On Minor Compounds Promoting the Growth of *Lactobacillus leichmannii* in Deoxyriboside Mixture Prepared from Acid Insoluble Fraction of Chick Embryo

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Summary

Although the bulk of the minor components in chick embry DNA appears to be 5-methylcytosine and uracil, the presence of some other minor components still remains possible. In order to obtain the clue for the possibility, this work was carried out.

The DNA Fraction extracted from acid insoluble fraction of 14 day old chick embryos was hydrolyzed with DNase I and then snake venom. The hydrolysate was fractionated into 14 fractions by celite column chromatography. The separation of the minor compounds which promoted the growth of *Lactobacillus leichmannii* ATCC 7830 (*L. leichmannii*) was carried out by the column procedure and the final purification was accomplished by paper chromatography or paper electrophoresis. For the detection of the minor compounds, the microbial assay method with *L. leichmannii* was applied.

Three minor compounds which promoted the growth of the organism were detected and examined the some properties. On the basis of the summation of the microbial assay, paper chromatographic, electrophoretic and spectral evidences, the compounds were supposed to be deoxyriboside and that differ from those of common or known components of animal DNA and the others.

The experimental results suggested the possibility that unknown minor components may present in the DNA.

INTRODUCTION

As a minor component of chick embryo DNA, 5-methylcytosine had been found by Smith and Stoker[1], but not by Kleinschmidt and Manthey[2]. Recently, with sensitive radioactive analysis Culp, Dore and Brown[3] confirmed the presence of 5-methylcytosine. The base was purified and identified by the present authors[4]. Also, the possibility that uracil and a novel base may be minor components in chick embryo DNA was suggested by the authors[4,5].

Although the bulk of the minor components in the DNA appears to be 5-methylcytosine and uracil from results of the experiment on the determination of base composition of the DNA[6], the presence of some other components still remains possible. In order to obtain the clue for the possibility that some other components may present in chick embryo DNA, the enzymatic hydrolysate of the DNA extracted from acid insoluble fraction of chick embryo was fractionated by celite column chromatography and analyzed mainly by microbial assay method with *Lactobacillus leichmannii*ATCC 7830 (*L. leichmannii*). Three minor compounds which promoted the growth of *L. leichmannii* were detected and examined the some properties.

The experimental results showed that the minor compounds were deoxyribosides, which differed from common components of animal DNA and the others. The present paper describes the detection, purification and preliminary examination on the properties of the minor compounds.

MATERIALS AND METHODS

Chick embryo. The eggs of white Leghorn hens with normal diet in Gifu Prefectural Poultry Breeding Station were used. The eggs were incubated under constant temperature and humidity for 14 days. Then, the eggs were opened and embryos were pooled and, as soon as possible, treated for the preparation of acid insoluble fraction.

Chemicals. Authentic deoxyribosides and bases were purchased from Nutritional Biochemical Corporation. DNase I (pancreatic crystalline enzyme) and snake venom (lyophilized powder venom prepared from *Crotalus atrox*) were obtained from Sigma Chemical Company.

Extraction of DNA from acid insoluble fraction. Acid insoluble fraction was prepared according to the method already reported[6]. The DNA was extracted from acid insoluble fraction after removing RNA with alkali. All operations were carried out at 0° to 4℃ unless otherwise stated.

The embryos were washed with 0.9% NaCl, and homogenized with a volume of ice cold 0.6 N perchloric acid (PCA), equal to four times of the weight of the embryos. Homogenate was centrifuged at 10,000 x g for 10 min. The precipitate was reextracted with ice cold 0.6 N PCA, and then centrifuged again. The precipitate was treated with ethanol and then ethanol-ether (3 : 1 v/v) to remove lipids according to STS method. The precipitate was incubated at 37℃ overnight with 0.3 M KOH. The alkaline solution was acidified to give a final concentration of 5% with conc. PCA, and centrifuged at 10,000 x g for 10 min. The precipitate was reextracted with 5% PCA three times to remove RNA components.

The precipitate was suspended in water and adjusted to pH 8.8 with 0.3 M KOH, and cooled overnight. Potassium perchloride and any other insoluble materials were removed by centrifugation at 800 x g for 5 min. The supernatant was adjusted to pH 7.0, and concentrated to give a final concentration of approximately 1 mg as DNA per ml.

