A NUCLEOTIDE-PEPTIDE ISOLATED FROM BOVINE LIVER

Thesis for the Degree of Ph. D.

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David Richard Wilken

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This is to certify that the

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presented by

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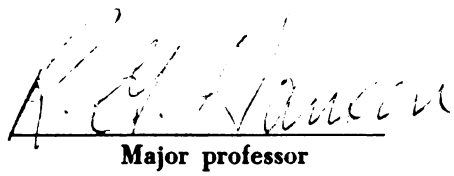

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By

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A THESIS

Submitted to the School for Advanced Graduate Studies of
Michigan State University of Agriculture and
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To Jo Anne

VITA

David R. Wilken was born on February 20, 1934, at Amarillo, Texas. He later moved to Alton, Illinois, where he graduated from Alton Senior High School. From 1951 to 1955 he attended Blackburn College where he received his B. A. degree in Chemistry. He then began his advanced training at the University of Illinois in the Department of Dairy Science where he received his M. S. degree in 1958. He transferred to Michigan State University to complete his formal academic training and his research requirements for the degree of Doctor of Philosophy in the Department of Agricultural Chemistry. He has been awarded a National Foundation postdoctoral research fellowship to continue his studies at the Institute for Enzyme Research at the University of Wisconsin.

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AN ABSTRACT

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ABSTRACT

Numerous reports have appeared in the recent literature concerning the detection of peptide derivatives of nucleotides in a variety of biological materials. A new nucleotide-peptide has been isolated from bovine liver extracts. It has been purified by resin column and paper chromatography. The purified compound has been shown to be homogeneous in several paper chromatographic and paper electrophoretic systems.

Analyses of the peptide moiety of the nucleotide-peptide indicate that it is composed of the amino acids, glutamic acid, glycine, β-alanine, cysteic acid, and taurine. In addition, a sixth ninhydrin reactive component has been detected but remains to be identified. The N-terminal amino acid of the peptide has been identified as glutamic acid.

The nucleotide moiety of the nucleotide-peptide has been identified as 3',5'-adenosine diphosphate by absorption spectrum, enzymatic studies, and paper chromatography of its hydrolysis products. The nucleotide-peptide contains a third phosphate moiety which presumably is a component of the peptide portion of the molecule. Although the nature of the nucleotide-peptide linkage has not been conclusively established, the number of possibilities has been considerably reduced.

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INTRODUCTION

INTRODUCTION

A number of reports have appeared in the recent literature concerning nucleotides and amino acids or peptides combined by covalent linkage. Many of the compounds reported can be placed into one of four generally recognized classes:

1) Nucleotide-peptide compounds of the type originally isolated from penicillin treated Staphylococcus aureus. This class of compounds is composed of various peptide derivatives of UDP-acetylmuramic acid[1] which are precursors of UDP-acetylmuramic acid-L-Ala-D-Glu-L-Lys-D-Ala-D-Ala. In this case the linkage of the nucleotidic and peptidic moieties is an amide involving the carboxyl group of UDP-acetylmuramic acid and the amino group of L-alanine, the N-terminal amino acid of the peptide. A similar compound in which diaminopimelic acid replaces L-lysine has also been reported. These compounds are thought to be involved in the synthesis of the bacterial cell wall. Recently their biochemical formation has been more fully elucidated.

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[1]The following abbreviations are used: Ala, alanine; AMP, ADP, and ATP, adenosine mono-, di-, and triphosphate; ADPR, adenosine diphosphoribose; ATPR, adenosine triphosphoribose; DNP, the 2,4-dinitrophenyl radical; DPN, oxidized diphosphopyridine nucleotide; DEAE-cellulose, N,N-diethylaminoethyl cellulose; FDNB, 1-fluoro-2,4-dinitrobenzene; Glu, glutamic acid; GMP, GDP, and GTP, guanosine mono-, di-, and triphosphate; IMP, inosine monophosphate; Lys, lysine; Pi, inorganic phosphate; PPi, inorganic pyrophosphate; PAPS, adenosine-3'-phosphate-5'-phosphosulfate; RNase, ribonuclease; UMP, UDP, and UTP, uridine mono-, di-, and triphosphate; UDPAG, UDPAH, UDH, and UDPGal, uridine diphosphate N-acetylglucosamine, N-acetylhexosamine, hexose, and galactose; TPN, oxidized triphosphopyridine nucleotide; EDTA, ethylenediaminetetraacetate; CpCpA, cytidylyl-(3'-5')-cytidylyl-(3'-5')-adenosine.

2) Nucleotides composed of a sulfur containing amino acid and 5'-deoxyadenosine which are chemically combined as thioethers or S-alkalated thioethers (sulfonium compounds). Representative compounds of these types are S-adenosylhomocysteine, S-adenosylethionine, and S-adenosylmethionine. The latter compound is biologically important in the formation of spermidine and also functions as a methyl group donor.

3) Nucleotides composed of individual amino acids and adenosine-5'-phosphate in an anhydride linkage. In this case, the amino acid and the nucleotide are combined via a mixed carboxyl-phosphate anhydride bond. These compounds, which normally are enzyme-bound, are believed to represent the initial product of amino acid activation required for protein synthesis.

4) Nucleotide amino acid ester complexes composed of various amino acids and soluble RNA (S-RNA). The ester linkage in S-RNA-amino acid compounds involves the carboxyl group of the amino acid and either the 2' or 3' hydroxyl group of the terminal nucleotide of the S-RNA. Such compounds appear to be intermediates in the protein synthetic sequence lying between initial amino acid activation and peptide bond formation.

Other reports have appeared in the literature concerning the detection of nucleotide-peptide complexes which probably are not intimate members of the above chemically defined groups. They appear to be diversified but apparently have one general feature in common, namely some type of nucleotide-amino acid or nucleotide-peptide association. Such compounds have been obtained from fish, chlorella, yeasts, bacteria, fungi, spores, and mammalian tissue. Although no

evidence concerning the nature of the nucleotide-peptide linkage has been reported in most cases, peptide hydroxamates have been observed after reacting some nucleotide-peptide preparations with hydroxylamine. In addition, some nucleotide-peptide preparations have been cleaved by treatment with aqueous hydrazine forming hydrazides of peptides, indicating covalently linked nucleotide-peptides. The question of the biochemical function of the above nucleotide-peptide compounds has not yet been answered.

The present report concerns the isolation and identification of a new nucleotide-peptide obtained from bovine liver.

LITERATURE REVIEW

LITERATURE REVIEW

Peptide Derivatives of UDP-Acetylmuramic Acid

The first report concerning peptide derivatives of UDP-acetylmuramic acid (Figure 1) came from Park and Johnson (1) who were attempting to elucidate the mechanism of action of penicillin. It had previously been observed that Staphylococcus aureus cells increased in size in a medium containing penicillin, although they did not divide. Therefore, it seemed of interest to ascertain whether or not this increased growth was a function of an increased amount of one or several biological intermediates or due to some other cause. Dry weight, nitrogen, phosphorus, nucleic acid phosphorus, and nucleic acid increased at comparable rates during growth in the presence of penicillin, although the increases in these constituents were only about one-half of the increase observed in untreated cells. In contrast, a rather marked change in the distribution of acid-soluble phosphate was observed. While the per cent increase in acid-stable phosphate was about equal in penicillin treated and non-treated cells, the amount of inorganic phosphate was decreased in treated cells. The most interesting change observed, however, was that while normal cells increased in acid-labile phosphate two fold, there was a three fold increase of acid-labile phosphate in penicillin treated cells. The acid-labile phosphate compounds were partially purified by precipitating the barium salts with alcohol. In addition to the presence of pentose and uracil moieties, such preparations contained one mole of

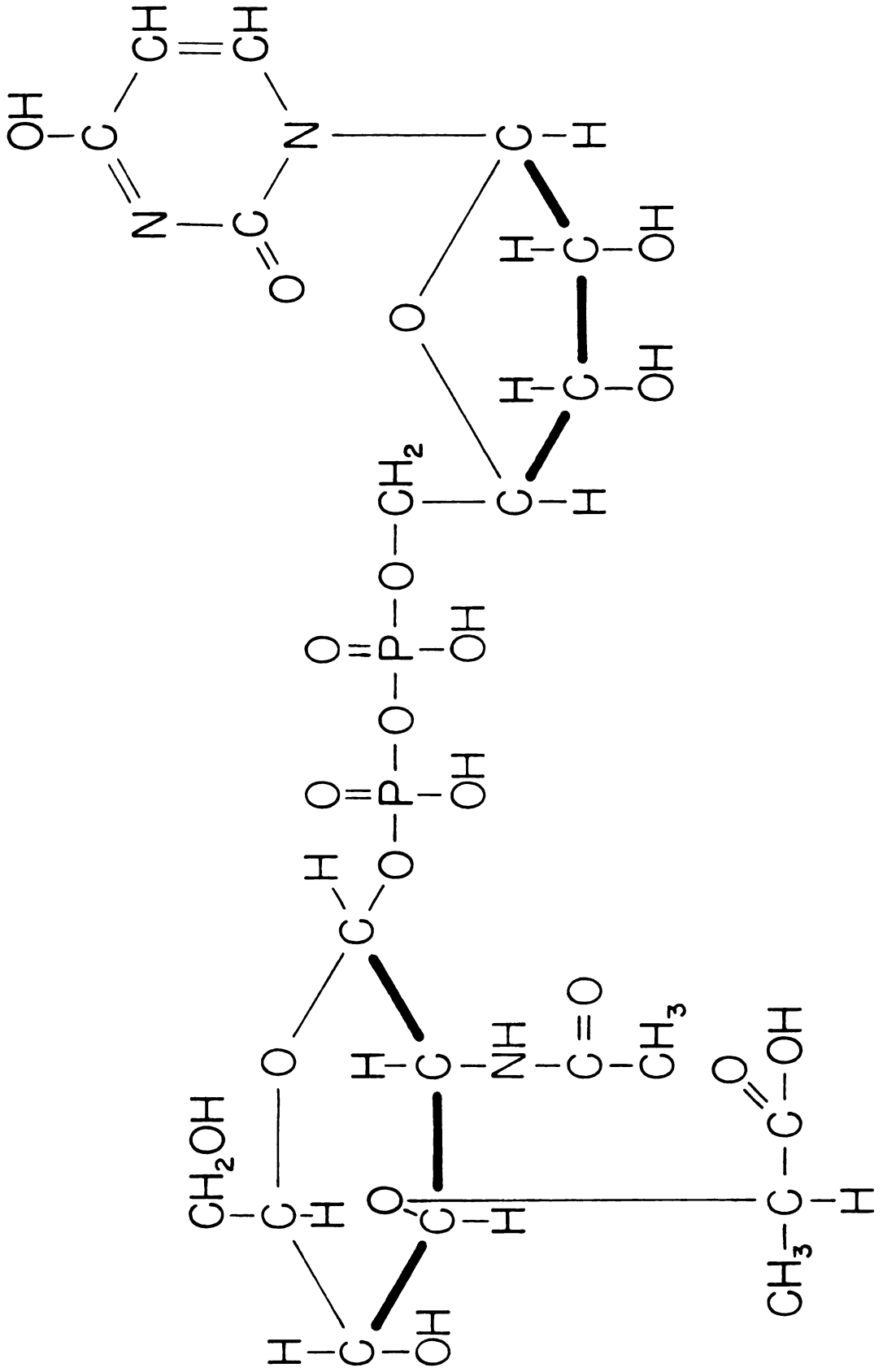


Figure 1 Uridine diphosphate acetylmuramic acid

acid-stable phosphate and 0.7 mole of potential reducing power per mole of acid-labile phosphate.

These several observations were the beginning of numerous investigations which in the past eleven years have led to elucidation of the structure, almost the entire biosynthetic pathway and a proposed function of the peptide derivatives of UDP-acetylmuramic acid.

Park (2) succeeded in purifying the acid-labile phosphate preparation described above and resolved it into three distinct components by means of chromatography. All were derivatives of uridine containing molar ratios of labile phosphate:stable phosphate:uridine: potential reducing power of 1:1:1:0.8. The major difference in the three compounds was their nitrogen content which approached molar ratios of 3, 4, and 9 for compounds 1, 2, and 3 respectively. The increased amount of nitrogen in compounds two and three was due to the presence of amino acids.

Compound one was studied in more detail by Park (3). He was able to establish that it was a derivative of uridine monophosphate and contained a pyrophosphate linkage as well as a glycosidic-phosphate linkage. The postulated structure was a uridine diphosphate-reducing substance in which the latter was combined with the terminal phosphate of UDP by an acetal-like linkage. Although the exact identification of the reducing substance, acetylmuramic acid, was not ascertained at that time, it was indicated that it was an acidic amino sugar with no <u>cis</u> hydroxyl groups and that the amino group was acetylated. The identification of the reducing sugar as acetylmuramic was reported by Strange (4) and the structure suggested by him

was later confirmed by synthesis (5).

