. Y.

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reaction with adenosine-5' triphosphate, to form the mixed anhydrides of the type I and the subsequent transfer of the aminoacyl groups to the terminal adenosine residues (partial structure II)<sup>4</sup> of relatively small molecular weight ribonucleic acids, designated variously as soluble, transfer, or amino acid acceptor

(4) For the system of short hand representation of polynucleotides 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.

ribonucleic acids.<sup>5</sup> That the aminoacyl group is indeed carried by the terminal adenosine unit and that it is involved in an ester linkage with the 2'- or 3'-hydroxyl group has been shown by isolation and



characterization of aminoacyl adenosine after pancreatic ribonuclease degradation of C<sup>14</sup>-labeled aminoacyl ribonucleic acids.<sup>6,7</sup> Detailed studies of the equilibrium constants of the enzymatic reactions<sup>8b,8-10</sup> leading to the aminoacyl ribonucleic acid formation show these reactions to be readily reversible.

The synthetic work on aminoacyl ribonucleosides described in this paper was performed in 1959 as a part of our interest in the general chemistry of the amino acid acceptor ribonucleic acids and particularly in developing approaches for separation of the individual amino acid acceptor ribonucleic acids utilizing the aminoacyl group as the handle. Furthermore, it was hoped that the availability of synthetic aminoacyl ribonucleosides and their protected derivatives would facilitate the study of the properties of this new class of compounds<sup>11</sup> and that this would in turn lead to a decision on the exact placement (2'- or 3'-hydroxyl group in II) of the  $\alpha$ -aminoacyl groups in the enzymatic reactions between aminoacyladenylates (I) and the terminal adenosine of the amino acid acceptor ribonucleic acids. The present paper describes a rather general method for the synthesis of 2' (or 3')-O-aminoacyl ribonucleosides.<sup>12,13</sup> The facile isomerization (2'-O  $\rightleftharpoons$  3'-O) of the carbobenzyloxyphenylalanyl group in 5'-O-trityladenine derivatives has been clearly demonstrated and it is concluded that interconversion of this type would likewise occur with an unprotected aminoacyl group, thus complicating the problem of determination of the location of such groups in the enzymatic reaction leading to II. Finally, the probable

reasons for the high lability of the aminoacyl ester of the nucleoside linkage are considered.

Experiments were first carried out with a pyrimidine ribonucleoside, uridine, and the derivative chosen was one that carried a suitable protecting group in the 5'-position. The steps involved are shown in Chart I. Carbobenzyloxy-DL-phenylalanine was converted, by reaction with dicyclohexylcarbodiimide, in excellent yield to the corresponding anhydride<sup>14,15</sup> III which was isolated in a crystalline form and characterized by elemental analysis, infrared spectrum and conversion to the previously described anilide. Compound III served as the acylating agent for 5'-O-tri-*p*-methoxytrityluridine<sup>16</sup> (IV) under basic catalysis provided by pyridine. The total mixture (V-VII) was treated briefly with aqueous acetic acid<sup>16</sup> at room temperature to remove the tri-*p*-methoxytrityl group and the resulting products were separated by partition chromatography on a silicic acid column. Carbobenzyloxy-DL-phenylalanyluridine, presumably a mixture of 2'- and 3'-isomers (VII and VIII), was characterized by elemental analysis and negative periodate test. So long as the carbobenzyloxy protecting group was present on the  $\alpha$ -amino group, the product was stable to storage in a solid state, there being no evidence of decomposition by paper chromatography. The same was the case in the adenosine analogs described below.

The removal of the carbobenzyloxy group from VII and VIII was accomplished by palladium-catalyzed hydrogenolysis in acetic acid at low temperature and the product, 2' (or 3')-O-(DL-phenylalanyl)-uridine (IX), was pure as determined by paper chromatography and paper electrophoresis. It was further characterized by quantitative spectrophotometric (for uridine) and ninhydrin (for phenylalanine) analysis.

The starting material for the corresponding adenosine esters was 5'-O-trityladenine<sup>17,17a</sup> (IV, R = adenine, R' = H). Reaction with the anhydride III as described above for the uridine derivatives gave the expected number of products, all of which could be separated (Fig. 1) by chromatography on a silicic acid column. Thus, peak I contained unreacted carbobenzyloxy-DL-phenylalanine; peak II, 2',3'-di-O-(carbobenzyloxy-DL-phenylalanyl)-5'-O-trityladenine; peak III, 3'-O-(carbobenzyloxy-DL-phenylalanyl)-5'-O-trityladenine; peak IV, the 2'-O-isomer of the preceding compound; and peak V contained a small amount of unreacted 5'-O-trityladenine. Closer examination of the products in peaks III and IV showed their facile interconversion. Thus when a mixture of peaks II and III (peak II served as the reference) was rechromatographed on a fresh column of silicic acid, some of the material corresponding to peak IV was formed at the expense of peak III (Fig. 2). When purified peak IV, the amount of which normally

(5) (a) P. C. Zanicnik, "Harvey Lectures," Vol. LIV, 256 (1958-1959); (b) F. Lipmann, W. C. Hülsmann, G. Hartmann, H. G. Boman and G. Acs, *J. Cellular Comp. Physiol.*, **54**, 75 (1959); (c) P. Berg, *Ann. Rev. Biochem.*, **30**, 293 (1961).

