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The Isolation and Identification of 7-Oxo-8-aminopelargonic Acid, a Biotin Intermediate*

Max A. Eisenberg and Ramon Maseda

ABSTRACT: The identity of a biotin vitamer which was previously shown to be an intermediate in biotin biosynthesis was made possible by its isolation in crystalline form. The purification was carried out by large scale column chromatography of 100 gal of culture medium from *Penicillium chrysogenum*. The compound was shown to be identical with 7-oxo-8-aminopelargonic acid by elemental analysis, mixture melting point, infrared spectrophotometry, and mass spectroscopy. Both the natural and synthetic compounds gave identical growth curves with yeast and were 65–95% as active as biotin.

In an early study with *Phycomyces blakesleeanus* an unknown biotin vitamer was shown to accumulate in the growth medium along with biotin and desthiobiotin (Eisenberg, 1963). Although present in very low concentrations, it was possible to show by electrophoretic analysis that the vitamer contained both an amino group and a carboxyl group with the charges widely separated. In addition the vitamer did not bind to the protein, avidin, indicating the absence of a ureido structure. The very low concentration of the compound precluded its isolation and identification at that time.

It was subsequently found that *Penicillium chrysogenum* produced the same biotin vitamer in much higher yields (Eisenberg, 1966). Additional information about its structure was obtained when it was found that $^{35}SO_4^{2-}$ was not incorporated into the vitamer (Eisenberg, 1965). This observation indicated the absence of the tetrahydrothiophene ring and therefore suggested an open-chain structure for the vitamer. Since an intermediary role in the biosynthesis of biotin was proposed for this vitamer, based on incorporation studies with 1,7-[^{14}C]pimelic acid, the open-chain structure indicated the vitamer was an early intermediate (Eisenberg and Maseda, 1966; Eisenberg, 1966). It was postulated that the condensation of either serine or alanine with pimelic coenzyme A would yield an open-chain compound with the observed properties. Attempts to determine which of the two amino acids was a precursor of the vitamer did not give definite results because incorporation of the labeled amino acids was extremely low. Since the vitamer comprised 60–80% of the total biotin activity of the medium, it was considered feasible that a large scale purification procedure could yield sufficient crystalline material for characterization. The present study describes the isolation of the vitamer in crystalline form by column chromatographic procedures and its identification as 7-oxo-8-aminopelargonic acid.

While this work was in progress, it was shown by Iwahara *et al.* (1965) that a compound with properties similar to those of the unknown vitamer was formed in the medium of *Bacillus sphaericus*. They were able to identify the compound as 7KAP[1] by chromatography in three solvent systems (Iwahara *et al.*, 1966). Soon after, the condensation of pimelic coenzyme A and L-alanine to form 7KAP was demonstrated in cell-free extracts of *Escherichia coli* by Eisenberg and Star (1968).

Materials and Methods

P. chrysogenum was grown in 100 gal of Czapek-Dox medium for 7 days with continuous aeration. The filtrate was pan-dried, yielding about 4 kg of hygroscopic material.

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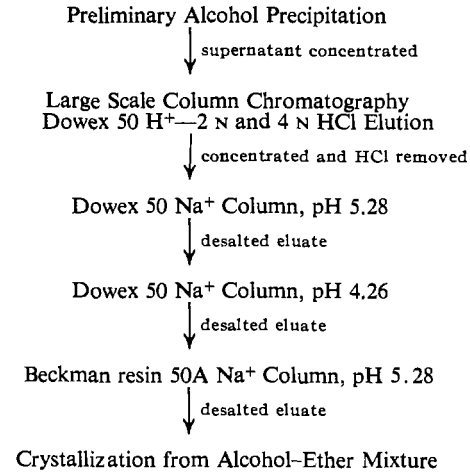
[1] Abbreviations used are 7KAP for 7-oxo-8-aminopelargonic acid and mmu for millimass unit.

Preliminary tests indicated that the vitamer yield on such a large scale culture was equivalent to that obtained from a 1-l. culture.

