# Nucleotides Containing Amino Acids in Bacteria

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The importance of nucleic acids in protein synthesis is well established [1,2]. In later years Hoagland *et al.*[3] and others [4-9] have presented evidence for the existence of amino acyl-adenylate compounds and amino acid — RNA complexes [10,11] as intermediates in the biosynthesis of proteins.

Since low molecular nucleotides containing amino acids may act as participants or carriers of amino acids to the active site of protein or peptide synthesis, our laboratory has for some time been actively engaged in the search for such substances in different bacteria, yeasts and mammalian cells under various conditions of growth.

The recent publications by Brown [12] on nucleotidebound peptides from *S.faecalis* and of similar reports [13-15] prompt us to describe some of our work.

As nucleotides containing amino acids might be labile and difficult to extract we have used different extraction procedures.

The present report concerns the isolation of nucleotides containing amino acids from *E. coli* and *B. subtilis* grown under aerobic condition and harvested in the logarithmic growth phase. In the case of *E.coli* the rapidly frozen cells were disrupted in a Hughes bacterial press and extracted with cold 0.6 M perchloric acid at 0°C. The perchloric acid was removed as the potassium salt. The cells of *B. subtilis* were extracted with 90 % phenol at room temperature. The extracted material was displaced into an aqueous solution by the addition of ether. Both extracts were adsorbed on a Dowex 1 column in the formate form and fractionated by gradient elution. Since the presence of weakly charged substances was anticipated, better resolution of these would be obtained by elution with weaker concentrations of formic acid than those usually employed. Details of the gradient chromatography are shown in Figs. 1 and 2.

Fraction III from *E. coli* (Fig. 1) was separated into 4 U.V. absorbing bands by paper chromatography in *iso*butyric acid-N ammonia-EDTA (100:60:1.6). The slowest band was DPN. The second slowest band ($III_2$) had the same $R_F$ value as AMP-5′

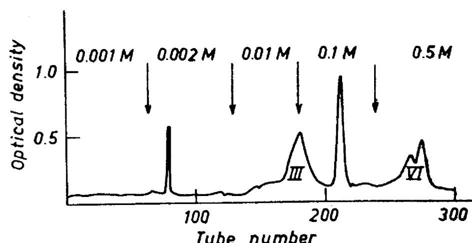


*Fig. 1.* Gradient elution of *E. coli* extract. Mixing volume 500 ml. Dowex 1 × 8 in the formate form (1.2 cm × 10 cm). Elution was started with 0.001 M formic acid followed by 0.002 M, 0.01 M, 0.1 M and 0.5 M as indicated by the arrows. Volume of individual fractions 5 ml. 2 190 optical units put on column.

in this solvent system and was further investigated. The substance had the same $R_F$ value as AMP-5′ in *n*-butanol-conc. ammonia-water (30:5:15) and *n*-butanol-acetic acid-water (50:25:25). By paper electrophoresis in 0.2 M ammonium acetate buffer pH 5.8 and in borate buffer pH 10, the mobility was identical to that of AMP-5′. In 0.05 M phosphate buffer pH 7.5, AMP-5′ moved slightly ahead of the substance. By elution with 0.1 N HCl from a paper chromatogram in butanol-acetic acid-water the absorption maxima and minima were the same as for AMP-5′, whereas the ratios 280/260 and 290/260 were 0.44 (AMP-5′ 0.25) and 0.22 (AMP-5′ 0.09), respectively. The U. V. absorbing

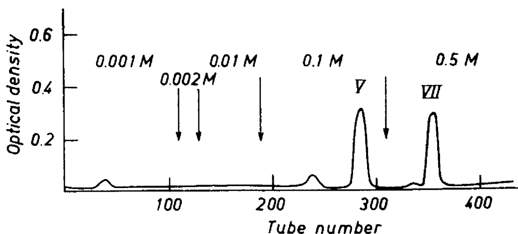


*Fig. 2.* Gradient elution of *B.subtilis* extract. Volume of mixing flask 500 ml. Dowex 1 × 4 in the formate form (20 cm × 1 cm). Elution was started with 0.001 M-formic acid in the reservoir flask followed by 0.002 M, 0.01 M, 0.1 M and 0.5 M as indicated by the arrows. Volume of individual fractions 5 ml. 216 optical units put on column.

substance gave a ninhydrine negative reaction. Chromatography in *n*-butanol-acetic acid-water (4:1:5) of material hydrolyzed with 6 N HCl for 18 h at 120°C gave four ninhydrine positive spots with the $R_F$ values 0.43 (alanine 0.47), 0.35 (glutamic acid 0.36), 0.29 (glycine 0.29) and 0.15.

After hydrolysis with N HCl for one hour at 100°C adenine was identified and three ninhydrine spots ($R_F$ 0.31, 0.26 and 0.14) were found by chromatography in *n*-butanol-acetic acid-water.

The elution diagram of *B. subtilis* is seen in Fig. 2. Each of the fractions V and VII were purified by successive chromatography in *iso*butyric acid-N ammonia-EDTA (100:60:1.6), ethanol-M ammonium acetate pH 7.5 (70:30), 0.1 M phosphate buffer pH 6.8-ammonium sulphate-*n*-propanol (Pabst) and gave only one U.V. absorbing spot with the same $R_F$ value as AMP-5′ in these solvent systems.

After purification in the third solvent, the eluates were absorbed on charcoal and eluted with ethanol-ammonia. After freeze-drying, their spectra were determined in 0.1 N HCl and compared with AMP-5′ treated in the same way.

