Pyridine Nucleotide Metabolism in *Escherichia coli*

II. NIACIN STARVATION*

Ronald Lundquist and Baldomero M. Olivera

From the Department of Biology, University of Utah, Salt Lake City, Utah 84112, and the Department of Biochemistry, College of Medicine, University of the Philippines, Manila, Philippines

SUMMARY

The effect of niacin starvation has been studied in a niacin-requiring auxotroph of *Escherichia coli*. If a culture is totally deprived of niacin, cells continue to divide until the total pyridine nucleotide content has fallen from 1.9×10^6 to 1.2×10^5 molecules per cell. During starvation, the relative proportion of the pyridine nucleotides changes greatly: the TPN:DPN ratio increases from 0.30 to over 2.0 and nicotinic acid mononucleotide accumulates until it is present at concentrations comparable to DPN. The changes in the distribution of the pyridine nucleotides suggest that DPN is turning over during niacin starvation and that the normally observed breakdown of TPN to DPN during exponential growth is inhibited. If cells are starved for niacin by balanced growth under limiting concentrations of niacin, less disproportion in the TPN:DPN ratio is observed, and growth occurs with a pyridine nucleotide content as low as 4×10^4 molecules per cell.

The fate of the niacin ring in *Escherichia coli* during normal exponential growth is metabolically limited. Once the niacin ring is taken up, it is not excreted into the medium. Inside the bacterial cell over 95% is found as pyridine nucleotides DPN and TPN even after many generations (1).

In the studies described in this series of papers, we have attempted to define the various pathways through which the niacin ring can flow in *E. coli* and to understand the factors which regulate the flow of the niacin ring through the various pathways. Our particular interest in these studies stems from the discovery that DPN is the cofactor for DNA ligase in bacterial cells (2, 4), a molecule of DPN being broken down to nicotinamide mononucleotide for each DNA-joining event. It is the ultimate aim of our work to examine DNA ligase activity under different physiological conditions by measuring the kinetic parameters of niacin metabolism *in vivo*.

In this paper, we have studied the effect of niacin starvation on pyridine nucleotide metabolism in *E. coli*. Niacin starvation

causes an obvious perturbation on pyridine nucleotide metabolism: we have examined how this perturbation affects other reactions involving the niacin ring within the cell. For example, in our previous studies, we found that the TPN:DPN ratio was maintained largely by the rates of interconversion of TPN and DPN. In these studies we show that the rates of these interconversion reactions appear to change with the total pyridine nucleotide concentration.

Niacin starvation has also been used by other workers to examine DNA-joining activity (4). These earlier studies examined the fate of the DNA during the starvation but did not examine the effect on the pyridine nucleotides. We are undertaking a more detailed analysis of this physiological condition here.

In the following paper (III) we show how the regulation of endogenous synthesis and exogenous uptake of the niacin ring are regulated *in vivo* to yield a relatively constant level of total pyridine nucleotide (5).

In Paper IV of this series, the turnover cycles of pyridine nucleotide metabolism are defined and measured (6).

EXPERIMENTAL PROCEDURES

Materials—Labeled niacin and other pyridine nucleotides were obtained from Amersham-Searle Corp. Nicotinic acid mononucleotide was a generous gift from Dr. H. C. Friedmann of the Department of Biochemistry, University of Chicago. Nicotinic acid ribonucleoside was prepared by treating the nicotinic acid mononucleotide with bacterial alkaline phosphatase. Bacterial alkaline phosphatase was obtained from Worthington Biochemicals. All other biochemicals and enzymes were obtained from Sigma.

Other materials are described in the previous paper (1).

Starvation Experiments—Nicotinic acid-requiring strains of *E. coli* were starved for niacin in the following way. The strain was grown in an M9 medium containing 8.1×10^{-6} M (1 μg per ml) [14C]niacin to a cell density of about 3×10^8 cells per ml and then filtered rapidly on a Millipore filter (Bact-T-Flex B6, Schleicher and Schuell), washed with M9 salts (with no glucose), and resuspended and grown in a 20-fold larger volume of M9 medium containing no niacin. The number of cells per ml, the absorbance at 595 nm, and the uptake of 14C were followed as a function of time.

Niacin-limited Growth Experiments—Cultures were grown under conditions of limiting niacin as follows. A culture of *E. coli* 15T⁻ nic⁻ was grown in M9 medium containing 4.1 ×

10^{-6} M (0.5 μg per ml) of unlabeled niacin. This culture was then used to inoculate (1:100 dilution) M9 media containing limiting levels of [^{14}C]niacin (0.16, 0.40, and 0.81 μM; specific activity, 2.33 × 10^7 cpm per μmole). Each culture was allowed to grow for at least five generations in this medium before being diluted at least 50-fold into an identical medium. The absorbance at 595 nm, cell density, and [^{14}C]niacin uptake was then followed. This series of dilutions was necessary to make sure that balanced growth was occurring at the limiting niacin concentrations.

Measurement of Bacterial Volume—Cell volumes were measured at various stages of niacin starvation. Electron microscopy was performed as described by Chai and Lark (7) on an RCA-2 electron microscope. Cells were also examined by phase contrast illumination in a Zeiss photomicroscope using an apochromatic oil immersion objective (100 ×). Micrographs were made using ADOXKB14 film. Volumes were estimated from well focused cells on positive prints at × 3000 magnification.

