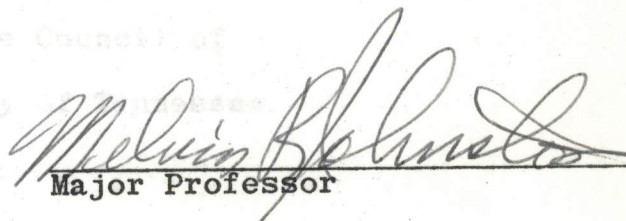


A 5'-PHOSPHODIESTERASE OF THE
STREPTOMYCES SPECIES

February 12, 1964

To the Graduate Council:

I am submitting herewith a thesis written by John Y. Lu entitled "A 5'-phosphodiesterase of the Streptomyces species." I recommend that it be accepted for nine quarter hours of credit in partial fulfillment of the requirements for the degree of Master of Science, with a major in Food Technology.


Major Professor

We have read this thesis
and recommend its acceptance:

Bruce M. Anderson.

Gerald D. Leman, Jr.

Accepted for the Council:


Dean of the Graduate School

A 5'-PHOSPHODIESTERASE OF THE
STREPTOMYCES SPECIES

A Thesis
Presented to
the Graduate Council of
The University of Tennessee

In Partial Fulfillment
of the Requirements for the Degree
Master of Science

by
John Yauyen Lu
March 1964

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33

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CHAPTER I

INTRODUCTION

The flavor enhancing property of inosine-5-phosphate (5'-IMP) was first reported by Kodama in 1913 (23).* Batzer and LandMann (7) also identified this compound as one of the important beef flavor precursors. Kuninaka (24) from his studies on the characteristics of 5'-ribomononucleotides found that guanosine-5-phosphate and xanthine-5-phosphate also had a seasoning effect similar to 5'-IMP.

Snake venom (21) and intestinal mucosa (13) were known as sources of 5'-phosphodiesterase which was able to catalyze the cleavage of phosphate ester linkage of RNA to produce 5'-ribomononucleotides. However, it has been discovered recently (25, 33, 35) that many microorganisms also are capable of producing 5'-phosphodiesterase and of degrading nucleic acid into 5'-nucleotides. Therefore, the production of 5'-mononucleotides by means of a microbial enzyme has become possible.

With these ideas in mind, a microorganism was isolated from soil and this study was made in an attempt:

(1) to identify the microorganism which is capable of degrading ribonucleic acid (RNA) into 5'-ribomononucleotides;

*()Numbers refer to Bibliography.

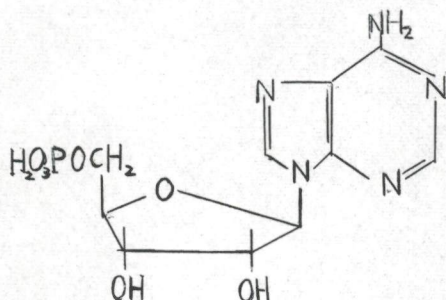
(2) to identify the variety of 5'-ribomononucleotides produced from the hydrolysis of RNA by this organism.

CHAPTER II

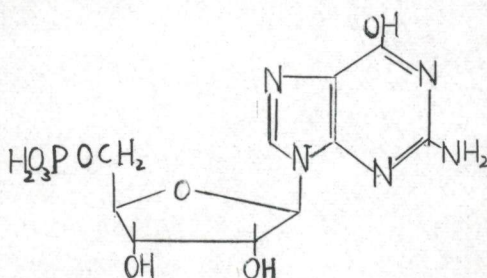
REVIEW OF THE LITERATURE

Chemistry of 5'-Ribomononucleotides

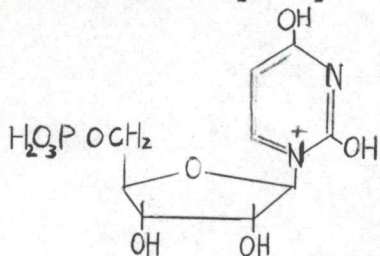
The 5'-ribomononucleotides are compounds which consist of ribose, purine and pyrimidine bases and phosphoric acid. The structures of these compounds are shown as follows:



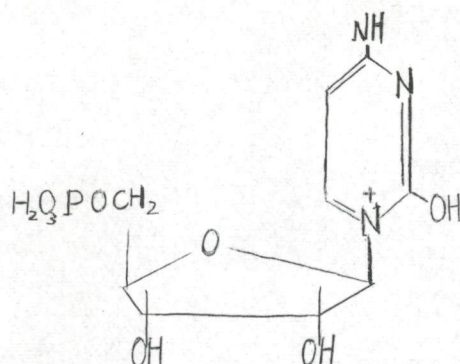
Adenosine-5-phosphate (5'-AMP)



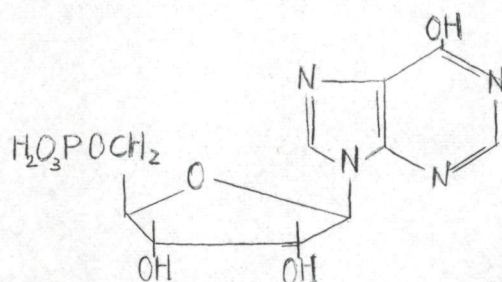
Guanosine-5-phosphate (5'-GMP)



Uridine-5-phosphate (5'-UMP)



Cytidine-5-phosphate (5'-CMP)



Inosine-5-phosphate (5'-IMP)

The chemical structures of 5'-ribomononucleotides have been characterized by the periodate oxidation method (16, 22) and confirmed by chemical synthesis (3, 4).

Some of the important chemical reactions of the 5'-ribomononucleotides are as follows:

(1) Orcinol reaction. The 5'-ribonucleotides will react with orcinol to form a reaction product. Albaum and Umbreit (1) reported that color developing rate of 5'-ribomononucleotides could be distinguished from that of 2', 3'-isomers due to slightly higher resistance of 5'-phosphodiester linkage to acid hydrolysis.

(2) Carbazole reaction. Dische and Landsberg (17) indicated that the reaction products of 5'-nucleotides had

two absorption maxima, at 710 m μ and 600 m μ , and gave a blue color, whereas, 3'-nucleotides had an absorption maximum at 540 m μ and gave a purple color.

(3) Periodate reaction. The 5'-ribomononucleotides are distinguished from their 2', 3'-isomers by their susceptibility to the periodate reaction which indicates the presence of two adjacent cis-hydroxyl groups in the ribose moiety. This specific property has been utilized by Todd and Davoll (16) and Khym and Cohn (22) in elucidating the structure of the 5'-nucleotides and 5'-nucleosides. The color reaction between aldehydes, periodate and dinitrophenylhydrazine (26) or Schiff's base (10) provide a specific method for determining 5'-ribomononucleotides in the presence of the 2' and 3'-isomers.

