*SOME OBSERVATIONS ON THE MECHANISM OF THE ACYLATION PROCESS IN PROTEIN SYNTHESIS**

By Beverly E. Griffin and C. B. Reese

UNIVERSITY CHEMICAL LABORATORY, CAMBRIDGE, ENGLAND

Communicated by Lord Todd, January 2, 1964

During recent years much attention has been given to the mechanism of synthesis of proteins in biological systems.[1] Although the process is by no means fully understood, some of the steps involved appear to be well established.[2–4] Before amino acids can be incorporated into polypeptide chains they must first be "activated." The "activation" process involves ribonucleic acids of comparatively low molecular weight, known as "transfer" or "soluble" ribonucleic acids (sRNA), adenosine-5′ triphosphate (ATP) as an energy source, and a specific enzyme for each amino acid. In the first step of the "activation" process, the amino acid reacts with ATP to form a mixed anhydride with adenosine-5′ phosphate (AMP) releasing inorganic pyrophosphate; this mixed anhydride then acylates a specific sRNA on its terminal nucleoside (adenosine) residue to yield a 2′ (or 3′)-aminoacyl ester—the "activated" amino-acid. These processes are reversible.

$$\text{ATP} + \text{amino acid} \rightleftharpoons \text{aminoacyl-AMP} + \text{pyrophosphate} \quad (1)$$

$$\text{sRNA} + \text{aminoacyl-AMP} \rightleftharpoons \text{aminoacyl-sRNA} + \text{AMP} \quad (2)$$

Beyond this point our knowledge of the process of polypeptide synthesis is less certain. The "activated" amino acids are believed to be transferred to the ribosomes—the site of assembly of polypeptide chains—and then the amino acids become linked together in a genetically controlled order to synthesize a specific protein.[5] Although there is as yet no definite evidence regarding this final step, it is frequently assumed that it involves a simple acylation as indicated in step (3).

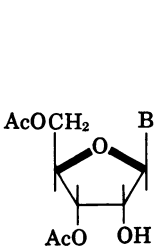
$$\text{Aminoacyl-sRNA} + \text{peptide} \rightarrow \text{aminoacyl-peptide} + \text{sRNA} \quad (3)$$

If this assumption is correct, then the peptide chain-lengthening step is simply the common reaction between an ester and an amine to form an amide. Although it is well known that esters of amino acids are usually more susceptible to nucleophilic attack than those of simple carboxylic acids,[6] it was clear from a variety of observations that aminoacyl-sRNA possessed a remarkable reactivity, behaving rather like an acid anhydride.[2] Several previous studies have indicated but not clarified some possible reasons for this. Wieland *et al.*[7] found that 2′- and 3′-leucyl esters of 5′-ribonucleotides were cleaved more rapidly by hydroxylamine than was the monoleucyl ester of *cis*-cyclopentane-1,2-diol. This result suggested that the known substantial effect of a *cis*-vicinal hydroxyl group on hydrolysis rates of esters, which has been studied by Bruice and Fife,[8] could not of itself account for the observed high reactivity of the aminoacyl-nucleotides. Wieland *et al.*[7] suggested that both the glycosidic nitrogen and the ring-oxygen of the sugar residue might be involved. Zachau[9] reported that valyl-ribonucleotides and -nucleosides were hydrolyzed by alkali at about the same rate as valyl-sRNA so that the macromolecular character of the latter seemed unimportant; he also found that the nature of the pyrimidine or purine base in the nucleotide had little effect. Zachau and Karau[6] further showed by experiments using esters of various

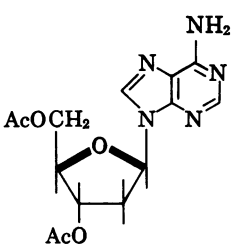
model alcohols that the presence of a ring-oxygen atom in the alcohol increased the reactivity toward hydroxylamine of esters containing a *cis*-vicinal hydroxyl group.

The importance of the aminoacyl-sRNA's in protein synthesis seemed to us such that further examination of this problem was warranted. In particular we were struck by the fact that although there is believed to be a specific sRNA corresponding to each individual amino acid, all of them have the same terminal sequence . . . pCpCpA, the amino acid becoming attached to the adenosine residue in the "activation" process.[1] It was therefore decided to examine the whole problem of the "activated" esters afresh, and the present paper describes studies carried out on the reaction of various acyl-nucleosides with morpholine in ethanol solution. If the biological polypeptide synthesis really proceeds by the mechanism indicated in step (3), then such studies are highly relevant. In the experiments described below, simple acetyl derivatives were used since they could be obtained in the pure crystalline state, and since the same factors must necessarily have operated as in the case of aminoacyl-esters. Morpholine (pK_a 8.4) was chosen as a suitable model for the terminal amino group of a peptide chain, and ethanol was used as a solvent; it cannot of course be stated with certainty whether morpholine rather than ethoxide ion was the actual nucleophile involved in the observed reactions.