For exponentially growing cultures, the cells were assumed to be cylindrical with two half spheres at both ends of the cylinder. Measurements were then taken of the length and width of the cylindrical portion of the cell and of the radii of the hemispheric ends. The cells that had been severely starved for niacin were more nearly spherically shaped, and the diameter of the sphere was measured on the photographs. We are indebted to Dr. David Wolstenholme for advice regarding these measurements, and for taking the photographs of the bacteria.

Other Methods—Methods for measurements of radioactivity taken up by the cell, for preparation of cell extracts, and for chromatography of the pyridine nucleotides are described in the previous paper (1).

Bacterial Strains—All niacin-requiring strains of *E. coli* used were described previously (1).

RESULTS

General Effects of Starvation for Niacin—The behavior of niacin auxotrophs of *E. coli* during niacin starvation is shown in Fig. 1. In this experiment, cells are grown in a medium containing [^{14}C]niacin, and at the time indicated, the culture is filtered, washed, and transferred to a medium without niacin. After transfer, the cells continue to divide with an unchanged or slightly faster growth rate for over 2 hours, after which cell division stops. During late starvation, substantial leakage of the intracellular radioactivity into the medium takes place. This is not due to cell lysis as there is no measurable decrease in viability. The leakage is a specific function of niacin starvation which stops when unlabeled niacin is added to the medium.

Cells deprived of niacin were examined by electron and photomicroscopy. Normal cells are more elongate than the almost spherical starved cells. Normal cells and starved cells were measured as described under "Experimental Procedures" and a continuous decrease in cell volume was found during starvation. Cells that were no longer dividing were found to be 30% of normal volume. Electron micrographs show that while exponentially growing cells generally have two nuclear regions, starved cells have one. Nozawa and Mizuno (4) have reported the formation of unusually long forms during niacin starvation in the auxotroph they have investigated. These forms do not occur to any significant extent upon starvation of *E. coli* 15T$^-$ nic$^-$.

The growth characteristics of *E. coli* during niacin starvation are generally reproducible in the four niacin-requiring strains (15T$^-$ nic$^-$, nic A, nic B, and nic C) which we have tested al-

though a certain amount of variation in the amount of growth after transfer to the medium lacking niacin, as well as in the rate of leakage of the radioactivity into the medium has been observed. However, in all cases, for 2 hours after deprivation of niacin, the rate of cell division was equal to or greater than the normal rate.