(4) The reaction for phosphate. Any of the standard methods, such as Fiske and Subbarow's (19), Allen's (2) or Bartlett's (6), are available for the estimation of phosphorous content. The ratio of phosphorous to ribose and purine or pyrimidine bases is one of the important criteria used for the determination of the monophosphate nucleotide, the diphosphate nucleotide or some other compound.

The compounds which contain purine or pyrimidine bases absorb ultra-violet light characteristically. This property is due to the conjugated double bond systems of

purine and pyrimidine rings and it is very useful for both qualitative and quantitative analyses of nucleic acid and its derivatives (40).

The ratio of the relative extinctions at two wavelengths affords a quantitative measure of the shape of the spectrum which is related to purity and characteristics of the substances in question. The pH values of the solution will influence the ionized forms of the substances, therefore, the extinction values should be measured under defined conditions. The detailed spectral data of purines, pyrimidines and their derivatives have been described elsewhere (8, 11, 13).

Chromatography of Nucleotides

Paper chromatography is a rapid and convenient method for determining nucleic acid and its derivatives. Various solvent systems have been proposed to resolve the hydrolytic products of nucleic acid by one- and two-dimensional chromatography (9, 27, 39). For the detection of purines, pyrimidines and related substances, the following techniques may be applied: Phosphate reagents for the nucleotides (15); silver chromate for the purines (38); periodate for nucleosides and 5'-ribonucleotides (10); ultra-violet light for all purine and pyrimidine containing compounds (20).

For large quantities of nucleotides, column

chromatography is desirable. Cohn and Volkin (13, 14) separated the enzymatic digests of RNA with Dowex 1 and Dowex 2 anion exchange resins. Nakajima and Saito (30) employed a Dowex 1-formate column to separate and identify naturally occurring 5'-ribomononucleotides in foods. Recently, Nakao and Ogata (32) demonstrated the separation and identification of nucleotides resulting from the hydrolysis of RNA by Aspergillus quercinus with a Dowex 1-formate column and formic acid and sodium formate solvent system.

Hydrolysis of Nucleic Acid to 5'-Nucleotides by Microorganisms

Plackett (35) reported that Asterococcus mycodes excreted a 5'-phosphodiesterase in the culture broth. The organism was incubated at pH 8, and at 37°C in the presence of 0.3 N NaCl. Magnesium ion had an inhibitory effect on this enzyme.

Kuninaka, et al. (25) found that Penicillium citrinum was capable of hydrolyzing RNA into 5'-ribomononucleotides. The highest enzyme activity was observed at pH 5 and at 60°C.

A study of the distribution of 5'-phosphodiesterases in microorganisms has been made by Ogata, et al. (33). They found that the enzymes were produced by various microorganisms belonging to Streptomyces, Bacillus, Fungi imperfecti

such as Fusarium, and Helminthosporium, and Ascomycetes such as Neurospora, Glomerella, and Aspergillus. Nakao and Ogata (32) observed that Aspergillus quernicus possessed two kinds of RNA depolymerases, a phosphodiesterase, a phosphomonoesterase and an adenylic acid deaminase. The formation of 5'-nucleotides or 3'-nucleotides depends on the range of pH employed for the hydrolysis. At a pH lower than 6, 3'-nucleotides were formed, while at a pH higher than 8.5, 5'-nucleotides were produced. Nakao and Ogata (31) also reported that many microorganisms which were capable of degrading RNA into 5'-mononucleotides, produced the enzymes that hydrolyze DNA into 5'-deoxymononucleotides, whereas, the microorganisms which produced such enzymes that degrade RNA into 3'-nucleotides, in general, did not produce the enzyme to degrade DNA.

Classification of Streptomyces Species

The members of the genus Streptomyces are spread widely in nature. The taxonomical study of this group of microorganisms has become important, because so many strains in this genus have been isolated after the discovery of antibiotics. However, the task of classifying Streptomyces at a specific level is still complicated by many difficulties because of lack of agreement on the classifying methods and descriptions.

Baldacci, et al. (5) proposed the separation of Actinomyces (Streptomyces) species into "Series" based on colors of vegetative growth and aerial mycelia and on soluble pigment produced by organisms on oatmeal agar.

Pridham, et al. (36) subdivided the Streptomyces into seven sections primarily depending on the morphology of sporophores. Pridham and Gottlieb (37) also made a study of the utilization of carbohydrates by the Streptomyces to aid in the differentiation of species.

According to Waksman's system (41, 42), the genus is subdivided into subgenera and into series based on both morphological and cultural characters. Each series is subdivided, on the basis of specific cultural and biochemical properties, into species. Formation of soluble pigments, pigmentation, and antibiotic producing characters are employed as the important criteria for the species classification.

CHAPTER III

EXPERIMENTAL

Identification of Streptomyces Species

An unknown species of Streptomyces was isolated from soil in Formosa in 1962.

A study on the typing of this Streptomyces species was carried out according to Waksman's system (41). Various media which were suggested by Waksman (42), and Pridham and Gottlieb (36) were prepared for the characterization of the species in question. In order to avoid making an erroneous description, several types of cultures, such as S. albus (NRRL-B-2208), S. grieseus (NRRL-B-150), S. aureofaciens (NRRL-2209), S. flaveolus (NRRL-B-2209) were incubated together with the unknown species, and the cultural characteristics of their growth were checked with the descriptions in the literature (37, 42).

Preparation of Media

Media of the following compositions were prepared for the cultural and morphological study of the Streptomyces species.

(1) Sucrose nitrate agar (Czapek's)

sucrose	3.0 %	KCl	0.05%
NaNO ₃	0.2	FeSO ₄	0.01

K_2HPO_4	0.1 %	agar	1.5
$MgSO_4 \cdot 7H_2O$	0.05		

The medium was adjusted to pH 7.0, and sterilized at 120°C for 5 minutes.

(2) Glucose-asparagine agar (Krainsky's)

glucose	1.0 %
asparagine	0.05
K_2HPO_4	0.05
agar	1.5

The medium was adjusted to pH 6.8, and sterilized at 120°C for 5 minutes.

(3) Nutrient agar

peptone	0.5%
meal extract	0.5
NaCl	0.5
agar	1.5

The medium was adjusted to pH 7.2, and sterilized at 120°C for 15 minutes.

(4) Gelatin

Sixteen grams of gelatin were dissolved in 100 ml. of water; the pH was adjusted to 7.4, and the medium was sterilized at 110°C for 30 minutes.