Hydrolysis of DNA fraction. For detection and separation of the minor compounds promoting *L. leichmannii* growth, enzymatic hydrolysis of the DNA with DNase I and snake venom was employed as mentioned in the previous paper[4].

Celite column chromatograhy. The fractionation of the deoxyriboside mixture was carried out by celite column chromatography. The procedure was based on that of R. H. Hall[7]. But, the use aqueous formic acid as the component of the solvent system was confind to the separation of compound A from fraction 3 (F-3) as mentioned later.

Analytical Methods.

Paper chromatography. Paper chromatography was performed with Tōyō No. 50 paper. Solvent systems used were n-butanol-water-28% aqueous ammonia (255 : 45 : 0.5 v/v/v)[6] (A) and n-butanol saturated with water[8] (B). The final purification of the minor compounds was performed by triple or double development technique with solvent A or B.

Paper electrophoresis. Electrophoresis was performed on 4 x 60 cm strips of Tōyō No. 51 paper at 3000 V for 90 min with pyridine-acetic-acid-water buffer (1 : 10 : 89 v/v/v) at pH 3.5[9].

Spectrophotometry. Ultraviolet absortption spectra of the minor compounds were obtained by a Shimadzu double-beam spectrophotometer UV-150.

Microbial assay. Microbial assay with *L. leichmannii* was employed for hunting and detecting the compounds[10].

RESULTS

Detection of the minor compounds promoting the growth of L. leichmannii.

The separation of the growth promoting compounds from the deoxyriboside mixture, prepared by preliminary fractionation[4] of the enzymatic hydrolysate of the DNA extracted from the acid insoluble fraction (DNA preparation) was carried out by celite column chromatography. The fractions of 18

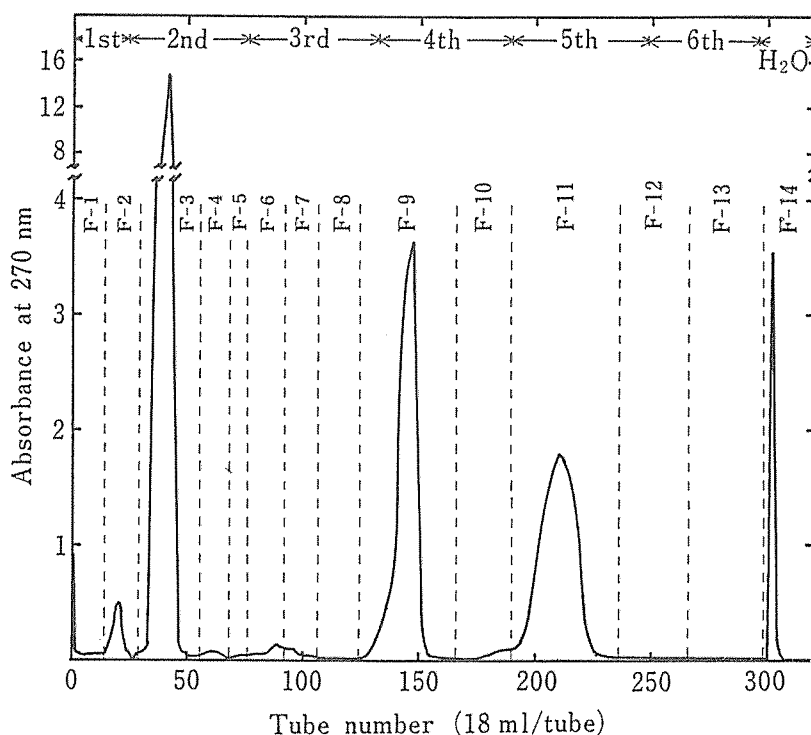

Fig. 1. Fractionation of Deoxyriboside Mixture obtained by Enzymatic Hydrolysis of DNA Preparation from Acid Insoluble Fraction of Chick Embryo.