Changes in Intracellular Distribution of Pyridine Nucleotides during Niacin Starvation—Fig. 2 shows the intracellular distribution of pyridine nucleotides at various times after transfer to a medium lacking niacin. A marked transition takes place
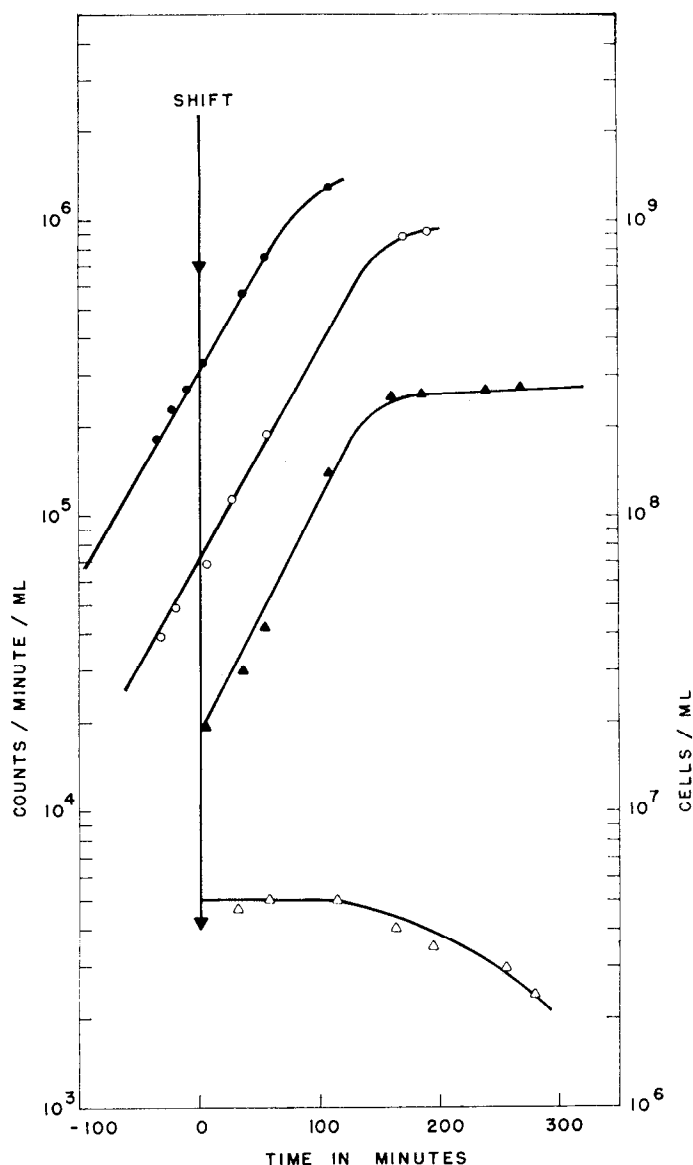
Fig. 1. Niacin starvation of *Escherichia coli* 15T$^-$ nic$^-$. A culture of *E. coli* 15T$^-$ nic$^-$ was grown in an M9 medium containing 8 × 10^{-6} M [^{14}C]niacin (59.1 Ci per mole). At the time indicated by the *arrow* an aliquot of the culture was filtered on a Millipore membrane filter, washed with M9 salts solution without glucose (M9-G), and transferred to an M9 medium without niacin at a 15-fold dilution. The cell density was determined at regular intervals by counting aliquots of the culture on a Coulter counter. Uptake of [^{14}C]niacin was measured by filtering a 0.1-ml aliquot of the culture on a membrane filter and washing with M9-G containing 40 μg per ml of unlabeled niacin. The *open circles* and *triangles* are measurements of [^{14}C]niacin uptake in the culture with and without niacin, respectively. The *closed circles* and *triangles* are the cell density in the culture with and without niacin, respectively.
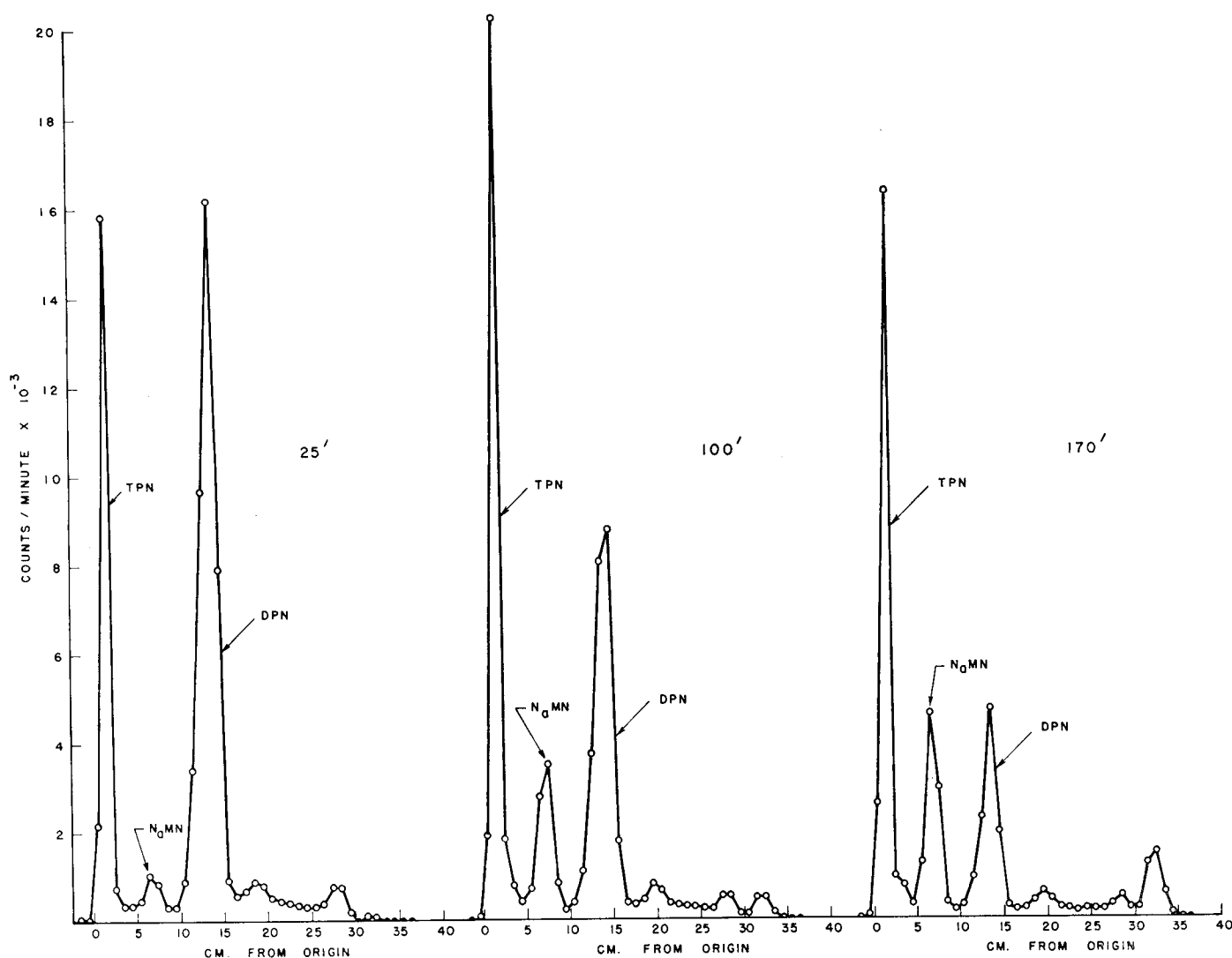
FIG. 2. Intracellular distribution of niacin during starvation. A culture of *Escherichia coli* 15T⁻ nic⁻ was grown in [¹⁴C]niacin and starved as described under "Experimental Procedures" and in the legend to Fig. 1. At 25, 100, and 170 min after transfer to the medium without niacin, samples for chromatography were collected, cell extracts were made and chromatographed on DEAE-paper for 5 hours ("Experimental Procedures"). Carrier DPN and TPN were added before chromatography; the identification of N_aMN is described in the text.

in the TPN:DPN ratio of the starved cells, which increases as much as 10-fold. In addition, a new peak of radioactivity is found (traveling between DPN and TPN on DEAE-paper). Three hours after the cells have been deprived of niacin, the amount of this new material and of DPN are approximately equal.

We have identified the new peak of radioactivity as nicotinic acid mononucleotide (N_aMN). The radioactivity isolated from the new peak traveled with authentic nicotinic acid mononucleotide on DEAE-paper chromatography. When both the radioactivity in the new peak and authentic carrier N_aMN were treated with bacterial alkaline phosphatase, the optical density and the radioactivity still traveled together upon chromatography, in a spot which moved just behind the solvent front (presumably nicotinic acid ribose). On treatment with alkali, most of the radioactivity, as well as the ultraviolet absorbing material derived from authentic N_aMN, traveled with the R_F of nicotinic acid (Fig. 3).

