STUDIES OF DEOXYRIBONUCLEIC ACID SYNTHESIS AND CELL GROWTH IN THE DEOXYRIBOSIDE-REQUIRING BACTERIA, *LACTOBACILLUS ACIDOPHILUS*

I. BIOLOGICAL AND CHEMICAL NATURE OF THE INTRA-CELLULAR ACID-SOLUBLE DEOXYRIBOSIDIC COMPOUNDS*

REIJI OKAZAKI AND TUNEKO OKAZAKI

Biological Institute, Faculty of Science, Nagoya University, Nagoya (Japan)

In recent years, remarkable progress has been made in the experimental approach towards an understanding of the mechanism whereby DNA** is synthesized in the cell.

KORNBERG *et al.*[1] succeeded in demonstrating incorporation of labeled thymidine or thymidine triphosphate into DNA-like polymers with the enzymes extracted from *Escherichia coli*. In their *in vitro* system, besides triphosphates of deoxyadenosine, deoxyguanosine and deoxycytidine and Mg^{++}, the presence of a "primer" (polymerized DNA) was required for the reaction. In agreement with this finding, the occurrence of di- and triphosphates of deoxycytidine and thymidine in calf thymus[2], and deoxyadenosine triphosphate in rat tumor[3], has been reported. The enzymes which catalyze the formation of some of these deoxyriboside polyphosphates were also found[4–7].

Some attempts to elucidate the *in vivo* pathway of DNA synthesis have been reported also. HECHT AND POTTER[8] studied the utilization of labeled orotic acid and cytidine for DNA synthesis in regenerating rat liver; they failed, however, to detect any deoxynucleotide even during the period of active synthesis of DNA. Occurrence of deoxycytidine was found, but its specific activity was not high enough to implicate deoxycytidine as a direct precursor of DNA. On the other hand, SCHNEIDER AND BROWNELL[9] observed, using the microbiological assay technique, an appreciable accumulation of deoxynucleotide-like compounds in regenerating liver. SCHNEIDER[10,11] demonstrated, moreover, the occurrence of pyrimidine deoxyribosides in a wide variety of rat and mouse tissues.

Lactobacillus acidophilus R-26 requires a deoxyriboside for its growth. Deoxynucleotides are also effective in supporting the growth of this bacterium while DNA is active only when added after depolymerization with deoxyribonuclease[10]. The bacterium, therefore, has been used as the assay organism of DNA and other deoxyribosidic compounds[9–22].

If one of the deoxyribosides which normally occur in DNA is added to the culture medium of this bacterium, the other three deoxyribosides may be formed by exchange

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** The following abbreviations are used: DNA, deoxyribonucleic acid; PCA, perchloric acid; DPN, diphosphopyridine nucleotide; AMP, adenosine monophosphate; UMP, uridine monophosphate; ADP, adenosine diphosphate; UDP, uridine diphosphate.

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of purine or pyrimidine base of the added deoxyriboside[23]. Accordingly, the deoxyribose moieties of both DNA and other deoxyribosidic compounds in this microorganism are derived exclusively from the deoxyriboside added to the medium. As a result, the rate of DNA synthesis and the level of intracellular pool of DNA-precursors could be controlled by changing the content of deoxyriboside in the medium. It will also be feasible to follow the process of DNA synthesis with labeled deoxyribosidic compounds in this microorganism. For these and other reasons, *L. acidophilus* R-26 appears to provide a favourable system for studying the mechanism of DNA synthesis and its relation to cell growth. In the present study, some features of acid-soluble deoxyribosidic compounds in this microorganism were investigated in an attempt to clarify the *in vivo* pathway of DNA synthesis.

METHODS

The microorganism and its growth

Lactobacillus acidophilus R-26 was maintained by weekly transfer in skimmed milk to which 0.5% of tomato juice, 0.5% of yeast extract (Daigo), and 3% of $CaCO_3$, were added.

The double-strength basal medium used both for culture and for microbiological assay contained the following constituents per 100 ml (slightly modified from that of HOFF-JØRGENSEN[14]): acid-hydrolyzed casein (Difco casamino acids), 2.5 g; enzyme-hydrolyzed casein (Difco bacto casiton), 1 g; DL-tryptophan, 20 mg; cysteine-hydrochloride, 26 mg; KH_2PO_4, 1 g; $Fe(NH_4)_2 \cdot 6H_2O$, 0.0003 g; NaCl, 0.002 g; $MnSO_4$, 0.008 g; $MgSO_4 \cdot 7H_2O$, 0.04 g; potassium acetate, 2.5 g; cytidylic acid, 5 mg; adenine sulfate, 2 mg; guanine hydrochloride, 2 mg; uracil, 2 mg; thymine, 2 mg; *P*-aminobenzoic acid, 0.25 mg; riboflavin, 0.25 mg; nicotinic acid, 0.25 mg; calcium pantothenate, 0.25 mg; vitamin B6, 0.25 mg; folic acid, 0.025 mg; thioglycolic acid, 10 mg; glucose, 3 g; tween 80, 0.1 g. The medium was adjusted to pH 6.7 with KOH. Enzyme-hydrolyzed casein was treated with charcoal before use.