Park's compounds two and three were found to contain one L-alanine residue and a peptide respectively (6). The glycopeptide portion of compound three was separated from the nucleotide moiety after mild acid hydrolysis and was found to be composed of three moles of alanine, one mole of L-lysine, and one mole of D-glutamic acid. Configurational analysis of the alanine residues of the peptide indicated a mixture of the D and L isomers. Recently it has been established that two of the alanine residues are D-alanine and the other residue is L-alanine (7).

Later experiments of Strominger (8) more clearly defined several of the parameters concerning the accumulation of the UDP-acetylmuramic acid derivatives in the presence of penicillin. Time-course studies indicated that the accumulation commenced immediately upon the addition of penicillin and that the half-time for maximum accumulation was about fifteen minutes. Several other antibiotics including aureomycin, terramycin, chloromycetin, and streptomycin at concentrations up to 100 times the amount of penicillin required to obtain accumulation of nucleotide derivatives caused little or no accumulation of these compounds. Streptomycin could cause some accumulation of the compounds, however 1000 times as much of it was required compared to penicillin. These two findings supported the idea that penicillin was acting in a relatively specific rather than a broad capacity. It was also observed that the accumulation could be induced in resting cultures supplied only inorganic salts, glucose, and either the amino acids L-lysine, L-alanine, and L-glutamic acid or

larger amounts of glutamic acid alone. This latter finding in conjunction with better isolation procedures has greatly aided more recent studies of these compounds.

The presence of the UDP-acetylmuramic acid derivatives in normal as well as in penicillin treated Staphylococcus aureus (8,9), Lactobacillus helveticus 335 (8) and in normal Streptococcus hemolyticus (10) indicated that these compounds might be of general importance in microorganisms. Indeed, the similarity of the molar ratios of the cell wall components of some bacteria to the amino acid and muramic acid content of UDP-acetylmuramic acid-peptide led Park and Strominger (11) to propose that this compound was a precursor of the bacterial cell wall. They found that hydrolysates of S. aureus cell wall contained molar ratios of D-glutamic acid:alanine:lysine:muramic acid of 1:3:1:1, the same as is present in the UDP-acetylmuramic acid-peptide.

The first estimates of the relative amounts of D and L isomers of alanine in the nucleotide-peptide (6) and in the cell wall hydrolysates (11) indicated approximately equal amounts of these components. In order for this finding to be consistent with the known number of alanine residues in UDP-acetylmuramic acid-peptide, namely three, it was necessary to consider the possibility that there were two nucleotide-peptides with similar properties. One such compound would have two L-alanine residues and one D-alanine residue, the other would have two D-alanine residues and one L-alanine residue. Such an interpretation would require the further postulate that the latter two hypothetical compounds be isolated in equimolar amounts and that they

be incorporated into cell wall in equal proportions. The above possibility seemed rather remote. Strominger and Threnn (7) therefore reinvestigated the D-alanine and L-alanine content of S. aureus cell wall and also the nucleotide-peptide. Indeed, they found that in both cases 67 per cent of the alanine was the D isomer and 33 per cent was the L isomer. This finding put the postulate that a single carbohydrate-containing peptide is incorporated into cell wall material on a more firm basis.

Although the function of the nucleotide-peptide had been proposed, the sequence of the amino acids in the peptide was not known. It was inferred that the N-terminal amino acid was L-alanine because it was assumed that UDP-acetylmuramic acid-L-alanine was an intermediate in the formation of the complete nucleotide-peptide. To establish the amino acid sequence in the peptide, Strominger (12) hydrolyzed the intact nucleotide-peptide with various concentrations of acid and succeeded in isolating several of the partial hydrolysis products. From the determination of the amino acid content of the various fragments, the number of possibilities for the amino acid sequence of the peptide could be reduced to four, namely,

1. Ala-Glu-Lys-Ala-Ala
2. Ala-Lys-Glu-Ala-Ala
3. Ala-Ala-Glu-Lys-Ala
4. Ala-Ala-Lys-Glu-Ala

Sequence 1 was determined to be the proper sequence on the basis of the isolation of a new UDP-acetylmuramic acid-peptide (12,13). This nucleotide-peptide accumulated when a resting culture of S. aureus

was placed in a lysine deficient medium. It contained one residue each of L-alanine and D-glutamic acid. The new compound was converted to the complete nucleotide-peptide when cells in which it had accumulated were transferred to a medium containing lysine. This indicated that the new nucleotide-peptide was an intermediate in the formation of the complete nucleotide-peptide. Furthermore, this series of findings were consistent only with the possibility that the intact nucleotide-peptide had the sequence: UDP-acetylmuramic acid-L-Ala-D-Glu-L-Lys-D-Ala-D-Ala. Subsequent isolation of a UDP-acetylmuramic acid-peptide (14) containing one mole each of L-alanine, glutamic acid, and lysine is consistent with the above peptide sequence.

The several nucleotide-peptides discussed above all appeared to be intermediates in the synthesis of the final nucleotide-peptide compound. Thus, it seemed probable that the amino acids were being incorporated singly and not as a preformed peptide unit. Recent experiments of Ito and Strominger (15) have elucidated this problem. These workers have separated the enzymes responsible for the incorporation of L-alanine, D-glutamic acid, and L-lysine singly and in that order into their respective substrates. In addition, an enzyme forming the dipeptide D-alanyl-D-alanine was also found and this dipeptide is incorporated intact into the complete nucleotide-peptide. Each reaction was found to require ATP as an energy source and a divalent metal such as Mn^{++}.

Although the entire sequence of the components of the complete UDP-acetylmuramic acid-peptide, as well as most of the structure and

most of the biosynthetic pathway is known, several problems remain to be answered. Insofar as structure is concerned the problems yet to be solved are: 1) the configuration of the lactic acid moiety of the molecule, and 2) the nature of the bonding of the glutamyl and lysinyl residues. It has not yet been established whether the linkages of these two amino acids are the same as normally found, that is that they involve the α-carboxyl andα-amino groups, or whether the amide linkages involve the γ-carboxyl group of glutamic acid and/or the ε-amino group of lysine. In addition, there appears to remain at present at least one missing step in the biosynthesis of the nucleotide-peptide, namely the synthesis of UDP-acetylmuramic acid. Strominger (16) has reported that cell-free extracts of S. aureus and E. coli are able to phosphorylitically condense phosphoenolpyruvate and UDPAG to form UDPAG-enol-pyruvate. This compound is a likely intermediate which would require the addition of a molecule of hydrogen to form UDP-acetylmuramic acid. Preliminary results indicate the presence of a UDP-acetylmuramic acid-peptide containing diaminopimelic acid in place of lysine (17). Further work will be required to elucidate its structure and biosynthesis.

S-Adenosylmethionine and Related Compounds

Although methylation of nicotinamide employing methionine as the methyl donor had previously been demonstrated under aerobic conditions, Cantoni (18) was the first to obtain a cell-free preparation in which the methylation could be carried out under anaerobic conditions. In this case, an ATP generating system or ATP itself could replace the previously required oxygen dependent systems. Later experiments by

Cantoni (19) demonstrated that an unidentified intermediate required for the transmethylation reaction was formed by incubating ATP, Mg^{++}, and methionine with a pig liver protein fraction. This finding was soon followed by isolation of the intermediate and presentation of a proposed structure (20,21) (Figure 2). The proposal of this structure was based to a large degree on the following findings: 1) The compound exhibited an absorption spectrum identical with adenosine and had a ratio of adenine:labile methyl groups:pentose of 1:1:1. When it was enzymatically prepared from C^{14} or S^{35} labeled methionine, it contained radioactivity. 2) Acid hydrolysis liberated three detectable compounds: adenine, homoserine, and an unidentified sulfur containing compound. 3) Evidence in favor of the postulated sulfonium ion included electrophoretic migration as a cation at pH 7.8 and failure to react with L-amino acid oxidase. This enzyme appears to attack only amino acids whose dipolar ions have their charges close together (22,23). On this basis, S-adenosylmethionine, like the methyl sulfonium derivative of methionine (24), would not be expected to be attacked by L-amino acid oxidase. Additional support for the proposed structure was gained when it was found that during heating at 100^{o} at pH 7 S-adenosylmethionine was cleaved to homoserine and adenine thiomethylribose, and that the untreated compound reacted with periodate which indicated no 2' or 3' hydroxyl substitution (25). In addition, treatment with 0.1 N alkali for ten minutes at 25^{o} yielded almost entirely adenine and S-ribosylmethionine (26). The final support for the structure has been its complete chemical synthesis (27).

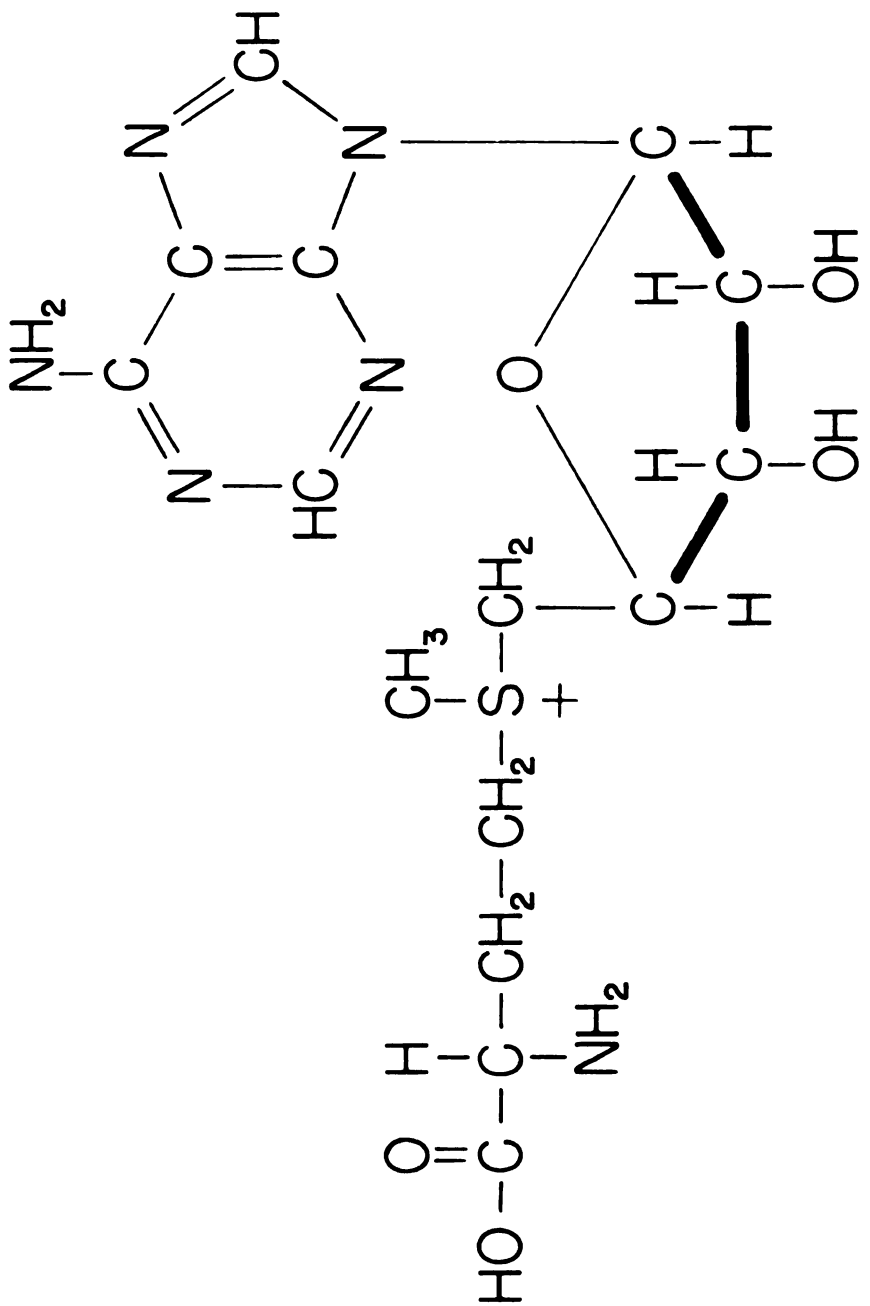


Figure 2 S-Adenosylmethionine

The chemically synthesized S-adenosylmethionine is not completely enzymatically active because presumably all four of the possible sterioisomers (D-L methionine, ± sulfonium ion) are formed in the synthesis employed and the various methylpherase enzymes are relatively specific for one of the isomers (28). A newer synthesis of S-adenosylmethionine combining enzymatic and chemical methods has reduced the number of sterioisomers obtained to two (28). In this synthesis, S-adenosyl-L-homocysteine was first prepared enzymatically from adenosine and L-homocysteine employing a rat liver condensing enzyme which is specific for L-homocysteine (29). Methylation of the enzymatic product yielded a preparation of (±) S-adenosyl-L-methionine which was 50% enzymatically active. Using S-adenosylmethionine prepared in this manner, it was demonstrated that the (-) S-adenosylmethionine is the substrate for guanidoacetic acid and catechol methylpherases and also for the yeast enzyme which cleaves S-adenosylmethionine to form 5'-methylthioadenosine and 2-amino-4-butyrolactone. This appears to be the first biological resolution of a sulfonium ion.