As shown in Fig. 1, a substantial fraction of the intracellular niacin leaks out into the medium during niacin starvation. After 4 hours of starvation, 50% of the radioactivity is lost to the medium. This radioactivity which is excreted into the medium has been examined by DEAE-paper chromatography (Fig. 4), and it is seen to travel just behind the solvent front as a single peak. This material has been tentatively identified as nicotinic acid ribonucleoside since it travels on chromatography with the R_F of authentic nicotinic acid ribonucleoside.

The relative distribution of the [¹⁴C]niacin label as a function of the time of starvation is shown on Fig. 5. The intracellular and extracellular levels of the pyridine nucleotides at various times after starvation are summarized on Table I.

Recovery from Starvation—The effect of adding back niacin to a culture of starved cells has been examined. In Fig. 6, it is seen that 30 min after niacin is added, normal intracellular levels are reached and cell division begins to take place. Cells return to their normal size during this 30-min lag period. The results of chromatography of the pyridine nucleotides during starvation and after niacin has been added back are shown in Fig. 7. This is a double label experiment in which the cells are first grown in the presence of [¹⁴C]niacin before starvation and [³H]niacin is added after starvation. It is seen that the [³H]niacin (which is taken up after starvation) assumes the intracellular distribution char-
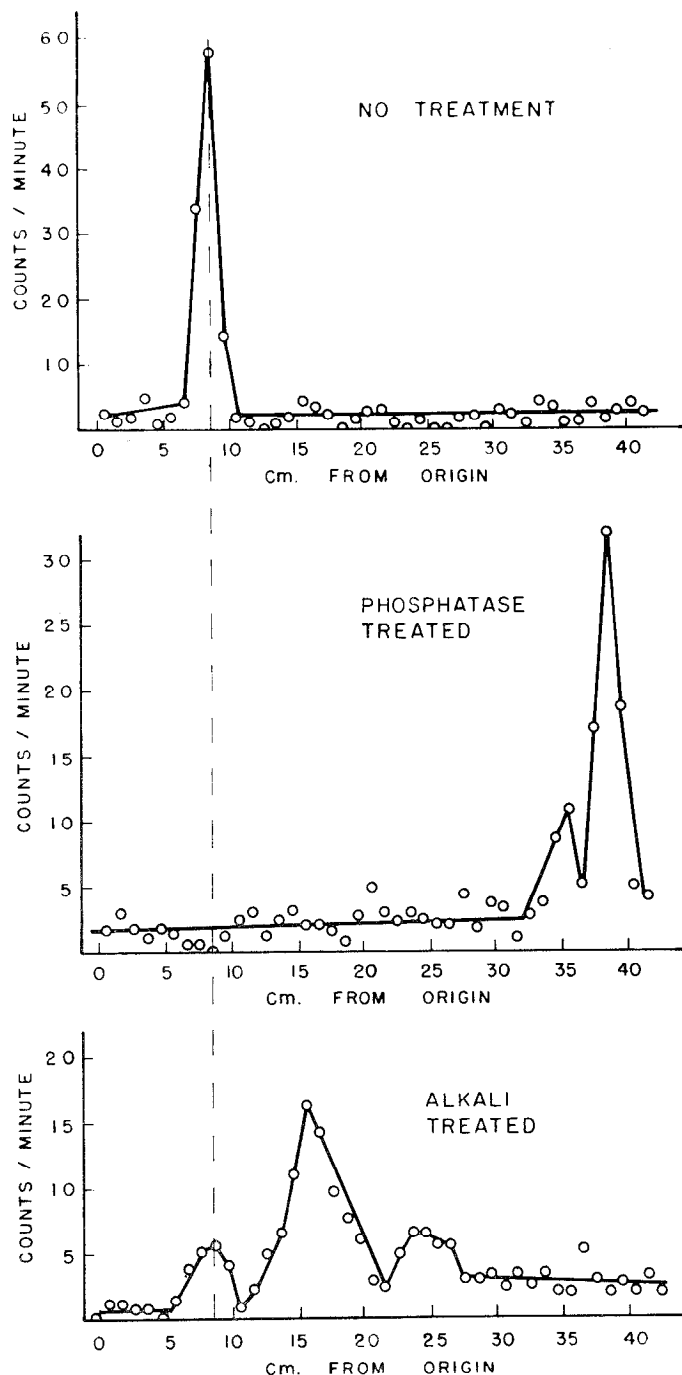
FIG. 4. Chromatography of extracellular radioactivity from a niacin-starved culture. A culture of *Escherichia coli* 15T⁻ nic⁻ was grown in [^{14}C]niacin and then starved for niacin for 330 min as described under "Experimental Procedures." One milliliter of the culture was filtered on a membrane filter and the filtrate was collected. The filtrate (0.1 ml) was chromatographed directly on DEAE-paper with added nicotinic acid and nicotinamide as carriers. The peak of radioactivity traveled with the same R_F as nicotinic acid ribonucleoside. Nicotinamide ribonucleoside and N-methyl nicotinamide traveled right with the solvent front, while nicotinamide mononucleotide traveled a few centimeters behind nicotinamide.