A loopful of the stock milk culture was transferred to a test tube containing 4 ml of the inoculum medium, which was prepared by adding 5 to 25 mμmoles thymidine per ml to the basal medium. After incubation at 37° for 22 to 26 h, the bacteria were sedimented by centrifugation and resuspended in 4 ml of sterile saline. The nutrient medium was inoculated with approximately 10^{-3} vol. of this suspension, and incubated at 37°. Growth was followed by turbidity measurement at 650 mμ in a Beckman model DU spectrophotometer. The number of cells per unit volume of culture was determined by counting the cells in an appropriately diluted suspension in a Thoma haemocytometer. To collect bacteria, the culture was chilled at 5° and cells were centrifuged down, washed twice in saline, and frozen with dry ice–ethanol mixture. An aliquot of cell suspension in saline was withdrawn for the estimation of total nitrogen.

Preparation of acid-soluble and nucleic acid fractions

The frozen cells were thawed and extracted with ice-cold 0.5 *N* PCA, precipitated and washed twice with 0.2 *N* PCA. The washings were combined with the original supernatant solution to give the acid-soluble fraction. The extract was neutralized with KOH to pH 7.0, and the resulting precipitate of $KClO_4$ was removed by centrifugation. After removal of lipids with ethanol and hot ethanol–ether (3:1) from the cold PCA-insoluble residue, nucleic acids were extracted with hot 5% trichloroacetic acid according to the method of SCHNEIDER[24]. The resulting residue was used for the determination of protein nitrogen.

Assay of deoxyribosidic compounds in acid-soluble fraction

Prior to the assay, an aliquot of neutralized sample was treated with crude snake venom (*Agkistrodon blomhoffii*)[20] at the concentration of 0.5 mg per ml in maleic acid–NaOH buffer (pH 6.5) containing 10^{-2} *M* $MgSO_4$. The enzyme digestion was carried out for 10 to 15 h at 37°, under a layer of hexan. The reaction was stopped by heating at 100° for 10 min. The venom-treated and untreated acid-soluble extracts of *L. acidophilus* were assayed for their deoxyribosidic growth effects using the same bacteria as the test organism.

For the microbiological assay, a loopful of the stock culture of the bacteria was put into 4 ml of the inoculum medium containing 5 mμmoles thymidine per ml. After 20 to 26 h incubation at 37°, the bacteria were centrifuged and resuspended in 2 ml of sterile saline. One drop of the suspension was diluted in 10 ml of saline. Each tube containing a total volume of 4 ml of medium was inoculated with one drop of this diluted suspension. After 36 to 40 h incubation at 37°, turbidity was measured at 650 mμ in the Beckman model DU spectrophotometer. A standard

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response curve to graded levels of thymidine was prepared each time. The amount of deoxyribosidic compounds was determined from the standard curve, and was expressed as mμmoles thymidine equivalent. Each value presented is the average of at least three tubes run in parallel.

Chromatographic fractionation of acid-soluble nucleotides

An acid-soluble extract was prepared from 10 litres of culture at the late logarithmic phase. The neutralized extract was lyophilized. The residue was taken up in a small volume of cold water, and the precipitate of $KClO_4$ was removed by centrifugation. The supernatant solution was adjusted to pH 7.5 with a small amount of aqueous ammonia. Part of the concentrated extract was used for the measurement of ultraviolet absorption and the assay of deoxyribosidic compounds.

A large aliquot was added to a column (0.8 $cm^2 \times 18$ cm) of Dowex-1 (formate) anion-exchange resin (X-10, 200–400 mesh). Adsorbed nucleotides were fractionated by using the extended gradient-elution chromatography with the "formic acid system" of HURLBERT *et al.*[25]. After washing the column with 25 ml water, elution was carried out by introducing successively the following eluents into a 500 ml mixing flask previously filled with water; 120 ml of 1 *N* formic acid; 450 ml of 4 *N* formic acid; 500 ml of 0.2 *M* ammonium formate in 4 *N* formic acid; 300 ml of 0.4 *M* ammonium formate in 4 *N* formic acid; 540 ml of 1 *M* ammonium formate in 4 *N* formic acid. The flow rate was 15 to 20 ml per h, and samples of effluent containing approximately 5 ml were collected automatically. The extinction at 260 mμ and 280 mμ were measured for each tube in the Beckman DU spectrophotometer. Effluent fractions corresponding to the peaks or portions of peaks were pooled and lyophilized to remove formic acid and ammonium formate. The resulting residues were dissolved in a given volume of water, and brought to pH 7.0 with a small amount of dilute NaOH. The ultraviolet absorption of each pooled fraction was determined, and the deoxyribosidic compounds were assayed microbiologically as described above.

Rechromatography of the fraction containing the bulk of deoxyribosidic compounds was performed by extended gradient-elution chromatography with ammonium formate (pH 5) as the eluent[25]. The resin column, Dowex-1 (formate) X-10, 200–400 mesh, 0.39 $cm^2 \times 10$ cm and 160 ml mixing flask were used. The following eluents were introduced successively into the mixing flask: 200 ml volumes of 1 *M* ammonium formate, 2 *M* ammonium formate, and 0.75 *N* formic acid in 2 *M* ammonium formate. Samples of effluent containing 5 ml were collected at 20 to 30 min intervals. Fractions were pooled, lyophilized, and used for the determination of their contents of deoxyribosidic compounds, spectrophotometric analyses, etc.

Chemical analyses

DNA was estimated by the *P*-nitrophenyl hydrazine reaction according to WEBB AND LEVY[26]. For the determination of nitrogen the method of LEVY AND PALMER[27] was used.

