CONTROLLING EDTA TREATMENT TO PRODUCE PERMEABLE *ESCHERICHIA COLI* WITH NORMAL METABOLIC PROCESSES

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Treatment with ethylenediaminetetraacetate (EDTA) causes a permeability change in *E. coli*, with the result that molecules normally excluded can be tested for their effect on intracellular metabolic processes (Leive, 1965a,b). For instance, actinomycin can enter, permitting studies of RNA and protein synthesis impossible in intact *E. coli*. Such studies are valid only if these permeable cells are otherwise normal, so a controlled method of EDTA treatment that yields cells with an apparently normal rate of growth, RNA, and protein synthesis, was devised (Leive, 1965a). Recently, however, it has been found that certain very similar procedures involving EDTA can injure RNA metabolism, causing release of the nucleotide pool and inducing RNA breakdown (Neu, Ashman and Price, 1966, 1967). It is therefore important to know exactly which steps of EDTA treatment are critical to avoid these and other intracellular injuries. The present paper will show that the previously described method does not cause such defects, and that permeable cells with normal metabolic processes, including a normal nucleotide pool, can be prepared if the following conditions of EDTA treatment are met: (a) chilling cells in the presence of Tris is avoided (b) EDTA treatment is for 2 minutes or less (c) treated cells are used experimentally before they repair their permeability barrier.

Materials and Methods - Two strains of *E. coli* were employed: AB 1105, previously used in studies of EDTA treatment, permeability, and RNA synthesis (Leive, 1965a,b) and a strain of K 12 donated by Dr. L. A. Heppel, previously used in studies of osmotic shock (Neu and Heppel, 1964, 1965). Both were

grown at 37° with aeration on a Tris-based minimal medium (Leive, 1965c) with 0.5% glucose as the carbon source. Viability was assayed by diluting and plating (Leive, 1965a).

Treatment with EDTA was as previously described (Leive, 1965a): cells growing exponentially were harvested at a density of 6 x 10^8/ml, centrifuged and washed once at room temperature with Tris-Cl pH 8, 0.12 M, and resuspended at a density of 4 to 6 x 10^9/ml at 37° in the same buffer. EDTA, 2 x 10^{-4} M final concentration, was added and 2 min later the cells were diluted 10-fold with prewarmed complete medium. Details of variations in procedure are given in the legends.

RNA synthesis was measured by incubating cells at 37° in medium containing uracil-2-^{14}C (1 mc/m mole; 50 μM final concentration). At various times during a 20 min incubation, during which time the rate was linear, portions were removed and added to TCA, 5% final concentration. The precipitates were collected on Millipore filters and counted in a scintillation counter as described previously (Leive, 1965c).

Actinomycin sensitivity was determined by measuring the rate of ^{14}C-uracil incorporation in the presence and absence of actinomycin (10 μg/ml final conc). The actinomycin insensitivity of a sample is defined as

$$\frac{\text{cpm incorporated per minute in the presence of actinomycin}}{\text{cpm incorporated per minute in the absence of actinomycin}} \times 100$$

The actinomycin sensitivity of a sample equals 100 minus the actinomycin insensitivity.

The perchloric acid (PCA)-soluble pool was measured by suspending cells in 0.4 M PCA at 4° for 10 min, centrifuging at 4°, and measuring the absorbance at 260 mμ of the supernatant. The remaining ultraviolet (uv)-absorbing material of the cell was assayed by hydrolyzing the pellet in 0.1 N HCl for 30 min at 95°, centrifuging, and measuring the absorbance at 260 mμ of the supernatant.

Results - (a) Time course of EDTA action.

As soon as EDTA is added to cells in Tris-Cl buffer at 37° (Figure 1)