The disc assay procedure of Genghof *et al.* (1948), with yeast as the responsive organism, was used to monitor the chromatographic procedures. The turbidity assay was a modified form of the procedure of Dhyse and Hertz (1958). Infrared analysis was performed with a 1.5-mm micro KBr disk in a Perkin-Elmer Model 221 spectrophotometer. Mass spectral analysis was carried out with a Hitachi RMU-6A high resolution spectrometer. Peak matching was done with the CEC 21-110B high resolution mass spectrometer.

7KAP was synthesized according to the methods of Suyama and Kaneo (1963).[2] Dowex 50-X8 resin (100–200 mesh) was purchased from Baker and Co., and BioRad AG 50W-X8 resin (200–400 mesh) was obtained from Calbiochem. All other chemicals were reagent grade. Thin-layer chromatography was carried out on silicic acid coated polyester sheets (Eastman Kodak) using 1-butanol–acetic acid–water, 60:15:25 (v/v), as developing solvent.

SCHEME I



Results and Discussion

Purification. The large scale chromatographic separation procedure was based on studies of the behavior of the unknown vitamer in the amino acid analyzer (Eisenberg and Maseda, 1966). However, the pan-drying process introduced certain difficulties as the result of alterations to certain ingredients in the basal medium. The columns rapidly became occluded and it was difficult to regenerate them. To circumvent these difficulties, the protocol finally adapted is shown in Scheme I.

Preliminary Precipitation. In order to remove interfering substances, 300 g of the pan-dried material was dissolved in a final volume of 1 l. of water and 3 l. of 95% alcohol was added with stirring. After standing for 1 hr at 0°, the heavy precipitate was removed by centrifugation. The alcohol and water were removed in a flash evaporator at 50° leaving a syrupy residue.

Dowex 50-X8 (100–200 mesh) H^+ Column Chromatography. The syrupy residue was taken up in water to a final volume of 2 l. and adjusted to pH 2.2. The dark brown solution was introduced into a column, 15 × 30 cm, filled to a height of 20 cm with 10 lb of Dowex 50-X8 (100–200 mesh) H^+ form resin. The column was washed overnight with 18 l. of distilled water to remove neutral and acidic compounds. The column was then eluted with 6 l. of 2 N HCl, collecting 500-ml fractions, and then with 20 l. of 4 N HCl, collecting 2-l. fractions. The disc assay showed that all the biological activity was associated with the latter eluent as shown in Figure 1A. Approximately 10–12 mg of biotin equivalents was obtained from 300 g of starting material, representing a recovery of 50–60%. The contents of those flasks showing more than 0.1 mg of biotin equivalent were combined and taken to dryness in the flash evaporator. The contents were stored at −20° until three to five such samples had been accumulated. The samples were then combined, made 6 N with respect to HCl, and refluxed for 6 hr. This procedure was introduced to facilitate the subsequent column operations by removing tar-like material. Previous studies had shown that the unknown vitamer was stable under these conditions (Eisenberg and Maseda, 1966). The hydrolysate was taken to dryness in the flash evaporator. The residue was made up to 1 l. with water and adjusted to pH 2.2, and the solution was filtered. The filtrate was then rechromatographed on the Dowex 50 H^+ column in the same manner described.

Column Chromatography at pH 5.28. The residue obtained from the previous chromatographic procedure was dissolved in 60 ml of distilled water and adjusted to pH 2.2 and one-half added to a Dowex AG 50W-X8 (200–400 mesh) Na^+ form resin in a jacketed column, 3.8 × 56 cm. Two runs were necessary to avoid overloading of the column. Elution was carried out at 50° with 0.38 N sodium citrate buffer, pH 5.28. Buffer (6 l.) was pumped through the column at the rate of 8.0 ml/min, collecting 500-ml fractions. Assay of each fraction gave the sharp profile shown in Figure 1B. Those fractions showing biological activity were pooled and the entire contents passed through a Dowex AG 50W-X8 (100–200 mesh) H^+ form resin in a column, 3.4 × 20 cm, for desalting. The column was first washed with 2 l. of 0.5 N HCl and the vitamer was then eluted with 4 N HCl. The HCl was removed in the flash evaporator at 50°.