(6) H. G. Zachau, G. Acs and F. Lipmann, *Proc. Natl. Acad. Sci. U. S.*, **44**, 885 (1958).

(7) J. Preiss, P. Berg, E. J. Ofengand, F. H. Bergmann and M. Dieckmann, *ibid.*, **45**, 319 (1959).

(8) P. Berg and E. J. Ofengand, *ibid.*, **44**, 78 (1958); P. Berg, F. H. Bergmann, E. J. Ofengand and M. Dieckmann, *J. Biol. Chem.*, **236**, 1726 (1961).

(9) R. S. Schweet, F. C. Bound, E. Allen and E. Glassman, *Proc. Natl. Acad. Sci. U. S.*, **44**, 173 (1958).

(10) I. D. Raake, *Biochim. Biophys. Acta*, **27**, 416 (1958).

(11) The presence of amino acids in ester linkages with hydroxyl groups of carbohydrate moieties of naturally occurring polymers appears to be of more general significance. Thus *D*-alanine widely occurs in ester linkage with ribitol or glycerol hydroxyl groups in teichoic acids and glycerol phosphate polymers: See, e. g., J. Baddiley, J. G. Buchanan, R. O. Martin and U. L. RajBhandary, *Biochem. J.*, **85**, 49 (1962).

(12) A preliminary report was published in 1960: D. H. Rammler and H. G. Khorana, *Federation Proc.*, **19**, 349 (1960).

(13) Using a different method, other workers have since recorded the synthesis of 2' (or 3')-O-leucyladenosine<sup>6</sup> and 2' (or 3')-O-valyl-ribonucleosides [H. G. Zachau, *Chem. Ber.*, **93**, 1822 (1960)].

(14) (a) H. G. Khorana, *Chem. Rev.*, **53**, 145 (1953); (b) ref. 4, Chapter 6.

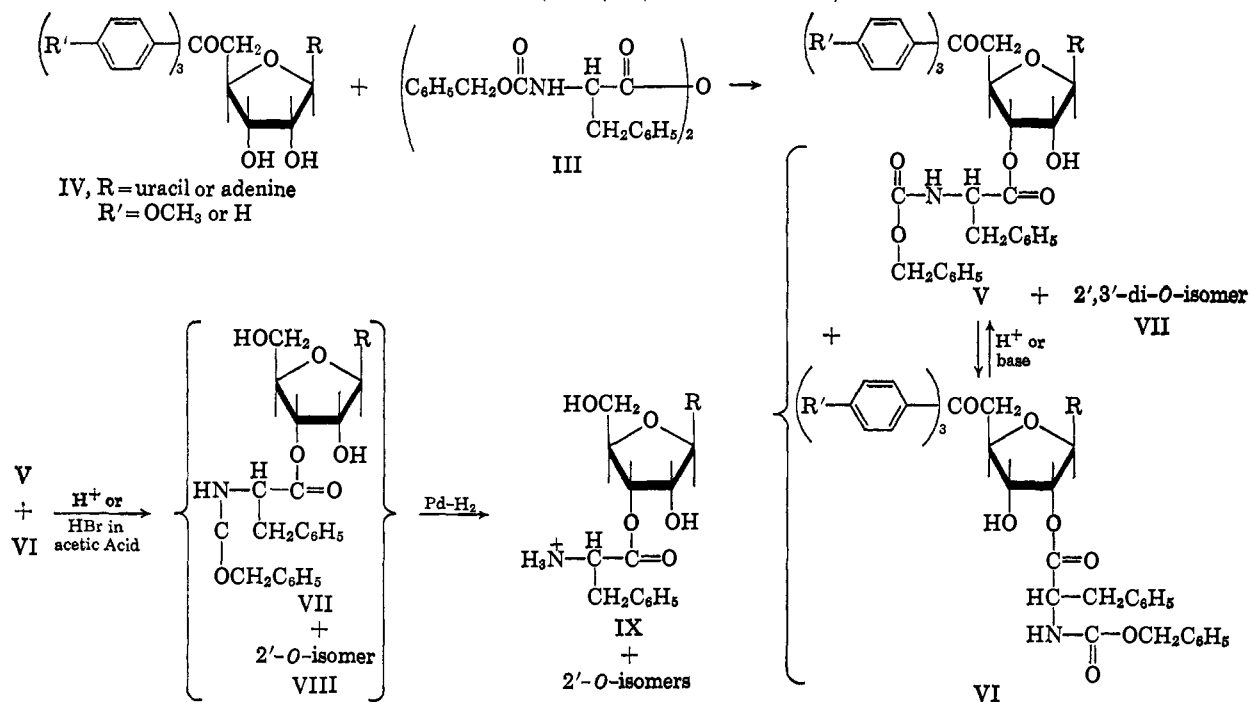
(15) Other workers have independently reported on the preparation of symmetrical anhydrides of *N*-acylamino acids by the carbodiimide method. I. Muramatsu and A. Hagitani, *J. Chem. Soc. Japan, Pure Chem. Section (Nippon Kagaku Zasshi)*, **80**, 1497 (1959); H. Schüssler and H. Zahn, *Chem. Ber.*, **95**, 1076 (1962).