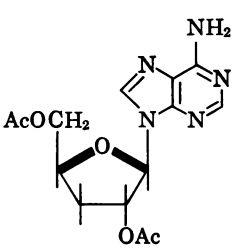
Ethanolic solutions (*ca.* 10^{-2} molar) of 3′,5′-diacetyladenosine[10] (I; B = adenine-9), 3′,5′-diacetyl-2′-deoxyadenosine[11] (II), and 2′,5′-diacetyl-3′-deoxyadenosine (III) [m.p. 159–160° (Found: N = 21.1%. Calc. for $C_{14}H_{17}N_5O_5$: N = 20.9%), prepared by acetylation of 3′-deoxyadenosine, kindly supplied by Dr. B. R. Baker] were each heated under reflux with one molar equivalent of morpholine. In each case, the course of reaction was followed by paper chromatography (in the upper phase of butan-1-ol:acetic acid:water, 5:1:4). After five hr, 3′,5′-diacetyladenosine (I; B = adenine-9) was *ca.* 95% converted to a product which was identified by paper chromatography and paper electrophoresis (borate buffer, pH 8) as 5′-acetyladenosine.[10] After 8 hr, paper chromatography indicated that the starting material had been completely converted to 5′-acetyladenosine containing, at most, traces of adenosine. However, both 3′,5′-diacetyl-2′-deoxyadenosine (II) and 2′,5′-diacetyl-3′-deoxyadenosine (III) were entirely unchanged after they had been heated with one molar equivalent of morpholine under the same conditions for 8 hr. The striking effect of the *cis*-vicinal hydroxyl group was thus demonstrated; there appeared to be no appreciable difference in the reactivity of the isomeric 2′- and 3′-esters of the deoxynucleosides, i.e., (III) and (II), under these conditions.



(I)



(II)



(III)

In order to examine the possible influence of the heterocyclic base, ethanolic solutions (*ca.* 0.03 molar) of 3′,5′-diacetyladenosine (I; B = adenine-9) and 3′,5′-diacetyluridine[12] (I; B = uracil-3) were each heated at 50° with one molar equivalent of morpholine. The solutions were examined after various intervals by paper chromatography, and the ratio of starting materials to products was estimated spectrophotometrically. The results are given as follows:

Time (hr)	0	2	5	9
% 3′,5′-diacetyladenosine (I; B = adenine-9)	100	55	36.5	20
% 5′-acetyladenosine	0	45	63.5	80
% 3′,5′-diacetyluridine (I; B = uracil-3)*	100	66	39	25
% 5′-acetyluridine[12]	0	34	61	75

* *Note added in proof:* Some recent studies by Mr. D. R. Trentham suggest that the percentages estimated after 2, 5, and 9 hr relate to mixtures of 2′, 5′- and 3′, 5′-diacetyluridine, i.e., acyl-migration has accompanied deacylation.

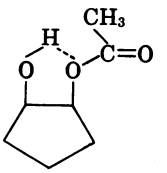
The above shows clearly that within experimental limits, 3′,5′-diacetyladenosine and the corresponding uridine derivative were converted to the respective 5′-acetates at virtually the same rate; the assays after a reaction period of 5 hr are the mean of three values and are the most accurate. Therefore, if adenine participates at all in the deacetylation of 3′,5′-diacetyladenosine under these conditions, then it seems that uracil must participate to the same extent in the corresponding uridine derivative. The possibility must be considered[13, 14] that the aminoacyl group may be attached to the 2′- and not to the 3′-position of the adenosine residue in aminoacyl-sRNA. However, in the attempted deacylation of the two diacetyl deoxyadenosines (II) and (III), no enhanced reactivity of the secondary acetoxy group in the latter compound was observed.

