Effect of Ethylenediaminetetraacetic Acid-Tris(hydroxymethyl)aminomethane on Release of the Acid-soluble Nucleotide Pool and on Breakdown of Ribosomal Ribonucleic Acid in *Escherichia coli*

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Received for publication 21 December 1966

Brief treatment of *Escherichia coli* with 2×10^{-4} M ethylenediaminetetraacetic acid (EDTA)-0.12 M tris(hydroxymethyl)aminomethane (Tris), pH 8.0, or 0.12 M Tris alone resulted in the release of the acid-soluble nucleotide pool at 3 or 23 C. Exposure to EDTA-Tris for up to 90 min at 3 C did not result in the release of increasing amounts of 260-m μ -absorbing material. At 23 and 37 C, EDTA-Tris resulted in a steady increase in acid-soluble 260-m μ -absorbing material. Previous growth environment did not alter the release. There appeared to be degradation of 23S ribonucleic acid (RNA) after 10 min of exposure at 23 C. In addition, there was degradation of nucleotides to nucleosides and bases. This occurred either within the cells with altered permeability or in the periplasmic space. This occurred in the presence of EDTA and Tris but was not seen with EDTA-phosphate. The mechanism of this degradation is unclear, since it occurs in ribonuclease I-deficient strains. Exposure to Tris buffer for long periods of time at 23 C resulted in release of the nucleotide pool and in degradation of RNA and nucleotides. These studies point out that the EDTA-Tris effect on *E. coli* must be divided into two parts, an early (4 to 5 min) change in permeability and a later phase of actual RNA breakdown and nucleotide degradation. Studies utilizing EDTA and Tris as agents altering permeability must thus be viewed with caution. Although the cells are viable, they have lost their acid-soluble nucleotide pool and have undergone degradation of some ribosomal RNA.

A variety of studies have recently been published on the effect of treatment of gram-negative bacilli with ethylenediaminetetraacetate (EDTA), usually in a tris(hydroxymethyl)aminomethane (Tris) buffer system. These studies have shown that a number of surface enzymes can be released by the use of EDTA-Tris and osmotic shock of *Escherichia coli* (18, 20) and that the acid-soluble pool of *E. coli* is probably released (19). Leive demonstrated that EDTA-treated cells are permeable to actinomycin D (11, 13), and Sellin, Srinivasan, and Borek (21) showed that such cells admit puromycin. Buttin and Kornberg (3) reported that deoxyribonucleoside triphosphates are able to penetrate whole cells

treated with EDTA. Leive (12) and Ashbell and Eagon (1) have shown that lipopolysaccharide is released by EDTA treatment. Several other investigators have applied the EDTA treatment for long periods of time, with conflicting results (5, 16).

These studies were undertaken to clarify the role of EDTA-Tris and Tris treatment in releasing the acid-soluble nucleotide pool of *E. coli*. It is clear that the acid-soluble pool is rapidly released by both EDTA-Tris treatment and by Tris alone. The studies show that the EDTA-Tris treatment causes damage to the cells as well as nonselectively altering permeability (12, 13). It is also shown that cellular ribonucleic acid (RNA) is affected by long exposure to EDTA-Tris and that exposure to EDTA-Tris

¹ Deceased 29 May 1966.

must be for short periods if the cells are to be used as models of RNA metabolism.

MATERIALS AND METHODS

Bacteria and media. *E. coli* K-15, K-12, K-37, and MRE 600, a ribonuclease (RNase) I-deficient strain, were used. Stock cultures were maintained on nutrient agar slants. A modified minimal medium of Levinthal et al. (14), the high-phosphate medium "C" of Neu and Heppel (20), or Difco Antibiotic Medium 3 were used. The carbon source was either 0.5% glucose or glycerol. Platings for survival were done on nutrient agar with 10^{-3} M Mg^{++} , 10^{-4} M Ca^{++} content.