(16) M. Smith, D. H. Rammler, I. H. Goldberg and H. G. Khorana, *J. Am. Chem. Soc.*, **84**, 430 (1962).

(17) While the use of the 5'-O-tri-*p*-methoxytrityl group in the uridine series enabled the very facile removal of this protecting group, the use of the more stable trityl derivative in the adenosine series made possible the repeated chromatography on silicic acid columns of the protected phenylalanine esters without the loss of the trityl group. This in turn enabled the orientation of the esterifying group on the 2'- or the 3'-hydroxyl by the phosphorylation of the sole remaining hydroxyl in the adenosine moiety and ready identification of adenosine-2' or -3' phosphate.

(17a) NOTE ADDED IN PROOF.—We have recently become aware of the independent synthesis of 2' (or 3')-O-carbobenzyloxyphenylalanyl-5'-O-trityladenine described in Russian literature: E. Ya. Dreiman, V. A. Dmitrieva, S. G. Kamzolova, Z. A. Shabarova and M. A. Prokof'ev, *Zh. Obshch. Khim.*, **31**, 3899 (1961); *Chem. Abstr.*, **57**, 9936 (1962).

CHART I. SYNTHESIS OF 2'(OR 3')-O-(DL-PHENYLALANYL)-RIBONUCLEOSIDES



was less than that of peak III, was rechromatographed, both peaks III and IV were again obtained (Fig. 3), the two products being present in about equal amounts. The interconversion appeared to be catalyzed by the acidity provided by the column and/or by the solvent.<sup>18</sup> Under the conditions used the conversion of peak IV to III (3'-O-carbobenzyloxy-DL-phenylalanyl ester) appeared to be faster than the reverse process. (This migration was found to occur also under basic catalysis as shown below.)

Phosphorylation of peak III in pyridine with a mixture of  $\beta$ -cyanoethyl phosphate and dicyclohexylcarbodiimide<sup>19</sup> and subsequent removal of the protecting groups gave mainly (85%) adenosine-2' phosphate.<sup>20</sup> Phosphorylation of the isolated peak IV gave adenosine-3' and -2' phosphates in about equal amounts and it seems very probable that in this case base-catalyzed migration occurred in favor of the 3'-O-isomer.<sup>21</sup> From the total of these results the conclusions are drawn that peak III contained 3'-O-(carbobenzyloxy-DL-phenylalanyl)-5'-O-trityladenosine (IV, R = adenine, R' = H) and peak IV the isomeric 2'-O-ester. Interconversions are facile and are probably catalyzed both by acid<sup>22</sup> and base.<sup>21</sup>

(18) In one experiment, purified peak III was heated in reagent grade chloroform for 1 hr. and the product rechromatographed under the standard conditions. Both peaks III and IV were present.

(19) G. M. Tener, *J. Am. Chem. Soc.*, **83**, 159 (1961).

(20) During the alkaline treatment to remove the carbobenzyloxyphenylalanyl group and to eliminate the  $\beta$ -cyanoethyl group, the former must have come off first and the possibility of some transesterification to form the 2',3'-cyclic phosphate<sup>4</sup> while the cyanoethyl group was still present cannot be ruled out. In fact it could have contributed to the small extent of randomization of the phosphate group that was encountered in this experiment. The other possibility for the presence of some 3'-phosphate is the acid-catalyzed isomerization before the phosphorylation actually took place.

(21) The results are consistent with those recorded previously [D. H. Rammler and H. G. Khorana, *J. Am. Chem. Soc.*, **84**, 3112 (1962)] on the phosphorylation of N,O<sup>6</sup>,O<sup>5</sup>-tribenzoyl- and N,O<sup>2</sup>,O<sup>5</sup>-tribenzoylcytidines. The former gave only cytidine-2' phosphate while the latter gave, in addition to the 3'-phosphate, some of the 2'-phosphate. There, the migrations were probably base-catalyzed and occurred in favor of the 3'-O-benzoyl derivatives.

(22) Acyl group migrations under acidic conditions have been demonstrated by a large number of investigators in the carbohydrate and other fields. Only a few references are given here: A. Doerschuk, *J. Am. Chem. Soc.*, **74**, 4202 (1952); R. K. Ness and H. G. Fletcher, *J. Org. Chem.*, **22**, 1470 (1957); Lohnizen and P. E. Verkade, *Rec. trav. chim.*, **79**, 133 (1960); R. C. Hockett, *J. Am. Chem. Soc.*, **68**, 928 (1946); E. E. van Tamelen, *ibid.*, **73**, 5773 (1951).