Column Chromatography at pH 4.26. The combined fractions from the previous chromatographic procedure were dissolved in distilled water and adjusted to pH 2.2. The entire sample, 35 ml, was run into the jacketed column described above which had been regenerated and equilibrated with 0.38 N sodium citrate, pH 4.26. The column was first eluted with the same buffer at 30° for 12 hr, collecting 6200 ml in one batch which contained no biologically active material. The temperature of the water jacket was then rapidly raised to 50° and the elution continued for another 18 hr, collecting 500-ml samples. Assay of these fractions gave the elution pattern shown in Figure 1C. The failure to obtain a sharp profile is typical for this column because of the prolonged elution time and is also seen for arginine in the amino acid analyzer under the same conditions. The fractions with biological activity were combined and desalted in the manner previously described. A sample of this material

[2] The 7KAP was synthesized from L-alanine and therefore assumed to be the L isomer.

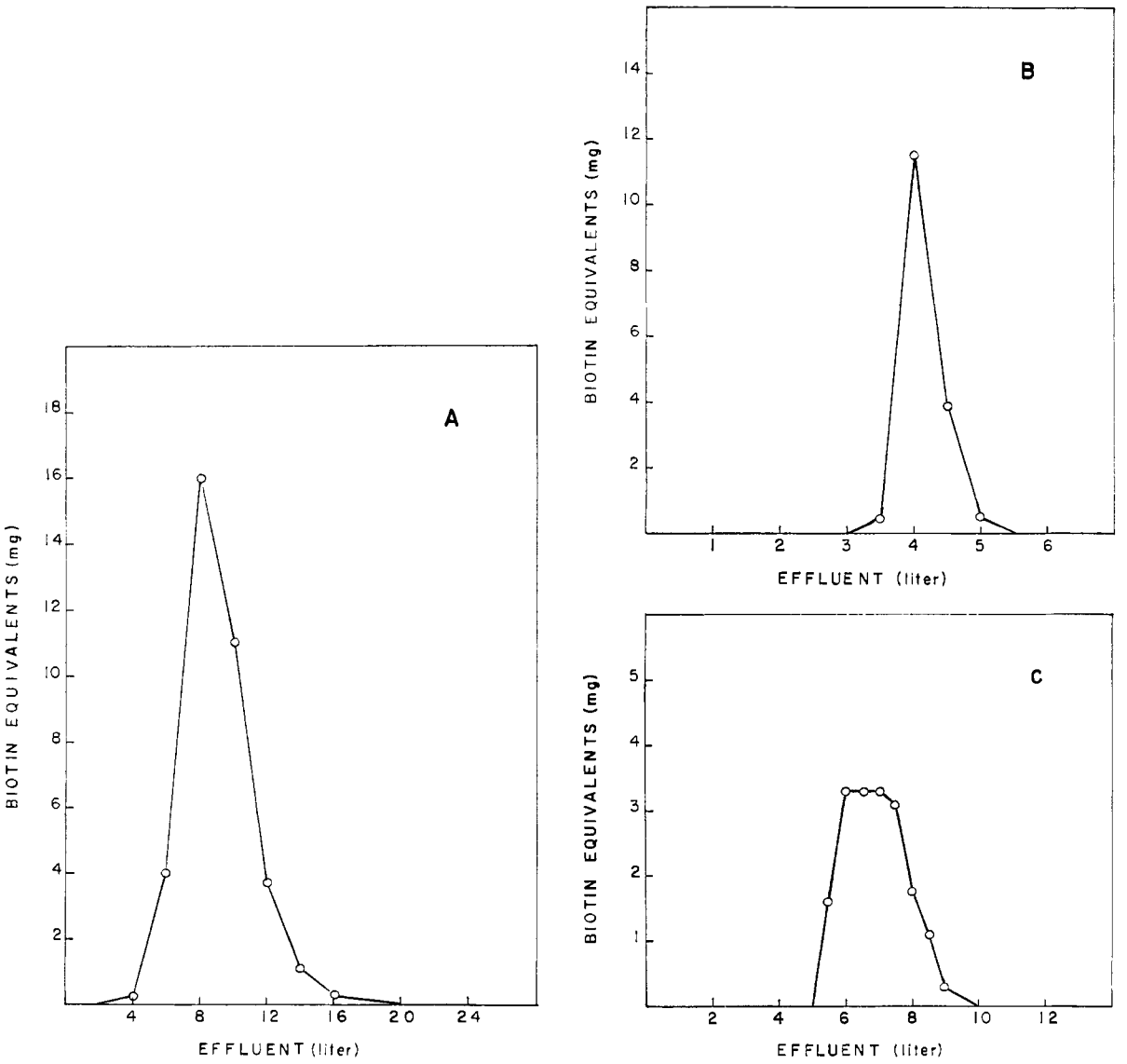


FIGURE 1: (A) Column chromatography with 4 N HCl. (B) Column chromatography at pH 5.28. (C) Column chromatography at pH 4.26.

when analyzed in the Spinco amino acid analyzer at pH 5.28 showed a small number of amino acids eluting within the first 30 min and a major peak appearing at 203 min. A sample of synthetic 7KAP showed a peak emerging at 206 min.

Column Chromatography at pH 5.28 and Crystallization. Since the amino acid analyzer indicated the presence of small amounts of neutral or acidic amino acids, the chromatography at pH 5.28 was repeated. A small preparative column, 1.8 × 56 cm, was utilized and the finer