These experimental results indicate that under the conditions used the contribution of the *cis*-vicinal hydroxyl group toward the unusual lability of the acyl group in the acyl-ribonucleosides is considerable and far outweighs any other effect; it could well account for the "anhydride character" of aminoacyl-sRNA as indicated[2] by the reversibility of step (2) and for the involvement of ribonucleic rather than deoxyribonucleic acids in the "activation" step in protein biosynthesis. Its bearing on the later stages of protein synthesis is less clear. If the peptide chain-lengthening step is simply a reaction between aminoacyl-sRNA and the terminal amino group of a peptide, then only the effect of the *cis*-vicinal hydroxyl group need be seriously considered; and in such a case the nature of the terminal nucleoside residue in sRNA would be of no importance in the reaction. This need not be true if there were one or more intermediate steps between the initial "activation" of the amino acids and the final polypeptide synthesis. The complexity of the enzyme system involved in the process makes the existence of some more subtle series of reactions than direct amide formation at least conceivable. The possibility must also be considered that the specific terminal sequence . . . pCpCpA might be required for step (2), i.e., for the acylation of sRNA. However, in the absence of more detailed evidence the question must remain open.

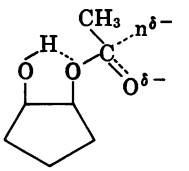
Our results do not help in deciding whether the aminoacyl residue in aminoacyl-sRNA is attached to $C_{2'}$ or $C_{3'}$ of the terminal (adenosine) residue of sRNA. However, the work of Bruice and Fife[8] suggests that were this group attached to the 2′-position, the 3′-hydroxyl group would be just as effective in facilitating nucleophilic attack upon it as is the 2′-hydroxyl group in activating the 3′-acetoxy

group in 3′, 5′-diacetyladenosine. It is possible that step (2) might lead in the first instance to 2′-aminoacyl-sRNA which could then rearrange to the 3′-isomer before step (3) occurred. This has some support in chemical evidence which indicates that in 5′-acetyladenosine[15] and 5′-acetyluridine[12, 16] the 2′-hydroxyl group is more nucleophilic than the corresponding 3′-hydroxyl, yet both compounds are acetylated exclusively on the 3′-position[10, 12] by acetic anhydride. The phenomenon of base-catalyzed acyl migration in polyols is well known,[17, 18] and it may well occur also in the acyl-ribonucleosides. Further investigations bearing on the position of the acyl group in aminoacyl-sRNA and other aspects of this problem are in progress in this laboratory and will be reported later.

Although the work described above supports the view that the *cis*-vicinal hydroxyl group promotes attack on the aminoacyl group of aminoacyl-sRNA, it does not help elucidate the detailed mechanism involved. However, Bruice and Fife,[8] and Kupchan *et al.*[17] have recently discussed the mechanism of the participation of vicinal hydroxyl groups in ester solvolysis. The neighboring hydroxyl group can hydrogen-bond to the ether oxygen (as illustrated in (IV) for *cis*-cyclopentane-1,2-diol monoacetate) or to the carbonyl oxygen of the ester, making the group more open to nucleophilic attack. Hydrogen-bonding to the ether oxygen would tend to increase the infrared carbonyl stretching frequency, whereas hydrogen-bonding to the carbonyl oxygen would tend to lower it.[8] Both types of hydrogen-bonding could help stabilize the tetrahedral transition state resulting from the attack of a nucleophile (n^-). This is illustrated, again with hydrogen-bonding to the ether oxygen, for *cis*-cyclopentane-1,2-diol monoacetate in (V).



(IV)



(V)

We examined the infrared spectra of our compounds (in chloroform solution) and observed no significant difference in carbonyl absorption between esters with or without a *cis*-vicinal hydroxyl group; 3′,5′-diacetyladenosine (I; B = adenine-9) had ν_{max} 1750 cm^{-1}, 3′,5′-diacetyl-2′-deoxyadenosine (II) had ν_{max} 1749 cm^{-1}, and 2′,5′-diacetyl-3′-deoxyadenosine (III) had ν_{max} 1750 cm^{-1}. These results make Michelson's report,[19] that comparison of the infrared spectra of 2′,3′- and 3′,5′-diacetyladenosine suggested hydrogen-bonding in the latter, difficult to understand. As our infrared spectral data cannot justify interactions exemplified by (IV), we concur with the views of Bruice and Fife,[8] and Kupchan *et al.*[17] and suggest that in our experiments the *cis*-vicinal hydroxyl group promoted nucleophilic attack by stabilizing the tetrahedral transition state either as in (IV) or through the polarized ester carbonyl group.

We wish to thank Lord Todd, F.R.S., for interesting us in this problem, and for his continued encouragement and advice.

* This work was supported in part by research grant CA-06066 from the National Cancer Institute, National Institutes of Health, U.S. Public Health Service.

