REPLICATION AND PROPERTIES OF DNA IN NICOTINAMIDE ADENINE DINUCLEOTIDE DEFICIENCY OF ESCHERICHIA COLI CELLS

BY RYUSHI NOZAWA AND DEN’ICHI MIZUNO

FACULTY OF PHARMACEUTICAL SCIENCES, UNIVERSITY OF TOKYO, HONGO, TOKYO, JAPAN

Communicated by Rollin D. Hotchkiss, May 14, 1969

Abstract.—When niacin-requiring E. coli was starved of niacin, a precursor of nicotinamide adenine dinucleotide (NAD), the cells elongated and synthesis of DNA was inhibited while syntheses of RNA and protein continued. The cellular NAD concentration decreased to less than one tenth of the normal level in niacin deficiency, but no change in DNA ligase level was detected. The apparent inhibition of DNA synthesis can be explained as due to reduction in the activity of the cofactor, NAD, for the enzyme.

The DNA synthesized in this state was small in an alkaline sucrose gradient but of normal size in a neutral sucrose gradient. The molecules had a double-stranded structure, as judged by hydroxyapatite chromatography. The small pieces of DNA were integrated into normal-size DNA molecules on addition of niacin, when the cellular NAD concentration was restored to the normal level but DNA synthesis was not. It is suggested that this small DNA is an intermediate in DNA replication.

DNA ligases which repair breaks in single strands of DNA duplicates have been isolated from phage-infected1, 2 and noninfected E. coli3–6 and mammalian cells.7 The enzyme requires ATP as a cofactor in phage-infected E. coli1 and mammalian cells,7 and NAD in E. coli.3–8

This enzyme is suggested to be important in DNA replication,9, 10 repair,11 and recombination.12 The possible role of the enzyme in vivo in DNA replication was proposed to be that of covalently joining DNA fragments which are units of discontinuous DNA replication.9, 10 These DNA fragments accumulated in the absence of a functional T4 ligase.13–15

We produced E. coli without functional DNA ligase and studied the in vivo function of the ligase with respect to replication of DNA. A part of this work was reported previously.16

Materials and Methods.—Bacteria and medium: E. coli W3110 (thymine−, niacin−) and the medium used were described previously.18

Cell cultivation: Cells were cultivated to an early logarithmic phase in medium with added niacin, harvested, washed once, and transferred to niacin-free medium.

Separation of 14C-NAD by electrophoresis: Cells labeled with 14C-niacin were extracted twice with 5% cold TCA, and the extract was washed repeatedly with ether.17 It was then spotted on Whatman no. 1 filter paper and subjected to electrophoresis in 0.015 M citrate buffer (pH 5.5) at 3000 v for 40 min. The radioactivity of the 14C-NAD fraction was counted with a gas-flow counter (Aloka).

Substrate and assay of DNA ligase: As substrate for E. coli DNA ligase, the homopolymer pair of dA18 (3000 nucleotides) and dT (70–100 nucleotides) were used.4 The 5′-phosphate end of the dT chain was labeled with 32P by polynucleotide kinase.19 The incubation mixture (0.1 ml) contained 10−3 M Tris-HCl, pH 8.1, 10−3 M MgCl2, 5 × 10−4 M EDTA, 6.5 × 10−6 M dA, 7.2 × 10−6 M 32P-dT, 1.5 × 10−4 M NAD, and en-
zyme. After incubation at 30° for 30 min, E. coli alkaline phosphomonoesterase was added and the mixture was incubated for 15 min at 85°. The fraction which was resistant to phosphatase was counted with a GM counter (Aloka), in accordance with the procedure of Olivera and Lehman.

**Purification of DNA ligase:** DNA ligase from E. coli was purified as described previously up to the step of ammonium sulfate fractionation.

**Extraction of DNA:** DNA was extracted by the procedure of Thomas.

**Zone sedimentation in sucrose gradients: Alkaline sucrose gradient:** Native DNA was denatured by incubation in 0.1 N NaOH containing 10^{-3} M EDTA at room temperature for 20 min and then layered on a 5-20% sucrose gradient containing 0.1 N NaOH, 0.8 M NaCl, and 10^{-3} M EDTA. The tubes were spun in a Hitachi RPS 40 rotor. Denatured DNA was also prepared by another method in which spheroplasts were placed directly on the top of the alkaline sucrose gradient. Phage particles of T4 or λ, labeled with 14C-thymine or 3H-thymidine, were used as an internal marker of size in the alkaline sucrose gradient.