The major function of S-adenosylmethionine appears to be its ability to serve as a source of methyl groups in transmethylation reactions, although it also undergoes other interesting biological reactions. Evidence has been obtained for its role as a methyl donor to form the following specific compounds or general types of compounds: a) N-methylnicotinamide (18), b) creatine (30), c) O-methylcatechols (31), d) ergosterol (32), e) N-methylamino purines (33), f) methionine (via transmethylation with homocysteine) (34), and g)

choline (35). S-adenosylhomocysteine has been identified as the remaining product in creatine synthesis (36), and it presumably is one of the products in the other transmethylation reactions described above. Its structure has been demonstrated by synthesis (37). Transethylation involving S-adenosylethionine has also been observed (38). This compound is found in Torulopsis utilis grown in the presence of ethionine. Formation of the S-adenosylethionine and subsequent transethylation may be due to incomplete specificity of the enzymes responsible for the analogous reactions involving methionine. In addition to its transmethylation function, S-adenosylmethionine is involved in spermidine synthesis (39). In this case it is the α, β, and γ carbons of the methionine moiety which are transferred to putrescine after an initial decarboxylation of the methionine moiety. S-adenosylmethionine also undergoes a biochemical cleavage (40,41) similar to one of its known chemical cleavage reactions (42). In both cases, the products are 5'-methylthioadenosine and 2-amino-4-butyrolactone. The mechanism of the enzymatic cleavage has been studied (43). Although no mechanism has been proved, an initial elimination of 2-amino-3-butenoic acid as an intermediate in the reaction has been excluded since no tritium from the aqueous medium was incorporated into the 2-amino-4-butyrolactone formed. A second possible mechanism which has not been tested is a nucleophylic attack on the carbon adjacent to the sulfur atom by a carboxyl oxygen with the elimination of 2-amino-4-butyrolactone.

One of the most interesting aspects of S-adenosylmethionine metabolism is its biological formation. The enzyme(s) catalyzing

its formation from ATP and methionine and the proper metals have been partially purified from rabbit liver (44) and yeast (45) and their properties have been compared. Enzymes from both sources require high concentrations of Mg^{++} for optimal activity and both require monovalent cations such as NH_4^+ or K^+ for activity. Some differences between the enzymes have been found, such as the observation that the liver enzyme specifically requires Mg^{++} while the yeast enzyme responds, although less well, to other divalent cations. In addition, while fluoride inhibits the rabbit liver enzyme, it appears to have no effect on the yeast enzyme even at higher concentrations than required to demonstrate inhibition of the liver enzyme. Although differences do exist between the enzymes, the overall reaction appears to be the same. It has been demonstrated that the products of the reaction are inorganic phosphate and pyrophosphate as well as S-adenosylmethionine. The inorganic phosphate arises from the γ or terminal phosphate of ATP and the pyrophosphate arises from the α and β phosphates of ATP. Although inorganic phosphate is liberated from the terminal phosphate of ATP during the reaction, it has not been possible to demonstrate any participation of free ADP in the reaction. Furthermore, neither has it been possible to separate the overall reaction into two or more fractions nor to demonstrate an exchange of Pi^{32} into ATP or PPi^{32} into ATP either with or without methionine in the presence of the purified enzyme. Incorporation of C^{14}-labeled methionine into S-adenosylmethionine in the presence of the purified enzyme also could not be demonstrated. Observations reported thus far indicate that the entire reaction may be catalyzed

by a single enzyme, however, confirmation of this possibility must await absolute purification of the enzyme(s). Aside from the interesting elimination of Pi and PPi, the reaction represents a rather unique utilization of ATP, namely as an adenosine donor.

Amino Acid Adenylates

Although other reports had appeared in the literature which demonstrated the incorporation of radioactive amino acids into protein, Zamecnik and Keller (46) were the first to demonstrate incorporation under anaerobic conditions. Their system required an ATP generating system, amino acids, a soluble, heat-labile, non-dializable fraction, and a microsome-rich fraction into which amino acids were incorporated. Later systems were simplified in that the membranous material could be removed from the microsomes leaving ribonucleoprotein particles into which amino acids were incorporated (47). In ascites tumor cell preparations it was observed that ATP could replace the previously required ATP generating systems (47). As the overall protein incorporating system was refined, it became evident that GDP or GTP also played some role in the synthetic process (48). Study of individual components required for protein synthesis has lead to the discovery of amino acid adenylates and the S-RNA-amino acid complexes.

The first report of amino acid carboxyl group activation came from Hoagland and co-workers (49,50) as a result of their studies on protein synthesis. The enzymatic activation was dependent upon ATP, amino acids, and a protein fraction obtained from the supernatant (soluble fraction above) of rat liver homogenates by precipitation

at pH 5.2. Enzymatic reactions were measured either by exchange of PPi^{32} into ATP or by the formation of amino acid hydroxamates in the presence of hydroxylamine. In the latter case the hydroxylamine acts as an acceptor of the activated amino acid. Although net accumulation of the activated amino acid could not be demonstrated, a concomitant equimolar formation of PPi and amino acid hydroxamate could be observed in the presence of hydroxylamine. Failure of C^{14}-labeled AMP to exchange with ATP under conditions in which PPi^{32} rapidly exchanged with ATP indicated that the activated amino acid and AMP were enzyme-bound. The above findings were consistent with the postulated intermediate, namely, a tightly enzyme-bound amino acid adenylate in which the mode of linkage was a carboxyl-phosphate anhydride bond (Figure 3).

It was demonstrated that the pH 5 fraction supplemented with GTP could incorporate amino acids into microsomal protein in the presence of ATP. Thus the carboxyl activation represented the initial step in protein synthesis. Of several amino acids tested none showed competitive inhibition of activation of other amino acids. Instead, an additive PPi^{32} exchange with ATP or hydroxamate formation was observed. This finding, as well as partial separation of leucine, alanine, and methionine-activating enzymes indicated that each amino acid has a specific activating enzyme. Support for this hypothesis has come in the form of purification of several of the specific amino acid-activating enzymes (51-57).

Support for the carboxyl-phosphate anhydride structure of amino acid adenylates has come from several laboratories in various forms.

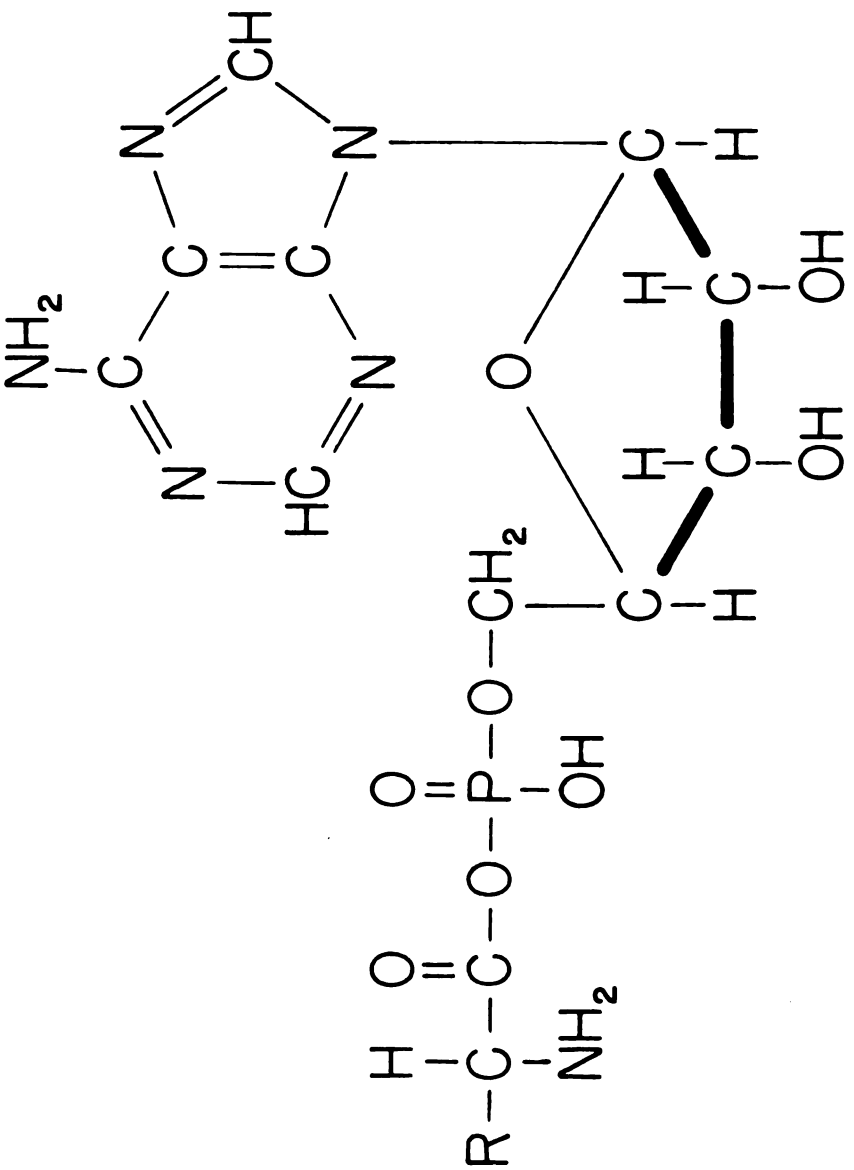


Figure 3 An amino acid adenylate

De Moss et al. (57) were able to synthesize leucine adenylate using the disilver salt of AMP and the acid chloride of L-leucine. The stability of the product decreased with increasing pH between pH 5.1 and 8.0. In the presence of the synthetic compound, PPi, and a purified leucine-activating enzyme from E. coli, a net appearance of ATP could be observed with a concomitant equimolar decrease of the leucine adenylate. This demonstrated that the proposed amino acid adenylates could react in the reverse direction of amino acid activation and thus could represent the product of amino acid activation. Reversibility of this reaction had previously been indicated by the PPi^{32} exchange into ATP (49). The formation of ATP from L-leucine adenylate and PPi appeared to be specific for L-leucine adenylate since neither D-leucine adenylate nor L-alanine adenylate showed appreciable capacity to form ATP although the latter had been active with crude extracts. These findings were consistent with the suggestion of specific amino acid-activating enzymes. The fact that the synthetic material reacted with hydroxylamine at lower concentrations than required in the enzymatic reaction, combined with the observation that there was no net breakdown of ATP in the enzymatic reaction of ATP, leucine, and enzyme even in the presence of inorganic pyrophosphatase, emphasized that the reaction product was tightly enzyme-bound.

Additional confirmation of the proposed carboxyl-phosphate anhydride structure of amino acid adenylates has come from isotope studies. Hoagland et al. (58) and Bernlohr and Webster (55) have studied the fate of carboxyl labeled O^{18} of tryptophan and alanine.

In both experiments a partially purified specific amino acid-activating enzyme was used. The enzyme, ATP, amino acid, and hydroxylamine were incubated and the inorganic pyrophosphate and phosphate from AMP which were formed were collected for determination of O^{18} content. It was found that O^{18} was incorporated into the phosphate oxygen of AMP in nearly the theoretical amount and that no O^{18} was incorporated into the oxygen of the inorganic pyrophosphate. This finding was consistent with the idea that the oxygen of the carboxyl group condensed with the stable phosphate of ATP via a nucleophylic attack with concomitant release of inorganic pyrophosphate. The amino acid adenylate thus formed was then cleaved at the carbon oxygen linkage of the anhydride bond to form the amino acid hydroxamate and AMP containing O^{18}.

The final confirmatory evidence that the product of amino acid activation was an amino acid adenylate came from Karasek et al. (59). These workers found that in the presence of substrate amounts of tryptophan-activating enzyme, ATP, tryptophan, and inorganic phosphatase, tryptophan adenylate was formed and could be isolated from the incubation mixture. The isolated product had the electrophoretic mobility of synthetic tryptophan adenylate and reacted rapidly with hydroxylamine to form the expected tryptophan hydroxamate. This finding has been confirmed and extended by Kingdon et al. (60). These workers identified the enzymatic reaction product as being the same as synthetic material by electrophoresis, resin column chromatography, reactivity with hydroxylamine, and by its ability to undergo the reverse enzymatic reaction to form ATP. Only one other amino

acid adenylate, namely serine adenylate, has been isolated thus far (56).