FIG. 3. Identification of nicotinic acid mononucleotide. A culture of *Escherichia coli* 15T⁻ nic⁻ was grown in an M9 medium containing [^{14}C]niacin and then starved for niacin ("Experimental Procedures") for 275 min. The culture was harvested, a cell extract was chromatographed on DEAE-paper with added N$_a$MN as carrier ("Experimental Procedures" and legend to Fig. 2). The material which ran between TPN and DPN on DEAE-paper chromatography (the peak 7 to 8 cm from the origin in Fig. 2) was eluted from the chromatogram using 0.4 ml of saturated NH$_4$HCO$_3$. This region of the chromatogram contained the N$_a$MN ultraviolet spot. The eluate was evaporated to a volume of 0.1 ml in a vacuum desiccator. The eluate was divided into three portions: to two 0.025-ml aliquots, 0.05 ml of 0.05 M Tris, pH 7.8, was added. To one of these aliquots 0.1 unit of bacterial alkaline phosphatase (0.05 mg of Worthington BAP-C) was added. Both mixtures were incubated for 15 min at 37°. The remaining 0.05-ml aliquot of eluate was mixed with 0.05 ml of a 5 mg per ml solution of authentic N$_a$MN and 0.05 ml of 0.33 M NaOH. The mixture was then incubated for 10 min at 50°,
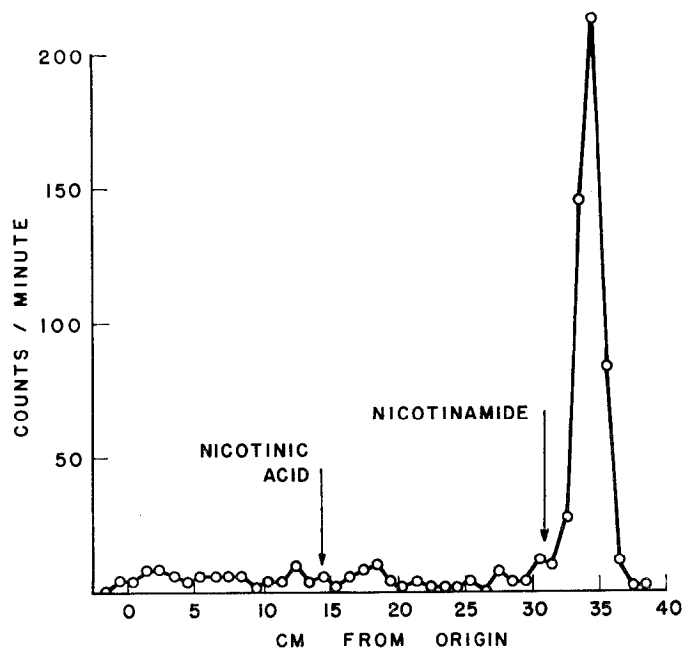
acteristic of exponentially growing cells (*i.e.* TPN:DPN = 0.3). The prelabeled niacin which is anomalously distributed as a result of starvation (TPN:DPN = 3.0) returns to the equilibrium TPN:DPN value of 0.57 expected of the DPN ⇌ TPN interconversion rates (1). The TPN:DPN ratio takes an hour before it returns to the equilibrium value; in contrast, the N$_a$MN present during starvation disappears after 25 min. The return of the TPN:DPN ratio from 3.0 to 0.57 confirms our previous suggestion (1) that there is only one pool of pyridine nucleotides in *E. coli*.

Growth of Niacin-requiring Mutants at Limiting Niacin Concentrations—We have also deprived *E. coli* 15T⁻ nic⁻ of niacin by growth under limiting concentrations of niacin. Under the growth conditions used, the niacin content per cell was found to be from 2 to 20% of normal levels. These cells divide with a significantly longer generation time than do normally growing cells (Table II).

A comparison of the intracellular niacin distribution in (*a*) cells starved for niacin by growing the cells in a relatively high niacin concentration followed by a shift to a medium without niacin and in (*b*) cells undergoing balanced growth in media containing very low niacin concentrations, is shown in Fig. 8.

cooled on ice, and cautiously neutralized with 1 M HCl to pH 7. After the above treatments, the three aliquots were chromatographed on DEAE-paper. Under these conditions, nicotinamide traveled 33 cm, niacin, 16 cm, and N$_a$MN, 8 cm from the origin. The ultraviolet peak was found to travel 7 to 9 cm in the control sample, 37 to 39 cm from the origin in the phosphatase-treated sample, and 15 to 18 cm in the alkali-treated sample.
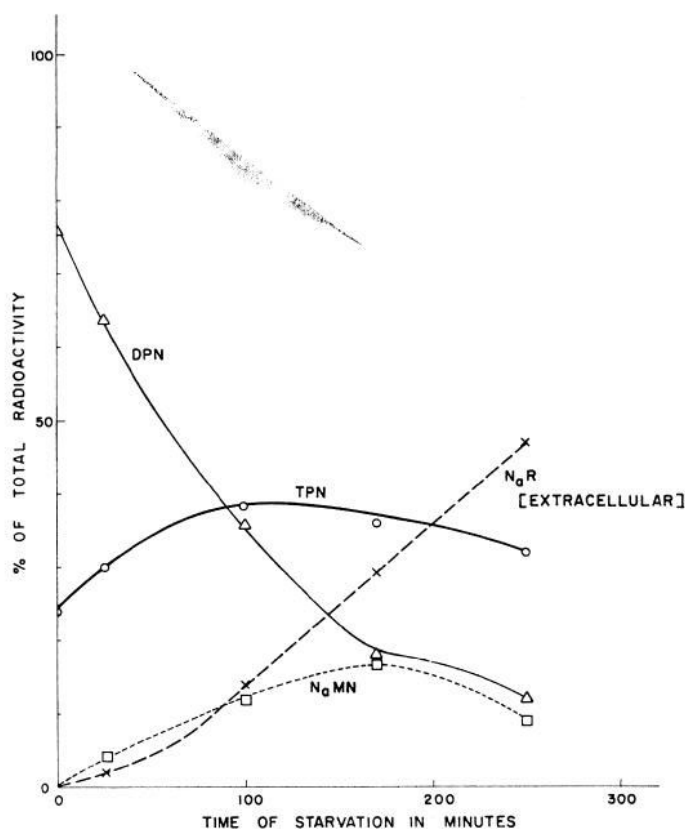
FIG. 5. The distribution of prelabeled niacin as a function of the time of starvation. The data were calculated from niacin uptake curves (Fig. 1), and chromatographic analysis of the intracellular pyridine nucleotides (see Fig. 2).