**Neutral sucrose gradient:** One-tenth ml of DNA was layered on a neutral 5-20% sucrose gradient containing 10^{-3} M NaCl and 10^{-3} M EDTA, pH 8. The tubes were spun in a Hitachi RPS 40 rotor.

**Chromatography of DNA on hydroxyapatite:** Hydroxyapatite was prepared by the method of Miyazawa and Thomas. DNA in 0.05 M phosphate buffer (pH 6.8) was adsorbed on a column of hydroxyapatite (1.3 × 12 cm). Elution was carried out with 200 ml of a linear gradient from 0.05 to 0.6 M phosphate buffer (pH 6.8). Fractions of 2.5 ml were collected.

**Reagents:** Radioactive materials were purchased from Daiiichi Kagaku Co. Pronase was from Kaken Kagaku Co. and lysozyme from Worthington Biochemical Co.

**Results.—Decrease of NAD during niacin starvation:** When niacin-requiring E. coli cells were grown with 14C-niacin and then transferred to niacin-free medium, the cellular content of 14C-NAD decreased with time to one tenth of the normal level after two hours of starvation (Fig. 1). This indicates that niacin starvation creates an NAD-deficient state in the cells. Upon addition of 14C-niacin to the culture after 2.5 hours of starvation, cellular 14C-NAD was rapidly restored to the normal level within 15 minutes (Fig. 1).

**Cells in NAD deficiency:** In NAD deficiency, the cell number did not increase, although the turbidity of the culture gradually increased. Cells of this state elongated to severalfold of the normal. When niacin was added after four hours of starvation, the cell number increased about twofold (Fig. 2). Thus, cell division seems to be inhibited by NAD deficiency.

**Inhibition of DNA synthesis:** The incorporation of 14C-adenine into the DNA and RNA fractions and of 14C-amino acid into proteins during niacin starvation

![Fig. 1.—Decrease of 14C-NAD during starvation. E. coli was cultivated in 70 ml of CG medium containing 14C-niacin (0.33 µg/ml) for three generations and then transferred to niacin-free medium. At the indicated times, 10 ml of cells were harvested, and their 14C-NAD was determined. At 2.5 hr 0.33 µg/ml of 14C-niacin was added.](image)
are shown in Figure 3. Inhibition of DNA synthesis occurred one hour after transfer, while RNA and protein syntheses continued. This indicates that the cellular concentration of NAD specifically affected the system for DNA synthesis. DNA ligase of *E. coli* requires NAD as a cofactor \(^3\), so NAD deficiency may cause apparent inactivation of DNA ligase followed by inhibition of DNA synthesis. This possibility was examined.

**NAD concentration and ligase activity:** The cellular concentration of NAD was calculated from the specific activity of \(^{14}\text{C}-\text{niacin} and the number of cells (Fig. 1); cells were assumed to be about 1 \(\mu\) long. Highly purified DNA ligase from *E. coli* requires more than \(1 \times 10^{-4} M\) NAD for full activity.\(^{20}\) The NAD concentration at zero time was calculated to be \(4.4 \times 10^{-4} M\) (Table 1), a value approximately coinciding with that reported previously.\(^{23}\) However, the concentration after two and 2.5 hours of starvation seemed to be sufficiently low to suppress the activity of DNA ligase (Table 1).

Cells from niacin-free and supplemented media were collected, and the DNA ligase in sonicated extracts of these cells was examined. However, the activity was too low to be detected, since this strain has endonuclease activity. Thus, the extracts were purified by streptomycin precipitation and ammonium sulfate fractionation to remove nuclease activity. In the presence of NAD, the activities of DNA ligase in the two extracts were similar (Fig. 4). Therefore, NAD deficiency

![Figure 2](image1.png)  
**Fig. 2.**—Viable cells during niacin starvation. Cells were transferred to niacin-free medium at 0 hr. At 4 hr niacin was added.