Growth and cell preparations. Samples of overnight cultures were used to inoculate fresh medium. Growth was followed by turbidity at 600 $m\mu$. The cells were grown with vigorous aeration on a rotary shaker at 37 C and harvested by centrifugation in early exponential phase when the cell concentration was 10^8 per milliliter. The cells were washed twice at 3 C with 40 volumes of either 0.85% NaCl or 0.03 M NaCl-0.01 M Tris (pH 7.2). The packed, washed cells were suspended in the appropriate medium at either 3 or 23 C at a concentration of 8×10^{11} cells per milliliter. This is equivalent to 1 g (wet weight) of cells in 80 ml of medium. Tris was 0.12 M, pH 8.0 (measured at 23 C) unless otherwise specified. EDTA concentration was varied from 2×10^{-4} to 5×10^{-3} M. The mixture was gently agitated on a rotary shaker, and portions were removed and centrifuged at 0 to 3 C at $12,000 \times g$ for 5 min. The clear supernatant fluid was removed, and absorbance at 260 $m\mu$ (A_{260}) was measured. Part of the sample was made 0.4 M with 1 M perchloric acid (PCA) to remove any acid-precipitable material. Another part was frozen for enzyme and protein assays. The PCA-soluble nucleotide pool was determined by suspending 0.5 g of cells, which had been washed once with 0.85% NaCl, in 30 ml of 0.4 M PCA at 0 C for 30 min. The trichloroacetic acid-soluble nucleotide pool was determined by treating 0.5 g of cells with 5.25% trichloroacetic acid at 0 C for 30 min.

Identification of products. The extent to which purine and pyrimidine material was released into the suspension medium was determined by absorbing the material on Norit. These compounds were eluted from Norit by 10% pyridine in 50% ethyl alcohol, and the eluates were evaporated to dryness. Recovery on the basis of A_{260} units was 60%. A portion of each concentrated Norit eluate was spotted on Whatman 3 MM chromatographic paper and developed bidimensionally with water-saturated *n*-butanol-ammonium bicarbonate vapors. Di- and polyanionic polynucleotides are immobile in this system. Another portion of the Norit eluate was subjected to two-dimensional chromatography in descending 0.5 M isobutyric acid- NH_4OH (5:3) for 21 hr and then in the other direction with descending 95% ethyl alcohol-1 M ammonium acetate, pH 7.5 (75:30), for 18 hr (24). All resulting spots of both systems were outlined by ultraviolet (UV) light, excised, eluted, and identified spectrally. The amount (micromoles) of

each compound per gram of cells was then calculated by use of their molar extinction at pH 7.0.

Preparation of ribosomes and RNA. Ribosomes were prepared according to the method of Tissieres et al. (23) from cells ruptured by sonic treatment. RNA was prepared by the phenol method of Grier and Schramm (9). Sucrose density gradients of 5 to 20% were made by the method of Britten and Roberts (2).

Assays. The assays for 5'-nucleotidase, RNase I, and β -galactosidase are those previously described (18). RNase II was measured according to Singer and Tolbert (22), and protein according to Lowry et al. (15).

RESULTS

It was essential that the processing of the cells did not result in substantial loss of the intracellular nucleotide pool. For this reason, we determined the concentration distribution ratio of nucleotides (Table 1). This is defined as the concentration of PCA-soluble A_{260} units in the intracellular space divided by the concentration of PCA-soluble A_{260} units in the extracellular space (suspension medium). The higher the value, the less nucleotide material has been lost. Rein-

TABLE 1. Nucleotide concentration distribution ratio^a

Treatment	Time of incubation	Concn distribution ratio	A_{260} units released/g of cells
Growth medium, 37 C.....	3.9 hr	477	
0.85% NaCl, 3 C.....	2 hr	119	
0.12 M Tris, pH 8, 24 C.....	10 min	16	
10^{-3} M EDTA, 0.12 M Tris, 3 C.....	10 min	11	45.6
10^{-3} M EDTA, 0.12 M Tris, 23 C.....	10 min	6	47.0
5% Trichloroacetic acid.....			44.7
0.4 M PCA.....			43.2

^a *E. coli* K-15 was grown to early exponential phase in the modified minimal medium with glucose as the carbon source, centrifuged, and resuspended in the growth medium or 0.85% NaCl at a concentration of 1 g per 80 ml. The 0.12 M Tris (pH 8) and 10^{-3} M EDTA-Tris (0.12 M, pH 8.0) samples had been washed once with 0.85% NaCl. At the noted times, the cells were centrifuged and the supernatant fluid at 0 C was made 0.4 M with respect to PCA by addition of a 1 M PCA. The acid-soluble A_{260} material released was then determined by reading against appropriate blanks. The A_{260} units released per gram of cells were determined for freshly harvested control cells by treating them with 5% trichloroacetic acid or 0.4 M PCA at 0 C.