FIG. 6. Recovery from niacin starvation. A culture of *Escherichia coli* 15T⁻ nic⁻ was grown in 8×10^{-6} M [¹⁴C]niacin and then starved for niacin as described under "Experimental Procedures." After 400 min of starvation, cells were transferred to an M9 medium containing 8×10^{-6} M [³H]niacin (787 mCi per mmole). Uptake of radioactivity and cell density were followed at regular intervals throughout the experiment. The *open circles* are ¹⁴C uptake points; *closed circles*, cell density; and *triangles*, [³H]niacin uptake.

TABLE I

Intracellular niacin distribution during starvation

Escherichia coli 15T⁻ nic⁻ was grown in an M9 medium containing [¹⁴C]niacin, and then starved as described under "Experimental Procedures" and in the legend to Fig. 1. Cell extracts were prepared (Fig. 2), and the intracellular pyridine nucleotide distribution calculated from the chromatographic data. Intracellular niacin and percentage of original niacin found extracellularly were determined from uptake and cell density curves.

| Time of starvation | Intracellular niacin | Intracellular niacin as: | | | Original niacin extracellular |
		DPN	TPN	$N_a MN$	
min	*molecules/cell*	%	%	%	%
0	1.90×10^6	76	24	0	0
25	1.16×10^6	65	31	4	2
100	0.23×10^6	42	44	14	14
170	0.12×10^6	25	51	24	29
250	0.07×10^6	23	60	17	47

This shows that although the total niacin per cell may be the same in cultures starved in different ways, the resulting distribution of the niacin is different.

A decrease in cell size was observed under all niacin-limited conditions of growth. A cell growing in 0.05 µg per ml of niacin with a generation time of 70 min had an average cell volume 50% smaller than cells growing with the normal generation time for this medium (40 min).
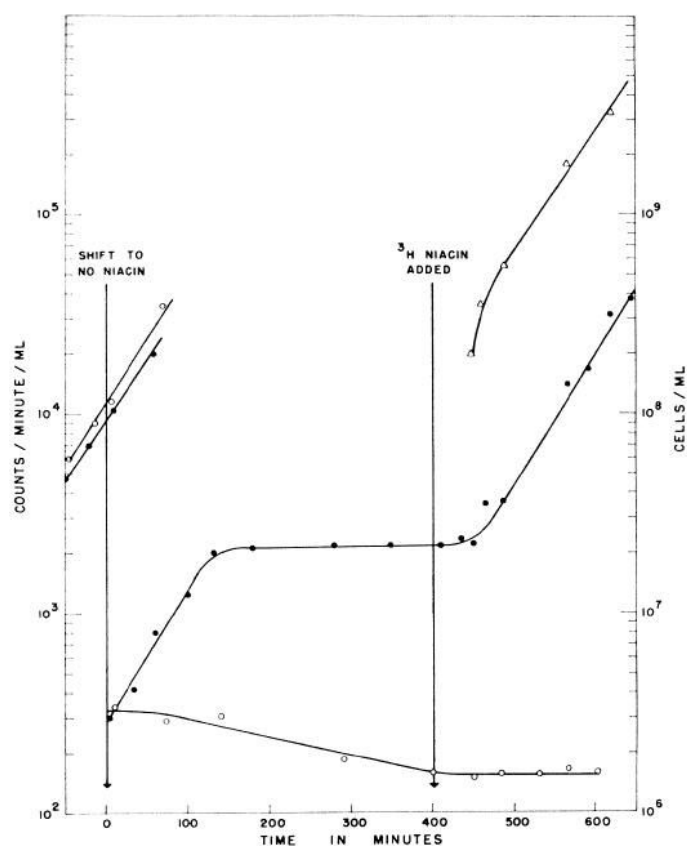
DISCUSSION

In previous studies (1), we found that the relative levels of DPN and TPN in *E. coli* were maintained by a steady state balance between the *de novo* synthesis of DPN, the synthesis of TPN from DPN, and the breakdown of TPN to DPN. It could be predicted that by altering the relative contribution of any one of the three factors, a change in the TPN:DPN ratio would occur. Thus, inhibiting the *de novo* synthesis of DPN should not only lower the levels of the total pyridine nucleotide in the cell, but change the proportion of TPN to DPN as well. We have inhibited *de novo* DPN synthesis by imposing niacin starvation on a mutant of *E. coli* which is unable to synthesize niacin endogenously. Our previous studies (1) lead to the prediction that if the rates of DPN and TPN interconversion remain constant the TPN:DPN ratio should increase from 0.30 to 0.57 during starvation.