![Figure 3](image2.png)  
**Fig. 3.**—Synthesis of DNA, RNA, and protein during niacin starvation. (a) At 0 time \(^{14}\text{C}-\text{adenine} (0.1 \mu\text{Ci/ml}) was added with nonradioactive adenine (10 \(\mu\text{g/ml}). \Delta-\Delta, \text{radioactivity in RNA fraction}; \bigcirc-\bigcirc, \text{in DNA fraction.} \ (b) At 0 time \(^{14}\text{C}-\text{amino acid} (0.5 \text{;Ci/ml}) was added. \bigcirc-\bigcirc, \text{radioactivity in protein fraction.}

<table>
<thead>
<tr>
<th>Time of starvation (hr)</th>
<th>Concentration of NAD ((M \times 10^6))</th>
</tr>
</thead>
<tbody>
<tr>
<td>0</td>
<td>44</td>
</tr>
<tr>
<td>1</td>
<td>14</td>
</tr>
<tr>
<td>2</td>
<td>4.4</td>
</tr>
<tr>
<td>2.5</td>
<td>2.8</td>
</tr>
</tbody>
</table>

The NAD concentration in cells at 0 time was calculated from Fig. 1, \(^{14}\text{C}-\text{niacin} (10.7 \text{mCi/mg}), 4.5 \times 10^6 \text{cells/ml. Other results were obtained by extrapolation of Fig. 1.}
Fig. 4.—Activity of ligase in niacin-starved cells; 10 ml of suspensions of normal cells and those after 3-hr NAD deficiency were used. DNA ligase was partially purified from the sonicated crude extract (1.4 mg of protein/ml) to the stage of ammonium sulfate fractionation.4 O··O, niacin-starved; ○-○, normal.

seems to cause suppression of ligase activity, rather than inactivation of the enzyme; the over-all effect is to cause apparent inhibition of DNA synthesis.

Characterization of DNA synthesized in NAD deficiency: DNA synthesized in NAD deficiency showed a reduced sedimentation rate in an alkaline sucrose gradient.18 When NAD-deficient cells were labeled with 3H-thymidine for two minutes and 30 minutes, similar profiles of sedimentation were obtained (Fig. 5). This smaller DNA was integrated into DNA of normal size ten minutes after addition of niacin (Fig. 5a), when DNA synthesis had not yet returned to the normal level16 but the normal NAD level had been restored (Fig. 1). Therefore, it seems that small DNA was integrated into larger DNA only in the presence of NAD, possibly by restoration of DNA ligase activity.

DNA was extracted by the procedure of Thomas21 from cells labeled with 3H-thymidine for five minutes after 75 minutes of niacin starvation. It was sedimented in neutral and alkaline sucrose gradients (Fig. 6). In the neutral gradient, the DNA synthesized in NAD deficiency was as large as control DNA (Fig.
6a), but in the alkaline gradient it was smaller (Fig. 6b). The size of the small fragments was calculated from the internal marker of λ and T4 DNA to be heterogeneous with a molecular weight of 16–38 × 10^6.

This DNA was analyzed by chromatography on a hydroxyapatite column (Fig. 7). Heat-denatured DNA was eluted with 0.17 M phosphate and native DNA with 0.22 M phosphate (Fig. 7a), while small DNA was eluted with 0.22 M phosphate (Fig. 7b), suggesting that it had a double-stranded structure.

DNA synthesized before transfer: Cells were labeled with H-thymidine for three generations in the presence of niacin and then transferred to niacin-free medium. The culture was then chased by addition of nonlabeled thymidine. Aliquots were removed at intervals, and DNA was analyzed in an alkaline sucrose gradient. It was shown clearly that no DNA synthesized before transfer was fragmented in NAD deficiency (Fig. 8), indicating that the small pieces of DNA accumulating (Fig. 5) were newly synthesized material.