Column (1.8 x 88 cm) contained 80 g of Celite No. 545 mixed with 32 ml of lower phase of the first solvent system. The deoxyriboside mixture (approximately 200 mg as DNA), dissolved in 3 ml of lower phase of the solvent, was mixed with 8 g of the celite. The elution was performed by stepwise addition of the each upper phase of solvent systems from the first to the sixth succesively and then water. Solvent systems used for fractionation were as follows ; first : ethylacetate saturated water, second : ethylacetate-n-butanol-water (12 : 1 : 1 v/v/v), third : ethylacetate-n-butanol-water (12 : 1.4 : 1 v/v/v), fourth : ethylacetate-n-butanol-water (12 : 2 : 1 v/v/v), fifth : ethylacetate-n-butanol-water (6 : 1 : 1 v/v/v) and sixth : ethylacetate-n-butanol water (4 : 1 : 1 v/v/v).

ml each were combined as shown in **Fig. 1**. Each combined fraction (F-1 to F-14) was evaporated to dryness in vacuo. The residue was dissolved in 300 μl of distilled water. The aliquots of each (5~10 μl) were applied on the papers and developed by the triple ascending technique with solvent A. Developed chromatograms were investigated by UV absorbing method and the microbial method. The compounds of the fractions (F-1 to F-14) were examined by comparison with standard deoxyribosides in *Rf* values, UV absorbing spectra and in elution patterns by celite column chromatography. The relative bacterial growth of the fractions were summarized in **Table I.** The main compounds of five fractions (frs. 3, 4, 9, 10, and 11) which were most or more active in promoting the bacterial

Table I. RELATIVE *L. leichmannii* GROWTH OF THE FRACTIONS OBTAINED FROM DEOXYRIBOSIDE MIXTURE

Fraction	Growth of *L. leichmannii*	Fraction	Growth of *L. leichmannii*
1	±	9	+++
2	±	10	++
3	+++	11	+++
4	++	12	+
5	+	13	+
6	+	14	+
7	+		
8	±		

¬, no growth ; ±, slight growth ; +, growth ;
++, good growth ; +++, heavy growth.

growth were identified as folluws ; F-3 was mixture of deoxythymidine and deoxyadenosine, F-4 was deoxyuridine, F-9 was deoxyguanosine, F-10 was 5-methyldeoxycytidine and F-11 was deoxycytidine[4].

In addition, the results of the preliminary analyses suggested the presence of the minor compounds, which promoted *L. leichmannii* growth, in F-3, F-6 to F-7 and F-11.

In order to confirm the presence of the minor compounds in the three fractions, namely F-3, F-7 and F-11 fractionated from deoxyriboside mixture obtained from the DNA preparation of approximately 100 g of the embryo were subjected to paper chromatographic analysis used A as solvent system. Chromatograms were investigated mainly with the microbial assay. The results were shown in **Fig. 2**. As shown in this Fig, the minor compounds, which promoted the growth of the bacteria, were detected in F-3, F-7 and F-11. For convenience of the description, the tentative names compound A, B and C were given for the minor compounds from F-3, F-7 and F-11, respectively.

Separation and purification.

For the separation of the compounds, the deoxyriboside mixture of the DNA preparation from approximately 1300 g of the embryo was fractionated by the column procedure. The each pooled fraction containing the minor compounds was subfractionated by the column procedure, and the compounds were purified described below.

Compound A. The pooled F-3 was concentrated in vacuo, and subfractionated with upper phase of ethylacetate-ethylcellosolve- 2% aqueous formic acid (4 : 1 : 2 v/v/v) by the column procedure. The each subfraction was assayed by paper chromatography and then with the microbial method. It was found that compound A was eluted out accompany with deoxyadenosine. The pooled fraction was concentrated in vacuo. The final purification was accomplished by means of paper chromatography with solvent A. The positive sections for the bacterial growth of the chromatograms were cut out and eluted with distilled water. The eluate was concentrated in vacuo. To purify further, the procedure was repeated again. The chromatograms were investigated by UV absorbing and the microbial assays. The result is shown in **Fig. 3**.