RESULTS AND DISCUSSION

1. The results of assay of acid-soluble deoxyribosidic compounds in the culture grown in the medium containing a sufficient quantity of thymidine (25 mμmoles per ml) are shown in Fig. 1*. It will be noted first that the majority of the deoxyribosidic compounds present in the acid-soluble fraction of *L. acidophilus* were in such a state that they were active for the growth test only when added externally after snake-venom digestion. The same remarkable situation was found previously in the acid-soluble extracts of sea urchin eggs[20,21]. DNA also becomes active towards the growth of *L. acidophilus* if treated with snake venom. Secondly, the content of the deoxyribosidic compounds related to nitrogen was higher in actively growing cells than in cells which had ceased proliferating. In the logarithmic phase the proportion of acid-soluble deoxyribosidic compounds corresponded to approximately 5% of the deoxyribosides contained in DNA.

It would be inferred that the deoxyriboside added to the medium is incorporated into the cell and converted to the more complex acid-soluble deoxyribosidic com-

* In the bacteria grown in a medium containing only a small quantity of thymidine (0.75 mμmole per ml) the content of acid-soluble deoxyribosidic compounds expressed per mg N as well as that expressed per litre culture were significantly lower.

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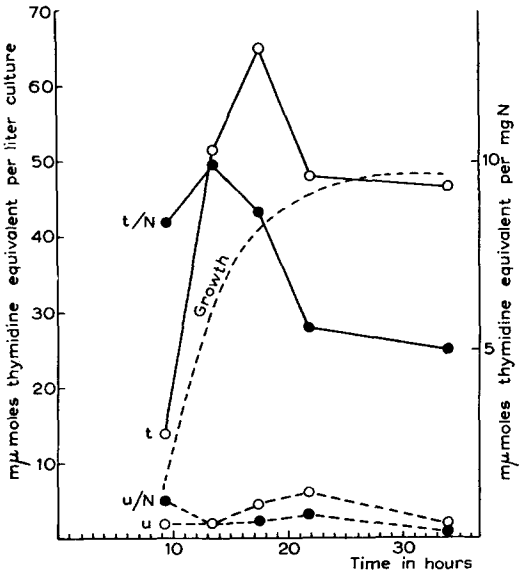

Fig. 1. Content of acid-soluble deoxyribosidic compounds in a culture grown in the medium containing excess thymidine (25 mμmoles per ml). u: assayed before venom-treatment and expressed per litre culture. t: assayed after venom-treatment and expressed per litre culture. u/N: assayed before venom-treatment and expressed per mg total nitrogen. t/N: assayed after venom-treatment and expressed per mg total nitrogen.

pounds, which are inactive as external growth factors unless digested with snake venom. The deoxyribosidic compounds thus formed in the cell might serve as the building blocks of cellular DNA, in view of the fact that their concentration in the growing cells significantly exceeds that in the resting cells.

2. For testing this possibility, the following experiment was performed. The bacteria were grown up to the late logarithmic phase in a medium containing excess* thymidine. They were then washed with saline and resuspended in a larger volume of the fresh medium containing a "limiting"* quantity of thymidine. When the bacteria were transferred to such a medium, growth ensued. However, the rate of DNA synthesis and cell division were found to be much slower than in thymidine-rich medium because of the limited supply of deoxyriboside from the medium**. Under such conditions, the level of intracellular pool of DNA-precursors, if any, is expected to be lowered as the DNA synthesis proceeds.

Figs. 2 (a) and (b) show the changes in the amount of acid-soluble deoxyribosidic compounds when cells were transferred from the thymidine-rich (25 mμmoles thymidine per ml) to thymidine-poor (1.75 mμmoles thymidine per ml) medium. In Figs. 3 and 4 are presented the increase in DNA and total nitrogen in the same

* Under these experimental conditions the amount of DNA synthesized when the cells were suspended in a fresh medium with large quantities of thymidine corresponded to approximately 15 mμmoles deoxyriboside per ml culture. Further, if graded quantities of thymidine were tested, the maximum DNA synthesis was reached at about 15 mμmoles thymidine per ml. Therefore, a quantity of thymidine above 15 mμmoles per ml culture medium was considered to be "excess". A quantity of thymidine much lower than this value may safely be regarded as the "limiting" quantity.

** The increase in mass and in protein content was little affected (see Fig. 4). Even if the bacteria were suspended in a fresh medium free of deoxyriboside an increase in total nitrogen or in turbidity as much as approximately 70% of that found in the medium containing excess thymidine was observed. This is quite different from what was observed when the culture media were lightly inoculated, as in the microbiological assay of deoxyribosidic compounds (test for deoxyribosidic growth effect). In the latter case the increase in turbidity was approximately proportional to the concentration of thymidine in the medium up to about 2 mμmoles thymidine per ml.