Soluble RNA-Amino Acid Complexes

The discovery of S-RNA-amino acid complexes as well as part of the structural identification of these complexes came as the result of continued study of protein synthesis. These complexes have been found to be the product of the transfer of the amino acid of amino acid adenylates to S-RNA. This represents the second step in protein synthesis.

Hoagland et al. (61,62) were the first to demonstrate that RNA present in the amino acid-activating enzyme of rat liver (usually termed soluble RNA, S-RNA, or transfer RNA) became labeled when it was incubated with the pH 5 enzyme (from the soluble fraction above), ATP, Mg^{++}, and C^{14}-amino acid. Berg (63) has indicated that the amino acid-activating enzyme may also catalyze the transfer of the activated amino acid to S-RNA. This suggestion came from the observation that during approximately a hundredfold purification of methionine-activating enzyme the ratio of amino acid activation to incorporation into S-RNA was constant. Incorporation of a mixture of amino acids into S-RNA was additive (62) as in the case of amino acid activation and indicated the existence of a specific S-RNA for each amino acid. Furthermore, it was found that preformed S-RNA-leucine could transfer the amino acid to microsomes in the presence of GTP and an ATP generating system. Since GTP was not required for incorporation of amino acids into S-RNA, it appears that it functions after the formation of the S-RNA-amino acid complex. Some component

of the pH 5 fraction also seemed to be necessary for the transfer of the amino acid of the S-RNA-leucine to ribonucleoprotein, since carefully washed microsomes incorporated the amino acid moiety only poorly unless the pH 5 fraction was also present. That the pH 5 fraction was not merely reconverting the S-RNA-leucine to leucine adenylate which in turn might transfer the amino acid residue to the microsomes was indicated by the failure of C^{12}-leucine to lower labeling of the microsomes, and by the failure of S-RNA-leucine pretreated with mild alkali to incorporate the amino acid into microsomes. Treatment of the S-RNA-amino acid complex with mild alkali liberates the amino acid from the S-RNA.

Several other properties of the S-RNA-amino acid complex were studied (62). It was noted that the compound was non-dializable and stable against water, 10 per cent NaCl, and 8 M urea. In addition, the amino acid was liberated by treatment with 0.01 N alkali for twenty minutes at room temperature, however, at pH 4 to 6 it was relatively stable. It reacted with anhydrous hydroxylamine to form the respective amino acid hydroxamate indicating carboxyl activation. Since several of these properties were similar to the amino acid adenylates, it seemed possible that a carboxyl-phosphate anhydride linkage was present although other possibilities were not excluded. Among other possibilities mentioned by Hoagland *et al.* (62) was an ester involving the carboxyl group of the amino acid and the 2' or 3' hydroxyl group of the S-RNA (Figure 4). It is now known that this is the mode of linkage and that the nucleotide moiety involved is the adenosine terminal nucleotide of the S-RNA.

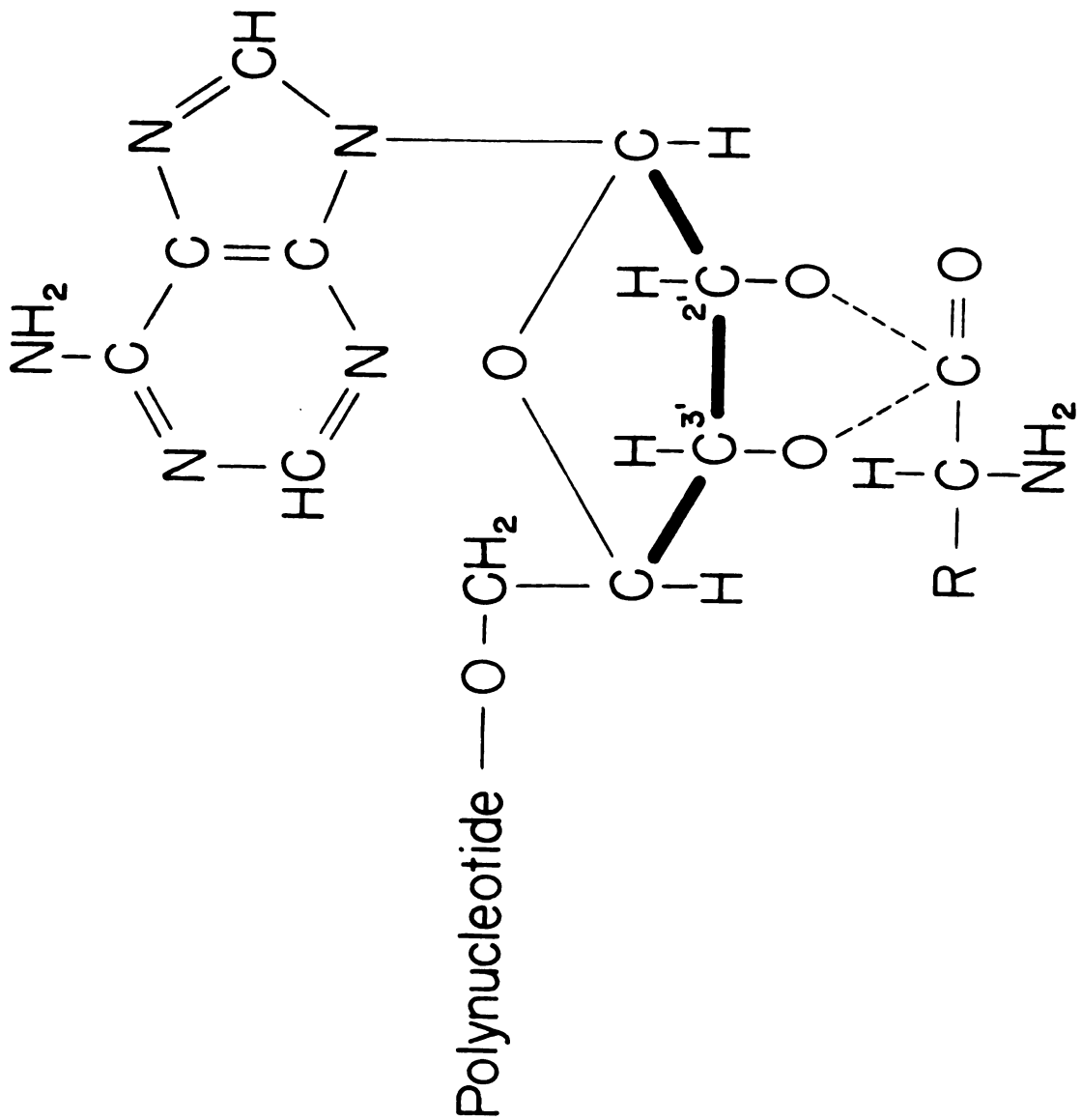


Figure 4 A soluble RNA-amino acid complex

Confirmation of the incorporation of amino acids into S-RNA and evidence for the existence of a S-RNA for each amino acid have come from other laboratories. The S-RNA required for tyrosine and leucine have been partially separated (64,65). One of the earliest and clearest demonstrations that there was a specific S-RNA for each amino acid was reported by Priess et al. (66). S-RNA was charged with leucine, valine, or methionine and the resultant S-RNA and S-RNA-amino acid were treated with periodate to destroy all of the S-RNA except that protected by the incorporated amino acid. The resulting S-RNA-amino acid was then freed of its amino acid and tested for its capacity to accept leucine, valine, and methionine. The results indicated that the S-RNA obtained by these treatments would react only with the amino acid with which it had been originally charged, thus confirming the existence of specific S-RNAs.

Another elegant demonstration of specific acceptor S-RNAs should be mentioned because the methodology employed represents a potentially powerful tool for the separation of these compounds. Brown et al. (67) found that at the proper pH only histidine and tyrosine reacted with a polydiazostyrene resin at an appreciable rate. Taking advantage of this property, they placed a preparation of S-RNA fully charged with all of the common amino acids on such resin. Only S-RNA-histidine and S-RNA-tyrosine remained bound to the resin while the other S-RNA-amino acids were washed free of the resin. The S-RNAs for histidine and tyrosine could then be liberated from the resin by mild treatment at pH 10. The resulting mixture of the two S-RNAs was resolved by charging it with tyrosine using a partially purified

tyrosine-activating enzyme and then treating this mixture with the polydiazostyrene resin. During the second resin treatment, only S-RNA-tyrosine was retained by the resin; the rest of the S-RNA was washed free of the resin. The tyrosine-specific S-RNA was then removed from the resin by alkaline treatment. When the two purified S-RNAs were tested they were found to be relatively specific for their respective amino acids. Preliminary experiments attempting to charge the valine-specific S-RNA with tyrosinylvaline appeared to be successful. If this is true then application of this modification of the method may be used for preparing specific S-RNAs for all the other amino acids.

Several types of evidence have been advanced to demonstrate that the amino acid is attached to the S-RNA by an ester linkage involving the 2' or 3' hydroxyl position of the terminal adenosine unit of S-RNA. Priess et al. (66), as discussed above, demonstrated that S-RNA charged with amino acid was stable to periodate while uncharged S-RNA was attacked by periodate. This suggested that the amino acid was linked to the 2' or 3' hydroxyl group of the terminal adenosine unit because this unit is the only known site in the S-RNA molecule sensitive to periodate. Treatment of S-RNA-C^{14}-amino acid with RNase converted the amino acid to an acid-soluble form which migrated differently from either the free amino acid or adenosine. Treatment of this compound with mild alkali liberated adenosine and the free amino acid, thus the amino acid was in some way combined with the adenosine. Hecht et al. (68) noted that the stability of the S-RNA-amino acid compound was greater than that of amino acid

adenylates. For example, S-RNA-valine was stable to heating at 100° in 10 per cent NaCl at pH 4-5 and failed to react rapidly with ammonia to give the amino acid amide. This behavior was in marked contrast to valine adenylate. It was also found that borate inhibited the incorporation of C^{14}-valine into S-RNA possibly by complexing with the free 2', 3' hydroxyl groups of the terminal adenosine unit. Another indication of 2' or 3' hydroxyl substitution in the S-RNA-amino acid complex came from the application of Whitfield's (69) observation that after oxidation with periodate the terminal nucleoside of a di- or trinucleotide is readily split off at pH 10 while ordinary phosphodiesters are stable. When this principle was applied to equal amounts of S-RNA and S-RNA charged with amino acids, it was found that the amino acid derivative was considerably protected from attack by periodate compared to the S-RNA not charged with amino acids. Perhaps the most convincing arguments for esterification of the 2' or 3' hydroxyl of the terminal adenosine unit of S-RNA has been presented by Zachau et al. (70). These investigators studied the relative per cent decomposition of various leucine esters in the presence of hydroxylamine at pH 5.5 and 0°. They found values for leucine ethylester, 0; 2'/3' leucine ester of AMP, 28; C^{14}-leucyl-RNA, 41; and leucine adenylate, 100. The lability of the leucyl-RNA was much nearer to that of synthetic 2'/3' leucine ester of AMP than leucine adenylate. In addition, when S-RNA charged with C^{14}-leucine was treated with RNase it liberated 92 per cent of the leucine as 2'/3' leucyl-adenosine which had the same electrophoretic mobility as the synthetic compound. Since this product failed to

react with periodate, it was concluded that the ester linkage involved either the 2' or 3' hydroxyl of the adenosine moiety. It should be pointed out that as yet it has not been ascertained to which position, the 2' or 3' hydroxyl of the terminal nucleoside of S-RNA, the amino acid is combined.

Although progress has been made in determining the mode of linkage between the S-RNA and amino acid of the S-RNA-amino acid complex, as well as establishing the identity and terminal sequence of three nucleotide units of S-RNA, namely CpCpA (68,71), further sequencial nucleotide analysis will undoubtedly be performed in the future. Establishment of the complete structure of the S-RNA-amino acid complex may provide the answer to the problem of the specificity of each S-RNA for a particular amino acid. The tools discussed above for the separation of various S-RNAs will undoubtedly help solve this problem.

