Excretion of 5'-Nucleotides by Bacteria*

Part II. Formation of 5'-Nucleotides by Degradation of Endogenous Ribonucleic Acid from Washed Cells of a *Bacillus*

By Kôichi OGATA**, Akira IMADA and Yoshio NAKAO

Research Laboratories, Takeda Chemical Industries, Ltd., Osaka

Received June 4, 1962

For the purpose of clarifying the mechanism of extracellular formation of 5'-nucleotides during cultivation of a *Bacillus*, which had been reported previously¹⁾, attempts were made to form 5'-nucleotides by non-proliferating cells of this *Bacillus*.

It was found that this *Bacillus* rapidly degrades endogenous RNA and consequently forms 5'-nucleotides extracellularly, when the washed cells are incubated in alkaline salt or buffer solutions.

Comparison of the process of the excretion of 5'-nucleotides from washed cells with that of the accumulation of 5'-nucleotides during cultivation revealed that both were brought about by the same mechanism, i.e., the degradation of endogenous RNA.

Various factors which affect the degradation of endogenous RNA were also examined and the biological significance of this kind of RNA-degradation was discussed.

INTRODUCTION

In the previous paper¹⁾, the authors reported that 5'-nucleotides were accumulated in the culture fluid of a *Bacillus*. Since the accumulation took place in parallel with the reduction of intracellular RNA during the cultivation and since the composition of nucleotides accumulated in the culture fluid resembled that of intracellular RNA, these nucleotides were postulated to be breakdown products of intracellular RNA.

The present work was carried out to substantiate the deduction mentioned above.

At first, we studied the process of degradation of endogenous RNA and formation of 5'-nucleotides by non-proliferating cells of this *Bacillus*. Then we showed some evidences that the accumulation of 5'-nucleotides during cultivation is brought about by the same mechanism as the excretion of 5'-nucleotides from non-proliferating cells of this *Bacillus*.

At the same time, various factors which affect the degradation of endogenous RNA were examined and their effects were discussed.

Biological significance of this kind of excretion was also discussed.

MATERIALS AND METHODS

Biological Methods.

Bacterial Strains: *Bacillus* sp. No. 17-5, a soil bacterium, which has the ability to form 5'-nucleotides in the culture fluid during cultivation was used. Its characteristics were described in the preceding paper¹⁾. Other strains were obtained from the Institute for

Abbreviations: RNA, ribonucleic acid; DNA, deoxyribonucleic acid; AMP, GMP, CMP and UMP, adenylic, guanylic, cytidylic and uridylic acid; ADP, GDP, CDP and UDP, ribonucleoside 5'-diphosphates of adenine, guanine, cytosine and uracil; PCA, perchloric acid; Tris, tris (hydroxymethyl) aminomethane; EDTA, ethylenediamine tetra-acetic acid.

* Degradation of Nucleic Acids and their Related Compounds by Microbial Enzymes. Part XIV.

** Present address: Department of Agricultural Chemistry, Kyoto University, Kyoto, Japan.

1) K. Ogata, A. Imada and Y. Nakao, This Journal, **26**, 586 (1962).

Fermentation, Osaka.

Cultivation: The procedure for growth experiments was much the same as that reported previously¹³. Usually peptone-glycerol medium (PG-medium) in which Polypeptone (Daigo Nutritive Chemicals, Ltd., Osaka) was substituted for Casamino acids (Difco, vitamin-free) in G-medium¹³ was used for the purpose of obtaining log-phase cells. Constituents of the other media are indicated in Results.

Analytical Methods.

Preparation of Suspensions of Intact and Disrupted Cells. *Bacillus* sp. No. 17-5 was grown in PG-medium at 28°C unless otherwise specified, and the cells were harvested in the later logarithmic phase of growth (12 to 16 hours after inoculation) by centrifugation, washed twice with cold distilled water and resuspended in distilled water to give a density of 12.5 to 15 mg dry cells per ml (suspension of intact cells). In some cases this cell suspension was sonicated by a Sakuma SV-505 sonic oscillator at 10 kc for 10 minutes in the cold, and centrifuged at 16,000 r.p.m. in a Kubota KR-6L centrifuge for 30 minutes at 0°C. The resulting supernatant fluid (suspension of disrupted cells) contained more than 90% of the nucleic acids originally present in the suspension of intact cells.

Excretion of 260 m μ -Absorbing Material. The suspension of intact washed cells was mixed in a centrifuge tube with an equal volume of buffer, and incubated at 37°C unless otherwise mentioned. After an adequate period (15 to 300 minutes) of incubation, the mixture was cooled by immersing the tube in an ice bath and centrifuged in the cold.

The supernatant fluid thus obtained was designed as "excreted solution".

The rate of excretion was routinely calculated as follows: both the excreted solution and the mixture before incubation of cell suspension and buffer were mixed with an equal volume of 10% PCA and extracted for 30 minutes at 80°C. The resulting precipitates were centrifuged off and the absorbance of both supernatants at the wave length of 260 m μ were measured with a Hitachi EPU-IIA spectrophotometer. The rate of excretion was calculated from the proportion of the absorbance of the excreted solution to that of the mixture of cell suspension and buffer.

Since among cellular 260 m μ -absorbing materials, RNA predominated over DNA and acid-soluble compounds to a great extent (see Results), the rate of

excretion calculated in this way roughly approximated the degree of degradation of intracellular RNA.