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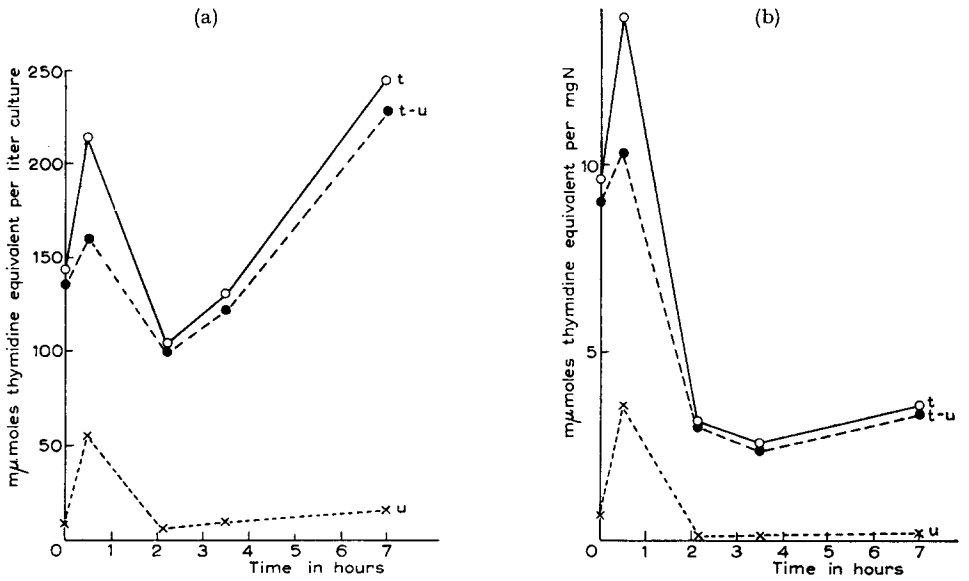


Figs. 2 (a) and (b). Changes in the intracellular pool level of acid-soluble deoxyribosidic compounds upon incubation in the medium with a limiting quantity of thymidine. 200 ml of bacterial culture grown to about 10^8 cells per ml in the medium containing 25 mμmoles thymidine per ml, were washed with saline, resuspended in 960 ml of the fresh medium containing 1.75 mμmoles thymidine per ml, and incubated at 35°. Fig. 2 (a): quantity of deoxyribosidic compounds per litre culture. Fig. 2 (b): quantity of deoxyribosidic compounds per mg nitrogen. t: venom-treated. u: untreated. t—u: venom treated minus untreated.

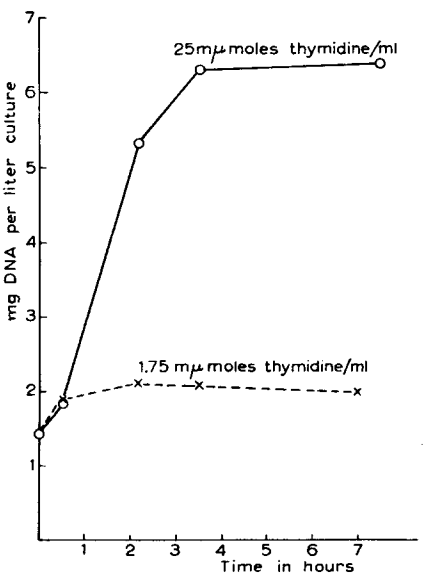


Fig. 3. DNA synthesis upon incubation in the thymidine-rich and thymidine-poor media. The bacteria previously grown in the medium containing 25 mμmoles thymidine per ml were transferred either to the medium containing excess thymidine (25 mμmoles per ml) or to the medium with the limiting quantity of thymidine (1.75 mμmoles per ml). The same experiment as Figs. 2 (a) and (b) and 4.

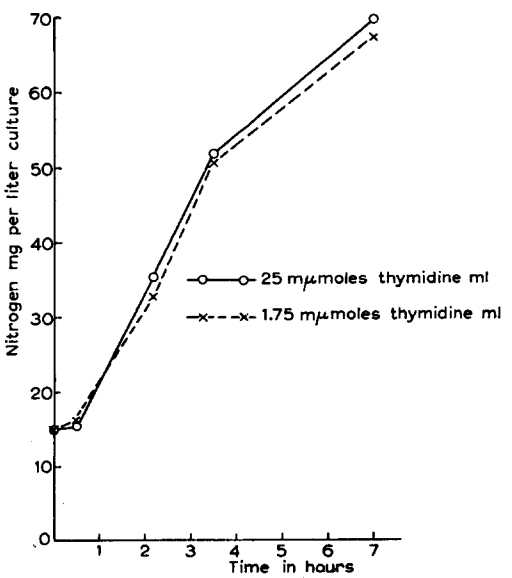
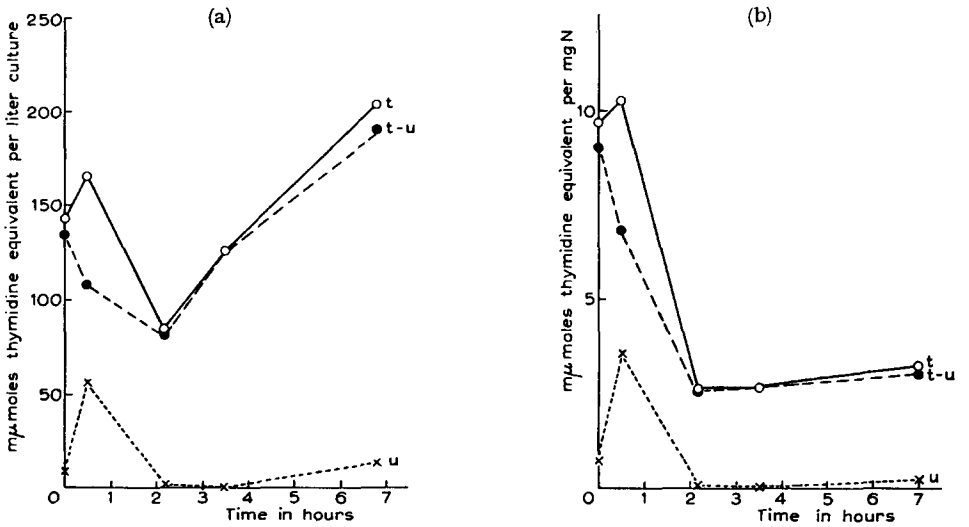


Fig. 4. Increase in total nitrogen in the thymidine-rich and thymidine-poor media. The same experiment as Figs. 2 (a), (b) and 3.

culture, together with those observed when cells were transferred to the fresh medium with excess thymidine (25 mμmoles thymidine per ml). Fig. 3 shows that during the period from 30 to 130 min after transfer to the thymidine-poor medium, the thymidine content of the medium was limiting the rate of DNA synthesis. Precisely during this period, as seen in Figs. 2 (a) and (b), a marked decrease was observed in the content of acid-soluble deoxyribosidic compounds inactive in the growth test unless treated with the venom (t — u) as well as of those active without the pretreatment (u).