Fractions V, VII and AMP-5′ had the same absorption maxima (257 m$\mu$) and minima (232 m$\mu$), whereas the 280/260 ratios were: 0.33, 0.32, and 0.25, respectively. By paper electrophoresis in 0.2 M ammonium acetate buffer pH 5.8 fraction V had 33 % and fraction VII 85 % of the mobility of AMP-5′.

After hydrolysis with 6 N HCl for 20 h at 120°C 10 ninhydrine positive spots were found by chromatography in *n*-butanol-acetic acid-water (50:25:25).

The following amino acids were tentatively identified: lysine, aspartic acid, glycine, glutamic acid, alanine and valine.

Hydrolysate from substance VII gave four ninhydrine positive spots by chromatography in *n*-butanolformic acid-water (75:13:12). Three of these were tentatively identified as glycine, aspartic acid and alanine.

After hydrolysis in N HCl at 100°C for one hour adenin was identified.

Since the two substances from *E.coli* behave electrophoretically similar to AMP-5′ it is surprising that they are eluted more easily from the column than AMP-5′ (identified in fraction VI). The reason for this is not known for the present time, but the structure of the substances is being investigated.

The substances are possible intermediates in peptide or protein synthesis, but may also be formed by degradation of more complex substances containing nucleotides and amino acids.

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# A New Acidic Ultraviolet Absorbing Substance Containing Amino Acids in Bacteria, Yeasts and Spore Preparations

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In recent years nucleotides containing amino acids have been isolated from extracts of mammalian tissues [1] and bacterial cells [2]. In a previous paper [3] we have described such substances from *E. coli* and *B. subtilis* harvested in the logarithmic growth phase. We now wish to report the occurrence and properties of an unidentified substance containing amino acids and with a characteristic ultraviolet absorption spectrum.

The substance has been isolated by gradient elution of cell extracts adsorbed on a Dowex 1 × 8 column in the formate form. Fig. 1 shows a typical elution of an extract

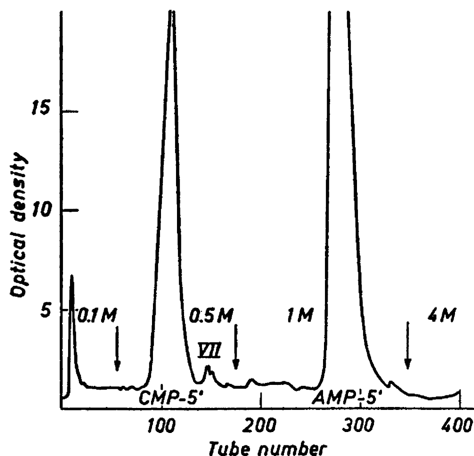


*Fig. 1.* Part of the gradient elution diagram of *B. cereus* extract. Volume of mixing flask 750 ml. Dowex 1 × 8 in the formate form (3 cm × 18 cm). Elution was started with 0.001 M formic acid in the reservoir flask followed by 0.002 M, 0.02 M, 0.1 M. The arrows indicate concentrations of 0.5 M, 1.0 M and 4 M formic acid. Volume of individual fractions. 29 600 optical units put on column.

from *B. cereus*. The unknown substance was eluted in fraction VII close to CMP-5′ when using 0.5 M formic acid in the reservoir. It was purified by successive paper chromatography and paper electrophoresis.

The substance has been found in *E.coli, B. cereus* spores and vegetative cells and in *Candida tropicalis* var. *lambica*. It was present in acid-soluble extracts from cells disrupted in the Hughes bacterial press and was also released by boiling the cells in distilled water for 10 min. The substance could be adsorbed on activated charcoal and again eluted at 0°C with 50 % ethanol containing 2 % conc. ammonia. The amount varied from 0.3–0.6 % of the total U.V. absorption at 260 mμ fo the crude extracts.

The most conspicuous feature of the ultraviolet absorption spectrum of the highly purified material (Figs. 2 and 3) is the ratio 280/260 in 0.1 N HCl. This is on an average 0.1 and is therefore lower than that of known nucleotides. The ratio 250/260 is usually 0.60. While the point of maximum absorption has constantly been found at 261–262 mμ, the inflection points at about 258 mμ and 268 mμ (Fig. 2) have been more or less pronounced and may be almost missing as shown in Fig. 3. Figs. 2 and 3 represent the extremes of the variation in the absorption spectrum and the variation is not caused by U.V. absorbing impurities since it can be experienced in eluates from two identical chromatograms.

No significant change in the U.V. absorption was found after hydrolysis with formic acid at 175°C for 2 h or with 6 N HCl at 120°C for 18 h.

In paper electrophoresis the unidentified substance moved towards the anode at pH values above pH 4.0. In acetate buffer at pH 5.9 it had approximately the same mobility as orotic acid. In phosphate buffer of pH 7.4 the mobility was about 20 % greater than that of AMP-5′.

The $R_F$ values in paper chromatography was greater than those of the usual nucleotides. For instance in *iso*butyric acid-N-ammonia-EDTA (100:60:1.6) it had $R_F$ 0.78 whereas the $R_F$ for AMP-5′ is 0.52. The mobility was unchanged after hydrolysis. The U.V. absorption of the substance and its hydrolysate could not be reduced by treatment with sodium amalgam [4].

No ribose was found by the orcinol test using 20 min of heating at 100°C. Tests on phosphorus on material hydrolyzed with 6 N HCl were negative.

The purified U.V. absorbing substance gave a negative ninhydrine test while