Compound B. The pooled F-6 and F-7 were combined and concentrated in vacue, and subfractionated into fives with using the upper phase of ethylacetate-n-butanol-water systems (12 : 0 : 1, 12 : 1 : 1 and 12 : 1.4 : 1 v/v/v) by the column procedure. The each subfraction was analyzed by the same methods as described above. The fraction contained compound B was rechromatographed on celite column with using the same solvent system. The compound rich fraction was concentrated in vacuo. The concentrate was subjected to paper chromatography with solvent system A and investigated by UV absorbing and the microbial methods. The positive sections corresponding to *Rf* value

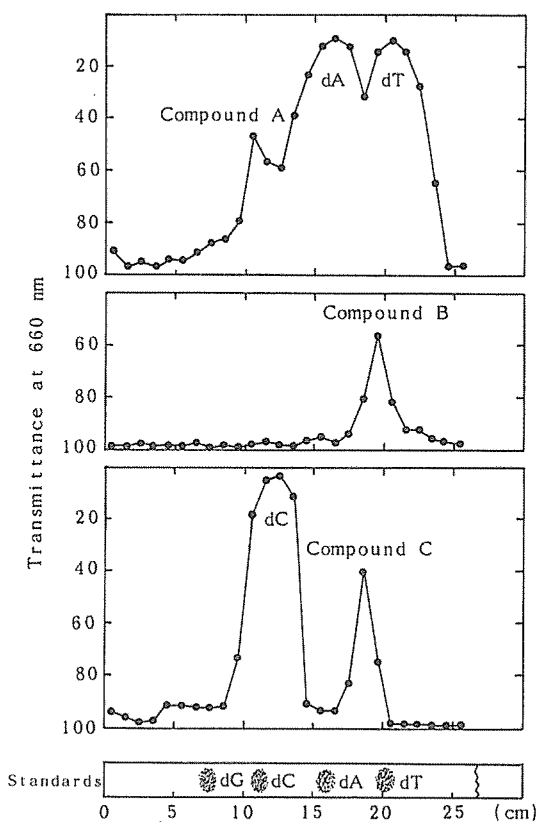

Fig. 2. Detection of *L. leichmannii* Growth Promoting Compounds from Fractions 3, 7 and 11.

Aliquotes of the each concentrate prepared from three fractions (Frs, 3, 7 and 11) as described in the text were applied on Tōyō No. 50 filter paper and developed by triple ascending technique with solvent A at 25℃. Developed chromatograms were investigated by the microbial assay with *L. leichmannii*. The symbols dG, dC, dA and dT are abbreviations for deoxyguanosine, deoxycytidine, deoxyadenosine and deoxythymidine, respectively.

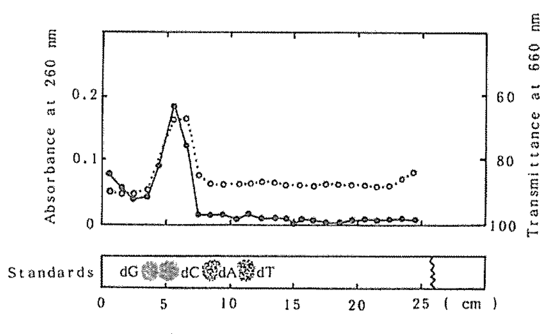

Fig. 3. Paper Chromatography of Compound A.

Aliquotes of Compound A purified from fraction 3 were applied on Tōyō No.50 filter paper and developed by ascending technique with solvent A. The chromatograms were examined by UV absorbing and the microbial assay methods.

●——●, transmittance at 660 nm.

○·······○, absorbance at 260 nm.

The abbreviations are same as in Fig. 2.

of the minor compound were cut out and eluted with distilled water. The eluate was concentrated in vacuo, and subjected to paper electrophoresis to purify further. The electropherograms were investigated by the same methods. The result is shown in Fig. 4.

Compound C. F-11 was pooled and rechromatographed by the column procedure with using upper phase of ethylacetate-n-butanol-water (6 : 1 : 1). The eluate from the column was divided into three fractions (F-A, F-B and F-C). Each subfraction was concentrated in vacuo, and subjected to paper chromatography and the microbial assay. A small peak (F-C) following to the deoxycytidine peak (F-B) contained the minor compound. Compound C rich fraction (the pooled F-C) was concent-

rated in vacuo, and then rechromatographed on celite column with using the same solvent. The rechromatography is shown in **Fig. 5**. The peak obtained was evaporated to dryness in vacuo. The residue was dissolved in a small quantity of distilled water. The aliquots were developed on the paper and investigated by the same methods described above. The result is shown in **Fig. 6**.