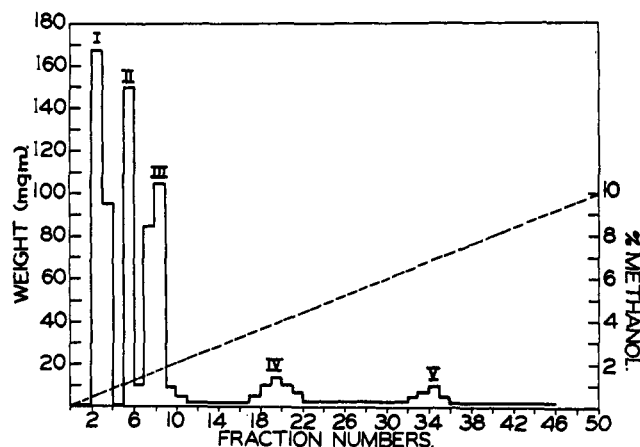


Fig. 1.—Products of the reaction of 5'-O-trityladenosine with carbobenzyloxy-DL-phenylalanyl anhydride; chromatography on a silicic acid column; conditions as described in text: peak I, carbobenzyloxy-DL-phenylalanyl; peak II, 2',3'-di-O-(carbobenzyloxy-DL-phenylalanyl)-5'-O-trityladenosine; peak III, 3'-O-(carbobenzyloxy-DL-phenylalanyl)-5'-O-trityladenosine; peak IV, the 2'-O-isomer of the preceding compound; peak V, 5'-O-trityladenosine.

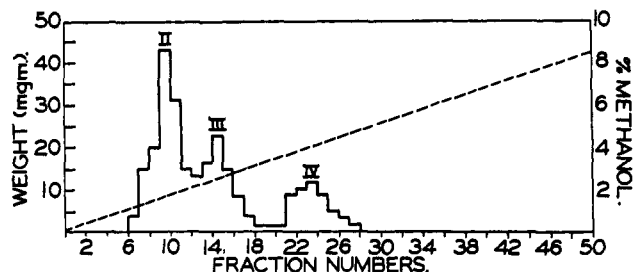


Fig. 2.—Rechromatography of combined peaks II and III of Fig. 1 on a silicic acid column; note the appearance of peak IV.

Brief treatment of 3'-O-(carbobenzyloxy-DL-phenylalanyl)-5'-O-trityladenosine with hydrogen bromide in acetic acid removed the trityl group and the product (VII + VIII, R = adenine) was purified by chromatography on a silicic acid column and characterized by

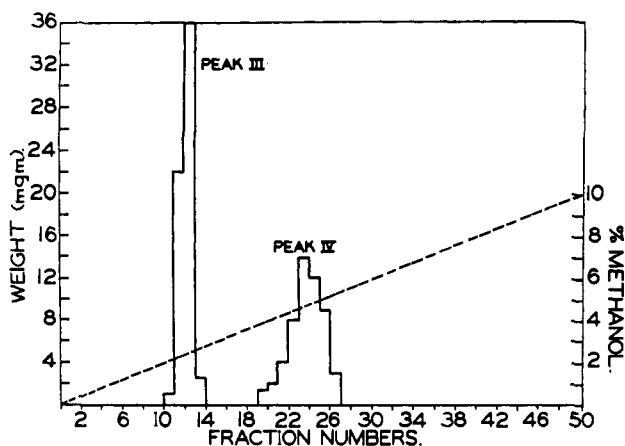


Fig. 3.—Partial conversion of purified peak IV into peak III; chromatography on a standard silicic acid column.

paper chromatography and by elemental analysis. Removal of the carbobenzyloxy group was accomplished by hydrogenolysis and 2'-(or 3')-O-(DL-phenylalanyl)-adenosine was pure as determined by paper electrophoresis at acid pH. Full characterization was accomplished as described above for the uridine analog.

In contrast with the stability of the carbobenzyloxy-DL-phenylalanyl esters, the free aminoacyl esters showed high lability. The rate of hydrolysis was followed in 0.1 M phosphate buffer at 25° and 34° by following the release of periodate-sensitive nucleoside. The half-life of DL-phenylalanyl ester of adenosine was 48 min. at 25° and 22 min. at 34°. The reaction followed first-order kinetics for the major part (up to about 70–80%) of the hydrolysis, but some deviation from this order occurred in the remaining part. The results are in general agreement with those of a number of previous workers<sup>5b, 6–10, 13</sup> in that they show the ease of hydrolysis of the aminoacyl ester linkage.