cubating cells in the growth medium and washing cells with 0.85% NaCl did not result in appreciable loss of nucleotide material. Washing cells with 0.01 M Tris (pH 7.2)-0.03 M NaCl also did not release significant UV-absorbing material. The material which was released in the absence of EDTA-Tris had an extinction maximum at 255 $m\mu$ and a minimum at 240 $m\mu$ at pH 7.0. It was mainly free purines and pyrimidines, with the major component being hypoxanthine. Thus, the cell membranes retained their impermeability to nucleotides during processing. On the other hand, cells exposed to 0.12 M Tris (pH 8) at 24 C or Tris-EDTA (10^{-3} M) at 3 and 23 C showed a very low intracellular concentration of nucleotide material. The material released showed a UV maximum at 260 $m\mu$ and a minimum at 235 $m\mu$ at pH 7.0. All of the material released was soluble in PCA and trichloroacetic acid. It could not be absorbed to membrane filters and was diphenylamine-negative, which speaks against oligonucleotide release or deoxyribonucleic acid (DNA) breakdown. It was quantitatively adsorbed to acid-washed Norit. Table 1 further shows that the A_{260} material released into the EDTA-Tris medium was equal to the A_{260} material released by treatment of control cells with 5% trichloroacetic acid or 0.4 M PCA. These values are consistent with those previously obtained by other investigators (4, 7, 10). Thus, exposure to 0.12 M Tris (pH 8.0) for 10 min at 23 C or to 0.12 M Tris- 10^{-3} M EDTA at 3 or 23 C released the cells' acid-soluble nucleotide pool. The cells exposed to 0.12 M Tris (pH 8.0)- 10^{-3} M EDTA were able to take up ^3H -actinomycin in the manner of Leive (13), but the 3 C sample was not checked in this regard. Similarly, the cells could take up radioactive ribonucleotide diphosphates at 23 C. When Tris buffer alone was used, the sample at 23 C did not allow the uptake of either actinomycin or nucleotides.

At 0 to 3 C, the release of the "true" acid-soluble nucleotide pool was complete by 10 min, with no appreciable rise due to polymer breakdown in the next 90 min. Cells exposed to 5×10^{-4} EDTA-0.12 M Tris (pH 8.0) for 60 min at 3 C lost 5 to 10% of their phenol-extractable RNA. Table 2 compares the release of A_{260} material at 3, 23, and 37 C. At 3 C, there was only a 6% rise in A_{260} material between 9 and 25 min. At 23 C, the rise was 23%. Most significantly, the A_{260} material released in 3 min at 37 C exceeded the "true" acid-soluble pool. The purine and pyrimidine material released at 23 and 37 C is due to generation of an "abnormal" acid-soluble pool due to RNA breakdown. Exposure to EDTA-Tris at 37 C must be for less than 5 min if RNA breakdown is to be avoided.

Figure 1 follows the release of A_{260} material after the initial phase. It is clear that at 23 C there was a progressive release of material into the Tris-EDTA medium. The increased release with Tris alone was less impressive, but was significant when compared to Tris or Tris-EDTA at 3 C.

TABLE 2. Time course of release of A_{260} material^a

Temp	Time of incubation (min)		
	3	9	25
C			
3	6.9	7.8	8.3
23	7.8	9.0	11.6
37	13	34	57

^a The release of A_{260} material by EDTA-Tris treatment, expressed as A_{260} units per sample. *E. coli* K-37, a mutant originally derived from K-10 by Zinder, was grown to early exponential phase in the high phosphate medium with glucose, harvested by centrifugation, and washed once with 0.85% NaCl at 3 C. The cells were resuspended in 0.12 M Tris (pH 8.0)- 5×10^{-4} M EDTA at the indicated temperatures. Samples were removed, at the times indicated, by rapid centrifugation. The supernatant fluid was treated with 0.4 M PCA, and the acid-soluble A_{260} material released was determined.

FIG. 1. Release of 260- $m\mu$ -absorbing material by EDTA-Tris. MRE 600, RNase I-deficient, was grown in high phosphate medium with glucose as the carbon source and harvested. The twice-washed cells were rapidly suspended in the indicated media at 5×10^{-4} M EDTA and gently agitated at 3 or 23 C. Samples were removed and centrifuged at the indicated times. The supernatant fluid was read immediately at 260 $m\mu$.