(5) Starch agar

soluble starch	1.0 %	NaCl	0.05%
$NaNO_3$	0.1	$MgCO_3$	0.1
K_2HPO_4	0.03	agar	1.5

The medium was adjusted to pH 7.0, and sterilized at 120°C for 5 minutes.

(6) Medium for nitrate reduction

peptone	0.5%
meat extract	0.3
KNO ₃	0.1

The medium was adjusted to pH 7.0, and sterilized at 120°C for 15 minutes.

(7) Medium for hydrogen sulfide

peptone iron agar (Difco)	3.6%
yeast extract	0.1

The medium was sterilized at 120°C for 5 minutes.

(8) Tyrosine agar

yeast extract	0.1%
l-tyrosine	0.1
NaCl	0.85
agar	1.5

The medium was adjusted to pH 7.0, and sterilized at 120°C for 5 minutes.

(9) Cellulose medium

A piece of filter paper, saturated with Czapek's solution free from carbohydrate, was used.

(10) Litmus milk

Ten grams of dry litmus milk (Difco) were dissolved in 100 ml. of water, and sterilized at 120°C for 10 minutes.

(11) Potato plug

Plugs of potatoes of desirable shape were placed in distilled water and sterilized at 120°C for 15 minutes.

(12) Oatmeal agar

One liter of distilled water was added to 60 grams of

rolled oats, cooked to a thin gruel, filtered; then, 1.8% agar was added. The medium was adjusted to pH 7.2 and sterilized at 120°C for 5 minutes.

(13) Glycerol-calcium malate agar

glycerol	1.0 %	K ₂ HPO ₄	0.05%
calcium malate	1.0	agar	1.5
NH ₄ Cl	0.05		

The medium was adjusted to pH 7.0 and sterilized at 120°C for 5 minutes.

(14) Carbon nutrition medium

carbon source	1.0 %	CuSO ₄ .5H ₂ O	0.00064%
ammonium sulfate	0.264	FeSO ₄ .7H ₂ O	0.0001
KH ₂ PO ₄	0.2388	MnCl ₂ .4H ₂ O	0.00079
K ₂ HPO ₄	0.565	ZnSO ₄ .7H ₂ O	0.00015
MgSO ₄ .7H ₂ O	0.1	agar	1.5

A variety of carbon sources was used: glucose, sucrose, mannitol, inositol, arabinose, xylose, salicin, rhamnose, lactose, raffinose, maltose. Each medium was adjusted to pH 7.0 and sterilized at 120°C for 5 minutes.

Cultivation and Observation of Organisms

Inoculation was carried out by transferring one loopful of one week old sporulating cultures which were grown on Emerson's media (34), into 5 ml. of sterilized distilled water and shook for 5 minutes. All the media of various compositions were inoculated and incubated at 28° to 30°C for two to three weeks. For the morphological study, petri-dish cultures on Czapek's agar, asparagine agar, oatmeal

agar, nutrient agar and starch agar were prepared. The forms of aerial mycelia, sporephores, spores and structures and branching of sporephores were observed with the microscope. The colors of aerial mycelia, reverse-side growth, formation of soluble pigment and pigmentation were thoroughly examined for all media. The proteolytic activity of the organism was observed in gelatin and litmus milk. All cultures were incubated for two weeks, and the degree of liquefaction of gelatin, and the peptonization of litmus milk were recorded. After incubating the organisms on starch agar for 10 to 14 days, 2 ml. of Lugol's iodine solution were added. A colorless zone around the colony of the organism indicates that the diastatic activity is positive. Difco's iron peptone agar was used to test the organism's ability to produce hydrogen sulfide. A bluish-black color indicates the production of hydrogen sulfide. The ability to reduce nitrate to nitrite was determined by adding one or two drops of sulfanilic acid reagent (34) to the media after two weeks' incubation. A pink color indicates a positive reaction. The utilization of carbohydrate by the organism was determined by growing on Pridam and Gottlieb's (37) basal medium which was supplemented with individual carbon compounds at the 1.0% level. A control without a carbon source was included.

RNA Degradation

Enzyme Preparation

A medium for growing the organism and producing RNA degrading enzymes was prepared with the following composition:

soluble starch	4.0 %	K ₂ HPO ₄	0.1 %
yeast extract	0.5	MgSO ₄ · 7H ₂ O	0.05
bacto-peptone	1.0	CaCl ₂	0.01

The pH of the medium was adjusted to 7.0 and a 30 ml. portion of the medium was poured into 250 ml. Hinton flasks and sterilized at 120°C for 10 minutes. The organisms were grown in the flasks on a rotary shaker (150 r.p.m.) for 72 hours at 30°C. The culture broth was centrifuged and the clear supernatant was employed as the enzyme preparation.

RNA Depolymerase Activity

McDonald's method (29) with some modification was employed in this experiment to determine RNA depolymerizing activity of the organism. The reaction mixture was prepared as follows:

2% RNA solution	2 ml.
0.5 M tris buffer (pH 8)	1 ml.
enzyme preparation	1 ml.

The reaction mixture was incubated at 37°C for 18 hours, and the reaction was stopped by the addition of 2 ml. of McFaden's reagent (0.25% uranyl acetate dissolved in 2.5% HClO₄) to precipitate oligonucleotides and undecomposed RNA.

One tenth ml. of the supernatant was diluted to 4 ml., and the optical density at 260 mμ was measured with a Beckman DU spectrophotometer. To set up 100% RNA degradation, 2 ml. of 2.0% RNA were incubated with 1 ml. of 1 N KOH at 37°C for 18 hours. The digest was adjusted to pH 3.6 with 10% HClO₄. The precipitate of KClO₄ was removed, then the digest was diluted to 6 ml. with water. One tenth ml. of the solution was diluted to 4 ml., and the optical density at 260 mμ was measured. For the preparation of the blank for ultra-violet measurement, McFaden's reagent was added to the enzyme preparation before incubation with RNA solution. The amount of degradation was calculated as follows:

$$\frac{A}{B} \times 100 = \text{the per cent of enzymatic degradation}$$

A: the optical density of the enzyme digestion
measured at 260 mμ.

B: the optical density of the KOH digestion
measured at 260 mμ.

Identification of 5'-mononucleotides

A reaction mixture of the following composition was prepared:

0.1 M Na ₂ HAsO ₄	2 ml.
4% RNA solution	2 ml.
1 M tris buffer (pH 8.4)	4 ml.
enzyme preparation	3 ml.

The hydrolysis of RNA was carried out at 37°C for 16 hours,

then the reaction was stopped by heating in boiling water for 15 minutes. After cooling, the reaction mixture was centrifuged to obtain a clear supernatant. In this degradation reaction, Na_2HAsO_4 was used as an inhibitor of the phosphomonoesterase activity of the organism.