**Discussion.**—The method chosen for the synthesis of the aminoacyl esters is general as demonstrated for a pyrimidine and a purine ribonucleoside. The amino group in the adenine ring caused no interference in selective acylation at the 2'- or 3'-hydroxyl groups with the protected amino acid anhydride. The results are consistent with the extensive studies reported from this Laboratory on the rates of acylation of the hydroxyl groups and the amino group in adenine nucleosides. The method herein described is simpler and gives much higher yields than those previously reported.

Clear demonstration has been given of the facile migration of carbobenzyloxy-DL-phenylalanyl group between the 2'- and 3'-hydroxyl functions under acidic catalysis. The results are consistent with our own previous findings in the cytidine series and indeed with a large body of earlier work in the carbohydrate field.<sup>22</sup> Although we have not attempted to demonstrate similar migration in the unprotected aminoacyl esters, it is our conclusion that the migration there would be even more rapid, being facilitated at acidic or neutral pH by the inductive effect of the protonated  $\alpha$ -amino group.<sup>23</sup> This complication would apply directly to the determination of the position of the aminoacyl group in the enzymatic formation of aminoacyl ribonucleic acids.

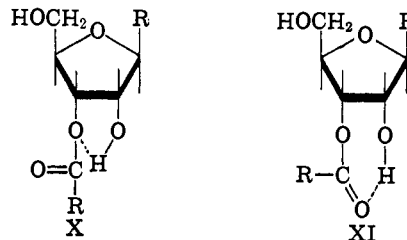
A large body of data is now available which shows the rather striking lability of the aminoacyl ester linkage.<sup>24</sup> The finding has prompted a great deal of

(23) E. S. Gould, "Mechanism and Structure in Organic Chemistry," Henry Holt and Co., New York, N. Y., 1959, p. 207.

(24) The equilibrium constants of the over-all reactions involving adenosine-5' triphosphate,  $\alpha$ -amino acids and amino acid acceptor ribonucleic

investigation into model systems<sup>25</sup> and a combination of inductive effects, steric effects and hydrogen bond formation with the neighboring hydroxyl group has been invoked as possible explanations for the high rate of hydrolysis.

As pointed out earlier,<sup>26</sup> a major factor responsible for the high lability of the aminoacyl ester linkage in ribonucleosides is the presence of the free neighboring hydroxyl group. Marked labilization of ester linkage in monoesters of *cis*-diols has been demonstrated by a number of workers.<sup>27</sup> Hydrogen bonding as in X<sup>27, 28</sup> or as in XI<sup>26b</sup> could facilitate the approach of a hydroxyl ion or a water molecule and cause stabilization of the



transition state associated with the attacking species.

Labilization of the amino ester linkage must also be caused by the inductive effect of the protonated  $\alpha$ -amino group.<sup>23, 29</sup> (It is highly probable that the species undergoing hydrolysis is that in which the  $\alpha$ -amino group is protonated.) Direct support for this conclusion was provided in the present work by the finding that the ester linkage was much more stable while carbobenzyloxy group protected the  $\alpha$ -amino group.<sup>30</sup>

### Experimental<sup>31</sup>

**General Methods.**—Reagent grade pyridine dried over calcium hydride was used. Evaporations were carried out *in vacuo* at below 40° bath temp. Silicic acid chromatography was performed using Mallinckrodt analytical grade silicic acid (100 mesh). The standard conditions used were: A column of 2 cm. dia. and containing 30 g. of silicic acid was used. Elution was performed using a linear gradient of a polar solvent, methyl alcohol in chloroform or ether. The mixing vessel contained 1 l. of pure chloroform (or ether) and the reservoir contained 1 l. of chloroform plus 5–10% methyl alcohol as specified in individual experiments. A flow rate of about 1 ml. per minute was maintained by application of a slight positive pressure of nitrogen.

Periodate oxidations were followed by the spectrophotometric method described elsewhere.<sup>32</sup> Quantitative amino acid analyses were performed using the ninhydrin method.<sup>33</sup>

Paper chromatography was performed by the descending technique using Whatman No. 40 (double acid-washed) paper. Nucleosides and related compounds were detected by viewing under an ultraviolet lamp; amino acid esters were detected by

acids<sup>8, 9–10</sup> show ready reversibility. Indeed, the result is as would be expected on the basis of the value previously established for the free energy of hydrolysis of a simple carboxylic ester, ethyl acetate [F. H. Carpenter, *J. Am. Chem. Soc.*, **82**, 1111 (1960)].

(25) (a) H. G. Zachau and W. Karau, *Chem. Ber.*, **93**, 1830 (1960); (b) T. Bruice and T. H. Fife, *J. Am. Chem. Soc.*, **84**, 1973 (1962), and the references cited therein; (c) Z. A. Shabarova, N. A. Hughes and J. Baddiley, *Biochem. J.*, **83**, 216 (1962).

(26) H. G. Khorana in *J. Cell. Comp. Physiol.*, **54**, Suppl. 1, p. 85 (1959).