NaCl-EDTA, buffered to pH 7.5 with 5×10^{-3} M potassium phosphate at 23 C, showed a continued release of A_{260} material; in other experiments, this was consistently much less than with Tris alone at 23 C. This experiment is of particular interest because the *E. coli* strain used lacks RNase I, which might be postulated as the cause of the RNA degradation. Evidence for a combined effect of Tris buffer and EDTA in altering cell permeability has been seen in studies with *Pseudomonas aeruginosa* (1) and *Azotobacter vinlandii* (6).

The material released by EDTA-Tris was of a similar general composition to that extracted by PCA (Table 3). It was possible to isolate and identify the following compounds: adenosine mono-, di-, and triphosphates, uridine diphosphoglucose, uridine monophosphate, uracil, guanine, and hypoxanthine. The amount of each released by EDTA-Tris was closely similar to that released by 0.4 M PCA.

Although there was more base and nucleoside material released by the EDTA-Tris treatment of cells, the percentage as nucleotide material was the same as that extracted by 0.4 M PCA.

E. coli cells were exposed to EDTA-Tris for 10 and 60 min at 3 and 23 C to evaluate the effect of time and temperature (Table 4). At both 3 and 23 C, the A_{260} values for 60 min were greater than the 10-min values. A substantial amount of A_{260} material was released by Tris alone after 60 min at 23 C. However, maximal release of nucleotide material occurred after 10 min at 3 C. The percentage of the A_{260} material that was nucleotide in type was actually less after 10 min at 23 C, and declined to the lowest level at 60 min at 23 C. The percentage of nucleotide material after 60 min of exposure to Tris at 23 C was also less than

the optimal value, seen with 10 min of EDTA-Tris exposure at 3 C. The reason for this is seen in Tables 5 and 6. At 23 C, there was breakdown of the nucleotides, and the nucleosides and bases were more readily released, accounting for the high A_{260} -released values. Breakdown of nucleotides could not have been caused by alkaline phosphatase, since its formation was suppressed by the high phosphate growth medium. 5'-Nucleotidase is not released into the medium, and, at the pH 8 of the medium, acid phosphatase is inactive. Thus, the breakdown of the nucleotides must occur in the cytoplasm or in the periplasmic space.

Although viability was good after 10 min of treatment (70 to 90%), long exposures to EDTA-Tris resulted in marked decreases in viability. This occurred even without significant loss of cellular protein or evidence of cell lysis. In other

TABLE 3. Composition of material released by EDTA-Tris^a

Treatment	A_{260} units/sample		Per cent nucleotides
	Bases, nucleosides	Nucleotides	
PCA (0.4 M).....	0.492	4.23	89.6
EDTA-Tris, 3 C.....	1.218	6.12	83.5

^a PCA-soluble samples from a 30- and a 60-min incubation of *E. coli* K-15 in 10^{-3} M EDTA-0.12 M Tris (pH 8.0) at 3 C were pooled and adsorbed to Norit. Analysis is based on the fact that di- and poly-anionic nucleotides are immobile in *n*-butanol saturated with water in the presence of NH_4HCO_3 while the nucleosides and bases are mobile.

TABLE 4. Effect of time and temperature of EDTA-Tris treatment^a

Treatment	Time	Temp	A_{260} units/g of cells	A_{260} units as nucleotides	Protein released	5'-Nucleotidase	Viability
	min	C		%	mg/g	units/g	%
Tris-EDTA	0	3	24.1	66.7	10.1	71	100
	10	3	47.1	86.0	11.2	222	65
	60	3	77.5	85.3	13.6	275	43
	10	23	85.0	52.4	12.8	109	70
	60	23	196.3	40.0	18.0	147	40
Tris	60	23	121.7	44.5	14.6	110	88

^a *E. coli* K-15 cells were prepared as in Table 1. Cells were suspended in 0.12 M Tris (pH 8.0) and 2×10^{-4} M EDTA at a level of 1 g to 80 ml of medium. A_{260} units were calculated from the PCA-soluble cell supernatant fluid. The percentage as nucleotides was based on chromatographic separation. Viability was from samples diluted in 10^{-2} M Mg^{++} , 10^{-3} M Ca^{++} medium, and plated on nutrient agar. A sonic extract of the cells yielded 3,900 units/g of 5'-nucleotidase, and the EDTA-Tris osmotic shock method (19) releases 90 to 100% of the 5'-nucleotidase. The 0-min sample actually represents 2 to 3 min, the time needed to suspend and centrifuge the cells. The PCA-soluble A_{260} units per gram of cells is 42.