The supernatant was spotted on Whatman No. 1 or Whatman No. 3 MM filter papers, and the papers were developed for 18 to 20 hours at room temperature by the ascending method with the following solvent systems:

- (1) isobutyric acid: concentrated NH_4OH : H_2O = 661: 10: 329 (v/v) (9).
- (2) saturated $(\text{NH}_4)_2\text{SO}_4$: 2-propanol: H_2O = 79: 2: 19 (v/v) (28).
- (3) 2-propanol: concentrated NH_4OH : H_2O = 7: 1: 2 (v/v) (39).

The positions of nucleotides were located by an ultra-violet lamp equipped with a glass filter which permits high transmittance of ultra-violet light at 253.7 m μ . The ultra-violet absorbing spots, their R_f values equivalent to the standard 5'-ribomononucleotides were cut from the paper and eluted with 3 ml. of water.

The eluents were subjected to the periodate oxidation, followed by dinitrophenylhydrazine* (DNPH) reaction to

*A saturated DNPH reagent was prepared by adding 20 gm. of a 2,4-dinitrophenylhydrazine to 200 ml. of 2N HCl.

determine whether or not they contained 5'-ribomononucleotides. The procedure was described as follows:

Three ml. of eluent were added to 1 ml. of 0.01N HIO_4 , and the solution was heated at 70°C for 3 minutes. One ml. of 0.05N Na_2HAsO_3 was added to stop the oxidation reaction. This was followed by addition of 1 ml. of concentrated HCl and 1 ml. of DNPH reagent. The reaction mixture was heated in boiling water for 10 minutes. The appearance of bright orange color precipitation indicates the reaction is positive.

Column Chromatography of RNA Hydrolysate

It is quite difficult to separate adequately all ribomononucleotide compounds with one dimensional paper chromatography, therefore Dowex 1 anion exchange resin was employed for the further confirmation and identification of the variety of 5'-ribomononucleotides from the hydrolysis of RNA by the organism.

Preparation of Column

Dowex 1 anion exchange resin (200-400 mesh, chloride form) was washed with water to remove suspended solids and packed as a wet slurry in a 12 mm I. D. glass column to a height of approximately 200 mm. The resin was changed into the hydroxyl form by treating with three bed volumes of 1 N

NaOH. The column was washed thoroughly with distilled water and was placed in the formate form by the addition of three bed volumes of 6 N formic acid. Again the column was washed until the pH of the effluent was above 5.0.

Preparation of Sample

A treatment was given to the sample before it was absorbed on the column. Lento and Denton's (26) method was employed in this experiment. Ten ml. of 20% trichloroacetic acid were added to the 10 ml. of RNA hydrolysate, followed by addition of 30 ml. of 95% alcohol. A proteinous precipitate was removed. The trichloroacetic acid and ethanol which remained in the supernatant solution were extracted with three aliquots of diethyl-ether. The aqueous phase was adjusted to pH 8.0 with ammonia and absorbed on the formate column. The column was washed with distilled water until the effluent reading at 260 mμ approached 100% transmission.

Chromatographic Solutions

The solvent system proposed by Nakajima, et al. (30) was employed in this experiment. The eluting reagents are listed as follows:

H ₂ O	
0.005 N	NCOOH
0.01 N	HCOOH
0.1 N	HCOOH
0.1 N	HCOOH + 0.05 N HCOONa
0.1 N	HCOOH + 0.1 N HCOONa

Instrumentation

A Rinco automatic fraction collector was used to collect each 10 ml. effluent from the chromatographic column at the rate of 0.5 ml. per minute. A Beckman DU spectrophotometer was employed to measure the optical density of the fractions.

Identification of Varieties of 5'-Ribomononucleotides

Each 10 ml. aliquot of ultra-violet absorbing fraction was collected. The absorption maximum and the spectral ratios at 250/260 and 280/260 of the collected fractions were measured to identify the variety of base moieties. DNPH reaction was carried out on a portion of the ultra-violet absorbing fraction, and the nucleotide component was further confirmed by paper chromatography with the developing solvents (1) and (2) (page 17).

CHAPTER IV

RESULTS AND DISCUSSION

Identification of Streptomyces Species

It is noted that the results of morphological and cultural studies of the unknown Streptomyces species show this particular strain has the following characteristic properties: (1) the formation of straight, wavy sporophores and oval to spherical spores as shown in Figure 1; (2) the color of aerial mycelium chalky white, cretaceous growth occasionally observed and the color of substrate growth usually colorless, becoming light yellow to light brown depending on the age of culture; (3) melanin negative; (4) no soluble pigment produced with the media described in the experiment; (5) a strong diastatic activity and nitrate reducing ability.

A detailed comparison was made with the type cultures, S. albus NRRL-2209 and S. griseus NRRL B-150. The result is shown in Table I. It will be noted that this isolate is more closely related to S. albus than to S. griseus chromogenetically and morphologically. The different characters observed between S. albus NRRL-2209 and the strain in



FIGURE 1
MORPHOLOGY OF THE STREPTOMYCES SPECIES

TABLE I

COMPARISON OF THE CULTURAL AND MORPHOLOGICAL CHARACTERS
OF THE STREPTOMYCES SPECIES WITH STREPTOMYCES
ALBUS NRRL-2209 AND STREPTOMYCES
GRISEUS NRRL-B 150

The cultural and morphological characters			
	<u>S. albus</u> NRRL-2209	<u>S. griseus</u> NRRL B-150	Unknown species
Morphology	Coiled chains of spores. Cretaceous to pinkish cretaceous aerial mycelium. Yellowish vegetative growth. Concentrated ring colonies formed.	Straight to flexuous chains of spores, olive buff or yellowish gray aerial mycelium. Diffuse type growth significant.	Straight to wavy sporophores. Colorless to light yellowish vegetative growth. White cretaceous aerial mycelium. Concentrated ring colonies formed.
Sucrose nitrate agar	Poor growth, white aerial mycelium, colorless growth.	Growth medium, spreading, becoming olive-buff.	Growth not vigorous. Aerial mycelium white, colorless.
Asparagine glucose agar	Growth medium, aerial mycelium becoming light brown.	Good growth, powdery, becoming yellowish green. Brownish soluble pigment formed.	Good growth. Aerial mycelium white. Substrate growth colorless, becoming light yellowish green. No soluble pigment.