(27) H. B. Henbest and B. J. Lovell, *J. Chem. Soc.*, 1965 (1957); S. M. Kupchan and W. S. Johnson, *J. Am. Chem. Soc.*, **78**, 3864 (1956); see also S. M. Kupchan, S. K. Eriksen and M. Friedman, *ibid.*, **84**, 4159 (1962).

(28) Hydrogen bonding in cyclopentane-*cis*-1,2-diol has been demonstrated by L. Kuhn, *ibid.*, **74**, 2492 (1952).

(29) The same effect causes dramatic lowering of the  $pK_a$  of the carboxyl group in glycine and other  $\alpha$ -amino acids, whereas acylation of the amino group returns the  $pK_a$ 's of the carboxyl groups to the normal range. E. J. Cohen and J. T. Edsall, "Proteins, Amino Acids and Peptides," Reinhold Publishing Corp., New York, N. Y., 1943, p. 116.

(30) See also K. Moldave, P. Castellfranco and A. Meister [*J. Biol. Chem.*, **234**, 841 (1959)], who noted similar effects in the mixed anhydrides of  $\alpha$ -amino acids and adenosine-5' phosphate.

(31) All melting points are uncorrected and elemental analyses were performed by W. Manser, Zurich, Switzerland.

(32) D. H. Rammler and J. C. Rabinowitz, *Anal. Biochem.*, **4**, 116 (1962).

(33) E. W. Yemm and E. C. Cocking, *Analyst*, **80**, 210 (1955).

spraying the chromatograms with ninhydrin or the hydroxamate reagent.<sup>34</sup> Paper electrophoresis was performed in an apparatus of the type described by Markham and Smith.<sup>35</sup> The medium used most frequently was 1 *M* acetic acid solution. The average potential was 15–20 volts/cm.

The solvent systems used for paper chromatography were: *n*-butyl alcohol–ethyl alcohol–water (4:1:5, solvent A); *n*-butyl alcohol–acetic acid–water (4:1:5, solvent B); isopropyl alcohol–concd. ammonia–water (7:1:2, solvent C); isopropyl alcohol–concd. ammonia–0.1 *M* boric acid (7:1:2, solvent D); saturated aqueous ammonium sulfate–1 *M* sodium acetate–isopropyl alcohol (80:18:2, solvent E).

**Carbobenzoyloxy-DL-phenylalanine Anhydride.**—To an anhydrous ether solution (3 ml.) of carbobenzoyloxy-DL-phenylalanine (0.328 g., 1.1 mmole) was added DCC (0.124 g., 0.6 mmole). The symmetrical anhydride as well as dicyclohexylurea precipitated from the solution immediately. After being kept at room temperature for 1 hour, the reaction mixture was cooled to 5° and the precipitate was collected by filtration. The anhydride was extracted with cold dry ethyl acetate (10 ml.) and filtered from the insoluble dicyclohexylurea. The solution was made opalescent with light petroleum ether (b.p. 30–40°). After 18 hours at 5°, this solution deposited needle-like crystals of the anhydride (0.21 g., 66%, m.p. 125–126°). A second crop of crystals (m.p. 122°) was obtained from the mother liquor. The total yield was 90%. The infrared spectrum of the product showed two carbonyl absorption bands (1132 cm.<sup>-1</sup> strong, 1752 cm.<sup>-1</sup> medium) characteristic of carboxylic acid anhydrides.<sup>36</sup>

*Anal.* Calcd. for C<sub>15</sub>H<sub>17</sub>O<sub>3</sub>N<sub>2</sub> (580.62): C, 70.3; H, 5.56; N, 4.83. Found: C, 69.49; H, 5.56; N, 5.10.

**Carbobenzoyloxy-DL-phenylalanine anilide** (0.01 g.) was obtained by treating the above anhydride (0.017 g.) with freshly distilled aniline (0.03 ml.) in dry toluene (2 ml.); m.p. 156–159° (Anderson, *et al.*,<sup>37</sup> quote m.p. 159–160°).

**2'(or 3')-O-Carbobenzoyloxy-DL-phenylalanyluridine.**—To a solution of 5'-*O*-tri-*p*-methoxytrityluridine (0.1 g., 0.17 mmole) in freshly distilled anhydrous tetrahydrofuran (3 ml.) was added anhydrous pyridine (0.03 ml.) followed by carbobenzoyloxy-DL-phenylalanine anhydride (0.125 g., 0.22 mmole). The mixture was kept for 18 hr. at room temperature with the exclusion of moisture. After this time, a chip of ice was added to destroy any residual anhydride and the solution was evaporated to dryness. The residue was dissolved in 80% aqueous acetic acid (3 ml.) and after 15 min. at room temperature the acetic acid was evaporated. After complete removal of the solvent, the residue was dissolved in a small amount of benzene and the insoluble uridine (about 1%) was removed by filtration. The clear benzene solution (about 5 ml.) was then passed onto a standard silicic acid column and elution (15-ml. fractions collected) was carried out as follows: A mixture of anhydrous ether (75%) and benzene (25%) eluted tri-*p*-methoxytritol in the first three fractions and carbobenzoyloxy-phenylalanine in the next three fractions. Elution was then continued using standard conditions with a linear gradient of 10% methyl alcohol in chloroform. The first nucleoside-containing peak eluted was 2',3'-di-*O*-carbobenzoyloxy-DL-phenylalanyluridine (11.2%) and it was followed by 2'(or 3')-*O*-carbobenzoyloxy-DL-phenylalanyluridine (80%). This product was obtained as a fine white powder by freeze-drying from a benzene solution containing a small amount of methyl alcohol.