Degradation of Endogenous Nucleic Acids. The suspension of intact or disrupted cells was mixed with buffer and incubated at 37°C as described previously, and the reaction was stopped by the addition of cold PCA to give a final concentration of 5% and centrifuged at 0°C. This acid supernatant was decanted into a test tube and heated for 30 minutes at 80°C and the absorbance at 260 m μ was measured (EX₂₆₀). Simultaneously the acid supernatant of the reaction mixture was obtained before incubation and the absorbance was measured just the same as before (EO₂₆₀). The difference of the absorbances between EX₂₆₀ and EO₂₆₀ was designated as ΔE_{260} . Total absorbance of the suspension was also measured as follows: the mixture of suspension of intact or disrupted cells and buffer was mixed with an equal volume of 10% PCA and heated for 30 minutes at 80°C, the precipitates were centrifuged off and the absorbance of the supernatant fluid was measured (ET₂₆₀). The degree of the degradation of nucleic acids was calculated using the equation below.

$$\text{Degree of degradation (\%)} = \frac{\Delta E_{260}}{ET_{260} - EO_{260}} \times 100$$

Extraction of Acid-soluble Compounds, Lipids and Nucleic Acids and Determination of Nucleic Acids. Schneider's method²³ was employed with slight modification. In our experiments, acid-soluble compounds were extracted with cold 5% PCA for 30, 15 and 5 minutes successively in the cold. The extraction of lipids and of nucleic acids was exactly the same as in Schneider's original report. RNA and DNA in the hot acid extract were determined as described in the previous paper¹³.

Phosphorus Determinations. Orthophosphate, acid-labile phosphate, total phosphate, 5'-nucleotide-phosphate and monoester-phosphate were determined by the same methods as in the previous paper¹³.

Ion-exchange Chromatography. The procedure was the same as reported previously¹³.

RESULTS

Degradation of Endogenous RNA and Formation on 5'-Nucleotides

Effects of Various Conditions on the Degradation of Endogenous Nucleic Acids.

Effect of pH. When the suspension of cells

2) W.C. Schneider, *J. Biol. Chem.*, **161**, 293 (1945).

was incubated for a short period in buffers of various pH values, ultraviolet absorbing materials were rapidly excreted into the suspending media. Their absorption spectra in the ultraviolet region had maxima around $260\text{ m}\mu$ and minima around $235\text{ m}\mu$ and they were similar to those of purine-pyrimidine mixtures (Fig. 1).

Degrees of the excretion (%) were 12.5, 16.3, 18.1, 49.5, 67.3 and 43.7 at pH 4.0, 5.0, 6.0, 7.1, 8.2 and 8.9, respectively, and the excretion occurred optimally in alkaline pH. For more precise optimal pH determination, the washed cells were incubated in 0.25 M Tris buffer and 0.25 M glycine-NaOH buffer between pH 7.0 and 10.5 and the degradation of nucleic acids was measured. As shown in Fig. 2, two pH optima, around pH 8.0 in Tris buffer and around pH 9.8 in glycine-NaOH buffer were observed for the degradation of nucleic acids. Although the presence of two apparent pH optima seemed to be attributed

FIG. 1. Ultraviolet Absorption Spectra of the Excreted Solutions.

Washed cells were suspended in 0.25 M acetate buffer of pH 4.0, 5.0 and 6.0 and in 0.25 M Tris buffer of pH 7.1, 8.2 and 8.9 and incubated for 30 minutes at 37°C .

FIG. 2. The Effect of pH on the Degradation of Intracellular Nucleic Acids.

Washed cells were suspended in buffers as shown and incubated for 40 minutes at 37°C .

—○— 0.25 M Tris buffer
—●— 0.25 M Glycine-NaOH buffer

to the difference in type of buffers and not to the presence of two routes of degradation, the degradation of nucleic acids in intact cells was often carried out at both pH optima.

Effect of Aeration. Washed cells were incubated in 0.25 M glycine-NaOH buffer, pH 9.55 without and with aeration by a Monod's apparatus for shaking culture.

As shown in Table I, the rate of degradation of the intracellular RNA as well as that of the decrease of $260\text{ m}\mu$ absorbing material from the cells were reduced by the aeration, while the rate of the increase of $260\text{ m}\mu$ absorbing material in the suspending medium was little affected by the aeration. This consequently resulted in the appearance of considerably larger amount of $260\text{ m}\mu$ absorbing material in the suspending medium than that disappeared from the cells, and disordered the stoichiometry, that is a coincidence of

TABLE I. THE EFFECT OF AERATION ON THE DEGRADATION OF RNA AND THE EXCRETION OF 260 m μ ABSORBING MATERIAL

Incubation period (hr)	Degradation of RNA (%)		Decrease of hot PCA soluble 260 m μ absorbing material in cells (O.D. 260 m μ per 13.7mg cells)		Increase of hot PCA soluble 260 m μ absorbing material in suspending medium (O.D. 260 m μ of suspending medium obtained from 13.7 mg cells)	
		Aerated		Aerated		Aerated
1	67.6	49.2	40.3	24.2	43.8	44.3
2	80.8	69.6	46.1	37.6	52.8	52.3
4	87.2	78.9	52.4	43.4	56.8	57.8

Washed cells were suspended in 0.25 M glycine-NaOH buffer, pH 9.55 to give a cell density of 6.84 mg per ml and incubated at 37°C with and without aeration.

the amount of 260 m μ absorbing material lost from the cells with that turned up into the suspending medium, which was observed under anaerobic state. This fact indicates that a net synthesis of 260 m μ absorbing material occurred during the aerobic incubation.

Effect of Reaction Temperature. The reaction was carried out between 10 and 70°C; and the excretion was found to occur optimally around 37 to 50°C. Therefore in the following experiments the incubations were carried out at 37°C.