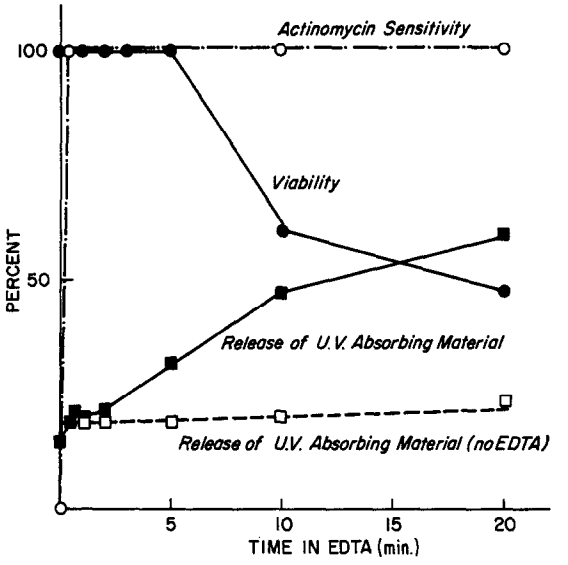

Fig. 1. Some effects of treatment with EDTA for various lengths of time. E. coli AB 1105 was washed and resuspended as described in the Methods. One portion received EDTA at 0 time and another served as a control. Just before addition of EDTA and at various times thereafter, samples were added to $MgCl_2$ (10^{-3} M final concentration) to terminate the treatment and were tested for actinomycin sensitivity and for viability. The viability of the control sample remained at 100%, and its actinomycin sensitivity remained at 0%, throughout the experiment. Portions of each sample were also centrifuged and the absorbance at 260 mμ of the supernatant was measured. The total uv-absorbing material of the culture was estimated as described in the Methods. The percent of uv-absorbing material released is defined as

$$\frac{\text{absorbance at 260 m}\mu\text{ of the supernatant}}{\text{total uv-absorbing material of the culture}} \times 100$$

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their permeability increases, as measured by actinomycin sensitivity, which becomes complete within 15 seconds. If EDTA treatment is prolonged, other facets of cell metabolism are affected. Thus, viability begins to drop after several minutes of treatment, and a large fraction of the uv-absorbing material of the cell is gradually lost. The size of this loss suggests that RNA is gradually broken down. RNA destruction caused by extended exposure to EDTA has also been observed by Neu et al (1967).

The above results show that, to preserve the metabolic integrity of the cell, EDTA treatment must be for 2 minutes or less.

(b) Minimizing loss of the nucleotide pool.

Neu et al (1966, 1967) using E. coli K 12, have found that under their conditions Tris alone, or Tris in combination with EDTA, can cause release of all the acid soluble uv-absorbing material of the cell even during a very short exposure at 37° such as the above 2 minute treatment. They therefore have questioned the advisability of using EDTA-treated cells in studies of RNA metabolism (Neu et al, 1966) since cells lacking a nucleotide pool can hardly be expected to synthesize RNA normally.

We find that EDTA or Tris treatment at 23° or 37° does not remove the acid-soluble pool, but Tris at 4° does cause loss of this pool. Since exposure to cold Tris is always part of the procedure of Neu et al (1966, 1967), it may explain their results. Table 1 (experiment 1) shows that when E. coli K 12 was prepared according to their procedure by washing in Tris-NaCl at 4°, and then was resuspended at 37°, the cells had a very low acid-soluble pool, regardless of whether they were resuspended in Tris, Tris-EDTA, or the usual growth medium. However, if they were centrifuged and washed at room temperature, their acid-soluble pool remained normal, again regardless of whether they were resuspended in Tris, Tris-EDTA, or the normal growth medium. Cells exposed to cold Tris also occasionally lost acid-precipitable uv-absorbing material, presumably RNA, which also is illustrated in Table 1. A variable amount of the lost uv-absorbing material was found in the Tris wash, and the remainder escaped when the cells were resuspended at 37°.

Other experiments indicated that washing with medium, or with NaCl, at either 4° or 23°, yielded cells with equivalent, apparently normal, acid soluble pools. It therefore proved possible to collect treated cells by centrifugation at 4° prior to assay if a 10-fold excess of medium was added to stop the action of Tris (e.g., Table 1 experiment 1). In some experiments Neu et al (1966, 1967) did not initially wash cells with Tris, but with NaCl, so those cells were probably not injured during washing. However, they always "terminated" the subsequent room temperature Tris or Tris-EDTA treatment by chilling the samples to 4° without adding medium or divalent cations to protect

TABLE 1

EFFECTS OF TEMPERATURE, TRIS, AND EDTA
ON RNA SYNTHESIS AND THE NUCLEOTIDE POOL IN *E. COLI* K 12

E. coli K 12 was harvested and washed at 4° or 23°. In experiment 1 the cells were washed with 0.03 M NaCl, 0.01 M Tris-Cl pH 7.2, and in experiment 2 they were washed with 0.12 M Tris-Cl pH 7.5. In both experiments they were then resuspended at 37° in (a) Tris-Cl 0.12 M pH 8 (b) Tris-Cl 0.12 M pH 8, EDTA 2×10^{-4} M (c) complete medium. Two minutes later the samples were diluted with a 10-fold excess of medium to stop the action of EDTA and/or Tris.

In experiment 1 the diluted samples were centrifuged at 4° and the PCA-soluble pool and remaining insoluble uv-absorbing material were assayed as described in the Methods. As a control, exponentially growing cells were centrifuged and washed with 0.85% NaCl at 4° and their pool and insoluble uv-absorbing material were then assayed as described in the Methods (*c.f.* Neu *et al*, 1967). The control value represents the average of 5 separate experiments ± the standard deviation.