Discussion.—In niacin starvation the cellular NAD content decreases to below the level necessary for DNA ligase activity (Table 1). NAD is mainly a coenzyme for dehydrogenases in the respiratory chain. Under our conditions synthesis of RNA and protein continued for at least three hours in niacin starvation (Fig. 3). This suggests that energy supply from the respiratory chain was not significantly affected when DNA synthesis was inhibited. These results might be interpreted as shown in Figure 9. In niacin starvation, NAD supply to compartments B and C would stop. In the respiratory chain, NAD is reduced to NADH which would be reconverted to NAD with the coupled system. On the other hand, in the ligase reaction NAD degrades to AMP and NMN, which would be one precursor of NAD. If NMN is converted to NAD in compartment

![Figure 7](image-url) DNA on hydroxyapatite column chromatography. (a) x-x, heat-denatured H-DNA; ••••, native DNA. (b) O—O, DNA from cells labeled with H-thymidine for 5 min in NAD deficiency. O—O, PO4.

![Figure 8](image-url) Sedimentation in alkaline sucrose gradient of DNA labeled before transfer to niacin-free medium. Cells were labeled with H-thymidine for three generations before transfer and collected at 0 time (O—O) 90 min (□—□), and 180 min (△—△) after transfer. Centrifugation was 30,000 rpm for 120 min.
A, supply of newly synthesized NAD to compartment B would be less than to compartment C (Fig. 9), so that the concentration of NAD would decrease preferentially in B. Compartment B is estimated to be a few per cent of the size of C. Assuming that DNA is replicated by coupling of DNA polymerase with DNA ligase, it is calculated that the NAD consumed in the ligase reaction is in the order of 2–4 per cent of the total. Thus, NAD deficiency seems to be a new system by which it is possible to study the function of DNA ligase in vivo.

It was suggested that in chromosomal replication small fragments were synthesized by DNA polymerase and then joined together by DNA ligase. These fragments were thought to be 1000 to 2000 nucleotides in length and single-stranded. The presence of a second intermediate which was double-stranded and a little larger than the first intermediate was also suggested. The DNA synthesized under our conditions was larger than either fragment and double-stranded (Fig. 7).

We showed that the apparent inhibition of DNA synthesis in NAD deficiency was due to suppression of cellular ligase activity. Thus, the final step of DNA replication seems to involve a process in which ligase functions in conversion of larger nucleotides to whole chromosomes. If this is the case, suppression of ligase activity by NAD deficiency mainly affects the final step of DNA replication, thereby inhibiting the initiation of DNA synthesis. The fact that larger DNA fragments accumulated in niacin deficiency and that they were integrated into normal chromosomes on addition of niacin suggested that they are intermediates of DNA as well as the second type described by Oishi, and that there is no significant inhibition of DNA polymerase under our conditions.

We wish to thank Dr. Y. Anraku for his valuable advice and suggestions.

1 Weiss, B., and C. C. Richardson, these PROCEEDINGS, 57, 1021 (1967).
4 Olivera, B. M., and I. R. Lehman, these PROCEEDINGS, 57, 1426 (1967).
5 Gefter, M. L., A. Becker, and J. Hurwitz, these PROCEEDINGS, 58, 240 (1967).
7 Lindahl, T., and G. M. Edelman, these PROCEEDINGS, 61, 80 (1968).
8 Olivera, B. M., and I. R. Lehman, these PROCEEDINGS, 57, 1700 (1967).
9 Okazaki, R., T. Okazaki, K. Sakabe, K. Sugimoto, and A. Sugino, these PROCEEDINGS, 59, 598 (1968).
10 Yudelevich, A., B. Ginsberg, and J. Hurwitz, these PROCEEDINGS, 61, 1129 (1968).
11 Pauling, C., and L. Hamm, these PROCEEDINGS, 60, 1495 (1968).
13 Sugimoto, K., T. Okazaki, and R. Okazaki, these PROCEEDINGS, 60, 1356 (1968).
15 Hosoda, J., and E. Mathews, these PROCEEDINGS, 61, 997 (1968).
18 Abbreviations used are recommended by J. Biol. Chem., 243, 1 (1968). The following abbreviations are used: dA, a homopolymer composed of deoxyadenylate residues; dT, a homopolymer composed of deoxythymidylate residues.
19 Richardson, C. C., these PROCEEDINGS, 54, 158 (1965).
26 Oishi, M., these PROCEEDINGS, 60, 329 (1968).
27 Ibid., p. 691.