TABLE 5. Nucleotides released by exposure to EDTA-Tris and Tris

Treatment	Time	Temp	Compound ^a (μmoles/g of cells)			
			AMP	ATP	NAD	UDPG
Tris-EDTA	<i>min</i>	<i>C</i>				
	0	3	Trace ^b	Trace	Trace	Trace
	10	3	0.0946	0.0314	0.0914	0.0680
	60	3	0.1174	0.0998	0.1811	0.1965
	10	23	0.1001	0.0446	0.0403	0.0594
	60	23	0.0744	0.0500	Trace	0.0783
Tris	60	23	Trace	—	—	—

^a Material that occupied the glucose diphosphate, uridine diphosphate, glucose triphosphate, and uridine triphosphate areas was not well separated in this run. The components listed were well separated and easily identified spectrally. AMP, adenosine monophosphate; ATP, adenosine triphosphate; NAD, nicotinamide adenine dinucleotide; UDPG, uridine diphosphogluconate.

^b Compounds detected by fluoroscopic examination but estimated to be present at less than 1 mole per 100 ml are listed as trace.

TABLE 6. Chromatographic separation of compounds released by EDTA-Tris^a

Treatment	Time	Temp	Compound (<i>A</i> ₂₆₀ units/sample)			
			Uracil	Guanine	Hypoxanthine	Nucleosides
Tris-EDTA	<i>min</i>	<i>C</i>				
	0	3	0.966	—	0.346	0.187
	10	3	0.608	0.008	0.186	0.160
	60	3	1.341	0.150	0.678	0.411
	10	23	5.520	1.032	2.532	1.293
	60	23	9.720	2.268	4.380	1.107
Tris	60	23	6.768	1.704	1.704	0.960

^a Samples were subjected to bidimensional chromatography in *n*-butanol. The individual spots were eluted and identified spectrally and in other solvent systems.

experiments, release of β -galactosidase was used as a measure of cell lysis.

Table 5 documents the amounts of four nucleotides in the medium after various periods of exposure to EDTA-Tris at 3 and 23 C. Table 6 shows that, with increasing time of exposure, there are increasing amounts of free bases and nucleosides.

The mechanism of the degradation of the RNA to yield the nucleotides and then nucleosides and bases is unclear. RNase I is not released into the medium by this exposure, in contrast to the situation with spheroplasts (19). However, it might be possible for RNase I to leave the periplasmic space, enter the cell cytoplasm, and thereupon degrade RNA. Against a role for RNase I is the fact that this process occurs in RNase I-deficient cells. The role of polynucleotide phosphorylase and RNase II under these conditions is unknown.

Further studies were performed to study the

effect of various ions on this release of the acid-soluble nucleotide pool. Several strains of *E. coli* were grown in a medium high in phosphate, Mg^{++} , and Ca^{++} and then subjected to EDTA-Tris treatment at 3 and 23 C. There were no differences that could be related to the previous growth medium. *A*₂₆₀ material of similar composition was released in all cases at similar rates. However, RNA breakdown was not studied. There was no significant change in the results if cells were washed with 0.85% NaCl or with 0.03 M NaCl-0.01 M Tris (pH 7.2). When EDTA was replaced by Ca-EDTA and 0.12 M Tris (pH 8.0) was used, the release of *A*₂₆₀ material still occurred.

The effect of pH on the nucleotide release was investigated with both Tris and phosphate as the buffer system (Table 7). At a more alkaline pH, there was greater release of *A*₂₆₀ material when Tris was the buffer. When phosphate buffers of comparable pH were used, this did not occur.

TABLE 7. Effect of pH upon the release of A_{260} material^a

Treatment	pH	A_{260} after incubation			
		2 min	10 min	30 min	60 min
Tris	7.2	0.085	0.389	0.567	1.391
Tris	7.5	0.266	0.478	0.978	2.208
Tris	8.4	0.198	0.580	1.681	3.154
K ₂ PO ₄	7.0	0.090	0.123	0.165	0.339
K ₂ PO ₄	7.5	0.066	0.099	0.178	0.508
K ₂ PO ₄	8.0	0.059	0.085	0.191	0.614

^a *E. coli* K-15 was grown in modified minimal medium with glucose as the carbon source. The cells were harvested in early exponential phase, washed twice, and suspended in 5×10^{-4} M EDTA and either 0.12 M potassium phosphate or 0.12 M Tris at 23 C. Samples were removed at the indicated times, and the A_{260} material released was determined.

Thus, it probably is not just an effect of better chelation at the alkaline pH, but is due to Tris itself.