resin, Beckman type 50A, used in place of the BioRad resin. The elution procedure and desalting were identical with that previously described for this columning. After removing the HCl, a slightly yellowish crystalline material remained on the walls of the flask. The material was extracted with small portions of absolute alcohol, leaving some of the yellow resin fines behind. A sample of the alcohol extract was chromatographed on thin-layer sheets and ninhydrin spray revealed a major component with an orange color typical of synthetic 7KAP and a minor component with a typical blue ninhydrin reaction.

Ether was added to the alcoholic extract to incipient precipitation. On standing in the cold, a small amount of a yellow flocculent precipitate formed which was removed by centrifugation. More ether was added to the supernatant until the solution appeared slightly turbid. The tube was tightly stoppered and the mixture left at 4° overnight. Compact white rosette shaped crystals were formed on the walls of the tube and were removed by centrifugation. The crystals were washed with ether and the washings added to the supernatant. The melting point of a single crystal using a Fisher–Johns melting point block was 133–136° uncorrected. Thin-layer chromatography of the crystalline material revealed only one component with the orange ninhydrin color.

The combined supernatant and ether washings was taken down to a small volume under a stream of nitrogen and more ether added until incipient turbidity. After standing overnight at 4°, a second crop of crystals was obtained with a melting point of 133–134°. The two crystal batches were

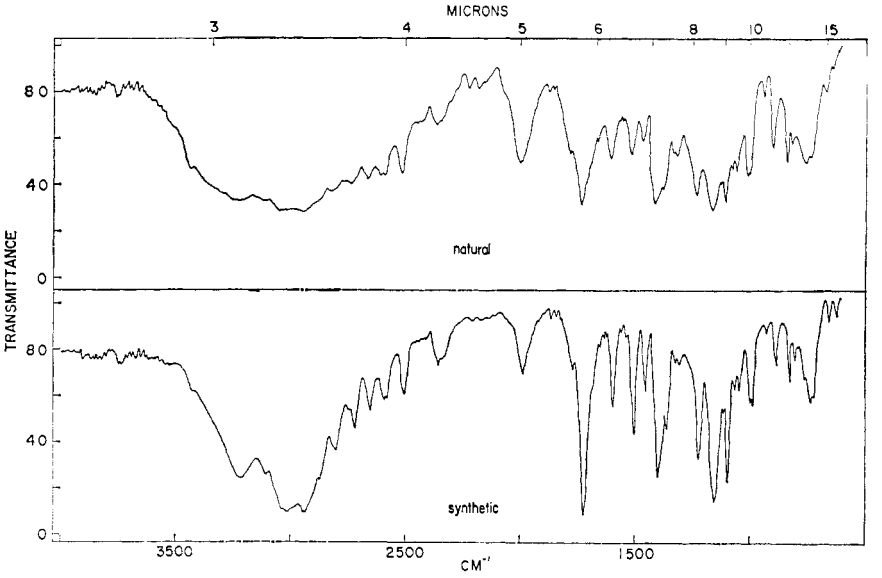


FIGURE 2: Infrared spectra of natural and synthetic 7KAP.