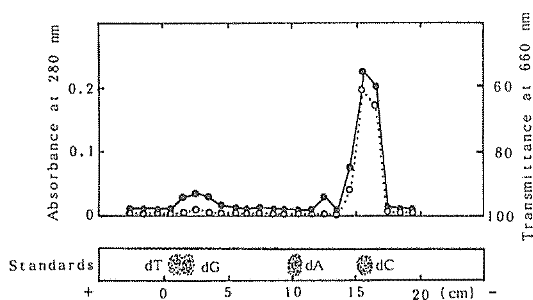

Fig. 4. Paper Electrophoresis of Compound B.

Aliquotes of Compound B purified from fraction 6 and 7 were applied on 4 x 60 cm strips of Tōyō No. 51 paper, and the electrophoresis was carried out at 2.3 mA/cm and 3000 V for 90 min at pH 3.5. The electropherograms were examined by UV absorbing and the microbial assay methods.

●——●, transmittance at 660 nm.

○·······○, absorbance at 280 nm.

The abbreviations are same as in Fig. 2.

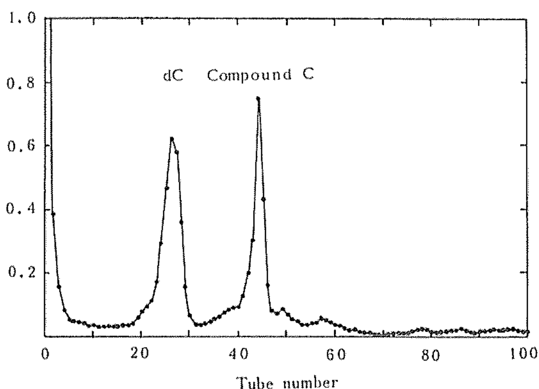

Fig. 5. Celite Column Chromatography of Compound C.

Compound C rich fraction obtained from Fraction 11 was rechromatographed on celite column (column size, 1.8 x 72 cm). N-butanol saturated with water was passed throught the column at a flow rate 7.2 ml/hr. The fractions of 5.7 ml each were collected and measured the absorbance at 280 nm.

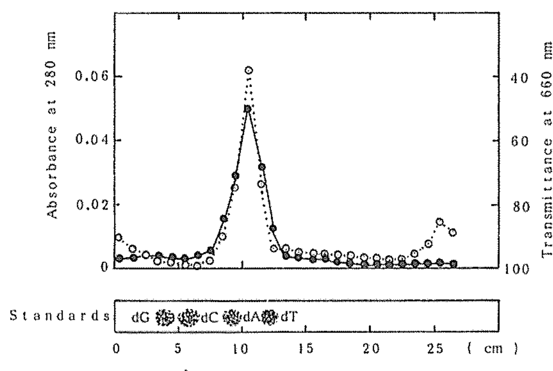

Fig. 6. Chromatography of Compound C.

Aliquots of Compound C purified from Fraction 11 were applied on Tōyō No. 50 filter paper and developed by ascending technique with solvent A. The chromatograms were examined by UV absorbing and the microbial assay methods.

●——●, transmittance at 660 nm.

○·······○, absorbance at 280 nm.

The abbreviations are same as in Fig. 2.

Properties.

Paper chromatography. Compounds, A, B, and C purified from F-3, F-6 - F-7 and F-11 were developed by ascending technique. The chromatograms were examined under an ultraviolet light. Also, the microbial assay was applied for the chromatograms of the compounds. The *Rf* values were presented in **Table II**.

Paper electrophoresis. The electropherograms were examined by the same methods. The results are presented in **Table III**.

Ultraviolet absorption spectra. Ultraviolet absorption spectra of the compounds are shown in **Figs. 7, 8 and 9**. As shown in those Figs, the minor compounds had strong ultraviolet absorption with a single maximum in water. The absorption characteristics of the compounds are listed in Table IV.