Thermostability of Excretion. Thermostability was measured by allowing the suspension of cells to stand at various temperatures for 5 minutes and then incubating in 0.25 M Tris buffer (pH 8.0) for 40 minutes at 37°C. The rate of excretion decreased as the temperature of treatment was raised, and was completely lost by treating the cells at 85 to 100°C for 5 minutes.

The behavior at pH 9.8 was the same as that at pH 8.0.

Effects of Concentration of Buffers and Salts. In the early experiments when buffers of low concentrations were employed the degradation of intracellular nucleic acids was poor, therefore its dependence upon the concentration of buffers was suggested.

Suspensions of intact and disrupted cells were incubated in Tris buffer (pH 8.05) of various concentrations and changes in absorb-

ance at 260 m μ corresponding to RNA, DNA and acid-soluble fraction were examined. The data shown in Fig. 3 indicate that the degradation of RNA either in intact or disrupted cells is greatly dependent upon the concentration of buffer and occurs optimally around 0.25 M. It is also clear that an increase of the absorbance in the acid-soluble fraction

FIG. 3. The Effect of Concentration of Buffer on the Degradation of Nucleic Acids in Intact and Sonicated Cells.

Intact and sonicated cells were incubated in Tris buffer, pH 8.05 of various concentration for 50 minutes at 37°C.

— Intact cell
 - - - - - Sonicated cell

took place at the expence of the RNA fraction, though little variation was observed in the DNA fraction.

Furthermore, since this behavior was much the same regardless of whether the cells were intact or disrupted, it was suggested that the concentration of buffer does not affect the permeability of the cell membrane but give an influence upon the susceptibility of endogenous RNA to the enzyme (or enzymes) for its degradation, or upon an activation of the enzyme (or enzymes).

Degradation is also promoted by the addition of various salts such as NaCl, KCl, ammonium sulfate, and sodium acetate etc., whereas MgCl_2 and CaCl_2 are inhibitory. Furthermore, other non-ionizing agents such as glucose or urea have no effect.

The effect of concentration of NaCl was more pronounced than that of Tris buffer as illustrated in Fig. 4, and the excretion occurred optimally at a concentration of around

NaCl concentration (M) in 0.05 M glycine-NaOH buffer, pH 9.4

FIG. 4. The Effect of Concentration of NaCl on the Excretion.

Washed cells were suspended in 0.05 M glycine-NaOH buffer, pH 9.4 containing various concentrations of NaCl and incubated for 30 minutes at 37°C.

TABLE II. THE EFFECT OF VARIOUS METAL IONS ON THE EXCRETION

Metal added	Concentration in M	
	10^{-2}	10^{-3}
None (Control)	100.0%	100.0%
MgSO_4	64.8	106.2
MgCl_2	64.3	101.0
BaCl_2	62.1	89.9
CaCl_2	49.7	102.4
MnCl_2	96.2	109.5
ZnCl_2	92.3	100.2
CuCl_2	60.5	84.3
CoCl_2	99.3	102.7
NiCl_2	104.4	98.8
FeCl_3	75.0	99.1
FeSO_4	75.0	85.0
HgCl_2	36.1	37.9

Washed cells were suspended in 0.01 M glycine-NaOH buffer, pH 9.4 containing 0.25 M NaCl and 10^{-2} or 10^{-3} M metals and incubated for 40 minutes at 37°C. Data are expressed in relative value to control experiment.

0.25 M.

Effect of Metal Ions. Table II shows the effect of various metal ions on the excretion. Among the metals tested, Hg^{++} shows the greatest inhibition and alkali earth ions generally show inhibitory effects. Cu^{++} , Fe^{++} and Fe^{+++} ions also have an inhibitory effect, but Mn^{++} and Zn^{++} have almost no effect.

Effect of Anions and EDTA. As shown in Table III, polybasic anions generally have some stimulatory effect. It is noteworthy that orthophosphate, among the polybasic anions tested, is distinctively stimulatory.

EDTA had an inhibitory effect.

Kinetic Study of the Excretion.

Washed cells were incubated in buffers and the increase of 260 $\text{m}\mu$ absorbing material in the suspending media and the shifts in various 260 $\text{m}\mu$ absorbing compounds in the cells during the incubation were examined.

Fig. 5 shows that RNA underwent rapid degradation within 60 minutes while DNA retained comparatively constant levels. The absorbance of the acid-soluble fraction in-

FIG. 5. Shifts in Amounts of RNA, DNA and Acid-Soluble 260 mμ-Absorbing Compounds During the Incubation of Washed Cells in Buffers.

Washed cells were incubated in a) 0.25 M Tris buffer, pH 8.2 and b) 0.25 M glycine-NaOH buffer, pH 9.8 at 37°C.

FIG. 6. Relationship Between the Decrease of Intracellular RNA and the Appearance of Extracellular 5'-nucleotides.

Data are expressed in phosphate of RNA (RNA-P) and that of 5'-nucleotide (5'-P). Experimental conditions are the same as in Fig. 5.

—○— Intracellular RNA degraded (as RNA-P)
—●— 5'-Nucleotide produced (as 5'-P)

TABLE III. THE EFFECT OF VARIOUS ANIONS ON THE EXCRETION

Anion added	Concentration in M	
	10 ⁻²	10 ⁻³
None (Control)	100.0%	100.0%
NaF	99.2	100.6
KCN	99.6	99.0
NaN ₃	100.2	101.0
Na ₂ HAsO ₄	103.8	103.3
Na-citrate	105.3	100.9
Na ₂ HPO ₄	114.4	100.4
K ₂ HPO ₄	112.4	100.2
Na-pyrophosphate	115.3	109.7
EDTA	52.4	65.2

Experimental details are indicated in Table II.

creased a little during the early stage of the incubation and then diminished to some extent towards the later stages.