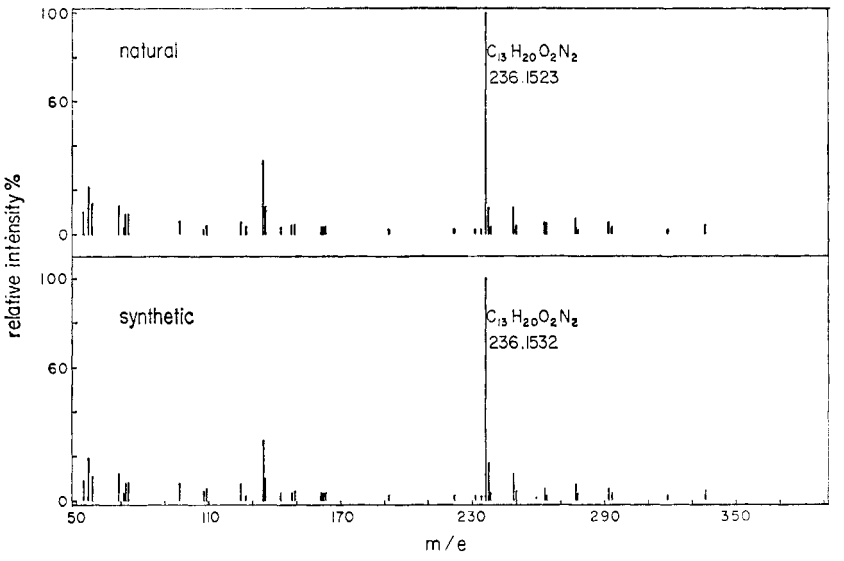


FIGURE 3: Mass spectra of natural and synthetic 7KAP.

combined and recrystallized twice more from alcohol–ether without any further change in melting point. Approximately 50 mg of the crystalline product was obtained from 100 gal of culture filtrate.

Identification of the Unknown Vitamer. Microanalysis of the crystalline compound gave the following results. *Anal.* Calcd: C, 48.30; H, 8.11; N, 6.26; Cl, 15.86; O, 21.47. Found: C, 48.09; H, 8.24; N, 6.19; Cl, 16.26; O, 21.22.

The calculated values were based on the formula for the hydrochloride salt of 7KAP, $C_9H_{17}O_3N \cdot HCl$. A mixture melting point with an authentic crystalline sample of 7KAP showed no depression.

An infrared spectral analysis of the natural and synthetic compound gave the results shown in Figure 2. The presence of the characteristic functional groups for this compound is evident in both the synthetic and natural compounds. Among these are the NH_3^+ adsorption at ≥ 2000 cm^{-1}, the carbonyl band at 1735 cm^{-1}, the absorption due to the free carboxyl function centering at 3000 cm^{-1}, and the absorption band at 720 cm^{-1} due to the presence of $(CH_2)_n$ where $n \cong 4$. Each peak observed with the natural compound can be matched with an identical peak in the synthetic 7KAP.