The fact that the phosphate buffer did not alter the permeability of the cells in regard to loss of nucleotide material is interesting in view of earlier work by Hurwitz et al. (10), who showed that phosphate promoted nucleotide excretion in the presence of streptomycin (which has a strong chelating action). It is important to use a minimal amount of EDTA, namely, 2×10^{-4} M, to obtain the highest viability, although permeability has been altered. Use of 5×10^{-3} M EDTA at 23 or 37 C in our hands has resulted in nonspecific damage to the cells with leakage of internal enzymes.

Figure 2 shows the resumption of growth in cells exposed to EDTA-Tris and Tris alone. There was a lag in the Tris-EDTA cells (90 min) and the Tris cells (70 min), compared to the NaCl control lag of 45 min. This was seen even in the 10^{-3} M Mg⁺⁺, 10^{-4} M Ca⁺⁺ medium. When cells were placed in a medium to which no Mg⁺⁺ was added, the lag was greatly prolonged. These results were seen whether the EDTA-Tris exposure was at 3 or 23 C. Examination of the return of alkaline phosphatase synthesis in cells grown in high phosphate, exposed to EDTA-Tris, and then placed in a low phosphate, high Mg⁺⁺ medium showed that the amount of alkaline phosphatase paralleled growth exactly but appeared earlier in the control cells.

Earlier studies (19) on the release of RNase I from *E. coli* by spheroplast produced with EDTA and lysozyme showed that the ribosomes of treated (10 min) and untreated cells were identical

in sucrose density gradient pattern as well as in RNA and protein content.

In the present experiments, the samples treated with EDTA-Tris at 3 C for from 10 to 60 min had a phenol-extractable RNA content ranging from 87 to 95% of the control sample. The 10 min, 23 C EDTA-Tris sample was 89% of the control, but the 60 min, 23 C sample had fallen to 66% of the control RNA. A 23 C, 60 min Tris sample had 89% of the control RNA.

Sucrose density gradients of the ribosomes and phenol-extracted RNA of control and EDTA-Tris-treated cells are shown in Fig. 3. Ribosomes from control cells and cells exposed to EDTA-Tris at 23 C for 60 min are quite similar. However, when the RNA of such cells is com-

FIG. 2. Resumption of growth after EDTA-Tris treatment. *E. coli* K-37₂, a mutant of K-10 having a low content of cyclic phosphodiesterase, DNA endonuclease, and glucose-6-phosphatase, was grown in high phosphate medium with glucose as the carbon source. Cells were harvested and washed as usual. They were suspended in 0.12 M NaCl, 0.12 M NaCl- 2×10^{-4} M EDTA, 0.12 M Tris (pH 8), or 0.12 M Tris (pH 8)- 2×10^{-4} M EDTA at a concentration of 1 g (wet weight) of cells to 80 ml of medium. After 30 min at 23 C, the cells were 25-fold diluted in Penassay Broth containing 10^{-3} M MgCl₂ and 10^{-4} M CaCl₂. They were then grown in a water bath-rotary shaker at 37 C. Change in turbidity at 600 mμ was followed.

FIG. 3. Sedimentation analysis of ribosomes and of RNA extracted from cells. Sucrose gradients were made of ribosomes and phenol-extracted RNA from control cells and cells treated with 0.12 M Tris ($\text{pH } 8.0$)- $2 \times 10^{-4}\text{ M}$ EDTA at 23 C for 60 min. The ribosomes were centrifuged for 2.75 hr at 34,000 rev/min. The RNA was centrifuged for 7.5 hr at 34,000 rev/min. The top figures represent control cells; the bottom figures, cells treated with EDTA-Tris.

pared, there is a change in the 23S component. At the same time, there is a minor rise in 4-5S material. Table 4 shows that, after 60 min of exposure to EDTA-Tris at 23 C , eight times the amount of A_{260} material is in the medium, compared with an increase in protein of less than twofold. These studies suggest that there is breakdown of the 23S RNA with long exposure to EDTA-Tris.

Other organisms. Studies were done with *Salmonella typhimurium* and *Proteus mirabilis*, showing similar release of A_{260} material by exposure to EDTA-Tris (Table 8). A variety of *Enterobacteriaceae* were studied (*Klebsiella-Aerobacter*, *Citrobacter*, *Paracolocentrum*, *Serratia marcescens*), all showing similar findings.