If cells were transferred to the medium containing 0.5 mμmole thymidine per ml, the pool level of acid-soluble deoxyribosidic compounds, which were inactive unless digested with venom (t — u), began to drop immediately (Figs. 5 (a) and (b)). In this case, the rate of synthesis of DNA also became limited immediately after transfer to the new medium.



Figs. 5 (a) and (b). Changes in the intracellular pool level of acid-soluble deoxyribosidic compound upon incubation in the medium with a limiting quantity of thymidine. 200 ml of bacterial culture grown to about 10^8 cells per ml in the medium containing 25 mμmoles thymidine per ml, were washed with saline, resuspended in 960 ml of the fresh medium containing 0.5 mμmole thymidine per ml, and incubated at 35°. Fig. 5 (a): amount of deoxyribosidic compounds per litre culture. Fig. 5 (b): amount of deoxyribosidic compounds per mg nitrogen. t: venom-treated. u: untreated. t—u: venom-treated minus untreated.

These observations are what would be expected from the reaction sequence:

$$\text{Thymidine} \longrightarrow X \longrightarrow \text{DNA} \qquad (\alpha)$$

where X represents the acid-soluble deoxyribosidic compounds that are fully active in the growth test only after enzymic digestion. However, the possibility is not necessarily excluded that X and DNA are independently formed from thymidine, *i.e.*

$$\text{Thymidine} \begin{cases} \nearrow X \\ \searrow \text{DNA} \end{cases} \qquad (\beta)$$

3. Assuming that the acid-soluble deoxyribosidic compounds which are active for the growth test only after venom-treatment represent intermediates in DNA

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synthesis (as shown in scheme a), it might be asked why they are incapable of supporting the growth when added to the medium. It is conceivable that these deoxyribosidic compounds fail to penetrate into the bacterial cell. However, in view of the observation described below, this appears not to be the case.

The bacteria were grown with excess thymidine until the late logarithmic phase. An aliquot of the bacterial culture was centrifuged, and the cells were washed and resuspended in a fresh medium containing an amount of acid-soluble extract of *L. acidophilus*. Another aliquot was treated in the same way and suspended in a medium containing the same amount of acid-soluble extract previously digested with venom. Parallel microbiological assay showed that this amount of untreated acid-soluble extract revealed the growth effect equivalent to 0.5 mμmole thymidine per ml, whereas the treated one had the growth effect equivalent to 8.0 mμmoles thymidine per ml.

As already mentioned, the transfer of bacteria from the thymidine-rich medium to thymidine-deficient medium causes a marked decrease in acid-soluble deoxyribosidic compounds in the cell. If the added acid-soluble deoxyribosidic compounds do not penetrate into the bacterial cell, the intracellular level of acid-soluble deoxyribosidic compounds should be depressed upon transfer of bacteria from the thymidine-rich medium to the medium containing only untreated acid-soluble extract. On the other hand, the amount of acid-soluble deoxyribosidic compounds in the cell should be increased when the bacteria are transferred to the medium containing venom-treated acid-soluble extract, because of the ample supply of activated deoxyribosidic compounds.

The results of assay of intracellular deoxyribosidic compounds at 130 min after transfer of bacteria to the new media are given in Table I. It is evident that a marked accumulation rather than depression of acid-soluble deoxyribosidic compounds took place when cells were incubated in the medium containing the untreated acid-soluble extract. It is also noted that the intracellular level of deoxyribosidic compounds was rather higher in the cells incubated with untreated acid-soluble extract than in the cells incubated in the medium containing the venom-treated extract. These results are contrary to what is expected on the basis of impermeability of the cell membrane to these acid-soluble deoxyribosidic compounds. It appears that the failure of the

TABLE I

CHANGES IN THE CONTENT OF INTRACELLULAR ACID-SOLUBLE DEOXYRIBOSIDIC COMPOUNDS UPON INCUBATION IN THE MEDIUM CONTAINING UNTREATED OR VENOM-TREATED ACID-SOLUBLE EXTRACT

Samples	*The amounts of the intracellular acid-soluble deoxyribosidic compounds revealed by the growth test (mμmoles thymidine equivalent)*			
	Per liter culture		*Per unit optical density (at 650 mμ)*	
	After venom-treatment	*Before venom-treatment*	*After venom-treatment*	*Before venom-treatment*
Before incubation	13.1	1.3	4.86	0.47
Incubated for 2 h 10 min in the medium containing untreated acid-soluble extract	32.9	5.3	8.08	1.3
Incubated for 2 h 10 min in the medium containing venom-treated acid-soluble extract	28.1	4.3	6.54	1.0

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deoxyribosidic compounds in question to support the growth is due to some other reason.