Since preliminary experiments showed that only 5'-nucleotides were present in the suspending media, increase in the amount of 5'-nucleotides in the suspending media was determined. The relationship between the increase of 5'-nucleotides and the reduction of intracellular RNA during the incubation is indicated in Fig. 6.

When the excretion was carried out at pH 9.8, most of the degraded RNA was converted to 5'-nucleotides, while at pH 8.2, 5'-nucleotides were produced during the early stages, however, 5'-nucleotides once produced were split into smaller fragments later.

Changes in the Viability and the Response of Gram-staining of Cells during the Excretion.

Relationship between the excretion of nucleotides and the viability of cells was examined. Although the result varied from one experiment to another, number of viable cells was greatly reduced during the excretion. In one experiment, more than 99% of cells were killed during the incubation of cells for 60 minutes in 0.25 M glycine-NaOH buffer, pH 9.8 and in 0.25 M Tris buffer, pH 8.0.

It was also observed that this *Bacillus* lost

its response to Gram-staining as a result of the degradation of endogenous RNA.

Analysis of Nucleotides in the Acid-soluble Fraction of Cells and Excreted into the Suspending Media.

After incubating the cells in 0.25 M glycine-NaOH buffer, pH 9.8 for 30 and 120 minutes at 37°C, the excreted nucleotides were analysed by ion-exchange chromatography on Dowex-1 × 8-formate. Analyses of nucleotides in the acid-soluble fraction of cells before the incubation and after incubating the cells for 15 minutes in 0.25 M glycine-NaOH buffer, pH 9.8 were also carried out.

The elution pattern from anion exchanger of nucleotides excreted when the cells were incubated in 0.25 M glycine-NaOH buffer, pH 9.8 for 120 minutes at 37°C is illustrated in Fig. 7, and the elution pattern of free nucleotides present in the acid-soluble fraction is illustrated in Fig. 8.

The nucleotides excreted at pH 9.8 were mainly composed of ribonucleoside 5'-monophosphates and of traces of nucleoside 5'-diphosphates. The molar proportions of 5'-monophosphates excreted in the suspending buffer were not very different from each other and were composed of almost equal molar amounts of 5'-AMP, 5'-GMP, 5'-CMP and 5'-UMP.

Among the nucleotides in the acid-soluble fraction, 5'-AMP predominated over other nucleotides to a great extent; the amounts of 5'-CMP and 5'-UMP were small. Furthermore, 5'-GMP was negligible in this fraction. The amount of nucleoside 5'-diphosphates was also small except for ADP.

The composition of nucleotides excreted in the suspending medium and remaining in the acid soluble fraction 15 minutes after the start of incubation in 0.25 M glycine-NaOH buffer of pH 9.8 was much the same as that of nucleotides excreted 120 minutes after the start of incubation.

FIG. 7. Separation of Nucleotides Excreted from Washed Cells by an Incubation in 0.25 M glycine-NaOH Buffer, pH 9.8 for 120 min. at 37°C by Anion Exchange.

Exchanger: Dowex-1×8-formate, 200~400 mesh, 0.79 cm²×6 cm.
Eluting solution: Formic acid (Formic) and sodium formate (Formate) as shown, 0.5 ml/min. Each 10 ml fraction was collected in a tube.

Sorbed material: Nucleotides excreted from washed cell.

(1) 0.005 M Formic, (2) 0.02 M Formic, (3) 0.1 M Formic, (4) 0.01 M Formic, 0.05 M Formate, (5) 0.1 M Formic, 0.1 M Formate, (6) 0.1 M Formic, 0.3 M Formate, (7) 0.1 M Formic, 0.4 M Formate, (8) 0.5 M Formic, 0.5 M Formate.

FIG. 8. Separation of Nucleotides in the Acid-Soluble Fraction of Washed Cells by Anion Exchange.

Exchanger and eluting solution: See Fig. 7.

Sorbed material: Nucleotides in the acid-soluble fraction of washed cells.

(1) 0.005 M Formic, (2) 0.02 M Formic, (3) 0.1 M Formic, (4) 0.01 M Formic, 0.05 M Formate, (5) 0.1 M Formic, 0.1 M Formate, (6) 0.1 M Formic, 0.3 M Formate, (7) 0.1 M Formic, 0.4 M Formate, (8) 0.5 M Formic, 0.5 M Formate.

The nucleotides excreted at pH 8.2 (in 0.25 M Tris buffer) were also composed of 5'-AMP, 5'-GMP, 5'-CMP, 5'-UMP and of traces of nucleoside 5'-diphosphates, however, in this case, much more 260 m μ -absorbing material, presumably bases or nucleosides, was eluted with 0.005 M formate by ion exchange chromatography.

Variation of the Ability to Degrade Intracellular Nucleic Acids with Conditions and Age of Culture.

FIG. 10. Shifts in the Ability to Degrade Intracellular Nucleic Acids in Washed Cells According to the Growth Stage.

Bacillus sp. No. 17-5 was cultured in PG-medium and the yield of dry cells, and content of nucleic acids were estimated (lower half of the figure).

The suspension of washed cells was prepared as in Fig. 9 then mixed with an equal volume of 0.5 M glycine-NaOH buffer, pH 10.0 and incubated for 15 minutes at 37°C. The amounts of RNA and DNA in the cells before and after the incubation in buffer were estimated and the degree of degradation was calculated.