*Anal.* Calcd. for C<sub>26</sub>H<sub>29</sub>O<sub>9</sub>N<sub>3</sub> (557.54): C, 58.3; H, 5.62; N, 7.53. Found: C, 58.29; H, 5.65; N, 7.29.

**2'(or 3')-O-DL-Phenylalanyluridine.**—Carbobenzoyloxy-DL-phenylalanyluridine (0.05 g.) was hydrogenated in ice-cold 80% aqueous acetic acid (6 ml.) using palladium-on-barium sulfate (0.05 g.) as the catalyst.<sup>38</sup> Hydrogenolysis of the carbobenzoyloxy group was complete in 90 minutes at 0°, and the catalyst was removed by filtration through a small Celite bed. The cold clear filtrate was examined as follows: An aliquot was subjected to paper electrophoresis in 1 *M* acetic acid. Only one ultraviolet-absorbing band was detected. This band moved under the conditions used toward the cathode at a rate of about 9 cm./hr., showing a net positive charge. The band gave positive reactions with ninhydrin and hydroxylamine sprays. Quantitative ninhydrin analysis and ultraviolet absorption measurement performed on an aliquot gave phenylalanine and uridine in the ratio 1. Another aliquot was diluted with water and freeze-dried to yield a powder which was kept in 1 ml. of concd. ammonia. After 2 hr. at 0°, the ammonia was removed by evaporation and the residue examined by paper electrophoresis. Uridine, phenylalanine and phenylalanine amide were identified as the products. The pres-

ence of phenylalanine amide was confirmed by paper chromatography (solvent A) using an authentic sample as marker.

**Isomeric (2' and 3')-O-Carbobenzoyloxy-DL-phenylalanyl-5'-O-trityladenosines.**—To a solution of 5'-*O*-trityladenosine (0.256 g., 0.5 mmole) in anhydrous pyridine (7 ml.) was added carbobenzoyloxy-DL-phenylalanine anhydride (0.435 g., 0.75 mmole). The reaction mixture was kept at room temperature for 18 hr. Pyridine was then removed by evaporation and the residue was dissolved in a small amount of benzene. This solution was chromatographed on silicic acid under the standard conditions, using a gradient of 10% methyl alcohol in anhydrous ether. The elution results (Fig. 1) were: peak I, carbobenzoyloxy-DL-phenylalanine, 0.168 g., fractions 2–4; peak II, 2',3'-di-*O*-carbobenzoyloxy-DL-phenylalanyl-5'-*O*-trityladenosine, 0.150 g., 28%, fractions 5–6; peak III, 3'-*O*-carbobenzoyloxy-DL-phenylalanyl-5'-*O*-trityladenosine, 0.198 g., 50%, fractions 7–10; peak IV, 2'-*O*-carbobenzoyloxy-DL-phenylalanyl-5'-*O*-trityladenosine, 0.04 g., 10%, fractions 17–22; peak V, 5'-*O*-trityladenosine, 0.027 g., 10%, fractions 32–36.

The compounds in the different peaks were characterized as: **2',3'-Di-*O*-carbobenzoyloxy-DL-phenylalanyl-5'-*O*-trityladenosine (Peak II, Fig. 1).**—Hydrogenolysis of the carbobenzoyloxy groups followed by alkaline hydrolysis gave adenosine and phenylalanine as judged by paper chromatography in solvent B. The analytical sample was obtained by crystallization from aqueous ethanol; m.p. 118–122°, with shrinking at 109°; λ<sub>max</sub> 259 mμ in methyl alcohol.

*Anal.* Calcd. for C<sub>63</sub>H<sub>57</sub>O<sub>10</sub>N<sub>7</sub> (1072.41): C, 70.6; H, 5.37; N, 9.16. Found: C, 69.88; H, 5.35; N, 9.52.

**3'-*O*-Carbobenzoyloxy-DL-phenylalanyl-5'-*O*-trityladenosine (Peak III, Fig. 1).**—A small amount of the material (0.005 g.) was heated in a boiling water-bath for 30 minutes in 80% aqueous acetic acid (5 ml.). The cooled solution was hydrogenated with palladium-on-barium sulfate as described above. After 2 hr., water (2 ml.) was added, and the precipitated triphenylcarbinol and the catalyst were removed by filtration through a Celite pad. The filtrate was evaporated to dryness and the residue used for quantitative determinations. The phenylalanine to adenosine ratio was found to be 0.97. An analytical sample was prepared by freeze-drying a benzene solution of the peak material. The sample had λ<sub>max</sub> (ethyl alcohol) at 259 mμ, the spectrum being very similar to that of adenosine. The equivalent weight as based on the adenosine chromophore was found to be 775.