Table II. *Rf* VALUES OF THE MINOR COMPOUNDS

Compound	Solvent system	
	A	B
Compound A from F-3	0.20	0.22
Compound B from F-7	0.43	0.33
Compound C from F-11	0.38	0.39
Deoxyadenosine	0.34	0.32
Deoxycytidine	0.21	0.21
Deoxyguanosine	0.13	0.14
Deoxythymidine	0.46	0.44

Table III. RELATIVE ELECTROPHORETIC MOBILITIES OF THE COMPOUNDS

Compound	Relative mobility
Compound A from F-3	0.10
Compound B from F-7	1.00
Compound C from F-11	0.94
Deoxyadenosine	0.60
Deoxycytidine	1.00[a]
Deoxyguanosine	0.16
Deoxythymidine	0.06

a : Deoxycytidine was assigned a mobility of 1.00 at 3000 V for 90 min at pH 3.5.

Table IV. ULTRAVIOLET ABSORPTION CHARACTERISTCS OF THE COMPOUNDS

Compound	λ max (nm)	λ min (nm)
Compound A from F-3	263[b]	229
Compound B from F-7	283[b]	245
Compound C from F-11	282[b]	247
Deoxyadenosine	260[a]	225
Deoxycytidine	271[a]	250
Deoxyguanosine	254[b]	223
Deoxythymidine	267[a]	235

a ; Data obtainned at pH 7.0. b ; Data obtained in water.

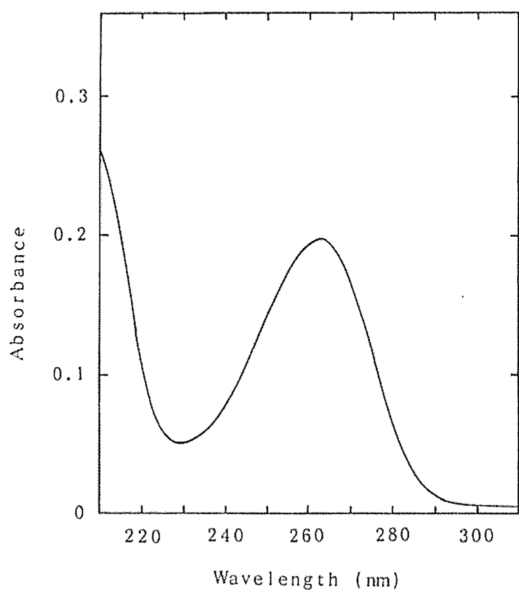

Fig. 7. Ultraviolet Absorption Spectrum of Compound A.

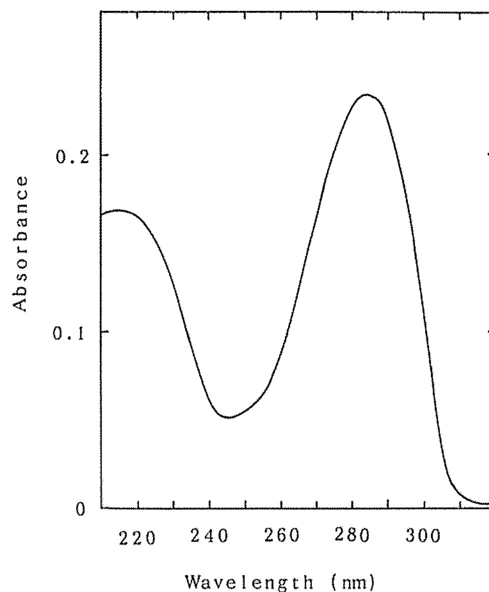

Fig. 8. Ultraviolet Absorption Spectrum of Compound B.

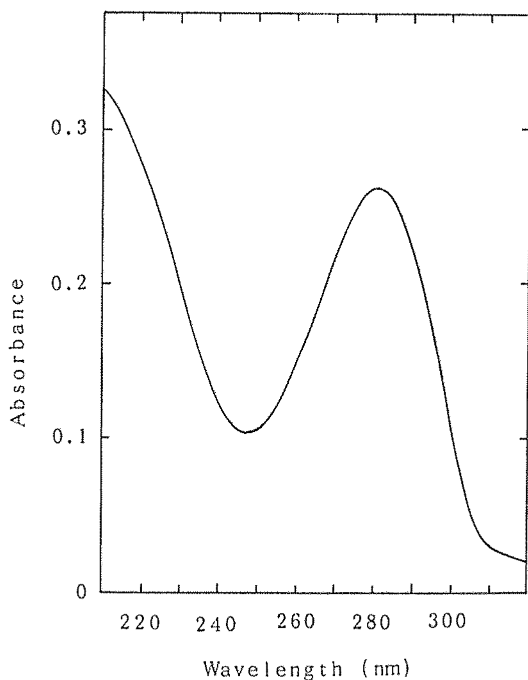

Fig. 9. Ultraviolet Absorption Spectrum of Compound C.