We in fact observe that the TPN:DPN ratio increases continuously beyond a value of 0.57. There is also a net conversion of DPN to nicotinic acid mononucleotide and nicotinic acid ribonucleoside. The conversion of DPN to $N_a MN$ and nicotinic acid ribonucleoside may be a consequence of normal pyridine nucleotide turnover. The results suggest that niacin starvation causes the inhibition of a metabolic reaction believed to be involved in pyridine nucleotide turnover (8), the $N_a AD$ pyrophosphorylase reaction, $N_a MN + ATP \rightarrow N_a AD + PP_i$.

If this reaction were differentially affected by starvation possibly because of the direct requirement for ATP, the normal turnover of DPN would then result in conversion of DPN to N_aMN. In addition, if N_aMN accumulated during starvation, there is a possibility that it would be acted on by a phosphatase and be transformed to nicotinic acid ribonucleoside. Since the latter compound is not phosphorylated, it could leak out of the cell.

The data suggest, however, that during starvation, there is also an inhibition of TPN breakdown. The plots in Fig. 5 indicate that although after 100 min of starvation, DPN and TPN are present in approximately equal amounts, by 250 min, the total TPN radioactivity has decreased only slightly (from 37 to 32% of the total radioactivity) despite the much larger decrease in DPN (from 36 to 11%). This would suggest that the TPN present at 150 min is quite stable; if it were breaking down at the normal rate (140 molecules per s (1)), then the TPN radioactivity should remain roughly proportional to DPN radioactivity. These experimental results suggest that TPN levels increase with respect to DPN because: (a) DPN is turning over in the cell but is not re-formed efficiently due to inhibition of the N_aAD pyrophosphorylase reaction, and (b) the breakdown of
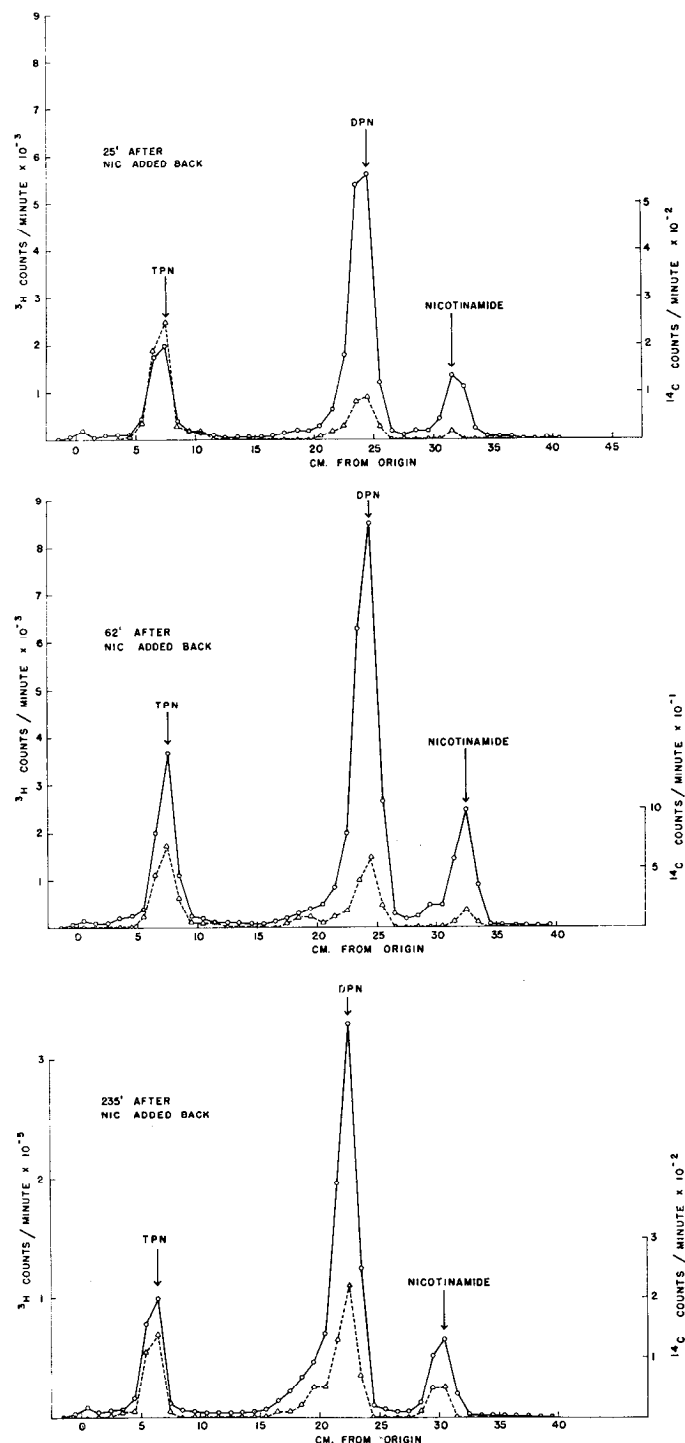

FIG. 7. Chromatography of intracellular pyridine nucleotide during recovery from starvation. Aliquots were taken for chromatography from the culture described in Fig. 6 at 25, 62, and 235 min after transfer to the culture containing [³H]niacin. Extracts were prepared and chromatographed as described under "Experimental Procedures." Radioactivity from ³H is shown by the *solid line* (*open circles*) and from ¹⁴C by the *dotted line* (*triangles*).

TABLE II

Intracellular niacin distribution under conditions of limiting niacin

Cultures of *Escherichia coli* 15T⁻ nic⁻ were grown under conditions of limiting niacin as described under "Experimental Procedures," to insure balanced growth. Intracellular pyridine nucleotide distribution was calculated from chromatographic data obtained from experiments similar to those described in Fig. 2.

