# THE RELEASE OF THE ACID-SOLUBLE NUCLEOTIDE POOL OF E. COLI BY EDTA-TRIS[1]

H. C. Neu[2], D. F. Ashman, T. D. Price[3]

Departments of Medicine, Biochemistry and Urology, Columbia University, New York

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In view of the current interest in the effect of EDTA-Tris treatment in altering the permeability of cells of Escherichia coli (Neu and Heppel, 1964b; Leive, 1965a,b,c; Girija and Sreenivasan, 1965; Moses and Sharp, 1966) we wished to extend and clarify some earlier observations on the release of acid-soluble nucleotide material from EDTA treated cells (Neu and Heppel, 1964a, 1965). At the same time we wish to point out that Tris-HCl itself alters the permeability of cells of E. coli as temperature is increased. Our studies suggest that the effect of EDTA-Tris treatment of E. coli is not as selective in altering permeability as originally thought (Leive, 1965c).

## Materials and Methods

E. coli K 15, K 12, and MRE 600, an RNase I-deficient mutant, were grown with vigorous aeration at 37°C on a minimal Tris-HCl medium or the high phosphate medium of Neu and Heppel, 1965. The carbon source was either 0.5% glucose or glycerol. Cells were harvested by centrifugation in early exponential phase when the concentration of cells was $10^8$ per ml. The cells were washed at 3°C with either 0.85% NaCl or 0.03 M NaCl, 0.01 M Tris-HCl pH 7.2. The packed cells were resuspended in the appropriate medium at either 3° or 23°C

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2. Research Scientist New York Health Research Council.

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at a concentration of 8 x 10" cells per ml, equivalent to 1 g wet weight of cells to 80 ml of medium. The concentration of Tris-HCl was either 0.1 or 0.12 M. The EDTA concentration was varied from 2 x $10^{-4}$ M to $10^{-3}$ M. At intervals cells were removed by rapid centrifugation at 0°C and the supernatant analyzed for absorbance at 260 mμ. Another sample of the supernatant was immediately incubated with 0.4 M perchloric acid (PCA) at 0° to remove any acid precipitable material. The supernatant fluid was assayed for release of RNase I, RNase II, β-galactosidase, 5'-nucleotidase and protein (Neu and Heppel, 1964b). Viability was determined by plating on tryptone agar. The PCA-soluble and TCA-soluble nucleotide pool of cells not exposed to EDTA was determined by treating washed cells with either 5% TCA or 0.4 M PCA at 0°.

The extent to which PCA-soluble purines and pyrimidines are released into the medium was determined by adsorbing the material on Norit. The compounds were eluted from Norit by 10% pyridine in 50% ethanol and evaporated to dryness. A portion of each concentrated Norit eluate was spotted on Whatman 3 MM chromatographic paper and developed with water saturated n-butanol: ammonium bicarbonate (solid) vapors. Another portion of the Norit eluate was subjected to two dimensional chromatography in descending isobutyric acid 0.5 M, $NH_4OH$ (5:3) for 21 hours and then in the other direction with descending 95% ethanol, 1 M ammonium acetate pH 7.5 (75:30) for 18 hours (Tsuboi and Price, 1959). All resulting spots of both systems were outlined fluoroscopically, excised, eluted and identified spectrally. The mole per cent was calculated using an average molar extinction coefficient of 12,000 for the mixture of nucleotides and bases applied to the chromatogram.

## Results and Discussion

All of the $A_{260}$ absorbing material was found to be acid-soluble and none could be adsorbed to millipore filters. The material was diphenylamine negative. The release of $A_{260}$ absorbing material by treating cells with $10^{-3}$ M EDTA in the presence of 0.12 M Tris-HCl pH 8.0 at 0°C is seen in Figure 1. There is a rapid release of material which is complete by 6-10 minutes and