DISCUSSION

The results presented here show that EDTA-Tris treatment of *E. coli* results in the release of the acid-soluble nucleotide pool. During growth,

as well as during processing in isotonic saline, this pool is held firmly inside the cell; on exposure to EDTA and Tris at 3 or 23 C , or Tris alone at 23 C , this pool is released. The process is rapid, essentially complete by 10 min; previous growth environment does not affect this release of nucleotide material. The action of the Tris buffer, which can by itself cause release of the pool, remains unexplained. Studies with *P. aeruginosa* showed an increased lethal effect of Tris-EDTA but not of Tris alone (1). Although Tris does release the acid-soluble nucleotide pool, viability remains high after periods as long as 60 min. It is clear that part of the Tris effect is pH-related, for there is greater loss of A_{260} material at $\text{pH } 8.0$ than at 7.2 . But pH is not the only factor, since phosphate buffer in the range of $\text{pH } 7.0$ to 8.0 showed no striking differences.

These studies point out that the EDTA-Tris effect must be broken down into two parts. The initial effect, which occurs in about 10 min at

TABLE 8. *Effect of EDTA-Tris on the acid-soluble pool of Salmonella typhimurium and Proteus mirabilis*^a

Organism	EDTA concn	<i>A</i> ₂₆₀ (units/g)
<i>S. typhimurium</i>	10 ⁻⁴	35
	10 ⁻³	43
<i>P. mirabilis</i>	10 ⁻⁴	37
	10 ⁻³	44

^a *S. typhimurium* and *P. mirabilis* were grown to early exponential phase in high phosphate medium with glycerol as the carbon source, harvested, and washed as usual. They were placed in 0.12 M Tris (pH 8.0) with the stated concentration of EDTA at 23 C for 10 min; the cell supernatant fluid was then removed and the PCA-soluble *A*₂₆₀ material determined. The PCA-soluble pool for *S. typhimurium* was 38 *A*₂₆₀ units and for *P. mirabilis*, 33 *A*₂₆₀ units.

3 C or in 4 to 5 min at 23 or 37 C, is the alteration of permeability, and we assume the release of wall lipopolysaccharide which Leive (12) and Ashbell and Eagon (1) have shown to occur; thereafter, there is probably actual breakdown of RNA and degradation of nucleotide material. There would appear to be loss of the 23S RNA in a manner analogous to the changes that Natori et al. (17) saw in Mg⁺⁺-depleted cells. The consistent lag in growth of such cells, even in the presence of high Mg⁺⁺ and Ca⁺⁺, tends to support this. The mechanism is unclear, since this occurs with cells lacking RNase I. The role of RNase II and polynucleotide phosphorylase is unknown. The breakdown of the nucleotides to free bases and nucleosides could occur either intracellularly or in the periplasmic space, since nucleotides released into the medium can re-enter the cell (3). The specific phosphatases or nucleotidases that do this are also not readily identified. It is possible that this does occur by activation of the periplasmic 5'-nucleotidase, but this is unproved. In the case of long exposure to Tris buffer alone, it would seem that degradative enzymes are activated to degrade the nucleotide pool and some RNA.

It is clear that caution must be exercised in the use of the EDTA-Tris model to alter the permeability of *E. coli* or other *Enterobacteriaceae*. If the exposure to EDTA is for a brief period of time, the model is justified. Longer periods, greater than 10 min, will result in changes in RNA metabolism and will not allow the cells to be used as a normal model. Even with short periods of exposure to EDTA in Tris medium, it should be noted that the acid-soluble nucleotide

pool is released and subsequent RNA metabolism is thus altered.

ACKNOWLEDGMENTS

This investigation was supported by a Career Scientist Award of the New York Health Research Council and by Public Health Service research grants AI-06840-01 from the National Institute of Allergy and Infectious Diseases and Ca-03179-09 from the National Cancer Institute.