4. Another possible explanation of the situation may be that the acid-soluble deoxyribosidic compounds which are inactive as external growth factors serve only as the building blocks of special parts of a DNA molecule, and therefore fail to show growth effect if they are added alone. When *L. acidophilus* is furnished with one sort of deoxyriboside, the other three deoxyribosides normally contained in DNA are formed by utilizing the deoxyribose of the added deoxyriboside, so that the DNA synthesis and cell growth are supported. Consequently, if the above assumption be true, upon simultaneous addition of a deoxyriboside, the deoxyribosidic compounds in question should be incorporated into DNA, resulting in an increase in the growth effect which cannot be accounted for by the added deoxyriboside alone.

The growth effect of the acid-soluble extract not pretreated with the venom was tested in the presence of graded amounts of thymidine. The results are shown in Table II. It will be seen that simultaneous addition of thymidine and the acid-soluble extract resulted in a noticeable augmentation of the growth. For example, the amount of the acid-soluble extract that showed the growth effect equivalent to 0.17 mμmole thymidine when tested alone, plus 3.5 mμmoles thymidine, caused a growth equivalent to 5.11 mμmoles thymidine. This value (5.11) was 1.44 mμmoles thymidine higher than the sum of the growth effects observed when thymidine and the acid-soluble extract were tested separately (3.67).

The results may be taken to indicate that the acid-soluble deoxyribosidic compounds which are less active in the ordinary growth test unless digested with the venom become more active in supporting the growth by simultaneous addition of thymidine. According to this interpretation, in the presence of sufficient thymidine, the growth effect of the acid-soluble extract was approximately ten times as much as that obtained in its absence (compare 1(d) and 4(d) in Table II). It can be also seen that the growth-supporting activity of the acid-soluble extract observed upon addition of a sufficient amount of thymidine reached above 60% of its full activity revealed after snake venom digestion (compare 4(d) and 5(a) in Table II).

TABLE II

GROWTH EFFECT REVEALED UPON SIMULTANEOUS ADDITION OF THYMIDINE AND THE ACID-SOLUBLE EXTRACT

(Expressed as mμmoles thymidine equivalent)

Samples	*(a)* *Observed growth effect*	*(b)* *Calculated sum of the growth effects obtained when thymidine and acid-soluble extract were tested separately*	*(c)* *Difference between observed growth effect and calculated sum ((a) — (b))*	*(d)* *Growth effect attributable to acid-soluble extract ((c) + 1(a))*
1. Untreated acid-soluble extract	0.017	0.017	0.000	0.017
2. Untreated acid-soluble extract + 0.1 mμmole thymidine	0.146	0.117	0.029	0.046
3. Untreated acid-soluble extract + 0.26 mμmole thymidine	0.397	0.267	0.130	0.147
4. Untreated acid-soluble extract + 0.35 mμmole thymidine	0.511	0.367	0.144	0.161
5. Venom-treated acid-soluble extract	0.263			

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It is suggested that the acid-soluble deoxyribosidic compounds, which show little growth effect in the ordinary growth test unless enzymically digested, are utilized for the synthesis of DNA if a deoxyriboside is added simultaneously. Although further substantial evidence is required for a definite conclusion, it is likely that these deoxyribosidic compounds serve as the building blocks of some definite parts of a DNA molecule. The scheme shown in Fig. 6 illustrates this hypothesis.

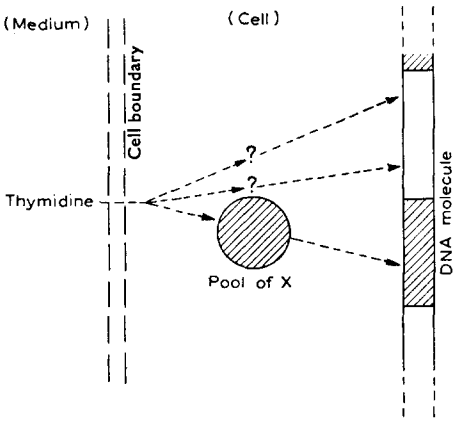

Fig. 6. Scheme to illustrate the possible role of the intracellular pool of acid-soluble deoxyribosidic compounds *X* which reveal little growth effect unless added together with thymidine or added after venom-digestion.

In the experiments mentioned in section 2 regarding the effect of deoxyriboside starvation on the intracellular deoxyribosidic compounds, it was observed that depression of the pool level of acid-soluble deoxyribosidic compounds was less remarkable upon incubation in the medium completely devoid of deoxyriboside than in the medium containing a small amount of thymidine. This fact may also be explained on the basis of the above hypothesis.

5. In order to study the chemical nature of deoxyribosidic compounds in *L. acidophilus*, an acid-soluble extract was prepared from 10 l of a late logarithmic-phase culture grown in the medium containing 25 mμmoles thymidine per ml. 18 ml of the neutralized and concentrated extract were applied to a column of Dowex-1 (formate), 0.8 cm^2 × 18 cm, and washed well with water to remove lightly adsorbed material. More than 98 % of the deoxyribosidic compounds present in the acid-soluble extract were adsorbed on the resin column, indicating that most of the acid-soluble deoxyribosidic compounds in this microorganism are not nucleosides.