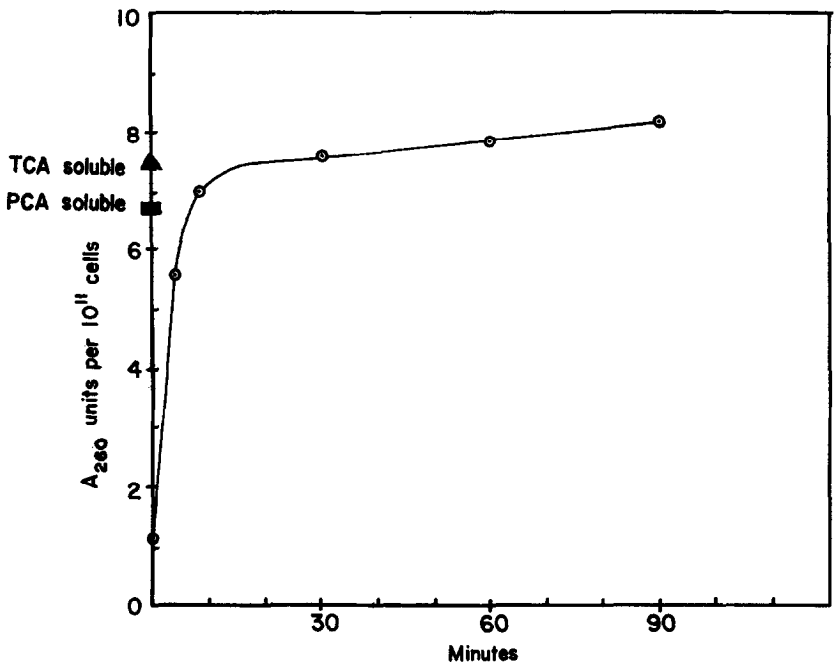


Figure 1. Kinetics of the release of acid-soluble UV-absorbing compounds from cells of E. coli K 15 suspended in $10^{-3}$ M EDTA, 0.12 M Tris-HCl pH 8.0 at 0°. TCA-soluble and PCA-soluble levels of nucleotides in untreated matched samples are indicated.

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remains fairly constant over the next 90 minutes. The nucleotide material is quantitatively equivalent to the TCA or PCA soluble nucleotide pool of the cells. Both the EDTA-Tris and PCA values for release of nucleotide material compare with previous studies of the PCA-soluble nucleotide pool of E. coli (Goldstein et al., 1960; Franzen and Binkley, 1961).

The material from the 30, 60 and 90 minute samples was pooled and subjected to chromatography and spectral analysis as outlined. This was compared with the PCA-soluble extract of matched cells. The per cent of $A_{260}$ material as nucleotides released by PCA treatment of control cells was 89.6% whereas in the EDTA-Tris treated cells at 0°C it was 83.5%. It would appear that the EDTA treatment does release the acid-soluble nucleotide pool of E. coli. Subsequent experiments on single time points show only minor differences between the 10 minute and 60 minute 0° samples. However, a 2 minute sample shows a

significantly lower per cent of nucleotides although the base and nucleoside components are equal. This would point to a diffusion process though a "leaky" cell which probably reaches maximum altered permeability at 0° by 4-10 minutes of exposure to EDTA-Tris.

The difference in mole per cent of various compounds, Table I, of the PCA-soluble pool and the EDTA-Tris released material may in part be due to

TABLE I

| Compound | Mole per cent of total | |
|---|---|---|
| | PCA-soluble | EDTA-Tris 0° extract |
| ATP | 3.94 | 1.60 |
| ADP | 3.43 | trace |
| AMP | trace | 8.5 |
| NAD | 5.8 | 4.9 |
| UDPG | 8.0 | 4.1 |
| UMP | trace | 3.5 |
| Uracil | 7.9 | 12.5 |
| Hypoxanthine | 4.6 | 5.2 |

Concentrated Norit eluates were chromatographed as described. Major components well isolated from others were eluted and estimated spectrally. The mole per cent was calculated using an average molar extinction coefficient of 12,000 for the mixture of nucleotides and bases applied to the chromatogram.

degradation of nucleotides. The base and nucleoside component released by EDTA-Tris is 2½ times greater than that released by PCA.

In view of the fact that this EDTA-Tris treatment fails to release surface enzymes as do the EDTA-lysozyme (Malamy and Horecker, 1964; Neu and Heppel, 1964a) or EDTA-osmotic shock methods of Neu and Heppel 1965, this degradation of nucleotides must occur intracellularly. Table II also demonstrates that the changes in the cell permeability are highly selective, for β-galactosidase is not released and viability remains high.

Following these studies at 0° we evaluated the effect of treating cells at 23° (Figure 2). A striking difference was immediately apparent. At 23° there is a progressive rise in the material absorbing at 260 mμ. This occurs with EDTA-Tris-HCl and Tris-HCl alone. However, at 0° no rise is seen in