TABLE I (continued)

	The cultural and morphological characters		
	<u>S. albus</u> NRRL-2209	<u>S. griseus</u> NRRL B-150	Unknown species
Glycerol calcium malate agar	White aerial mycelium. Poor growth.	Growth abundant. Aerial mycelium becoming gray- green, powdery.	Growth good. White aerial mycelium.
Nutrient agar	Chalky white, growth not good.	Powdery gray- white, good growth.	Chalky white, cretaceous. Growth abundant.
Starch agar	No growth.	Growth thin, white color, diastatic activity positive.	Growth good white color, diastatic activity strong.
Gelatin	Positive.	Strongly positive.	Positive.
Litmus milk	Peptonization fast, becom- ing deep blue color.	Heavy growth around the tube wall and the surface. Coagulation followed by peptonization, becoming alkaline.	Growth on the surface of media, white aerial mycelium, substrate growth colorless. Peptonization occurred, becom- ing blue color.
Tyrosine agar	Negative.	Negative.	Negative.
Nitrate reduction	Weakly positive.	Positive.	Strongly positive.

TABLE I (continued)

The cultural and morphological characters			
	<u>S. albus</u> NRRL-2209	<u>S. grisues</u> NRRL B-150	Unknown species
Hydrogen sulfide production	Negative.	Negative.	Negative.
Cellulose	Scant to no growth.	No growth.	Scant to no growth.
Potato plug	Growth lichenoid, cream to light brown color	Yellow to brownish substrate growth.	Yellow to light brown growth. Aerial mycelium scant.
Utilization of carbon sources			
D-Glucose	+	+	+
D-Xylose	(+)	+	+
L-Arabinose	(-)	-	+
Rhamnose	-	-	(+)
D-Fructose	(+)	(+)	(-)
Sucrose	(+)	(-)	(-)
Lactose	+	+	+
Maltose	+	+	+
D-Manitol	+	+	+
i-Inositol	-	-	-
Salicin	+	+	+
Sorbitol	-	-	-

*Symbols used: - no growth; (-) faint growth; (+) poor to fair growth; + good growth and positive utilization.

question are the form of sporophores, diastatic activity, nitrate reducing ability and the utilization of l-arabinose and rhamnose.

According to Waksman's (41) description of S. albus, this species is characterized by white aerial mycelium, spiral sporophores, oval to spherical spores, weak antagonistic property, ability to liquefy gelatin, to peptonize milk with production of an alkaline reaction and to form earthy or musty odor. He also states that the numerous strains of this species vary greatly in their proteolytic, diastatic and other cultural properties. The detailed description is shown in Table II.

From summation and comparison of the experimental results of the cultural and morphological studies of the organism, it indicates that this unknown strain is morphologically and chromogenetically related to S. albus. Some differences in biochemical properties, such as nitrate reduction, diastatic ability, are not considered major criteria for classifying this species; therefore, this unknown strain according to Waksman's system possibly is S. albus or a subspecies within this species.

RNA Degradation

RNA Depolymerase Activity

The experimental results indicate that this

TABLE II
DESCRIPTION OF THE TYPE CULTURE STREPTOMYCES
ALBUS IMRU 3005

The cultural and morphological characters	
Morphology	Sporophores produce long spirals, spores spherical to oval. Some strains produce straight sporephores depending on the composition of media.
Sucrose-nitrate agar	Substrate growth smooth, colorless. Aerial mycelium cottony to powdery; white to snow-white.
Glucose-asparagine agar	Aerial mycelium gray, becoming brownish.
Nutrient agar	Generally no aerial mycelium; chalky white deposited on old colonies.
Starch agar	Aerial mycelium white. Rapid hydrolysis of starch in some cultures; others show little or no hydrolysis.
Potato	Growth lichnoid, cream colored.
Milk	Surface ring cream colored. Aerial mycelium white, peptonization rapid.
Nitrate reduction	Positive.
Hydrogen sulfide formation	Negative.
Gelatin	Colonies gray. No soluble pigment. Strong liquefaction.

*The description comes from "Bergey's Manual of Determinative Bacteriology," 7th ed. 1957.

Streptomyces species hydrolyses RNA. The per cent of RNA degradation was calculated as follows:

$$A: 0.113 \qquad B: 0.385$$

$$A/B \times 100\% = 26.8\%$$

It would appear that the amount of RNA degradation was not always in accord with the formation of 5'-nucleotides, because the enzyme preparation might have contained various phosphatases. The RNA degrading conditions employed in the present study was for screening purposes, therefore, for a detailed study of the optimum conditions of RNA degradation for this strain such as temperature, pH, ionic strength of reaction mixture, cultivation phases of the organisms, length of RNA degrading time, composition of culture media for the growth of organisms, and presence of inhibitors or activators in the reaction mixture remain to be investigated.

Identification of 5'-ribomononucleotides

In general, a satisfactory chromatographic separation of nucleotides from nucleosides in the RNA hydrolysate was obtained with the solvent systems of Pabst (9), Smith and Drummund (39), and Markham, et al. (28). The R_f values were difficult to reproduce exactly since temperature, quality of solvents, type of papers, treatment of paper and many other factors influence these values. Therefore, authentic

nucleotides and nucleosides were developed with the unknown sample for a better comparison.

The results of chromatograms which depict the location of various ultra-violet absorbing spots, are shown in Figure 2. The spots which are numbered 2 and 4 of Figure 2-B; 5 and 6 of Figure 2-A gave positive reactions when they were subjected to the periodate reaction, followed by the DNPH color reaction. The results indicate that: (1) these spots were possibly purine or pyrimidine derivatives because they absorbed ultra-violet light strongly at 253.7 m μ ; (2) these spots might contain a sugar moiety, because of their positive reaction with the DNPH reagent; and (3) R_f values of the paper chromatograms coincided with the authentic 5'-ribomononucleotides. Therefore, on the basis of the above data, the spots may be identified as 5'- ribomononucleotides.

It was noted from the paper chromatograms (Figure 2) that there were many ultra-violet light absorbing spots and their R_f values corresponded to those of the nucleosides. The reasons possibly are: (1) this organism might excrete nucleosides into the culture broth, (2) the concentration of sodium arsenate in the reaction mixture was not enough to inhibit the phosphomonoesterase activity of the organism, and (3) the mononucleotides were further degraded by other phosphatase systems.