The adsorbed material was fractionated by the extended gradient-elution chromatography developed by HURLBERT *et al.*[25]. The ion-exchange chromatography was carried out in the cold room at 3–5°, in view of the lability of purine deoxynucleotides with respect to acid[29]. Effluent fractions represented by peaks or portions of peaks were pooled, lyophilized, and assayed for their content of deoxyribosidic compounds. In Fig. 7 the elution pattern and the amounts of deoxyribosidic compounds in each pooled fraction are given. Although no special effort was made to identify the fractions containing only small quantities of deoxyribosidic compounds, the following main components in some of the fractions may be suggested from the

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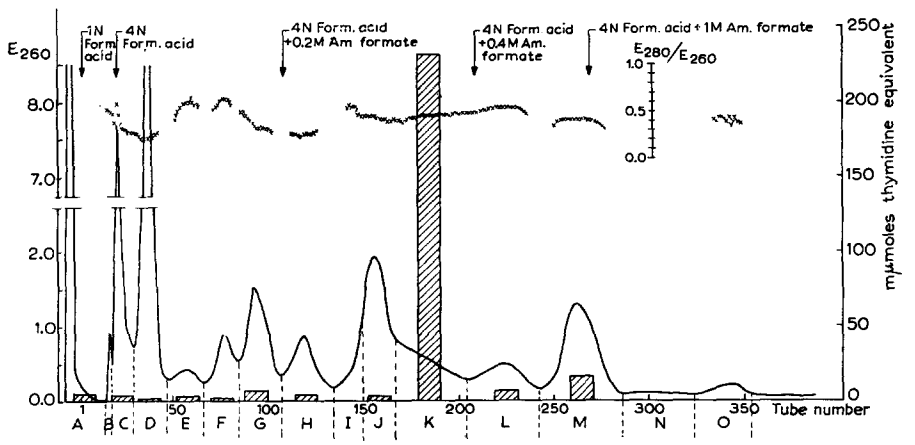

Fig. 7. Extended gradient chromatography of the acid-soluble extract of *L. acidophilus* with "formic acid system"[25]. Columns indicate the quantity of deoxyribosidic compounds found in each pooled fraction. Exchanger: Dowex-1 (formate), X-10, 200–400 mesh, 0.8 cm² × 18 cm. Volume of the mixing flask: 500 ml. Eluents were introduced successively into the mixing flask as indicated.

ratio of E_{280} to E_{260} and the location in the chromatogram: C, DPN; D, AMP; G, UMP; H, ADP; M, UDP.

It was found that 82 % of the deoxyribosidic compounds were recovered in fraction K, which was eluted in the region approximately corresponding to the location of uridine diphosphate *N*-acetylglucosamine and/or uridine diphosphate glucose contained in the acid-soluble fraction of rat liver[25]. Also in fraction K the full deoxyribosidic growth effect was revealed only after venom-digestion. However, the activity found before digestion expressed as percentage of the full activity, 27.8 %, was noticeably higher than 9.7 % found for the unfractionated acid-soluble extract. The location of fraction K in the chromatogram is different from those of di- or triphosphates of deoxycytidine and thymidine that have been isolated from the acid-soluble extract of the calf thymus[2]. The chromatographic behaviour of fraction K is also entirely different from that of deoxycytidine diphosphate choline recently found in sea urchin eggs[20,21]; the latter would be eluted near fraction B. It should be further noted that the nucleotides contained in fraction K cannot be any known simple nucleoside monophosphate which should be eluted faster than fraction I. In fraction M, which seems to contain UDP as the main component, 6 % of other deoxyribosidic compounds in the acid-soluble extract of the bacteria were found.

An aliquot of fraction K was adsorbed again on a smaller Dowex-1 column and rechromatographed by using the "ammonium formate system" of HURLBERT *et al.*[25]. As shown in Fig. 8, approximately 92 % of the deoxyribosidic compounds in fraction K added to the column were found in the main peaks designated as fraction Kc*. The absorption spectrum of this fraction revealed some similarities to that of uridylic acid, but the ratio of E_{280} to E_{260} (0.45 in acid and 0.425 in alkali) was considerably higher than that of uridylic acid.

SCHNEIDER AND POTTER[19] have recently reported that di- or triphosphates of

* The occurrence of deoxyribose in this fraction was confirmed by the cysteine-sulfuric acid test[28].

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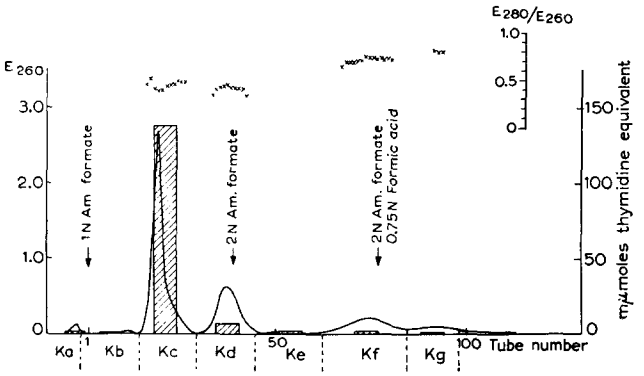

Fig. 8. Rechromatography of fraction K by "ammonium formate system"[25]. Columns indicate the quantity of deoxyribosidic compounds found in each pooled fraction. Exchanger: Dowex-1 (formate), X-10, 200–400 mesh, 0.39 cm^2 × 10 cm. Volume of the mixing flask: 160 ml. Eluents were introduced successively into the mixing flask as indicated.