Miscellaneous Amino Acid or Peptide Derivatives of Nucleotides or Polynucleotides

A relatively large number of reports have appeared in the literature concerning the detection of nucleotide-peptide complexes. Based on the very limited information available, the complexes as a group appear to be composed of different structural types, however, none appear to be intimate members of the four general classes discussed above. The one feature that they have in common is that they all appear to contain a nucleotide and amino acid or peptide in association, the mode(s) of which is as yet unknown. Attempts at a chemical definition of the nucleotide-amino acid or peptide associa-

tion have been made in only a few instances. These include the reports of Koningsberger et al. (72), Dirheimer et al. (73), and Szafranski et al. (74) who have observed the formation of hydroxamic acids of peptides after reacting nucleotide-peptide preparations with hydroxylamine. In addition, Weinstein et al. (75) have obtained hydrazides of peptides by treating liver nucleotide-peptide preparations with hydrazine. These reports tentatively indicate a nucleotide-peptide linkage involving the carboxyl group of the peptide. The function of all of these compounds is not clear as yet, but their biological importance may be indicated by their widespread distribution, namely in fish (76), chlorella (77-80), yeasts (72, 77-85), bacteria (73,85-88), fungi (73,89), spores (85), and mammalian tissue (73-75,83,90-93).

METHODS

METHODS

The method of isolating the nucleotide-peptide is described in detail under "Results" but the various procedures for examining the whole molecule or its components are outlined below. The paper chromatographic systems which have been used to indicate the homogeniety of the nucleotide-peptide are: a) isobutyric acid:H_2O:NH_4OH (66:33:1) (94), b) 95% ethanol:1 M ammonium acetate, pH 3.8 (7.5:3) (95), and c) isobutyric acid:1 N NH_4OH:EDTA (100:60:1.6) (88). The paper electrophoretic conditions of Wade and Morgan (96) were used, employing either 0.5 M butyrate buffer, pH 3.2 (5 hrs.) or a 0.05 M citrate buffer, pH 4.5 (6 hrs.). In both cases the voltage was maintained between 300 and 350 volts at a temperature of 0-5°. The nucleotide-peptide was detected on chromatograms by its ultraviolet absorption using a Mineralite lamp, model SL 2537, and by its reactivity with ninhydrin. The spray used was a 2 per cent ninhydrin solution in ethanol, collidine, and water (90:5:5). Photographs of ultraviolet absorbing spots on paper chromatograms were made from contact prints prepared by the following modification of the procedure of Markham and Smith (97). The chromatogram was placed over a sheet of "Kodagraph Contact Standard" paper, emulsion side up, and held in place by a thin sheet of polyethylene. The contact paper was then exposed to ultraviolet radiation for several seconds using the source described above.[2] Compounds containing phosphate were de-

[2]The procedure described was suggested by Dr. David S. Feingold, personal communication.

tected on paper chromatograms employing the reagents of Hanes and Isherwood (98) and the modified detection procedure of Bandurski and Axelrod (99). Compounds which react with periodate were detected on paper chromatograms by employing the method of Gordon et al. (100).

An extinction coefficient of 15.4 at pH 7 (94) was used to estimate adenosine nucleotide concentration at 259 mμ. Pentose was determined by the procedure of Mejbaum (101). Samples of pyrimidine nucleotides were first reduced with sodium amalgam (102) before being assayed for pentose by the orcinol test. Phosphorus was determined by the procedure of Fiske and Subbarow (103), and ninhydrin amino equivalents were determined according to Moore and Stein (104). Hexosamine determinations were carried out by a modified Elson-Morgan reaction (105). Hydroxamate reactions were performed by employing the methods of Lipmann and Tuttle (106) as well as the reaction conditions described by Raacke (107) in conjunction with the quantitative detection procedure of Schweet (108).

Amino acids were liberated from the peptidic moiety of the nucleotide-peptide by hydrolysis in 6 N HCl at 100-105° for 17 hours in a glass stoppered tube. They were identified by two dimensional paper chromatography employing water-saturated phenol followed by the butanol:propionic acid:water solvent of Flavin (109), and were detected using the polychromatic ninhydrin spray of Moffat and Lytle (110). DNP derivatives of the amino acids liberated by the above hydrolysis were prepared by the general procedures described by Fraenkel-Conrat et al. (111). After removing most of the dinitrophenol by vacuum sublimation (112), the residue was made 1 N with

respect to HCl. The DNP derivatives were then separated into ether extractable and ether nonextractable fractions before being submitted to paper chromatography. The DNP-amino acids were identified by two dimensional paper chromatography on phthalate-buffered paper using tert-amyl alcohol saturated with 0.05 M phthalate, pH 6.0 (113) followed by 1.5 M sodium phosphate, pH 6.0 (114). Authentic DNP derivatives of amino acids were prepared (111) or obtained from California Corporation for Biochemical Research.

Crotalus atrox venom was obtained from the Ross Allen Reptile Institute, Silver Springs, Florida, and was used as a source of 5'-nucleotidase (115). Rye grass 3'-nucleotidase was purified through step two of the method of Shuster and Kaplan (116). Since difficulties were experienced in the succeeding steps employing calcium phosphate and alumina Cγ gels, an alternative purification was employed. The enzyme from step two above was chromatographed on a DEAE-cellulose column using the elution system previously described by Maxwell et al. (117) for the purification of UDPGal-4-epimerase. This yielded a preparation with a specific activity (116) of approximately 220, and was used in experiments described below.

3',5'-adenosine diphosphate was prepared from coenzyme A according to the procedure described by Wang et al. (118).

RESULTS

RESULTS

Bovine Liver Nucleotides

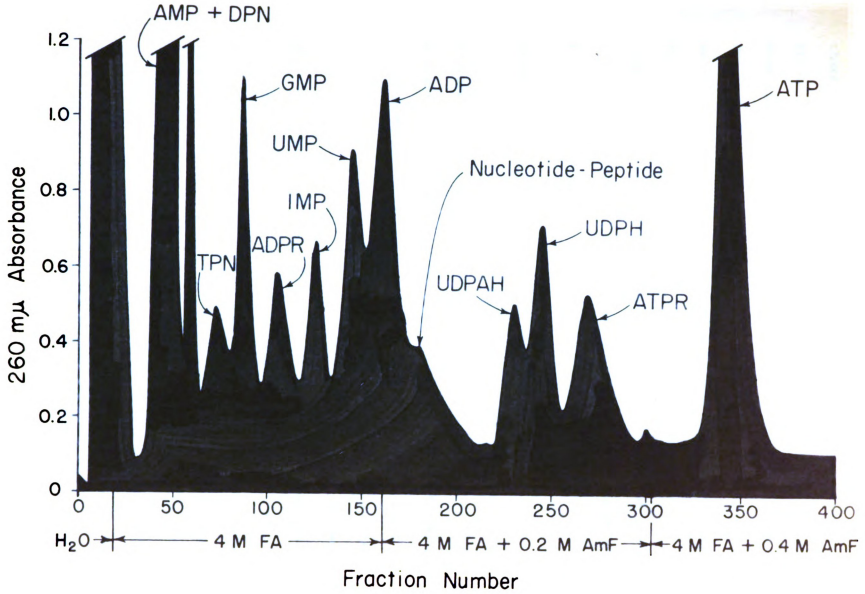
Bovine liver from freshly slaughtered animals was obtained from a local abattoir. The tissue was cut into small pieces and frozen with dry ice for transportation to the laboratory. The frozen tissue was placed in two volumes of cold 0.6 N perchloric acid and after the solution had warmed to -5°, the mixture was homogenized in a Waring Blendor. The extract was adjusted to pH 5-6 with 5 N potassium hydroxide and the potassium perchlorate which precipitated was removed by filtration. The filtrate was chromatographed on Dowex-1 (formate) resin employing a formic acid-ammonium formate gradient elution system similar to that of Hurlbert *et al.* (119). The separation of bovine liver nucleotides from 50 g of liver which were chromatographed on a 2.3 cm x 34 cm column of Dowex-1 (formate) is shown in Figure 5. The mixing flask originally contained 1000 ml of water. The eluting solutions which were run into the mixing flask before delivery to the resin column were: 1) 150 ml of water, 2) 2500 ml of 4 M formic acid, 3) 2500 ml of 4 M formic acid plus 0.2 M ammonium formate, and 4) 2500 ml of 4 M formic acid plus 0.4 M ammonium formate in that order. Approximately 18 ml fractions were collected.

The major component of several of the peaks shown in Figure 5 has been tentatively identified by absorption spectrum, pentose and phosphorus content, and by paper chromatography in isobutyric acid: H_2O:NH_4OH. The nucleotide base, pentose, and phosphorus content of

FIGURE 5

Chromatography of bovine liver acid-soluble nucleotides on a Dowex-1 (formate) resin column.

The separation shown represents the nucleotides obtained from 50 g of bovine liver. FA is formic acid and AmF is ammonium formate. For details see text.


AMP + DPN
GMP
ADP
ATP
UMP
Nucleotide - Peptide
IMP
UDPH
ADPR
TPN
UDPAH
ATPR
260 mμ Absorbance
1.2
1.0
0.8
0.6
0.4
0.2
0
0
50
100
150
200
250
300
350
400
H2O
4 M FA
4 M FA + 0.2 M AmF
4 M FA + 0.4 M AmF
Fraction Number

these compounds is shown in Table I. AMP and DPN were separated by preparative paper chromatography in the above solvent before being submitted to analysis. In addition to the compounds indicated in Figure 5, GDP and UDP-Hexuronic acid have been found to be present as minor components of the ATPR (120) and ATP peaks respectively. In general, the elution pattern of bovine liver nucleotides is qualitatively similar to those previously reported for rat liver (119), chicken liver (121), fish liver (122), and rat mammary gland (123).

Isolation and Purification of the Nucleotide-Peptide

For the isolation of the nucleotide-peptide, large columns were employed. Extract from approximately 450 g of liver was placed on a Dowex-1 (formate) resin column (5 cm x 55 cm). A 5000 ml mixing flask which was initially filled with water was employed. The elution was begun with 4 M formic acid in the reservoir and continued until the ADP peak was reached (usually 10-12 liters of effluent). Then 4 M formic acid plus 0.2 M ammonium formate was added until the nucleotide-peptide peak was eluted (usually 3-5 liters). In practice, collection of fractions of approximately 100 ml volume was begun after several liters of effluent had passed through the column. The effluent from appropriate tubes was combined and the nucleotides were adsorbed on charcoal and then eluted with 15 per cent aqueous pyridine (124). After extracting the pyridine with chloroform, the aqueous layer containing the nucleotides was lyophilized.

The lyophilized nucleotides from the nucleotide-peptide peak from approximately 450 g of liver were next placed on a Dowex-1

TABLE I

Chemical analysis of bovine liver nucleotides.

Nucleotide	Nucleotide base	Phosphorus	Pentose
AMP	.90	.90	1.00
DPN	1.15	2.14	2.00
TPN	1.13	2.80	2.00
GMP	1.05	.93	1.00
ADPR	1.03	2.18	2.00
IMP	.835	.884	1.00
UMP	1.22	1.19	1.00
ADP	1.06	1.87	1.00
UDPAH	.85	1.93	1.00
UDPH	.98	2.00	1.00
ATPR	1.20	3.00	1.57
ATP	1.05	3.21	1.00

(formate) resin column (1.8 cm x 40 cm) (Figure 6). The eluants were passed from a reservoir through a 500 ml mixing flask which contained water initially. Eluants were: 1) 200 ml of water, 2) 250 ml of 1 M ammonium formate, and 3) 1000 ml of 2 M ammonium formate. This chromatographic procedure eliminated almost all of a yellow material (presumably flavin) as well as some other ultraviolet absorbing contaminants (Peaks 1 and 2, Figure 6). Although the nucleotide-peptide located in peak 3 was not pure at this stage, it could be separated from the remaining contaminants by preparative paper chromatography. All of the fractions comprising peak 3 (e.g. fractions 77-125) were pooled and then adsorbed and eluted from charcoal as described above. The lyophilized nucleotides were dissolved in a small quantity of water and applied to Whatman 3MM paper in a narrow band. The separation of the nucleotides was achieved in 24 hours employing solvent a (Methods) (Figure 7). After locating the nucleotide-peptide by its ultraviolet absorption, and by its positive reaction with ninhydrin (using a small strip of the chromatogram), the compound was eluted from the paper with cold water (0-5°). Occasionally it was necessary to rechromatograph the nucleotide-peptide to remove traces of a slower moving compound which absorbed ultraviolet light but did not react with ninhydrin. The nucleotide-peptide either was used directly after elution from the chromatogram or it was adjusted to pH 2-2.5, extracted with ether to remove traces of isobutyric acid, and then readjusted to pH 5-6 before further use. From one kilogram of liver, the above procedure usually yielded approximately 8 to 10 μmoles of compound sufficiently pure for analyses.

FIGURE 6

Chromatography of the partially purified nucleotide-peptide on a second Dowex-1 (formate) resin column.

The nucleotide-peptide was present in peak 3. AmF is ammonium formate. For details see text.