DISCUSSION

The minor compounds, which gave positive results in a microbial assay with *L. leichmannii* and ultraviolet absorption were detected and purified from deoxyriboside mixture obtained by the enzymatic hydrolysis of the DNA preparation. Hence, the compounds appear to be deoxyribosyl compounds.

It has been known that in the absence of cobalamins *L. leichmannii* grow by adding a minute amount of deoxyribosides or deoxyribotides as a substitute for cyanocobalamin to medium containing the other essential nutrient compounds for growth of this bacteria[11~13]. However, the minor compounds detected in this study were distinctly different from deoxyribotides or cobalamins in chromatographic and electrophoretic behaviors and spectral properties. On the basis of the summation of the microbial assay, paper chromatographic, electrophoretic and spectral evidences, the compounds were supposed to be deoxyriboside. Of the compounds, compound C seems to be identical with deoxyriboside of a novel base in DNA of chick embryo[5]. The compounds appears to be different from deoxyribosides of common and known components of animal DNA[14~17], 6-methylaminopurine[18] and N^4-methylcytosine[19] in bacterial DNA, methylated guanines[3] in HeLa cell DNA or modified components in various phage DNAs[20~22].

Thus, the experimental results suggest the possibility that the minor compounds may be unknown component of the DNA. On the other hand, one might consider the possibility that the presence of the compounds are due to artifact during the preparation and enzymatic hydrolysis of the DNA preparation or are derived from the other substances of the DNA preparation. But, it seems that the possibility due to the both is very low, because the base composition (under deoxyribosyl level) of calf thymus DNA obtained by the enzymatic digestion agreeded well with the established value, and the deoxyriboside ratio of the DNA preparation extracted from acid insoluble fraction of chick embryo coincided with that of the purified DNA[6]. Also, it may be pointed out that all the enzymatic digestion and purification procedures employed were very similar with that reported by R. H. Hall[7].

In any event, it is the significant subject to elucidate whether the compounds are native one or not as the DNA constituents. For the sake of the elucidation, the more careful and detailed experiments must be done under more mild conditions.

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摘　　要

鶏胚酸不溶性画分から調製したデオキシリボシド中の微量 *L. leichmannii* 生長促進物質

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鶏胚 DNA の微量成分として，5−メチルシトシンとウラシルがあげられる。しかし，これ等化合物以外に，他の微量成分の存在する可能性が考えられ，その手がかりを得るため本研究を行なった。

鶏胚（孵卵14日目）の酸不溶性画分から抽出した DNA 画分を DNase Ⅰ，ついで蛇毒で処理してデオキシリボシド混合物を調製した。これを，セライトカラムクロマトグラフイーに供して14画分に分別した後，各画分中の主な *L. leichmannii* 生長促進物質をマイクロバイオアッセイおよび紫外線吸収法で同定する一方，微量の *L. leichmannii* 生長促進物質の検索を試みた。

その結果，アデニン，チミン，ウラシル，グアニン，5−メチルシトシンおよびシトシンの各デオキシリボシド以外に，微量の *L. leichmannii* 生長促進物質3種類をF−3，F−6〜F−7およびF−11から検出した。3画分のセライトカラムクロマトグラフイーによる細分画およびペーパークロマトグラフイー等により分離精製した各物質の Rf 値，電気泳動値および紫外線吸収スペクトルを検討した結果，これ等の物質はデオキシリボシドであることを知るとともに，各種 DNA の既知構成分のいずれにも該当しないと推定した。

以上の結果は，鶏胚 DNA には未知の微量成分の存在する可能性を示唆した。