*Anal.* Calcd. for C<sub>46</sub>H<sub>42</sub>O<sub>7</sub>N<sub>6</sub> (790.85): C, 69.9; H, 5.35; N, 10.63. Found: C, 69.34; H, 5.44; N, 10.53.

**Phosphorylation of 3'-*O*-Carbobenzoyloxy-DL-phenylalanyl-5'-*O*-trityladenosine (Peak III, Fig. 1) with β-Cyanoethyl Phosphate and DCC.**—To an anhydrous pyridine solution (0.5 ml.) of the material in peak III (0.010 g.) and pyridinium β-cyanoethyl phosphate (0.05 mmole) was added DCC (0.05 g.) and the sealed mixture kept at room temp. After 3 days, water (0.5 ml.) was added and the solution kept at room temperature for 12 hr. The solution then was evaporated to dryness several times with small amounts of ethanol to ensure complete removal of pyridine. The dry residue was dissolved in chloroform (10 ml.) and hydrobromic acid in acetic acid (0.1 ml., 0.380 mmole) was added. After 5 minutes at room temperature, the solution was evaporated to dryness and the residue was dissolved in a small amount of dioxane (1 ml.). To this solution were added 9 ml. of concd. ammonia. The solution was heated at 50° for 3 hr. and then evaporated to dryness. The residue was dissolved in a small amount of water and the solution filtered from the insoluble tritol and dicyclohexylurea. The total solution was chromatographed in solvent C. The products were found to be adenosine and the corresponding nucleotide. The nucleotide band was cut out and rechromatographed in solvent E. The major nucleotide component (85%) was identified as adenosine-2' phosphate and the minor component (15%) had an R<sub>f</sub> identical with that of adenosine-3' phosphate.

**2'-*O*-Carbobenzoyloxy-DL-phenylalanyl-5'-*O*-trityladenosine (Peak IV, Fig. 1).**—The analysis was performed on a sample obtained by freeze-drying the benzene solution of the material in this peak. The ultraviolet absorption characteristics were similar to those of adenosine. The equivalent weight as based on the extinction of adenosine was 826. After phosphorylation using exactly the conditions described above for peak III (Fig. 1) adenosine-3' phosphate and adenosine-2' phosphate were obtained in about equal amounts.

*Anal.* Calcd. for C<sub>46</sub>H<sub>42</sub>O<sub>7</sub>N<sub>6</sub> (790.85): C, 69.9; H, 5.35; N, 10.63. Found: C, 69.42; H, 5.55; N, 10.52.

**Interconversion of Peaks III and IV of Fig. 1.**—Peaks II (2',3'-di-*O*-carbobenzoyloxy-DL-phenylalanyl-5'-*O*-trityladenosine, 0.150 g.) and III (3'-*O*-carbobenzoyloxy-DL-phenylalanyl-5'-*O*-trityladenosine, 0.198 g.) from Fig. 1 were combined and the total dissolved in a small amount of benzene. The solution was applied to the top of a standard silicic acid column. (In this column, peak II was included as a marker.) The column was developed using a gradient of 10% methyl alcohol in anhydrous ether. The results

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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-trityl-adenosine (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-phenylalanyl-adenosine. 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-phenylalanyl-adenosine 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-phenylalanyl-adenosine 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.*<sup>39</sup>

**Hydrolysis of 2'(or 3')-O-DL-Phenylalanyl-adenosine.**—A solution of the compound (0.206  $\mu$ mole) in 0.1 M phosphate buffer (pH 7, 3 ml.) was treated at 25° and 34° with potassium periodate (0.42  $\mu$ mole). The rate of hydrolysis of the ester linkage<sup>40</sup> was followed by the decrease in absorption at 235 m $\mu$  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<sup>41</sup> (pH 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.

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

## Studies on Polynucleotides. XXI.<sup>1</sup> Amino Acid Acceptor Ribonucleic Acids (2). The Labeling of Terminal 5'-Phosphomonoester Groups and a Preliminary Investigation of Adjoining Nucleotide Sequences<sup>2</sup>

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The conversion of a phosphomonoester group to the corresponding 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 ( $C_3'-C_5' \rightarrow C_2'-C_5'$ ) 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.<sup>3</sup> The activation of the  $\alpha$ -amino acids occurs by pyrophos-

phorolysis of adenosine-5' triphosphate to form aminoacyladenylates. The latter donate the aminoacyl

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(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**, 73 (1959); (d) P. Berg, *Ann. Rev. Biochem.*, **30**, 293 (1961); (e) M. V. Simpson, *ibid.*, **31**, 333 (1962).