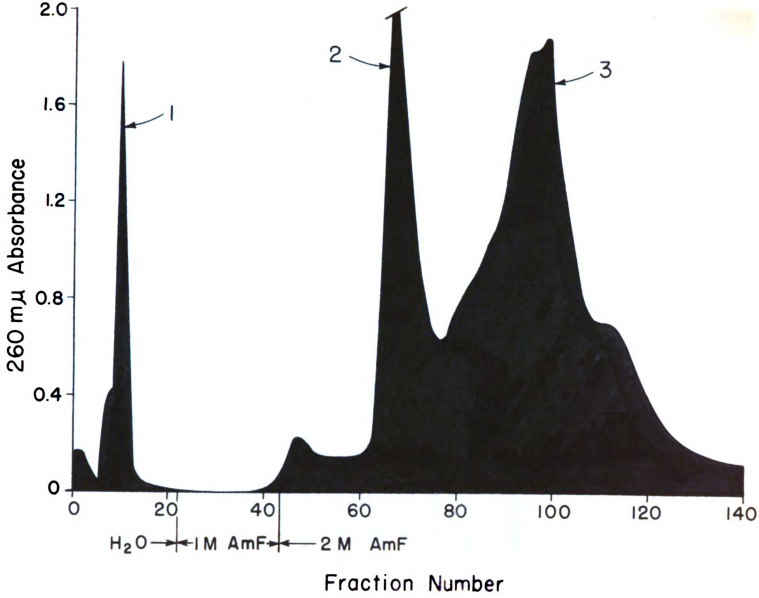
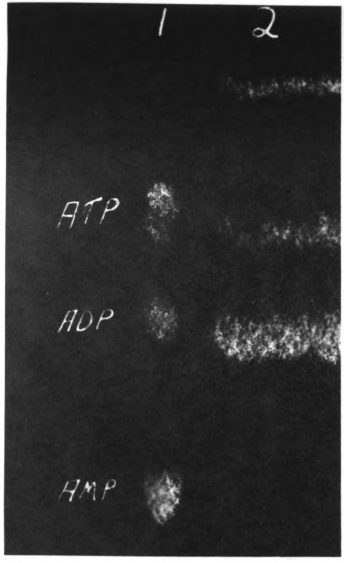

2.0
1.6
1.2
0.8
0.4
0
260 mμ Absorbance
1
2
3
0
20
40
60
80
100
120
140
H2O
1 M AmF
2 M AmF
Fraction Number

FIGURE 7

Preparative paper chromatography of the nucleotide-peptide.

The nucleotides from peak 3 of the second resin column (Figure 6) were chromatographed on Whatman No. 3MM paper, 24 hours, using isobutyric acid:H_2O:NH_4OH (66:33:1). Part A is a photograph showing ultraviolet absorbing compounds and Part B shows the same chromatogram after being treated with ninhydrin. ATP, ADP, and AMP standards are in column 1. The nucleotides from peak 3 are in column 2.



A

1 2

ATP

ADP

AMP

B

Homogeniety of the Purified Nucleotide-Peptide

Two properties of the molecule, ultraviolet absorption and reaction with ninhydrin, were found to exactly coincide in three paper chromatographic and two paper electrophoretic systems (Methods). This fact is demonstrated pictorially for the isobutyric acid:H_2O:NH_4OH chromatographic system (Figure 8), and the relative mobility of the nucleotide-peptide compared to AMP or ATP in all five systems is given in Table II. The homogeniety of the nucleotide-peptide during paper chromatography and electrophoresis, in addition to the coincidental migration of ultraviolet absorbance and positive reaction with ninhydrin during two different anion exchange chromatographic procedures, as well as adsorption and elution from charcoal, indicate that the nucleotidic and peptidic moieties do not occur in a fortuitous association but rather in chemical combination.

Since chromatography in the isobutyric acid:H_2O:NH_4OH solvent yielded the best resolution of standards and gave more compact spots than the other systems, it was used for all further chromatographic studies.

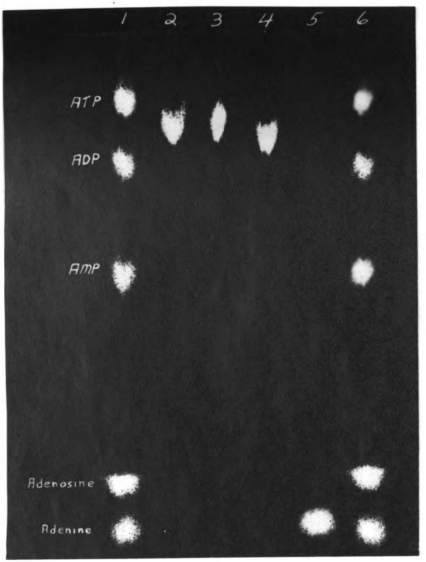
Components of the Nucleotide-Peptide

The nucleotide moiety of the nucleotide-peptide has an absorption spectrum identical to that of adenosine at pH 2 and pH 7 as shown in Figure 9. It exhibits the typical absorption shifts of adenosine derivatives with change in pH. In addition, after hydrolysis in 1 N HCl for 1 hour at 100° followed by chromatography in solvent a (Methods), only one ultraviolet absorbing compound could be detected; it had the mobility of adenine (Figure 8).

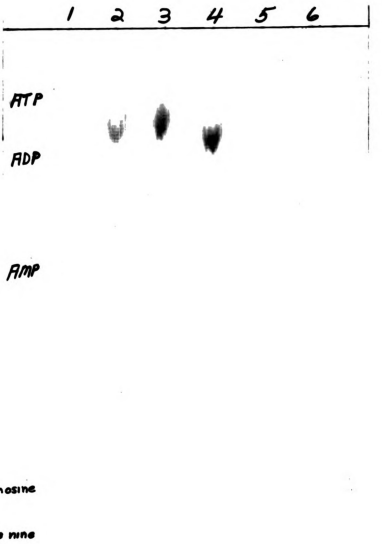
FIGURE 8

Acid and alkali stability of the nucleotide-peptide linkage.

After chromatography in isobutyric acid:H_2O:NH_4OH (66:33:1) for 17 hours, the chromatogram was photographed to reveal ultraviolet absorbing compounds (A) and then sprayed with ninhydrin to reveal ninhydrin reactive compounds (B). Columns 1 and 6 are ATP, ADP, AMP, adenosine, and adenine; and 2, 3, 4, and 5 are the products of the following treatments of the nucleotide-peptide: 2) untreated, 3) 0.1 N HCl, 30 minutes at 37°, 4) 0.01 N KOH, 20 minutes at room temperature, and 5) 1 N HCl, 60 minutes at 100°.


1
2
3
4
5
6
ATP
ADP
AMP
Adenosine
Adenine

A


1
2
3
4
5
6
ATP
ADP
AMP
Adenosine
Adenine

B

TABLE II

Paper chromatographic and electrophoretic mobility of the nucleotide-peptide.

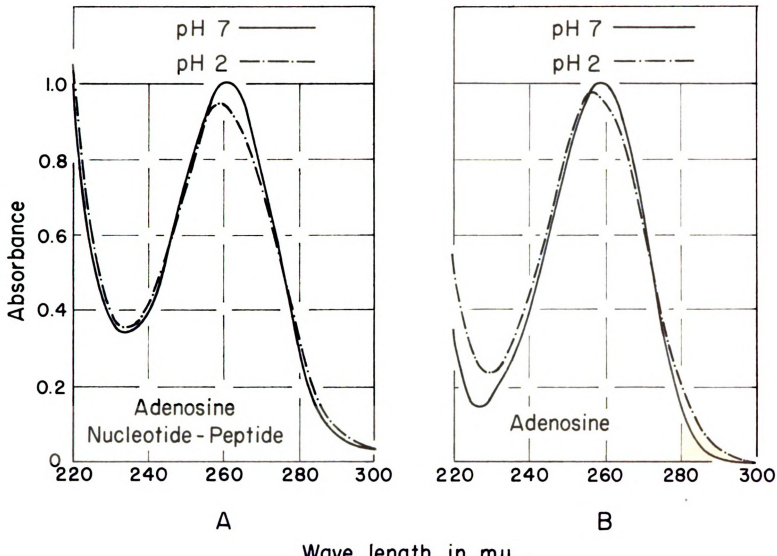
	Paper Chromatographic Systems	R_{AMP}
a)	isobutyric acid:H_2O:NH_4OH (66:33:1)	.61
b)	95% ethanol:1 M ammonium acetate, pH 3.8 (7.5:3)	.21
c)	isobutyric acid:1 N NH_4OH:EDTA (100:60:1.6)	.68
	Paper Electrophoretic Systems[1]	M_{ATP}
d)	0.5 M butyrate, pH 3.2	.21
e)	0.05 M citrate, pH 4.5	.87

[1]See Methods for experimental details.

FIGURE 9

Ultraviolet absorption spectrum of the nucleotide-peptide.

A small aliquot of a nucleotide-peptide stock solution was diluted with 0.01 N HCl or 0.02 N phosphate buffer, pH 7 for determination of the absorption spectrum at pH 2 and pH 7 respectively. Absorption measurements were taken on a Beckman model DU spectrophotometer at increments of 5 mμ except near the maximum and minimum where the increments were 2 mμ. The ultraviolet absorption spectrum of adenosine is shown for comparison.


pH 7
pH 2
Absorbance
1.0
0.8
0.6
0.4
0.2
0
Adenosine
Nucleotide - Peptide
220
240
260
280
300
A
pH 7
pH 2
Adenosine
220
240
260
280
300
B
Wave length in mμ

The quantity of the other components of the nucleotide-peptide relative to its adenosine content is shown in Table III. The compound contains one mole of pentose per mole of adenosine as determined by the orcinol reaction (101). Three moles of total organic phosphate (103) per mole of base are present. This organic phosphate is relatively stable to acid hydrolysis as treatment with 1 N H_2SO_4 for 10 minutes at 100° released only 0.2 of a μmole of inorganic phosphorus per mole of compound. This small quantity of phosphate released by dilute acid is suggestive of a 2'- or 3'-phosphate substitution on the pentose.

The positive reaction with ninhydrin indicates the presence of amino acids or a peptide. The ninhydrin amino equivalents were determined before and after acid hydrolysis (6 N HCl, 105°, 17 hrs.) to distinguish between these two possibilities. Before acid hydrolysis approximately one mole of ninhydrin amino equivalent per mole of base is observed, and after hydrolysis this number increases to between five and six suggesting that a peptide is linked to the adenosine nucleotide. The compound gave no test for hexosamine after hydrolysis for two hours in 2 N HCl at 100° followed by application of the modified Elson-Morgan hexosamine test (105).

Qualitative Amino Acid Content of the Peptide

The nucleotide-peptide was hydrolyzed with acid (6 N HCl, 105°, 17 hrs.) and the amino acid hydrochlorides were chromatographed and detected as indicated previously. The amino acids, glycine, glutamic acid, β-alanine, cysteic acid, and taurine were tentatively identified at this stage by their relative mobilities on chromatograms (Figure 10).

TABLE III

Molar ratios of the nucleotide-peptide components.