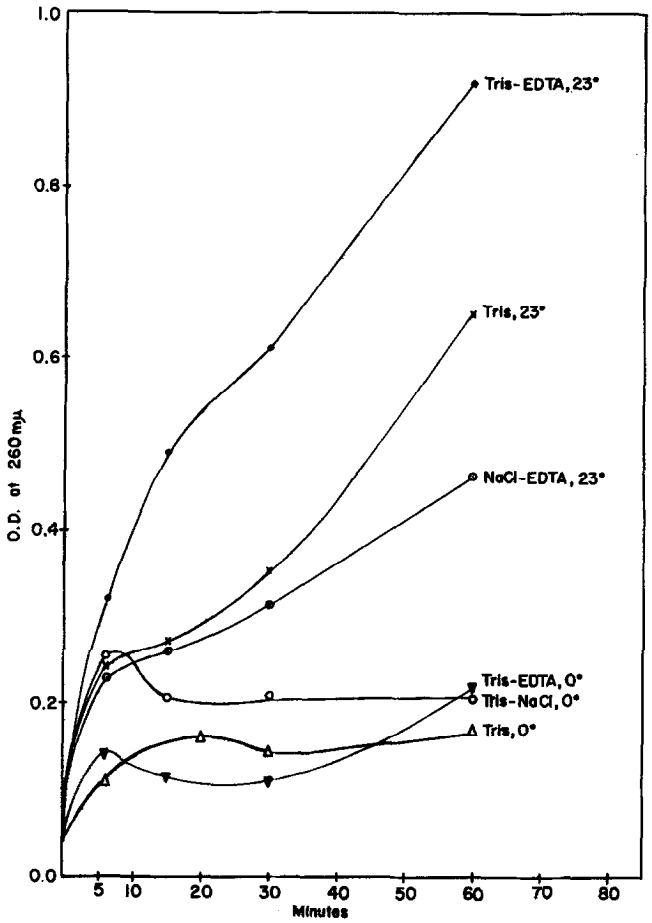


Figure 2. Release of $A_{260}$ absorbing material by E. coli K 12 exposed to $2 \times 10^{-4}$ M EDTA and 0.1 M Tris-HCl or NaCl at 23° or 0°. Samples obtained by removing the cells by rapid centrifugation in the cold.

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either case. When Tris is replaced by NaCl the rise is somewhat less rapid but this may be due to the less effective chelation at pH 7.0 than at pH 7.5. When potassium phosphate replaced Tris-HCl in a pH range from 7.0 to 8.0 no significant $A_{260}$ absorbing material was released at 23° for 90 minutes. Reducing the Tris concentration to 0.01 M and maintaining the osmolarity with sucrose also prevented the continued release of $A_{260}$ absorbing material. These observations suggest that the Tris-HCl is responsible in part for the changes in permeability and that pH or osmolarity are not the contributing factors.

Preliminary studies of the ribosomal and RNA patterns of the EDTA-Tris treated material show that the ribosomal patterns are not altered but that there appears to be loss of the 23s RNA with little effect on the 16s RNA. This would be in agreement with Natori et al. 1966 who found that ribosomes are rapidly degraded in the absence of exogenous $Mg^{++}$. There is in fact degradation of nucleotide material since with increasing time of exposure to the EDTA-Tris the amount of hypoxanthine, uracil, guanine and adenine rose while the per cent of nucleotide material released decreased. This clearly occurs within the cell without release of cell contents or degradative phosphatases.

TABLE II

| Treatment | | Units/g | | | Viability (per cent) |
|---|---|---|---|---|---|
| | | $A_{260}$ | -galactosidase | 5'-nucleotidase | |
| Tris | 0° | 7.1 | 0 | 48 | 92 |
| Tris-EDTA | 0° | 14.9 | 0.64 | 109 | 70 |
| NaCl-EDTA | 0° | 13.3 | 1.36 | 58 | 94 |
| Tris | 23° | 44.5 | 1.2 | 14 | 100 |
| Tris-EDTA | 23° | 23 | 9.4 | 124 | 65 |
| NaCl-EDTA | 23° | 33 | 8.4 | 53 | 90 |

E. coli, K 12, were exposed to 5 x $10^{-4}$ M EDTA and Tris-HCl or NaCl at 0.12 M at the specified temperatures. The acid-soluble $A_{260}$ and levels of $\beta$-galactosidase and 5'-nucleotidase were obtained from the cell supernatant at 40 minutes. Sonic extracts of matched untreated cells contained 1470 units/g of $\beta$-galactosidase and 2000 units/g of 5'-nucleotidase. RNase I and RNase II could not be detected in the supernatant fluid. Viability is expressed as per cent survival over a zero time sample. Dilutions were made into $10^{-3}$ M $Mg^{++}$, $10^{-4}$ M $Ca^{++}$ medium and plated on enriched agar.

The longer periods of exposure used in these experiments appear justified, for when we exposed cells to 2 x $10^{-4}$ M EDTA, Tris-HCl pH 8.0 for 4 minutes at 37°, the $A_{260}$ material released was equivalent to that released after 40 minutes at 23°. The material showed markedly increased amounts of free bases and we feel that at 37° degradation is great.

Although these experiments suggest that there is degradation of ribosomal material as well as of the nucleotides in the acid-soluble pool, the exact mechanism is unclear. Cells of a strain of E. coli lacking RNase I showed ex-

actly similar phenomena as other strains. RNase II and polynucleotide phosphorylase would be inhibited by the EDTA and resulting absence of $Mg^{++}$. The loss of RNA primarily from the 50s ribosomes suggests that there should be further study for an as yet undetected RNase.

Preliminary experiments on the effect of EDTA-Tris upon the acid-soluble nucleotide pool of other gram negative organisms, i.e. Salmonella typhimurium, Proteus vulgaris, Klebsiella-aerobacter and Paracolon yielded identical results.

These observations about the effect of EDTA and Tris-HCl upon the permeability of E. coli suggest that caution should be exercised in the interpretation of results from such cells. Although the cells are viable, they have lost their acid-soluble nucleotide pool and appear to have undergone degradation of some of the ribosomal RNA. Thus there may be significant changes in RNA metabolism which makes their use as normal models open to question.

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