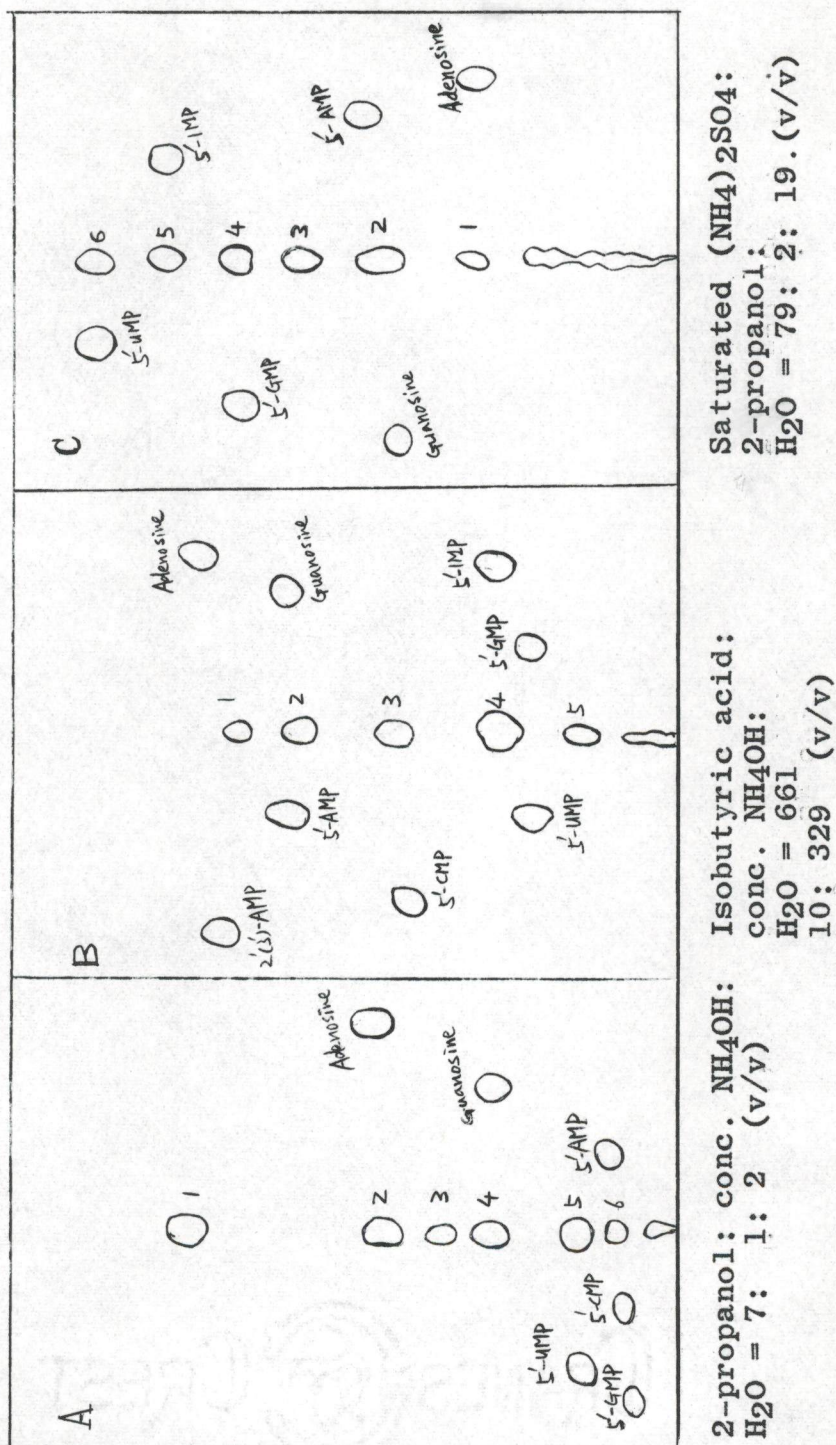


FIGURE 2

PAPER CHROMATOGRAMS OF ENZYMATIC DIGEST OF RNA

From this experiment, it is indicated that paper chromatography combined with ultra-violet light detecting technique and DNPH color reaction provides a simple and convenient method for identifying the presence of 5'-ribomononucleotides.

Column Chromatography of RNA Hydrolysate

Water and 0.005 N HCOOH were used to remove carbonyl compounds, purine and pyrimidine bases, nucleosides, carbohydrates and other non-ionic compounds prior to the elution of nucleotides absorbed on the column. These compounds would interfere with the DNPH color reaction and ultra-violet absorption measurement.

The chromatographic eluting solvent system employed in this experiment showed a good resolution for 5'-ribomononucleotides. The results are shown in Figure 3-B.

The elution curve of the enzymatic digest of RNA is shown in Figure 3-A. A comparison with the elution curve of 5'-ribomononucleotides (Figure 3-B) indicates that many ultra-violet light absorbing anionic compounds were produced together with 5'-mononucleotides. To each 260 m μ absorbing fraction of the curve, the λ max, spectral ratio, and DNPH color reaction were carried out to characterize the components in the fraction. The analytical results are shown in Table III.

A paper chromatographic technique also was employed to make a confirmation and identification of the variety of 5'-ribomononucleotides. The resulting chromatograms are shown in Figure 4 and Figure 5.

Combining and analyzing the information from the experimental results (Figures 3, 4, 5 and Table III), the component in each 260 mμ absorbing peak (Figure 3-A) was identified and confirmed as follows:

Fraction 1. Spectral data were identical to that of CMP, the DNPH color reaction was positive, R_f values on the paper chromatograms and the eluted position of this fraction from Dowex 1-formate column coincided with 5'-CMP. Therefore, it was concluded that this fraction was 5'-CMP.

Fraction 2. Spectral data of this fraction were not conclusive enough to characterize the nitrogen base moiety. The DNPH color reaction was negative. R_f values on the paper chromatograms did not match any of the authentic 5'-ribomononucleotides. This fraction remains to be identified.

Fraction 3. Absorption maximum and the spectral ratio were identical to that of AMP. The color reaction with DNPH was positive. The results of paper and column chromatography confirmed this fraction was 5'-AMP.