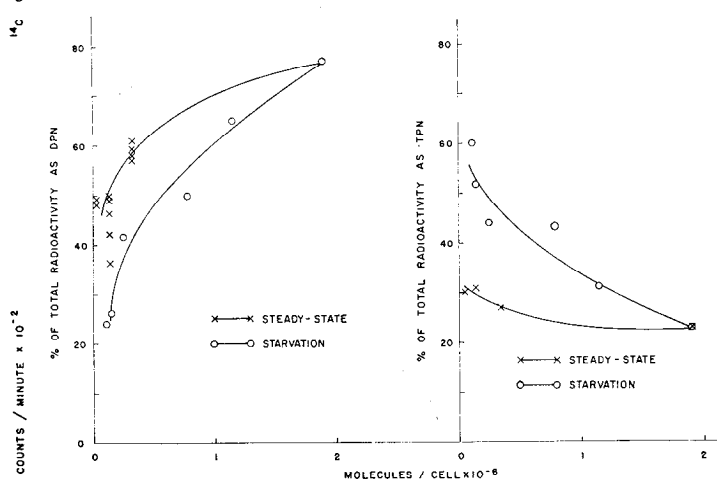| External niacin	Generation time	Intracellular niacin	Intracellular niacin as:		
			DPN	TPN	N_aMN
$\times 10^{-6}$ M	min	molecules/cell	%	%	%
8.1	40	1.90×10^6	76	24	0
0.81	47	0.32×10^6	60	27	13
0.40	70	0.18×10^6	50	31	19
0.16	120	0.04×10^6	49	30	21

FIG. 8. A comparison of the intracellular levels of DPN and TPN as a function of the total niacin content per cell under steady state conditions and starvation conditions. The fraction of DPN and TPN are calculated as a function of the total niacin content per cell for two types of growth conditions. The *open circles* ("*starvation*") represent cells grown in 8×10^{-6} M niacin and then starved. The different points are calculations at different times of starvation. $\times$ ("*steady state*") represents cells grown under balanced growth conditions in media containing limiting levels of niacin. The different points represent separate cultures grown in media containing different concentrations of niacin. The data on this figure are derived from Tables I and II.

TPN molecules to DPN is inhibited as the pyridine nucleotide concentration falls.

Two methods have been used for starvation, *i.e.* total deprivation of niacin or balanced growth under conditions of limiting niacin in the medium. This allows a comparison of two cells with the same amount of total pyridine nucleotide per cell, but which have been deprived of niacin in different ways. The comparison cannot be regarded as rigorous, since on the one hand cells are growing under balanced growth conditions, and on the other hand they are growing under unbalanced growth conditions. There is not as great a disproportionation of the TPN:DPN ratio in niacin-limited cells as there is in the totally starved cells, comparing cells that have the same total pyridine nucleotide content. This would be expected since the former cells are able to synthesize a little bit of DPN from the medium and therefore should have a relatively higher DPN level. An additional difference is the fact that the totally starved cells continue to divide at a normal rate (see Fig. 1), and then do not divide at all when the total pyridine nucleotide content has fallen below 120,000 molecules per cell. On the other hand, cells growing under limited niacin conditions have a lower rate of cell division but even cells with as low as 40,000 molecules per cell of total pyridine nucleotide still have the capacity to grow and divide. The unbalanced growth conditions and possibly the greater disproportion between TPN and DPN in the totally starved cells may account for the latter physiological observation.

Our results are consistent with the postulate that severe niacin starvation is accompanied by an ATP starvation. The accumulation of N_aMN under these conditions is of some interest in view of the unusual discovery by Friedmann and Cagen (9)

that N_aMN is a specific ribosyl donor in B-12 coenzyme biosynthesis. Since N_aMN is not detectable in normally growing *E. coli*, our results raised the interesting possibility that N_aMN may accumulate under other metabolic conditions related to an ATP deficiency, and therefore serve as a switch for B-12 biosynthesis.

The results above indicate that some care should be taken in interpreting experiments involving severe niacin starvation, since niacin starvation probably is accompanied by an ATP deficiency. Thus, the effects on DNA joining which have been reported due to niacin starvation (4) or the addition of cyanide (10) may not be due directly to lowering of DPN levels but due to the ATP deficiency. Recent studies suggest that optimal sealing of discontinuous chains requires ATP, in addition to the deoxynucleoside triphosphates and DPN.

REFERENCES

1. Lundquist, R., and Olivera, B. M. (1971) *J. Biol. Chem.* **246,** 1107
2. Olivera, B. M., and Lehman, I. R. (1967) *Proc. Nat. Acad. Sci. U. S. A.* **57,** 1426
3. Zimmerman, S. B., Little, J. W., Oshinsky, C. K., and Gellert, M. (1967) *Proc. Nat. Acad. Sci. U. S. A.* **57,** 1841
4. Nozawa, R., and Mizuno, D. (1969) *Proc. Nat. Acad. Sci. U. S. A.* **63,** 904
5. McLaren, J., Ngo, D. T. C., and Olivera, B. M. (1973) *J. Biol. Chem.* **248,** 5144
6. Manalapaz-Fernandez, P., and Olivera, B. M. (1973) *J. Biol. Chem.* **248,** 5150
7. Chai, N.-C., and Lark, K. G. (1970) *J. Bacteriol.* **104,** 401
8. Gholson, R. K. (1966) *Nature* **212,** 933
9. Friedmann, H. C., and Cagen, L. C. (1970) *Annu. Rev. Microbiol.* **24,** 159
10. Olivera, B. M., and Lundquist, R. (1971) *J. Mol. Biol.* **57,** 263