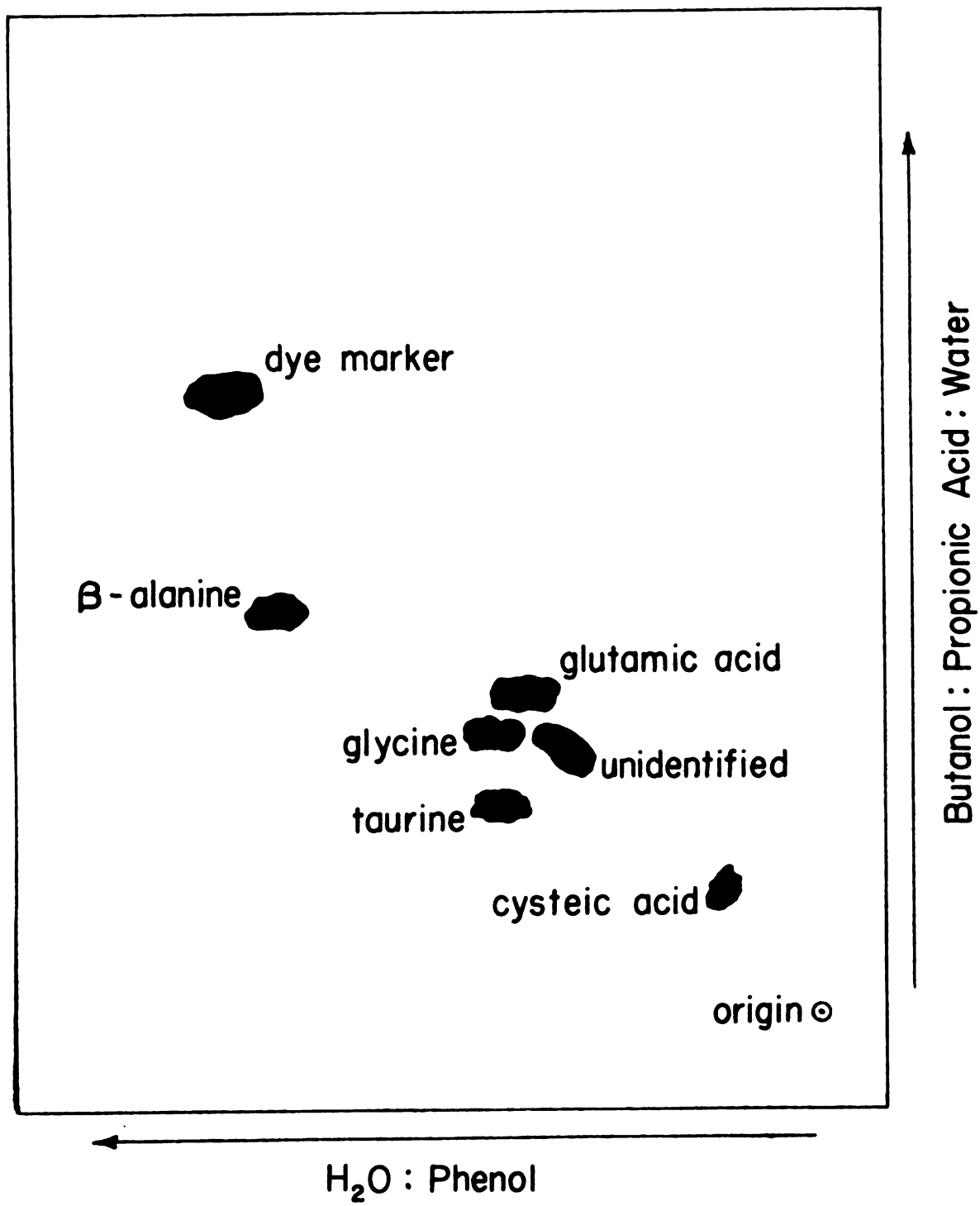
Adenosine	1.00
Pentose	0.97
Phosphate	
a) Total	3.03
b) Acid labile	0.21
Ninhydrin amino equivalents	
a) Before hydrolysis	1.04
b) After hydrolysis	5.41[1]

[1]This value represents an average from four preparations of nucleotide-peptide. All others represent an average from three preparations of nucleotide-peptide.

FIGURE 10

Chromatography of the amino acids obtained from the nucleotide-peptide.

The nucleotide-peptide was hydrolyzed in 6 N HCl at 105° for 17 hours and chromatographed on Whatman No. 1 paper. For details see text.


dye marker
β - alanine
glutamic acid
glycine
unidentified
taurine
cysteic acid
origin
Butanol : Propionic Acid : Water
H2O : Phenol

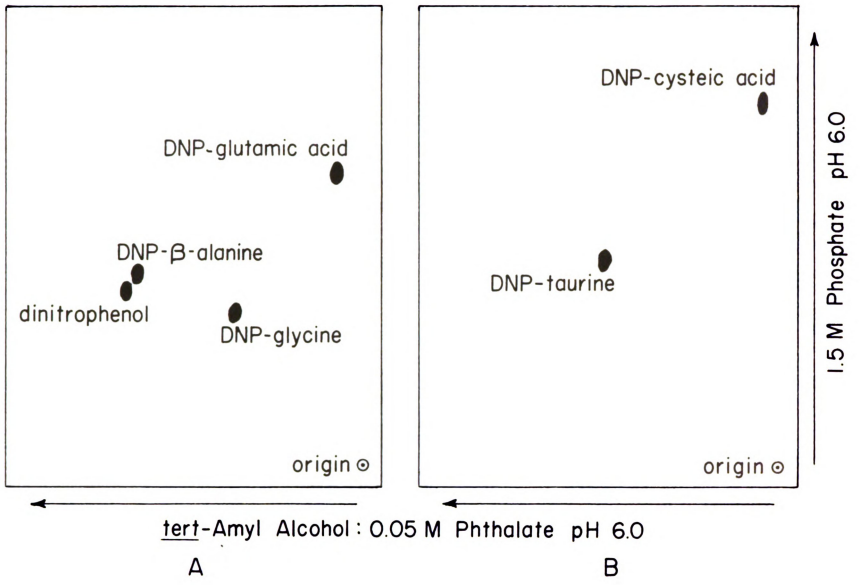
In addition, an unidentified ninhydrin positive compound with a mobility similar to serine was detected. This component is not serine since it does not cochromatograph with radioactive serine. Furthermore, their paper chromatographic behavior appears to eliminate - hydroxylysine, diaminobutyric acid, diaminopimelic acid, γ-methylene glutamic acid and agmatine, as well as all of the common amino acids.

For a more positive identification of the amino acids, a portion of an acid hydrolysate was used to prepare DNP derivatives (111) which were then separated into ether extractable and ether nonextractable fractions. Figure 11 shows the results of chromatographing these two fractions. The presence of the DNP derivatives of glutamic acid, glycine, and β-alanine in the ether extractable fraction, and the presence of the DNP derivatives of taurine and cysteic acid in the ether nonextractable fraction is consistent with the expected solubilities of these compounds (111). No DNP derivative of the unknown was detected. This finding remains unexplained; however, the possibility exists that the DNP derivative of the unknown is inseparable from one of the other DNP derivatives or dinitrophenol in the chromatographic system used. Some dinitrophenol was still present despite attempts to remove it by vacuum sublimation. In addition to their mobility on chromatograms as amino acid hydrochlorides and DNP derivatives, glycine and glutamic acid have been cochromatographed with radioactive standards, and glycine and β-alanine have been indicated by the unique colors which they produce with the polychromatic ninhydrin spray employed (110).

FIGURE 11

Chromatography of the DNP derivatives of the amino acids from the nucleotide-peptide.

Chromatogram A represents the DNP-amino acids extractable from 1 N HCl by ether. Chromatogram B represents the DNP-amino acids not extractable from 1 N HCl by ether. Standard DNP-amino acids were chromatographed on the edge of the chromatograms in each dimension to aid in the identification of the DNP derivatives from the samples. For details see text.


DNP-glutamic acid
DNP-β-alanine
dinitrophenol
DNP-glycine
origin
DNP-cysteic acid
DNP-taurine
origin
1.5 M Phosphate pH 6.0
tert-Amyl Alcohol : 0.05 M Phthalate pH 6.0
A
B

N-Terminal Amino Acid of the Peptide

The observation that the nucleotide-peptide contains one mole of ninhydrin amino equivalent indicated a free N-terminal amino acid. To identify this, the nucleotide-peptide was treated with FDNB and the DNP derivative formed was hydrolyzed with acid (6 N HCl, 105°, 12 hrs.). After removing the HCl *in vacuo* the residue was suspended in 1 N HCl and extracted with ether. Both the ether extract and the aqueous acid layer were concentrated and chromatographed two dimensionally. Only DNP-glutamic acid was detected.

Locations of the Phosphate Groups

When the nucleotide-peptide was subjected to the action of rye grass 3'-nucleotidase, approximately one mole of inorganic phosphate per mole of compound was released (Table IV) indicating that one of the three phosphate moieties is linked to the 3' hydroxyl of the pentose. As expected, in a control experiment almost no liberation of inorganic phosphate from either 2'-AMP or 5'-AMP was observed with the 3'-nucleotidase preparation.

The nucleotide-peptide also has been preincubated with 3'-nucleotidase followed by incubation with *Crotalus atrox* venom which contains 5'-nucleotidase activity (115). This treatment liberated two moles of inorganic phosphate per mole of compound (Table IV) and indicates that a second phosphate is esterified with the 5' hydroxyl of the pentose. Incubating the compound with only the 5'-nucleotidase preparation released a negligible quantity of inorganic phosphate. This finding is in accord with the specificity of the 5'-nucleotidase of *Crotalus adamanteus*, i. e., it hydrolyzes the 5'

TABLE IV

Cleavage of inorganic phosphate from the nucleotide-peptide by 3'- and 5'-nucleotidases.

Compound	Type of nucleotidase used[1]		
	3'	5'	3' and 5'
	μmoles inorganic phosphate released per mole of compound		
AMP 2'	0.04	---	---
AMP 3'	1.10	0.03	---
AMP 5'	0.04	1.15	---
Nucleotide-peptide[2]	0.92	0.13	1.93

[1]Rye grass 3'-nucleotidase and Crotalus atrox 5'-nucleotidase were used.

[2]Average of four experiments using three preparations of the nucleotide-peptide.

phosphate of a nucleotide monophosphate but shows no activity with nucleotide 2',5'- or 3',5'-diphosphates (118).

Paper chromatographic evidence has been obtained which confirms the above enzymatic finding indicating that the nucleotide-peptide contains a 3',5'-adenosine diphosphate moiety. It has been observed that treating the nucleotide-peptide with 0.01 N KOH at room temperature for 20 minutes followed by lyophilization without first neutralizing the sample causes a cleavage of the nucleotide-peptide. When the products of this reaction were chromatographed in solvent a (Methods), a compound with the mobility of 3',5'-adenosine diphosphate was detected (Figure 12A). Furthermore, when the products of the reaction of the nucleotide-peptide with 3'-nucleotidase or with 3'-nucleotidase followed by 5'-nucleotidase were treated with 0.01 N KOH as described above and then chromatographed, compounds having the mobility of 5'-AMP and adenosine, respectively, were detected (Figures 12A and 12B).

The enzymatic and paper chromatographic experiments described above conclusively demonstrate the presence of a 3',5'-adenosine diphosphate moiety in the nucleotide-peptide. The position of the third phosphate has not as yet been ascertained, however, it may be linked to the peptidic moiety of the molecule.

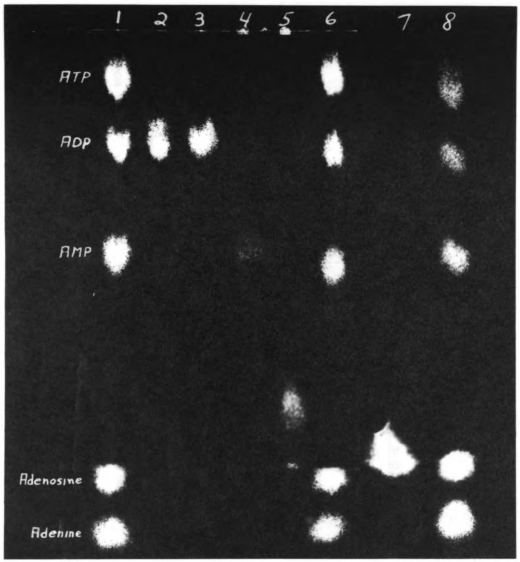
Retention of the Integrity of the Nucleotide-Peptide Linkage During Treatment with 3'-Nucleotidase.

It was demonstrated above that 3'-nucleotidase cleaves the 3' phosphate group from the nucleotide-peptide. In Figure 13 it is shown that although this cleavage changes the mobility of the pro-

FIGURE 12

Chromatographic identification of the nucleotide moiety of the nucleotide-peptide.

After chromatography in isobutyric acid:H_2O:NH_4OH (66:33:1) for 17 hours, the chromatograms were photographed to reveal ultraviolet absorbing compounds. Columns 1, 6, and 8 (A and B) each are ATP, ADP, AMP, adenosine, and adenine. Column 2 is standard 3',5'-ADP. Columns 3, 4, and 5 are the products of the following treatments of the nucleotide-peptide: 3) 0.01 N KOH, 20 minutes at room temperature followed by lyophilization without first being neutralized, 4) incubation with 3'-nucleotidase then treatment with 0.01 N KOH as in 3, and 5) preincubation with 3'-nucleotidase followed by incubation with 5'-nucleotidase then treatment with 0.01 N KOH as in 3. Column 7 (B) represents cochromatography of the ultraviolet absorbing spot in column 5 with authentic adenosine. The ultraviolet absorbing compound in column 5 was apparently retarded by salts during the first chromatographic procedure. See Figure 8 for the relative mobility of the untreated nucleotide-peptide.



A B

FIGURE 13

Effect of 3'-nucleotidase on the chromatographic mobility of the nucleotide-peptide.