—○— RNA
—●— DNA
—○— Growth

The organism was grown in four media in which it showed different patterns of nucleic acid metabolism. Cells were harvested at various stages of growth and the ability to excrete nucleotides was examined.

In Fig. 9, a) and b) are examples of cultures in which cellular nucleic acids vary in amount to a much greater extent than in c) and d) during the growth.

In cultures a) and b) the ability to excrete nucleotides was most extensive during early stages of growth and diminished towards the latter stages. However, no such variations were detected in c) and d). Moreover, in cultures c) and d) where nucleic acids remained rather at constant levels, this ability was comparatively low.

These facts are considered to show an intimate relationship between the process of the excretion and the metabolism of nucleic acids in vivo.

Here, a question may arise as to whether the excretion is brought about by the reverse reaction of RNA-synthesizing enzymes or by RNA-digesting enzymes. For the purpose of clarifying this point the variation of the ability to degrade endogenous RNA according to the age of the culture was examined.

As shown in Fig. 10, RNA degradation occurred most actively in the stationary phase of growth when cellular RNA was exponentially diminishing. Therefore enzymes participating in this degradation seemed to play a role in the degradation of RNA during growth in vivo.

Evidences for the Identity of Excretion of Nucleotides from Non-proliferating Cells and Accumulation of Nucleotides during Cultivation as the Result of the Same Metabolic Process.

In the previous paper¹⁾, it was reported that 5'-nucleotides were accumulated in the culture fluid of this *Bacillus* and in the present report it has been demonstrated that 5'-

FIG. 9. Variation in the Ability to Degrade Intracellular Nucleic Acids According to Culture Conditions.

Bacillus sp. No. 17-5 was cultivated in a) PG-medium, b) CSL-medium (2% of corn steep liquor was substituted for 1% of polypeptone in PG-medium), c) G-medium (1% of Casamino acids was substituted for 1% polypeptone in PG-medium) and d) ME-medium (1% of meat extract was substituted for 1% polypeptone in PG-medium). Cells were harvested at intervals, washed twice with water and resuspended in distilled water to give a cell density twice as much as that in the culture broth before harvesting the cells. The ability to excrete nucleotides was estimated by adding an equal volume of 0.5 M glycine-NaOH buffer, pH 9.4 to the suspension of washed cells followed by an incubation for 30 minutes at 37°C, then the degree of excretion was measured as indicated in the text.

Data are expressed as follows: Excretion (%) as in the text; growth, O.D. 660 mμ of the culture broth; and nucleic acids, hot PCA-extractable 260 mμ-absorbing materials in cells harvested from 1 ml of culture broth.

—●— Excretion
—○— Growth (O.D.)
—x— O.D. 260 mμ of cells

nucleotides are excreted from washed cells of the same organism when incubated in alkali-

line salt solutions. Therefore, comparative studies of these two phenomena have been

made from various points of views.

Comparison of Nucleotides "Accumulated" and "Excreted".

Bacillus sp. No. 17-5 was cultivated in G-medium¹⁾ and fractions "accumulated" and "excreted" were obtained as summarized in Fig. 11. Then nucleotides in these fractions were analysed by ion-exchange chromatography. Chromatograms obtained for both fractions were much the same as that shown in Fig. 7 and the molar ratio of nucleotides in these fractions are indicated in Table IV. The molar ratios of nucleotides in these fractions were very similar.

FIG. 11. The Procedures for Obtaining Fractions "Accumulated" and "Excreted".

A comparison of the base composition of these nucleotides with that of nucleotides of intracellular RNA is shown in Table V.

Here again a similarity is observed among base compositions, except that the proportion of adenosine nucleotides was larger than in the former two cases as compared with the

TABLE IV. MOLAR RATIOS OF NUCLEOTIDES "ACCUMULATED" IN THE CULTURE FLUID AND "EXCRETED" FROM THE WASHED CELLS

Nucleotide	"Accumulated"	"Excreted"
5'-CMP	0.76	0.83
5'-AMP	1.00	1.00
5'-UMP	0.58	0.56
5'-GMP	0.75	0.76
CDP (?)	0.09	0.08
UDP	0.20	0.20
GDP	0.26	0.21

Fractions "accumulated" and "excreted" were obtained as shown in Fig. 11, and nucleotides in them were analyzed by ion exchange chromatography. Data are expressed in comparative values against 5'-AMP.

TABLE V. COMPARISON OF COMPOSITIONS OF NUCLEOTIDES "ACCUMULATED" AND "EXCRETED" WITH THAT OF NUCLEOTIDES OF INTRACELLULAR RNA

Nucleotide	"Accumulated"	"Excreted"	Intracellular RNA
Adenosine nucleotide	1.18	1.10	0.99
Guanosine nucleotide*	1.20	1.07	1.18
Cytidine nucleotide**	1.00	1.00	1.00
Uridine nucleotide***	0.92	0.84	0.82

*Sum of 5'-GMP and GDP, **Sum of 5'-CMP and GDP(?), and ***Sum of 5'-UMP and UDP.

Composition of nucleotides of intracellular RNA was estimated as mentioned in the previous report¹⁾. Data are expressed in comparative values based on cytidine nucleotide.

base composition of intracellular RNA. However, this fact may be explainable on the basis that adenosine nucleotides predominate in the acid-soluble fraction of cells.

Effects of Phosphate, NaCl and CaCO₃ on the "accumulation" of Nucleotides during Cultivation.