The mass spectral analysis revealed a small peak at *m/e* 223, the theoretical molecular ion. Most significant was the appearance of a number of ions with masses greater than the parent ion. The ion with the greatest intensity was at *m/e* 236. Figure 3 shows all ions above *m/e* 50 with intensities greater than 1% of the 236 peak. It is apparent that the

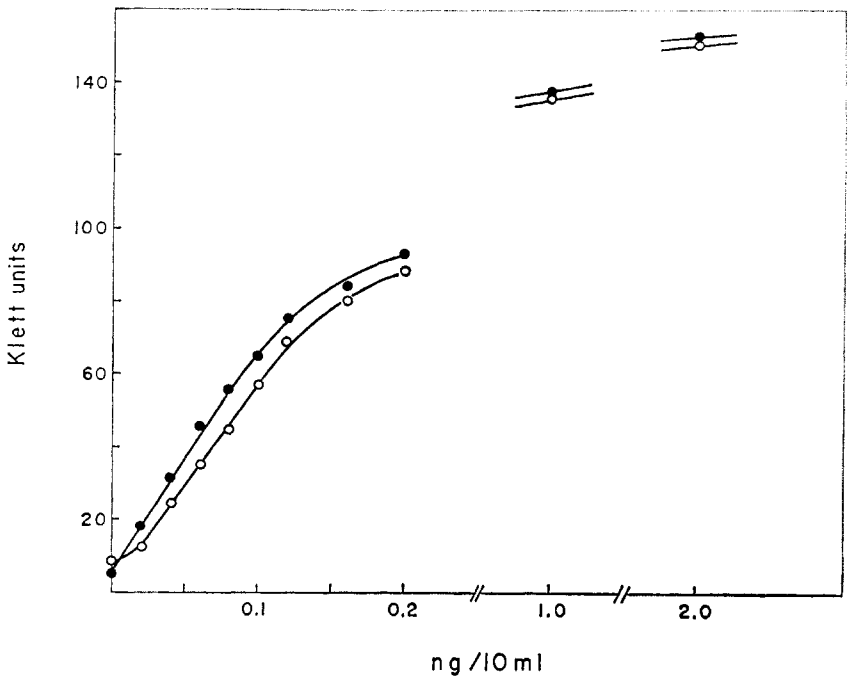
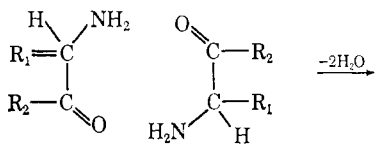


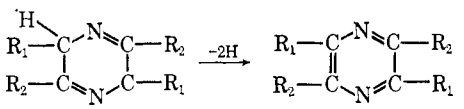
FIGURE 4: A comparison of the yeast growth curves for 7KAP and biotin; (●) biotin, (○) 7KAP.

fragmentation pattern and the observed intensities of the natural compound are identical with those of the synthetic 7KAP.

The presence of ions with masses greater than the molecular ion suggested the possible self-condensation of 7KAP under the conditions used. The self-condensations of α-amino ketones in alkaline solution to form dihydropyrazines is well documented in the literature. The dihydropyrazine is readily oxidized to the pyrazine compound in the presence of trace metals and oxygen (von Erlenmeyer *et al.*, 1964). The appearance of an ion at *m/e* 336 would correspond to the presence of a pyrazine compound arising by the following sequence of reactions.



7 KAP
m/e = 187



dihydropyrazine
m/e = 338

pyrazine
m/e = 336

$R_1 = CH_3$
$R_2 = (CH_2)_5COOH$

The peak at *m/e* 336 in the natural compound matched within 5 millimass units of that found in the synthetic compound and both agreed to within 10 mmu of the calculated formula weight of the pyrazine compound. When the major peaks, *m/e* 236, of both compounds were compared they agreed to within 10 mmu and the elemental composition of $C_{13}H_{20}O_2N_2$ was ascribed to this molecular ion. This would suggest that the pyrazine compound underwent a McLafferty rearrangement (Budzikiewicz *et al.*, 1967) in which one of the carboxylic acid side chains lost a fragment of *m/e* 100 (CH_2=$CHCH_2CH_2COOH$). That a similar reaction occurs to the other carboxylic acid side chain is evident from the peak at *m/e* 136.

Both the natural and synthetic compounds were capable of supporting the growth of yeast and a number of *E. coli* biotin mutants. The growth curves with yeast as the assay organism were superimposable and in Figure 4 are compared with that for biotin. The relative activity of 7KAP as compared to biotin varied somewhat over the concentration range used. On a molar basis, 7KAP had about 65% the activity of biotin below 0.06 ng/10 ml and from 80 to 95% in the range of 0.08–2.0 ng/10 ml.