[1] Cf. (*a*) Hoagland, M. B., in *The Nucleic Acids*, ed. E. Chargaff and J. N. Davidson (New York: Academic Press, 1960), vol. 3, p. 349; (*b*) Lipmann, F., W. C. Hulsmann, G. Hartman, H. G. Boman, and G. Acs, *J. Cell. Comp. Physiol.*, **54**, 75 (1959); (*c*) Berg, P., *Ann. Rev. Biochem.*, **30**, 293 (1961); (*d*) Simpson, M. V., *Ann. Rev. Biochem.*, **31**, 333 (1962); (*e*) Zamecnik, P. C., *Biochem. J.*, **85**, 257 (1962).

[2] Zachau, H. G., G. Acs, and F. Lipmann, these PROCEEDINGS, **44**, 885 (1958).

[3] Preiss, J., P. Berg, E. J. Ofengand, F. H. Bergmann, and M. Dieckmann, these PROCEEDINGS, **45**, 319 (1959).

[4] Hecht, L. T., M. L. Stephenson, and P. C. Zamecnik, these PROCEEDINGS, **45**, 505 (1959).

[5] Cf. refs. 1*c* and 1*d*.

[6] Zachau, H. G., and W. Karau, *Chem. Ber.*, **93**, 1830 (1960).

[7] Wieland, T., H. Merz, and G. Pfleiderer, *Chem. Ber.*, **93**, 1816 (1960).

[8] Bruice, T. C., and T. H. Fife, *J. Am. Chem. Soc.*, **84**, 1973 (1962).

[9] Zachau, H. G., *Chem. Ber.*, **93**, 1822 (1960).

[10] Brown, D. M., G. D. Fasman, D. I. Magrath, and A. R. Todd, *J. Chem. Soc.*, 1448 (1954).

[11] Andersen, W., D. H. Hayes, A. M. Michelson, and A. R. Todd, *J. Chem. Soc.*, 1882 (1954).

[12] Brown, D. M., Sir A. R. Todd, and S. Varadarajan, *J. Chem. Soc.*, 2388 (1956).

[13] Frank, W., and H. G. Zachau, *Hoppe-Seyler's Z. Physiol. Chem.*, **331**, 258 (1963).

[14] Rammler, D. H., and H. G. Khorana, *J. Am. Chem. Soc.*, **85**, 1997 (1963).

[15] Neilson, A. H., Ph.D. thesis (Cambridge University, 1957).

[16] Brown, D. M., D. B. Parihar, Sir A. R. Todd, and S. Varadarajan, *J. Chem. Soc.*, 3028 (1958).

[17] Kupchan, S. M., P. Slade, R. J. Young, and G. W. A. Milne, *Tetrahedron*, **18**, 499 (1962)

[18] Rammler, D. H., and H. G. Khorana, *J. Am. Chem. Soc.*, **84**, 3112 (1962).

[19] Michelson, A. M., *Ann. Rev. Biochem.*, **30**, 133 (1961).

---

*THE BIOSYNTHESIS OF THE BENZOQUINONE RING OF UBIQUINONE FROM p-HYDROXYBENZALDEHYDE AND p-HYDROXYBENZOIC ACID IN RAT KIDNEY, AZOTOBACTER VINELANDII, AND BAKER'S YEAST**

BY WILLIAM W. PARSON and HARRY RUDNEY†

DEPARTMENT OF BIOCHEMISTRY, WESTERN RESERVE UNIVERSITY SCHOOL OF MEDICINE, CLEVELAND, OHIO

Communicated by Harland G. Wood, January 6, 1964

In the photosynthetic microorganism *Rhodospirillum rubrum*, p-hydroxybenzaldehyde-U-C^{14} has been discovered to undergo a direct conversion into the benzoquinone ring of ubiquinone-10.[1] (U indicates the uniformly or randomly labeled compound.) The carbonyl carbon is lost in the course of this conversion, since carbonyl-labeled p-hydroxybenzaldehyde does not yield radioactive ubiquinone-10. It has been found that the methyl group attached to the ubiquinone ring is supplied by the one-carbon pool.[1, 2] It was of interest to determine whether the anaerobic pathway discovered in *R. rubrum* also occurs in species using oxygen as a terminal electron acceptor. This paper reports that a conversion of p-hydroxybenzaldehyde-U-C^{14} into the ring of ubiquinone occurs in aerated yeast and *Azotobacter vinelandii*, and in a mince of rat kidney cortex. In addition, p-hydroxybenzoic acid-U-C^{14} has been found to give rise to ubiquinone with about the same efficiency as does the aldehyde.

Materials and Methods.—C^{14}-labeled p-hydroxybenzaldehyde, prepared as described elsewhere,[1]