If the accumulation of nucleotides during cultivation was brought about by the same metabolic system as the excretion of nucleotides from washed cells, factors which stimulate or inhibit the excretion would be expected to affect the accumulation in a similar manner. Therefore, three factors which had some influences on the excretion were tested for their behaviors on the accumulation of nucleotides in the growth medium.

Potassium phosphate buffer, pH 7.0, was

added to G-medium¹⁾ from which phosphate had been excluded, to final concentrations of 0.055 M, 0.0275 M and 0.01375 M; and the organism was cultivated in these media. The

accumulation of 260 m μ absorbing material in the culture fluid increased with the increased concentration of phosphate, whereas intracellular nucleic acids remained at com-

TABLE VI. THE DEGRADATION OF INTRACELLULAR NUCLEIC ACIDS IN WASHED CELLS AND THE FORMATION OF 5'-NUCLEOTIDES AS A RESULT OF THE DEGRADATION BY VARIOUS BACTERIAL STRAINS

Bacterial strain	Medium*	Incubation in 0.25 M tris buffer, pH 8.0		Incubation in 0.25 M glycine-NaOH buffer pH 9.4	
		Degree of excretion	Formation of 5'-nucleotides	Degree of excretion	Formation of 5'-nucleotides
<i>Bacillus</i> sp. No. 17-5	A	###(86.3)**	###(58.0)***	###(89.4)**	###(63.4)***
<i>Aerobacter aerogenes</i>	A	++(61.5)	trace	++(41.0)	trace
<i>Azotomonas</i> sp.	A	+(32.1)	trace	++(60.1)	###(53.6)
<i>Bacillus megaterium</i>	A	###(89.2)	trace	###(90.3)	++(22.2)
<i>B. cereus</i>	A	++(61.2)	trace	###(86.0)	++(24.2)
<i>B. cereus</i> var. <i>mycoides</i>	B	###(79.1)	none	###(77.7)	+
<i>B. polymyxa</i>	S	###	++	###	###
<i>B. pumilus</i>	GB	###	++	###	none
<i>B. subtilis</i>	S	###	none	###	+
<i>Bacterium mycoides</i>	GB	###	###	###	+
<i>Corynebacterium sepeidonicum</i>	S	###(81.4)	none	###(80.1)	none
<i>Escherichia coli</i>	A	++(63.3)	trace	++(41.0)	trace
<i>Micrococcus flavus</i>	Mix.	+(15.5)	none	+(10.7)	none
<i>M. lysodeikticus</i>	A	+(21.8)	trace	+(11.3)	###(138.0)
<i>M. pyogenes</i> var. <i>albus</i>	S	++(35.1)	trace	++(42.3)	trace
<i>M. pyogenes</i> var. <i>aureus</i>	S	++(36.9)	none	+(30.0)	none
<i>Pseudomonas aeruginosa</i>	A	###(70.5)	++(24.8)	++(65.8)	###(51.5)
<i>Ps. coronafaciens</i>	Mix.	###	++	###	###
<i>Ps. fragi</i>	A	###(78.8)	++(21.8)	###(86.6)	###(45.5)
<i>Ps. graveolens</i>	A	++(63.5)	+(7.4)	++(65.4)	###(62.7)
<i>Ps. riboflavianus</i>	A	+(30.9)	trace	+(33.8)	trace
<i>Ps. striafaciens</i>	YB	###	###	###	###
<i>Sarcina albido</i>	A	+(27.7)	none	+(26.2)	trace
<i>Sarcina lutea</i>	A	+(16.2)	trace	+(17.2)	###
<i>Serratia marescens</i>	A	++(48.5)	trace	++(55.8)	trace
<i>Serratia polithicum</i>	A	+(20.1)	trace	+(23.0)	###(111.0)
<i>Proteus vulgaris</i>	B	###	none	###	+
<i>Vibrio percolans</i>	A	###	none	###	++

Various type cultures of bacteria were cultured in media indicated in the Table for 20 to 24 hours at 28°C, with shaking. The cells were harvested by centrifugation, washed twice with water and resuspended in water to give a cell density twice as much as that in the culture broth before harvest of cells. This cell-suspension was mixed with the same volume of 0.5 M Tris buffer, pH 8.0 and 0.5 M glycine-NaOH buffer, pH 9.4 and incubated for 2 hours at 37°C, and the degree of the excretion was measured as in the text.

* Media A, B, S, GB, YB and Mix. are as follows: A, acetic acid was used in place of glycerol in G-medium and neutralized with NaOH; B, acetic acid in A was neutralized with ammonium hydroxide; S, S-medium as described in the previous paper¹⁾; GB, nutrient broth containing 1% of glucose; YB, yeast extract was used in place of meat extract in GB; Mix., yeast extract was added to GB to 0.5%.

** Degree of excretion (%), calculated as described in the text.

+, the degree was less than 35%; ++, the degree was between 35 to 70%; ###, the degree was more than 70%.

*** Yield of 5'-nucleotide (calculated from the amount of 5'-P) per degraded nucleic acids.

+, the yield was less than 15%; ++, the degree was between 15 to 40%; ###, the degree was more than 40%.

paratively constant levels.

When NaCl was added to G-medium to a final concentration of 0.25 M, the accumulation was elevated to a great extent, especially during the early stages of growth.

When CaCO₃ was added to G-medium to a final concentration of 0.05 M, the accumulation of 260 mμ absorbing material in the culture fluid was extremely inhibited and nucleic acids became extraordinarily accumulated in the cells owing to the suppression of endogenous nucleic acids degradation by the metal.