LITERATURE CITED

1. ASBELL, M. A., AND R. G. EAGON. 1966. Role of multivalent cations in the organization, structure, and assembly of the cell wall of *Pseudomonas aeruginosa*. *J. Bacteriol.* **92**:380-387.
2. BRITTEN, R. J., AND R. B. ROBERTS. 1960. High-resolution density gradient sedimentation analysis. *Science* **131**:32-33.
3. BUTTIN, G., AND A. KORNBERG. 1966. Enzymatic synthesis of deoxyribonucleic acid. *J. Biol. Chem.* **241**:5419-5427.
4. FANZEN, J. S., AND S. B. BINKLEY. 1961. Comparison of the acid-soluble nucleotides in *Escherichia coli* at different growth rates. *J. Biol. Chem.* **236**:515-519.
5. GIRIJA, N. S., AND A. SREENIVASAN. 1965. Messenger RNA turnover and protein synthesis in actinomycin inhibited *Escherichia coli*. *Biochem. Biophys. Res. Commun.* **21**:404-411.
6. GOLDSCHMIDT, M. C., AND O. WYSS. 1966. Chelation effects on *Azotobacter* cells and cysts. *J. Bacteriol.* **91**:120-124.
7. GOLDSTEIN, D. B., B. J. BROWN, AND A. GOLDSTEIN. 1960. Amino acid starvation in an *Escherichia coli* auxotroph. *Biochim. Biophys. Acta* **43**:55-61.
8. GRAY, G. W., AND S. G. WILKINSON. 1965. The action of ethylenediamine tetra-acetic acid on *Pseudomonas aeruginosa*. *J. Appl. Bacteriol.* **28**:153-164.
9. GRIERER, A., AND G. SCHRAMM. 1956. Infectivity of ribonucleic acid from tobacco mosaic virus. *Nature* **177**:702-703.
10. HURWITZ, C., C. L. ROSANO, AND R. A. PEABODY. 1963. Excretion of ultraviolet-absorbing material by leaky and non-leaky strains of *Escherichia coli* exposed to streptomycin. *Biochim. Biophys. Acta* **72**:80-86.
11. LEIVE, L. 1965. Actinomycin sensitivity in *Escherichia coli* produced by EDTA. *Biochem. Biophys. Res. Commun.* **18**:13-17.
12. LEIVE, L. 1965. Release of lipopolysaccharide by EDTA treatment of *Escherichia coli*. *Biochem. Biophys. Res. Commun.* **21**:290-296.
13. LEIVE, L. 1965. RNA degradation and the assembly of ribosomes in actinomycin-treated *Escherichia coli*. *J. Mol. Biol.* **13**:862-875.
14. LEVINTHAL, C., A. KEYAN, AND A. HIGA. 1962. Messenger RNA turnover and protein synthesis in *B. subtilis* inhibited by actinomycin D. *Proc. Natl. Acad. Sci. U.S.* **48**:1631-1638.
15. LOWRY, O. H., N. J. ROSEBROUGH, A. L. FARR,

- AND R. J. RANDALL. 1951. Protein measurement with the Folin phenol reagent. *J. Biol. Chem.* **193**:265-275.
16. MOSES, V., AND P. B. SHARP. 1966. Effect of actinomycin on the synthesis of macromolecules in *Escherichia coli*. *Biochim. Biophys. Acta* **119**:200-203.
17. NATORI, S., R. NOZAWA, AND D. MIZUNO. 1966. The turnover of ribosomal RNA in *Escherichia coli* in a magnesium deficient stage. *Biochim. Biophys. Acta* **114**:245-253.
18. NEU, H. C., AND L. A. HEPPPEL. 1964. On the surface localization of enzymes in *Escherichia coli*. *Biochem. Biophys. Res. Commun.* **14**:215-219.
19. NEU, H. C., AND L. A. HEPPPEL. 1964. The release of ribonuclease into the medium when *Escherichia coli* cells are converted to spheroplasts. *J. Biol. Chem.* **239**:3893-3900.
20. NEU, H. C., AND L. A. HEPPPEL. 1965. The release of enzymes from *Escherichia coli* by osmotic shock and formation of spheroplasts. *J. Biol. Chem.* **240**:3685-3692.
21. SELLIN, H. G., P. R. SRINIVASAN, AND E. BOREK. 1966. Studies of a phage-induced DNA methylase. *J. Mol. Biol.* **19**:219-222.
22. SINGER, M. F., AND G. TOLBERT. 1965. Purification and properties of a potassium-activated phosphodiesterase (RNase II) from *Escherichia coli*. *Biochemistry* **4**:1319-1330.
23. TISSIERES, A., J. D. WATSON, D. SCHLESSINGER, AND B. R. HOLLINGWORTH. 1959. Ribonucleoprotein particles from *Escherichia coli*. *J. Mol. Biol.* **1**:221-233.
24. TSUBOI, K. K., AND T. D. PRICE. 1959. Isolation, detection and measure of microgram quantities of labeled tissue nucleotides. *Arch. Biochem. Biophys.* **81**:223-237.