After chromatography in isobutyric acid:H_2O:NH_4OH (66:33:1) for 17 hours, the ultraviolet absorbing areas were circled with pencil and then the chromatograms were sprayed with ninhydrin. Columns 1 and 4 are ATP, ADP, and AMP. Columns 2 and 3 are the products of the following treatments of the nucleotide-peptide: 2) untreated, 3) incubation with 3'-nucleotidase before chromatography. This figure is a composite photograph of two chromatograms.

duct of the reaction, the nucleotide-peptide linkage apparently remains intact. This conclusion is drawn since the ultraviolet absorption and the ninhydrin reactivity remain coincident during paper chromatography after treatment with 3'-nucleotidase. Figure 14 demonstrates that aside from the release of inorganic phosphate as a result of the action of 3'-nucleotidase on the nucleotide-peptide, the new nucleotide-peptide which is produced retains organic phosphate.

Nature of the Nucleotide-Peptide Linkage

Although the nature of the nucleotide-peptide linkage has not been unequivocally established, several possibilities have been eliminated. Since the absorption spectrum is identical with that of adenosine, a combination of the peptide with the amino group of the adenine moiety appears to be unlikely as such substitutions generally lead to a shift in the absorption maximum to a longer wave length (125).

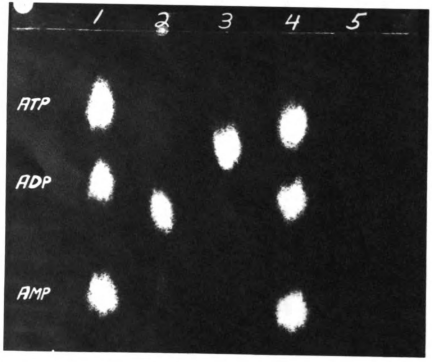
The possibility that the linkage is a carboxyl-phosphate anhydride analogous to the amino acid adenylates was tested using the procedure of Lipmann and Tuttle (106), in which case such anhydrides are rapidly cleaved to produce the corresponding hydroxamic acid of the carboxyl function. Under these conditions essentially no hydroxamic acid could be detected, therefore, a carboxyl-phosphate anhydride involving either the 3' or 5' phosphate of the adenosine 3',5'-diphosphate nucleotide moiety seems unlikely.

That the nucleotide-peptide linkage might be an ester analogous to the S-RNA-amino acid complexes was tested, again employing the

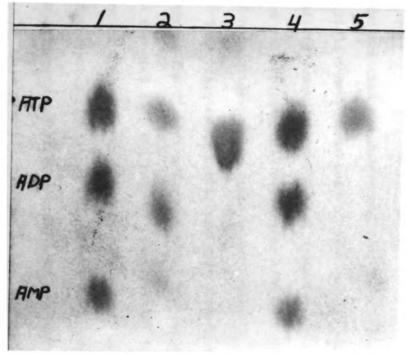
FIGURE 14

Retention of organic phosphate in the product of the reaction of the nucleotide-peptide with rye grass 3'-nucleotidase.

After chromatography in isobutyric acid:H_2O:NH_4OH (66:33:1) for 17 hours, the chromatogram was photographed to reveal ultraviolet absorbing compounds (A) and then sprayed with reagents to detect phosphorus (B) (99). Columns 1 and 4 represent standard ATP, ADP, and AMP, and column 5 is inorganic phosphate. Columns 2 and 3 are the products of the following treatments of the nucleotide-peptide: 2) incubation with 3'-nucleotidase and 3) same as 2 but without the enzyme.



A



B

hydroxylamine reaction but under conditions in which esters are cleaved to yield a hydroxamic acid of the carboxyl function (107). A small positive reaction was observed, however, the magnitude of the reaction was approximately 0.25 moles or less of hydroxamate per mole of nucleotide-peptide compared to authentic glycine hydroxamic acid. This reaction is consistent with a nucleotide-peptide ester linkage at the 2' hydroxyl position of the nucleotide, since it has been reported that the rate of reaction with hydroxylamine is dependent on the nature of both the alcohol and the amino acid moieties of the ester (107), and that different amino acid hydroxamates give different color intensities with the ferric chloride reagent (108). This possibility appears to be eliminated, however, by alkali stability data and the reaction with periodate described below.

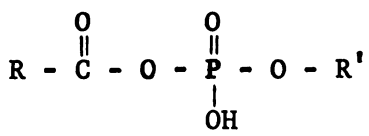
It has previously been demonstrated that the ester linkage of S-RNA-amino acid esters is completely hydrolyzed by treatment with 0.01 N KOH for twenty minutes (50) or less (63) at room temperature. Incubation of the nucleotide-peptide under these conditions for twenty minutes followed by neutralization, lyophilization, and chromatography has no noticeable effect (Figure 8).

If the 3' phosphate group were selectively removed from the nucleotide-peptide, then the possibility of substitution of the 2' hydroxyl could be determined by periodate reactivity of the 3'-dephosphorylated compound. A negative periodate reaction would indicate 2' hydroxyl substitution and a positive periodate reaction would indicate no 2' hydroxyl substitution. This type of experiment has

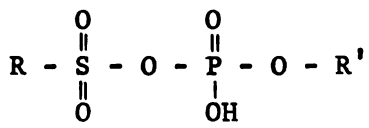
successfully been performed by removing the 3' phosphate substituent with 3'-nucleotidase as discussed above. The results are shown in Figure 15. It can be seen that the untreated nucleotide-peptide does not react with periodate (100) while the nucleotide-peptide which has been incubated with 3'-nucleotidase does react with periodate. These results indicate that the 2' hydroxyl appears to be unsubstituted.

Since reaction with 3'-nucleotidase cleaves the 3' phosphate group but apparently leaves the nucleotide-peptide linkage intact, it seems highly unlikely that the nucleotide-peptide linkage involves the phosphate group at the 3' position. If this is true, then the only possible linkage remaining would appear to include the 5' phosphate group of the nucleotide. From the known components of the peptide only the following linkages are readily apparent possibilities:

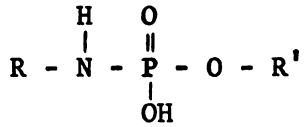
a) Carboxyl-phosphate anhydride



b) S-alkyl phosphosulfonic acid anhydride



c) N-alkyl phosphoramidate

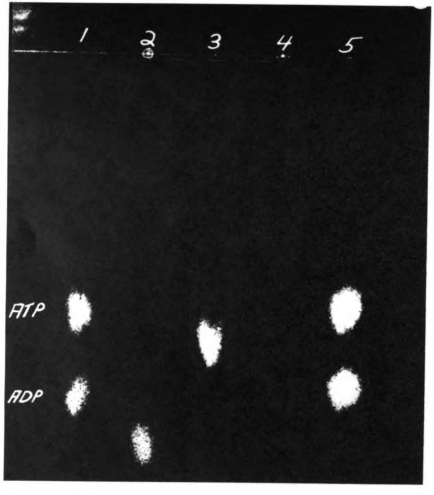


d) 0,0'-substituted pyrophosphate (involving the third phosphate group whose position has not been definitely established)

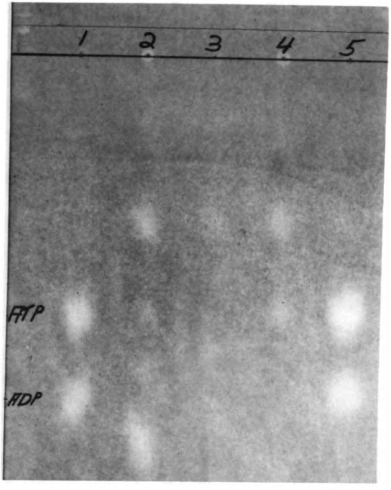
FIGURE 15

Periodate reactivity of the nucleotide-peptide before and after treatment with 3'-nucleotidase.

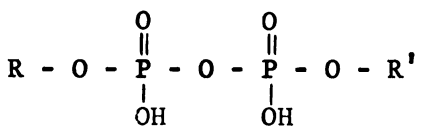
After chromatography in isobutyric acid:H_2O:NH_4OH (66:33:1) for 17 hours, the chromatogram was photographed to reveal ultraviolet absorbing compounds (A) and then dipped in periodate reagent (B) (100). Bleached areas represent a positive periodate reaction (B). Columns 1 and 5 are respectively 0.05 and 0.1 μmoles each of ATP and ADP. Columns 2, 3, and 4 are the products of the following treatments of the nucleotide-peptide: 2) incubation of 0.07 μmoles of nucleotide-peptide with 3'-nucleotidase, 3) incubation of 0.085 μmoles of nucleotide-peptide minus enzyme, and 4) incubation of enzyme with nucleotide-peptide omitted.



A



B



Where R = an amino acid component of the peptide, and R' = the 3'-phospho-5'-deoxyribosyladenine radical.

Of these four possibilities, the first seems unlikely on the basis of the hydroxamate experiments described above. Although direct evidence either for or against the other three possibilities has not been obtained, comparison of the acid stability of the nucleotide-peptide linkage with the acid lability of phosphosulfate and phosphoamide compounds deserves some comment. It has been reported that the phosphosulfate anhydride bond of PAPS is completely hydrolyzed in 30 minutes in 0.1 N acid at 37° (126). Also, it has been reported that the half-life of creatine phosphate at room temperature in 0.5 N acid is 4 minutes (127). It is demonstrated in Figure 8 that the nucleotide-peptide is stable to 0.1 N acid for 30 minutes at 37°. Although the S-alkyl phosphosulfonic acid anhydride (b) and N-alkyl phosphoramidate (c) linkages suggested above as the possible nucleotide-peptide linkage are not entirely analogous to PAPS and creatine phosphate respectively, they are similar enough structurally to anticipate that their acid labilities might be comparable. Assuming that this is true, it might be expected that the nucleotide-peptide would be entirely or at least partially hydrolyzed under the above acid treatment if it contained either of the suggested linkages (b and c). Since the nucleotide-peptide was stable to this acid treatment, a substituted pyrophosphate (d)

appears somewhat more attractive than either an S-alkyl phosphosulfonic acid anhydride (b) or an N-alkyl phosphoramidate (c), although the latter two possibilities cannot definitely be eliminated.

DISCUSSION

DISCUSSION

The adenosine nucleotide-peptide described appears to be rather unique. Although much is known about the compound, its function is not readily apparent from its composition. The nature of the nucleotide-peptide linkage appears not to be similar to that of the amino acid adenylates or S-RNA-amino acid derivatives which are intermediates in protein synthesis. In addition, at least three of the amino acids found in the peptidic moiety of the compound are not generally recognized as protein constituents. While these two factors do not eliminate the possibility that the nucleotide-peptide functions in some unrecognized phase of protein synthesis, this possibility is relatively unattractive at present.

Although direct evidence that the peptide is a phosphopeptide involving the third phosphate group of the nucleotide-peptide has not been obtained, the data indicates a phosphopeptide since apparently all of the other possible positions of substitution have been accounted for. Assuming that this is true, then the possible linkages of this phosphate group can be examined. The possibilities of a carboxyl-phosphate anhydride, phosphosulfonic acid anhydride, or a phosphoamide linkage is extremely unlikely because of their acid lability compared to the relatively acid-stable phosphate groups of the nucleotide-peptide. Only about 0.2 mole of inorganic phosphate is liberated from the nucleotide-peptide during hydrolysis in 1 N acid for 10 minutes at 100°. Acetyl phosphate (128) which contains

a carboxyl-phosphate anhydride bond, creatine phosphate (121) which contains a phosphoamide bond, and PAPS would be completely hydrolyzed under these conditions. Presumably a phosphosulfonic acid anhydride which is structurally similar to the phosphosulfate linkage of PAPS would also be completely hydrolyzed under these conditions. If these assumptions are correct, then a relatively acid-stable phosphate group such as an ester of a hydroxyamino acid must be considered. Although none of the known amino acids of the nucleotide-peptide are hydroxyamino acids, the unidentified ninhydrin-reactive component may contain a hydroxyl group. Little can be said about the identity of this component except that it appears not to be any of the common amino acids, nor any of the more rare amino acids which were tested.

Aside from conclusive evidence concerning the nature of the nucleotide-peptide linkage, the position of the third phosphate group, and the identification of the sixth ninhydrin-reactive component of the peptide, it would be interesting to know the sequence and configuration of the amino acids in the peptide. Some progress has been made concerning the latter problem, that is, it has been established that glutamic acid is the N-terminal residue. Definition of the function of the nucleotide-peptide as well as completion of its characterization must await further experimentation.

SUMMARY

SUMMARY

A new nucleotide-peptide has been isolated from bovine liver. It is a 3',5'-adenosine diphosphate-peptide derivative which also contains a third phosphate group presumably on the peptide. The peptide consists of glutamic acid, glycine, β-alanine, cysteic acid, taurine, and an as yet unidentified ninhydrin positive component. The N-terminal amino acid of the peptide is glutamic acid. The nature of the nucleotide-peptide linkage is discussed.

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