These three factors affected the accumulation of nucleotides during the cultivation stimulatory or inhibitory as well as on the excretion of nucleotides from washed cells.

Distribution of Homologous Phenomenon in Other Bacteria

From the above mentioned results, the degradation of endogenous RNA evoked by incubating washed cells of a *Bacillus* in alkaline buffer or salt solutions is suggested to be intimately connected with the degradation of RNA during growth. Moreover, since the degradation of RNA is generally observed during the cultivation of various microorganisms³⁻⁷⁾, this type of degradation could be expected to occur in other micro-organisms. Therefore, a variety of bacteria were tested for their ability to degrade their endogenous nucleic acids.

As depicted in Table VI, most of Gram-positive or -negative rods carried out an extensive degradation of endogenous nucleic acids and some of them produce 5'-nucleotides in the suspending media. On the other hand, cocci generally showed lesser degrees of excretion as compared with rods, however, 5'-

nucleotides were formed by some cocci.

It may therefore be concluded that this kind of degradation of intracellular nucleic acids when washed cells are incubated in alkaline buffers is a common phenomenon in many bacteria.

DISCUSSION

Degradation of Endogenous Nucleic Acids and Formation of 5'-Nucleotides. Our present work has demonstrated that 5'-nucleotides are excreted from washed cells of a *Bacillus* when incubated in alkaline salt or buffer solutions. These nucleotides were shown to originate from endogenous RNA.

Although many investigations have been made on the degradation of RNA in cells of various microorganisms either during the growth of them or during incubation in buffers, there have been few indications that 5'-nucleotides are formed.

In the past, Newton (1957)⁸⁾, Holden (1958)⁹⁾, Cohn et al. (1958)¹⁰⁾ and Herbst and Doctor (1959)¹¹⁾ studied the degradation of nucleic acids in *Pseudomonas aeruginosa*, *Lactobacillus arabinosus*, *Rickettsia mooseri* and on *Hemophilus influenzae*, respectively. However, nothing was mentioned about the breakdown products. Higuchi and Uemura (1959)¹²⁾ showed that *Saccharomyces cerevisiae* excreted mono-, oligo-, and polynucleotides possessing terminal phosphate in the 3' position, when the cells were incubated in slightly acid buffer in the presence of chelating agents such as citrate or EDTA. Stephenson and Moyle (1949)¹³⁾ found that mono-nucleotides were excreted by *E. coli* when incubated in carbonate buffer, pH 7.4, in the presence about 0.2 M of sodium lactate,

3) H.B. Levy, E.T. Skutch and A.L. Schade, *Arch. Biochem.*, **24**, 199, 206 (1949).

4) P.C. Caldwell, E.L. Mackor and Sir C. Hinshelwood, *J. Chem. Soc.*, **1950**, 3151.

5) H.E. Wade, *J. Gen. Microbiol.*, **7**, 24 (1952).

6) B.J. Katchman and W.O. Petty, *J. Bacteriol.*, **69**, 607 (1955).

7) J. Brachet, "The Nucleic Acids", Vol. II, Ed. E. Chargaff and J.N. Davidson, Academic Press, New York, 1955, p. 475.

8) B.A. Newton, *J. Gen. Microbiol.*, **9**, 54 (1953).

9) J.T. Holden, *Biochim. Biophys. Acta*, **29**, 667 (1958).

10) Z.A. Cohn, F.E. Hahn, W. Ceglowski and F.M. Bozeman, *Science*, **127**, 282 (1958).

11) E.J. Herbst and B.P. Doctor, *J. Biol. Chem.*, **234**, 1497 (1959).

12) M. Higuchi and T. Uemura, *J. Agr. Chem. Soc. (Japan)*, **33**, 304, 821, 826 (1959).

13) M. Stephenson and J.M. Moyle, *Biochem. J.*, **45**, vii (1949).

NaCl or KCl, and this work somewhat resembles our present investigation. However, no data were given concerning the kind of isomer these nucleotides might be.

The formation of 5'-nucleotides in the culture fluid of streptomycin inhibited cultures of *E. coli* was reported by Davis and his collaborators (1959, 1960)¹⁴⁻¹⁶ and Rosano et al. (1960)¹⁷. Whereas the former investigators clearly demonstrated that these nucleotides were not degradation products but formed *de novo*, the latter did not give any conclusion, however, they suggested the possibility that the accumulated nucleotides originated from intracellular RNA.

The work of Wade (1961)¹⁸, although published after the completion of the present investigation, is similar to ours. He studied the autodegradation of ribonucleoprotein of *E. coli* and found a route through which RNA is degraded into 5'-nucleotides at alkaline pH.

The degradation of endogenous RNA and the consequent excretion of 5'-nucleotides from washed cells of a *Bacillus* examined in the present work occurred optimally in an alkaline pH region. This excretion greatly depended upon the concentration of salt or buffer in the incubation medium. This dependence is considered to be an influence of these dilute salts or buffers upon the susceptibility of RNA to digestive enzymes or upon the enzyme activity itself. Stephenson and Moyle (1949)¹³ reported similar effects of salts on the degradation of RNA in *E. coli*. Apart from this there are also a number of indications that the degradation of RNA or polynucleotides by ribonuclease or other enzymes depends upon the presence of a variety

of salts; Fellig and Wiley (1960)¹⁹ reported that the ribonuclease of *Euglena gracilis* was activated by 0.5 M NaCl, Spahr and Hollingworth (1961)²⁰ showed that the ribonuclease in ribonucleoprotein of *E. coli* was activated by 0.25 M NaCl and Hendley and Beers (1961)²¹ observed an extensive acceleration of phosphorolysis of polyadenylic acid upon increasing the ionic strength of the incubation medium.