Fraction 4. Spectral data were similar to that of

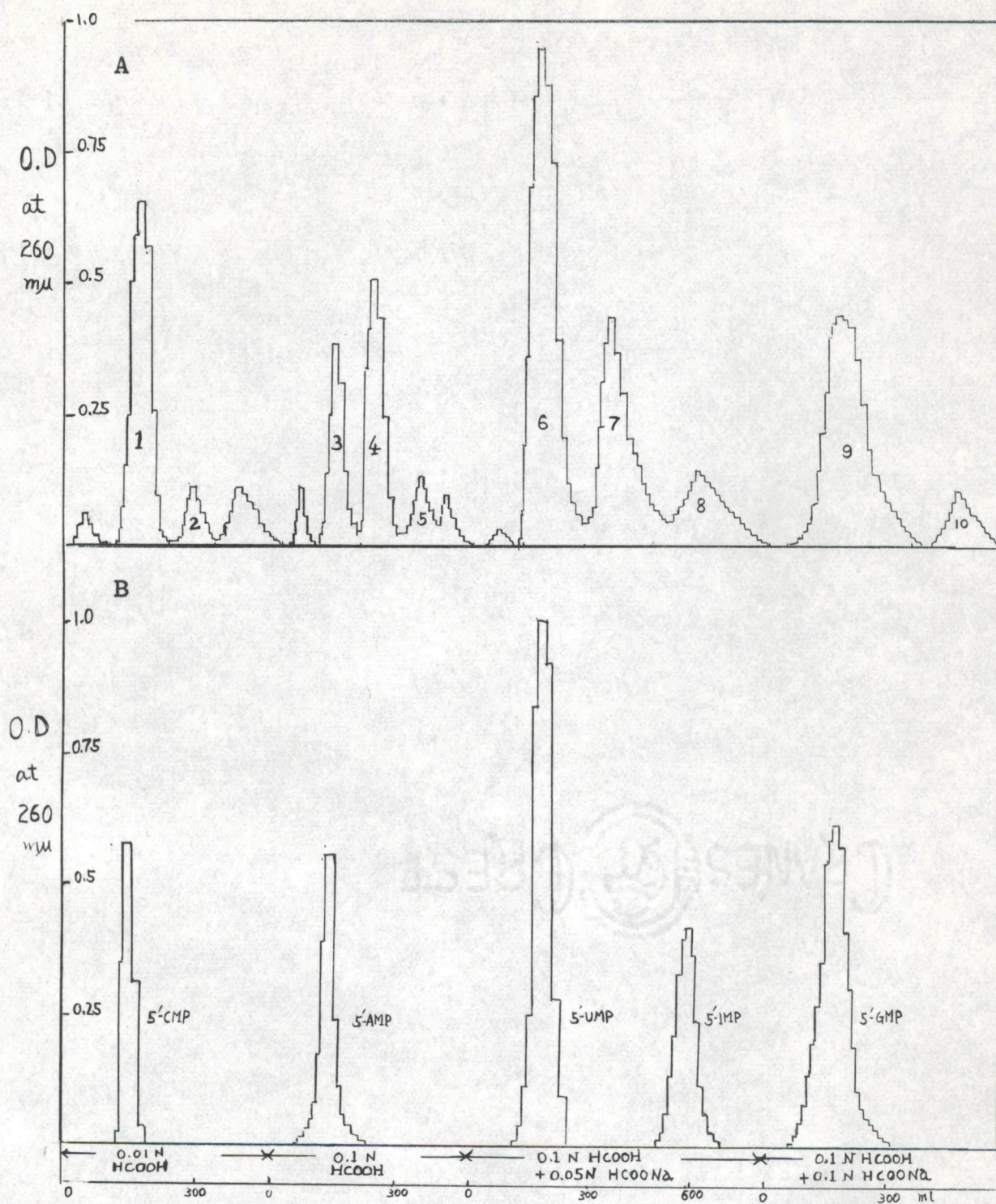


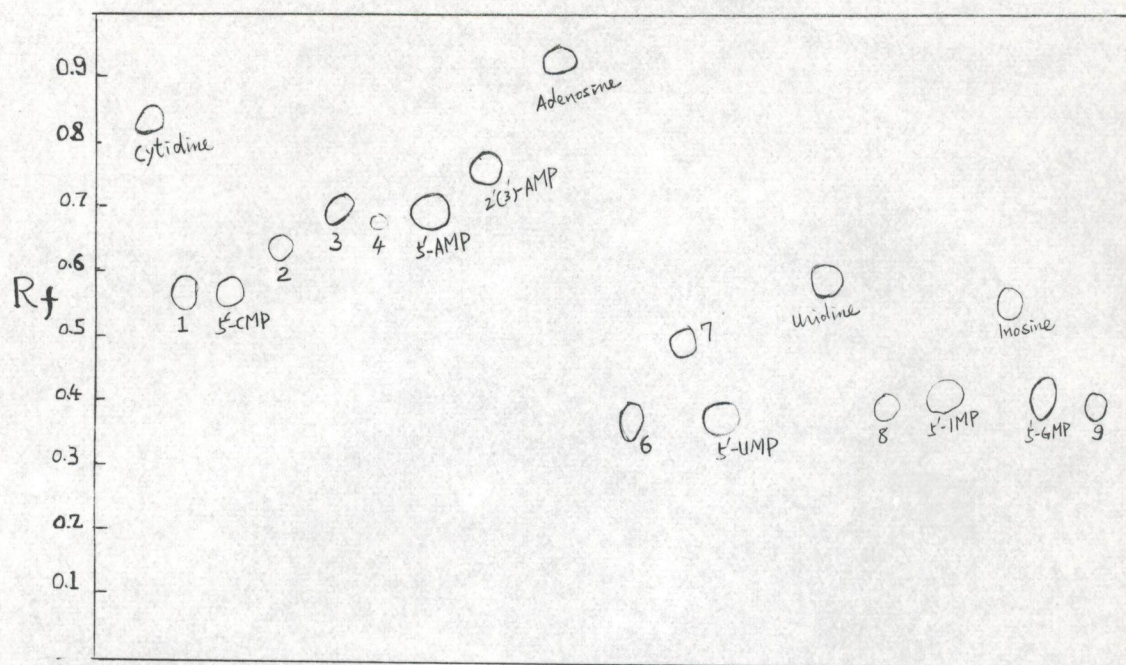
FIGURE 3

SEPARATION OF NUCLEOTIDES IN (A) ENZYMATIC DIGEST AND (B) 5'-RIBOMONONUCLEOTIDES BY ANION EXCHANGE RESIN

TABLE III
CHARACTERIZATION DATA OF EACH 260
m μ -ABSORBING FRACTION

No. of fraction	pH	$\lambda_{\max}$	$\frac{250^*}{260}$	$\frac{280^*}{260}$	$\frac{290^*}{260}$	DNPH reaction
1	2	280	0.42	1.96	1.4	+
	7	271	0.85	0.94	0.31	
2	2	260	0.52	1.49	0.92	-
	7	262	0.82	0.615	0.167	
3	2	258	0.82	0.212	0.0278	+
	7	259	0.76	0.143		
4	2	258	0.83	0.29	0.096	-
	7	259	0.75	0.18	0.0272	
5	2	258	0.83	0.30	0.07	-
6	2	261	0.75	0.38	0.049	+
	7	262	0.742	0.36		
7	7	262	0.71	0.44		-
8	2	249	1.63	0.27		+
	7	249	1.57	0.24		
9	2	255	0.95	0.67	0.47	+
	7	253	1.08	0.69	0.25	
10	2	256	0.94	0.69	0.50	-