Acknowledgments

The authors are grateful to Dr. T. Stoudt of Merck and Co. for providing us with 100 gal of culture filtrate. We also wish to thank Mr. George van Lear of Purdue University for the high resolution mass spectrum analysis and Dr. F. Abramson of CEC for the peak matching and elemental analysis. Mr. Carter Cook's assistance in interpreting the mass spectrometry data is greatly appreciated.

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Isolation and Characterization of Terminal Polynucleotide Fragments from Bacteriophage Ribonucleic Acids*

J. C. Lee, H. L. Weith, and P. T. Gilham

ABSTRACT: Procedures have been developed for the isolation and purification of polynucleotide fragments deriving from the terminals of large ribonucleic acids. The method depends on the selective periodate oxidation of the 2′,3′-diol groups of the terminal fragment after cleavage of the ribonucleic acid, and the specific binding of this fragment to aminoethylcellulose.

After the unoxidized polynucleotide fragments have been eluted from the cellulose the terminal fragment is released by a β-elimination reaction and is thus isolated in a form which lacks its terminal nucleoside. The method was developed initially with studies on dinucleoside phosphates and was then applied to the isolation of terminal polynucleotides from the ribonuclease T_1 digests of three bacteriophage ribonucleic acids. The fragments obtained from the bacteriophages, f2 and Qβ, have been characterized by alkaline and enzymatic hydrolyses and shown to have the compositions $(A_2U_2C_4)$-Cp and (C_9U_4)-Cp, respectively.

As an initial phase in a study of the relation between the primary structure and function of large RNA molecules, work in this laboratory has been directed toward the development of methods for the determination of nucleotide sequences near the terminals of these molecules (Lee and Gilham, 1965, 1966; Weith *et al.*, 1968; Weith and Gilham, 1967, 1969). It had been anticipated earlier that sequence studies near the terminals would be greatly facilitated by the fact that large ribonucleic acids such as those occurring in ribosomes and viruses possess terminals whose unique character can be maintained even after the molecules have been cleaved into small fragments. For example, if the RNA molecule is cleaved by one of the cyclizing ribonucleases or by alkali, the fragment which contains the left-hand terminal is distinguished by the fact that it possesses a phosphorylated 5′-hydroxyl group while the fragment corresponding to the right-hand end differs from all other fragments in that it contains an unphosphorylated 2′,3′-diol group. In the present work, the uniqueness of this terminal diol group together with the reactivity of its oxidation product are exploited in a method which involves the selective immobilization of terminal polynucleotides on aminoethylcellulose.

The specific oxidation of the terminal diol group of polyribonucleotides by periodate has been used in a number of laboratories as the basis for various methods of fractionation. For example, Zamecnik *et al.* (1960) have used this reaction in a method for tRNA fractionation where the change in solubility caused by the condensation of oxidized tRNA with a hydrazide allows the removal of those tRNA molecules containing free 2′,3′-diol groups from those containing a particular aminoacyl group at the 2′ or 3′ positions. A similar type of fractionation of a tRNA mixture was achieved when the tRNA molecules lacking an aminoacyl substituent were oxidized with periodate and then removed by absorption to aminoethylcellulose (Zubay, 1962). Yolles (1964) has also studied the binding of oxidized polyribonucleotides to a cellulose derivative (*p*-hydrazinobenzylcellulose) and, in addition, he investigated a procedure, involving displacement with benzaldehyde, for the recovery of the bound material. Unfortunately, this approach produced rather low yields in both the binding and recovery steps. Methods for the release of oxidized polynucleotides bound to aminoethylcellulose have also been studied (Habermann *et al.*, 1966; Temmerman, 1967). In these procedures the release was effected by elution of the cellulose with hydrochloric acid. However, in view of the expected instability of polynucleotides under such conditions, it seems that this approach may not be applicable to the isolation of polynucleotides of any considerable length.

The aim of the present work was to study methods for the

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