Furthermore, it has been known that the ribosomes of *E. coli* and other organisms contain a latent ribonuclease which is activated by the separation of the RNA from the protein moiety by means of an agent such as urea²²⁻²⁸.

The promotion of the degradation of RNA by salts or buffers in a *Bacillus* observed by us may be attributed to an influence of salts or buffers upon the physicochemical properties of ribosome or ribosomal RNA bringing about a higher sensitivity of the digestive enzymes, since as will be described in the forthcoming paper²⁹, RNA in heat denaturated ribosomes of this organism was rapidly degraded by its own intracellular enzyme system even at low buffer concentrations, whereas RNA in intact ribosomes was degraded only at higher concentrations.

Several polybasic anions more or less promoted excretion and alkali earth metals at relatively low concentrations (10 mM) inhibit

- 14) H. Roth, H. Amos and B.D. Davis, *Bacteriol. Proc.*, **1959**, 131.
- 15) N. Anand and B.D. Davis, *Nature*, **185**, 22 (1960).
- 16) H. Roth, H. Amos and B.D. Davis, *Biochim. Biophys. Acta*, **37**, 398 (1960).
- 17) C.L. Rosano, R.A. Peabody and C. Hurwitz, *Biochim. Biophys. Acta*, **37**, 380 (1960).
- 18) H.E. Wade, *Biochem. J.*, **78**, 457 (1961).

- 19) J. Fellig and C.E. Wiley, *Science*, **132**, 1835 (1960).
- 20) P.F. Spahr and B.R. Hollingworth, *J. Biol. Chem.*, **236**, 823 (1961).
- 21) D.D. Hendley and R.F. Beers, Jr., *J. Biol. Chem.*, **236**, 2050 (1961).
- 22) D. Elson, *Biochim. Biophys. Acta*, **27**, 216 (1958).
- 23) D. Elson, *Biochim. Biophys. Acta*, **36**, 372 (1959).
- 24) E.T. Bolton, R.J. Britten, D.B. Cowie, B.J. McCarthy, K. MaQuillen and R.B. Roberts, *Carnegie Institution of Washington, Annual Report*, 1959, p. 259.
- 25) Y. Otaka and K. Uchida, Proceedings for the Meeting of 9th Symposium on Nucleic Acids, and 1st Symposium on Microbial Genetics, February, 1961 (Tokyo).
- 26) P. Siekewitz and G.E. Palade, *J. Biophys. Biochem. Cytol.*, **4**, 309 (1958).
- 27) Y. Tashiro, *J. Biochem. (Japan)*, **45**, 937 (1958).
- 28) S.R. Dickman and K.M. Trupin, *Biochim. Biophys. Acta*, **30**, 200 (1958).
- 29) A. Imada, N. Nakao and K. Ogata, *Agr. Biol. Chem.*, **26**, 611 (1962).

excretion. This fact seems to show that effective anions may promote the degradation by removal of protective cations as reported by Holden (1958) in *L. arabinosus*⁸⁾. However, EDTA was inhibitory and a metal requirement was therefore expected.

It is noteworthy that phosphates are the most effective anion, suggesting that phosphates may act in a different way than other anions.

Kinetic studies revealed that the excretion of 5'-nucleotides was almost complete within one hour, when 70 to 90% of the RNA initially present in the cells had been consumed, whereas DNA was little degraded in the same interval. Therefore, the enzyme concerned with the excretion is presumed to be very specific for RNA.

Nucleotides excreted into the suspending media consisted mainly of ribonucleoside 5'-monophosphates and of traces of ribonucleoside 5'-diphosphates. However, no 2'- or 3'-isomers were found. These diphosphates were not present in the acid-soluble fraction of this organism and only appeared upon incubating the cells in buffers, therefore they were assumed to be degradation products.

The formation of ribonucleoside 5'-diphosphates suggests to us the participation of polynucleotide phosphorylase in RNA digestion. Such a concept is further supported by the stimulatory effect of phosphate and the inhibitory effect of EDTA. In the forthcoming paper, the existence of polynucleotide phosphorylase will be examined.

"Accumulation" of Nucleotides during Cultivation and "Excretion" of Nucleotides from Washed Cells.

The mechanism of the accumulation of nucleotides during cultivation of this organism in growth media seemed identical with that of the excretion of 5'-nucleotides during the incubation of washed cells in buffers from the following reasons: the composition of nucleotides formed in both processes resembled each other and also resembled that of nucleotides in intracellular RNA; and the addition of phosphate, and NaCl promoted both processes but Ca^{++} inhibited both processes.

Biological Significance of the Phenomenon. The ability to degrade endogenous RNA varied according to conditions or to the age of the culture. Generally, the ability was strong in stationary phase cells which were actively degrading endogenous RNA. Furthermore, when cells were grown in media in which the content of nucleic acids did not vary to a great extent throughout the growth cycle, the activity remained relatively low.

Thus the degradation of endogenous RNA studied in the present paper is intimately related to the catabolism of RNA in vivo in this *Bacillus*.

Furthermore, this type of degradation, with the occasional consequent formation of 5'-nucleotides occurred in a variety of bacteria, suggesting that it might be an essential process for the metabolism of RNA, therefore for the growth of bacteria.

Acknowledgement. We should like to express our thanks to Drs. S. Kuwada, K. Sato, S. Tatsuoka for their interests in this work and for their encouragements. We also thank Dr. E. Ohmura for his helpful advice during the course of this investigation.