TABLE III

EFFECT OF THE TREATMENT WITH 1 *N* PERCHLORIC ACID AT 100° FOR 15 MIN ON THE GROWTH EFFECT OF FRACTION Kc

Samples	*Growth effects*		
	Before venom-treatment (u)	*After venom-treatment (t)*	$t - u$
Fraction Kc not treated with PCA	39.9* (100)**	139.1* (100)**	99.2* (100)**
Fraction Kc treated with PCA	13.6* (34.1)**	115.2 (82.8)**	101.6* (102.2)**

* Expressed as mμmoles thymidine equivalent.
** Expressed as % of growth effects observed in the sample not treated with perchloric acid.

thymidine and deoxycytidine failed to show the growth effect towards *L. acidophilus* unless dephosphorylated to monophosphates by acid-hydrolysis.

In order to test whether a similar situation is found in the deoxyribosidic compound present in fraction Kc, an aliquot of the fraction was treated with 1 *N* PCA at 100° for 15 min*. After cooling in an ice-water bath it was neutralized with KOH, and the resulting $KClO_4$ was removed by centrifugation. The supernatant solution was assayed for its growth effects before and after venom-treatment. The data given in Table III reveal that the acid-treatment caused no increase in the growth effect, but on the contrary a considerable decrease. Further, by a subsequent venom-treatment, the hot PCA-treated sample was activated to the same extent as the one not pretreated with hot PCA (see $t - u$ in Table III). Thus it is clear that the treatment with 1 *N* PCA at 100° for 15 min entirely failed to replace the venom digestion in activating the deoxyribosidic compound in this fraction.

This fact may imply, in agreement with what was suggested from the chromatographic behaviour that the deoxyribosidic compound in fraction Kc differs from the known pyrimidine deoxyriboside polyphosphates which were tested by SCHNEIDER *et al.* However, the above experimental results also indicate that at least most of the

* This treatment was found to be sufficient to split the labile phosphates from adenosine triphosphate.

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bases occurring in the deoxyribosidic compound in fraction Kc are pyrimidines, since the purine-deoxyribose linkage is expected to be completely broken during hot PCA-treatment[12,17,29]. Further, as has already been mentioned, the deoxyribosidic compound in fraction Kc can be identical neither with deoxycytidine diphosphate choline nor with any simple deoxyriboside monophosphate. Thus it seems likely that the main deoxyribosidic compound in the acid-soluble extract of *L. acidophilus* is a deoxynucleotide-like compound but differs from hitherto reported deoxymononucleotides, including triphosphates of deoxyadenosine, deoxyguanosine, deoxycytidine, and thymidine, which have been reported to be the immediate precursors of DNA in the *in vitro* system[1].

Further efforts for the purification and identification of this microbial deoxyribosidic compound are being made.

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SUMMARY

Deoxyribosidic compounds in the acid-soluble fraction of the deoxyriboside-requiring bacteria, *Lactobacillus acidophilus* R-26, were studied using that bacteria as the test organism. It was shown that the majority of acid-soluble deoxyribosidic compounds in the cell were not nucleosides, and revealed a growth-supporting activity only after digestion with crude snake venom.

The contents of these deoxyribosidic compounds were higher in the growing cells than in the resting cells. When cells were transferred to the medium with a limiting amount of deoxyriboside, a decrease in the level of the intracellular pool of these deoxyribosidic compounds was observed as DNA synthesis proceeded. These deoxyribosidic compounds revealed little growth effect before venom-treatment if tested alone, but if administered with sufficient thymidine they showed a growth effect about ten times as great as that observed in the absence of thymidine. These observations are taken to suggest that these deoxyribosidic compounds are intermediates in DNA synthesis.

A preliminary attempt was made to isolate these compounds by using anion-exchange chromatography. Some properties of the deoxyribosidic compounds in a chromatographic fraction were studied. It is suggested that the main deoxyribosidic compound in the acid-soluble fraction of *L. acidophilus* is a deoxynucleotide-like compound which differs from the deoxymononucleotides hitherto found in the living material.

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Note added in proof

Further chromatography of fraction Kc on a cellulose column revealed that this fraction consisted of two components. The one component, having the absorption which was closely similar to that of thymidine within the range between 250 mμ and 290 mμ but much higher than thymidine at shorter wavelengths, showed a deoxyribosidic growth activity after snake-venom digestion. On the other hand, authentic TMP, TDP and TTP were eluted, upon chromatography on Dowex-1 with the formic acid system, at the positions corresponding to those of fraction H, M and O, respectively. It is evident, therefore, that the compound under consideration is not identical with those thymidine nucleotides. It is suggested that the main deoxyribosidic compound in the acid-soluble extract of *L. acidophilus* is a derivative of one of the thymidine nucleotides. The other compound present in fraction Kc appears to be a derivative of uracil ribonucleotide.

EFFECT OF NITROGEN AND SULFUR MUSTARD ON NUCLEIC ACID SYNTHESIS IN *ESCHERICHIA COLI*[*]

F. M. HAROLD[**] AND Z. Z. ZIPORIN

U. S. Army Medical Nutrition Laboratory, Denver, Colo. (U.S.A.)

INTRODUCTION

The mustards are prominent members of that group of substances whose biological activities are described as "radiomimetic". On the cellular level, both mustards and radiation inhibit cell division, induce chromosome fragmentation and gene mutation and may ultimately kill the cell. Thus it appears that the nuclear apparatus of the cell is particularly susceptible to the action of these agents and may well be the primary

[*] The opinions expressed in this paper are those of the authors, and do not necessarily represent the official views of any governmental agency.

[**] Present address: Division of Biology, California Institute of Technology, Pasadena, Calif.

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