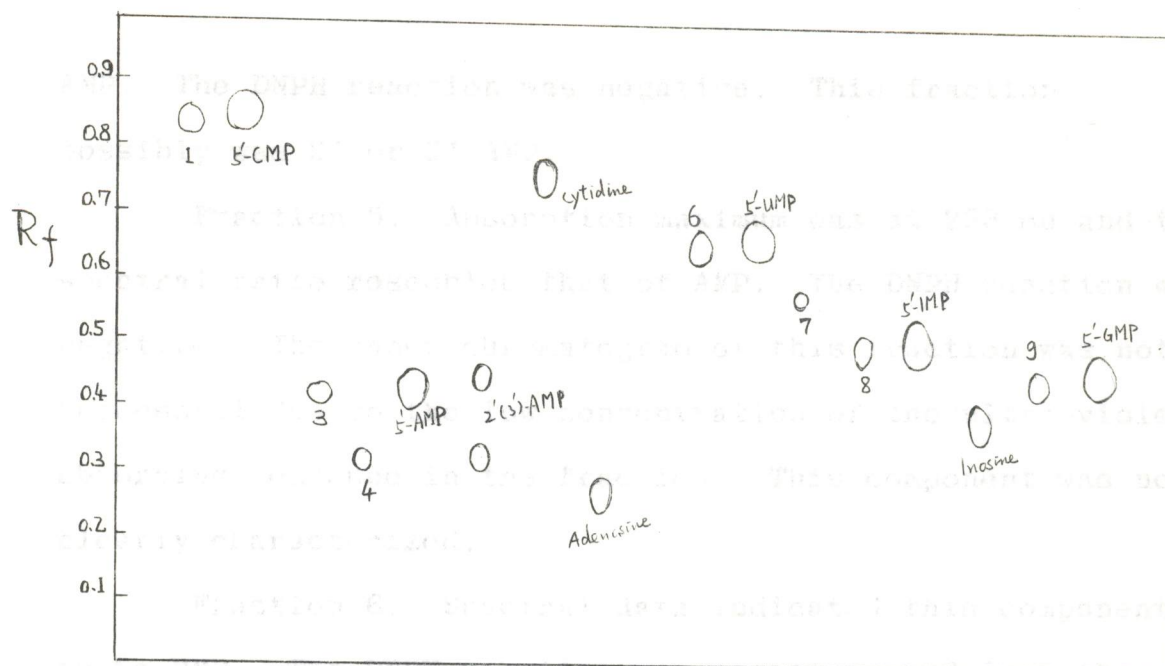
*The ratio of optical densities at 250 m μ , 280 m μ and 290 m μ vs. 260 m μ .



Isobutyric acid: conc. NH_4OH : H_2O =661 : 10: 329 (v/v)

FIGURE 4

PAPER CHROMATOGRAM OF ULTRA-VIOLET LIGHT ABSORBING
FRACTIONS SEPARATED FROM ANION EXCHANGER
WITH NO. 1 SOLVENT



Sat. $(\text{NH}_4)_2\text{SO}_4$: 2-propanol: H_2O = 79: 2: 19

FIGURE 5

PAPER CHROMATOGRAM OF ULTRA-VIOLET LIGHT ABSORBING
FRACTIONS SEPARATED FROM ANION EXCHANGER
WITH NO. 2 SOLVENT

AMP. The DNPH reaction was negative. This fraction possibly was 2' or 3'-AMP.

Fraction 5. Absorption maximum was at 258 mμ and the spectral ratio resembled that of AMP. The DNPH reaction was negative. The paper chromatogram of this fraction was not successful due to the low concentration of the ultra-violet absorbing compound in the fraction. This component was not clearly characterized.

Fraction 6. Spectral data indicated this component to be UMP. The DNPH reaction was positive, and from the paper and column chromatography, the component was identified as 5'-UMP.

Fraction 7. This fraction exhibited an absorption maximum at 262 mμ (pH 7). The spectral ratio resembled that of UMP. The DNPH color reaction was negative. The R_f value on the paper chromatogram did not match 5'-UMP; therefore, it appears to be 2' or 3'-UMP or others.

Fraction 8. Spectral data from this fraction were similar to that of IMP. The DNPH reaction was positive; and the paper chromatograms and the column chromatogram of this fraction also indicated that the component was 5'-IMP.

Fraction 9. Spectral data suggested this component was GMP. The DNPH reaction was positive. The results of paper and column chromatograms further confirmed this compound to be 5'-GMP.

Fraction 10. The data of spectral ratio and λ max. indicated the fraction to be GMP. The DNPH color reaction was negative and the eluted position of this fraction from the Dowex-1 column suggested that this fraction was possibly 2' or 3'-GMP.

The identification of 5'-ribomononucleotides, as described above, indicates the following:

(1) A 5'-phosphodiesterase was produced by the isolated *Streptomyces* species, which was able to degrade RNA into 5'-CMP, 5'-AMP, 5'-UMP, and 5'-GMP.

(2) A 5'-IMP was identified together with other 5'-ribomononucleotides. This fact indicates the possibility of the presence of an adenylic acid deaminase in the culture broth which converts 5'-AMP into 5'-IMP.

(3) The identification of some of 2' and 3'-mononucleotide-like compounds suggests that another enzyme system was involved in the enzymatic digestion of RNA. However, the relative amounts of these compounds were less than their corresponding 5'-ribomononucleotides (Figure 2-A).

CHAPTER V

SUMMARY

A study was made on the classification of a Streptomyces species isolated from soil and the ability of this strain to produce a 5'-phosphodiesterase in broth culture media.

Under conditions of the experiment reported in this paper, it was found that:

(1) The microorganism under study appears to be a Streptomyces albus or a subspecies within this species.

(2) The presence of 5'-phosphodiesterase was confirmed by detecting the formation of 5'-ribomononucleotides resulting from the enzymatic digestion of RNA. Paper chromatography combined with ultra-violet light detecting technique and periodate oxidation followed by DNPH color formation reaction were found to be a practical and convenient method for identifying 5'-ribomononucleotides.

(3) The ability of this organism to degrade RNA was confirmed. The amount of RNA degradation was 26.8%.

(4) The 5'-ribomononucleotides from the hydrolysis of RNA were identified as 5'-CMP, 5'-AMP, 5'-UMP, 5'-IMP and 5'-GMP. The formation of 5'-IMP indicated that this organism was also able to produce an adenylic acid deaminase.



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