In experiment 2 the diluted samples were incubated at 37° and incorporation of ^{14}C-uracil into acid-precipitable material measured as described in the Methods. As a control the incorporation of ^{14}C-uracil into the parent culture of normal *E. coli* K 12 growing exponentially at 37° was measured.

temperature during centrifugation & washing	resuspended at 37° in	Experiment 1: Absorbance at 260 mμ / mg dry weight, PCA soluble (A)	Experiment 1: Absorbance at 260 mμ / mg dry weight, PCA insoluble (B)	Experiment 1: pool as fraction of total $\left(\frac{A}{A+B}\right)$	Experiment 2: ^{14}C-uracil incorp. cpm per min / mg dry wt.
	control for expt. 1	0.42±0.04	8.9±0.5	0.045±0.003	—
	control for expt. 2	—	—	—	6700
4°	(a) Tris	0.16	7,6	0.020	1400
"	(b) Tris-EDTA	0.17	6.6	0.025	500
"	(c) medium	0.16	6.5	0.021	1100
23°	(a) Tris	0.37	8.9	0.041	6000
"	(b) Tris-EDTA	0.34	9.1	0.036	5800
"	(c) medium	0.39	9.3	0.040	5600

the cells, and so presumably lost the pool at this point in the procedure.

The destructive effect of cold Tris is reflected in its effect on RNA synthesis. In experiment 2 of Table 1 cells of *E. coli* K 12 were washed with

0.12 M Tris-Cl pH 7.5, a buffer in use in this laboratory for washing cells preparatory to EDTA treatment (Leive, 1965a). They were treated at 37° with Tris, Tris-EDTA, or medium for 2 minutes, and after they were diluted with medium their rate of RNA synthesis was assayed by measuring incorporation of labeled uracil into acid-precipitable material. As a control, the rate of RNA synthesis of an exponentially growing culture was measured. Cells washed at room temperature synthesized RNA at 85-90% of the control rate, and subsequent treatment with EDTA or Tris at 37° did not lower this rate. Cells washed at 4° could synthesize RNA at only a fraction (10-25%) of the control rate.

In the experiments of Table 1, no attempt was made to control for the change in pH of Tris caused by the change in temperature. In other experiments it was found that this pH change did not affect the results, which were independent of pH in the range of 7 to 8.

The above results show that EDTA-treated cells retain normal RNA synthetic ability if the cells are not chilled during washing with Tris.

(c) Regeneration of impermeability to actinomycin.

When EDTA-treated cells grow, they repair their permeability barrier, thus regaining insensitivity to actinomycin. Figure 2 shows the time course of this repair for E. coli AB 1105. Different strains vary slightly in their repair rate, and the rate is generally proportional to the growth rate. For most strains repair of the permeability barrier is complete after half a generation.

Thus, to perform metabolic studies employing actinomycin the cells must not be allowed to grow long enough to become insensitive to actinomycin.

Discussion - The underlined sentences above indicate three conditions that, if met, yield actinomycin sensitive cells with apparently normal RNA metabolism: EDTA treatment must be for a short period of time (between 15 seconds and 2 minutes), the cells should not be chilled during washing with Tris, and subsequently should not grow sufficiently long to become insensitive to actinomycin. These conditions are met in the previously described (Leive, 1965a) method of EDTA treatment.

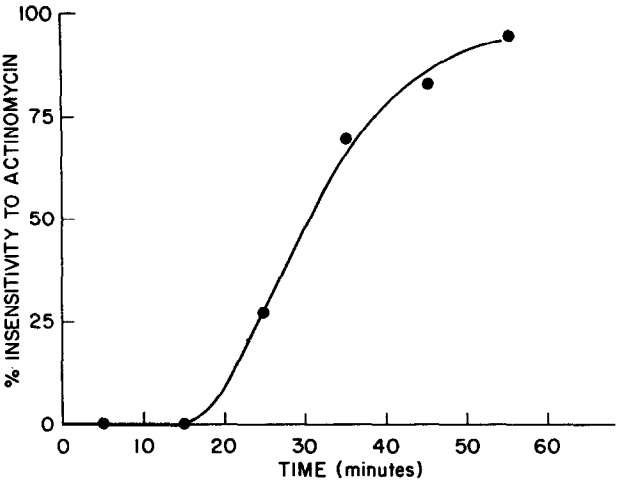

Fig. 2. Repair of the permeability barrier in EDTA-treated cells. E. coli AB 1105 was washed, resuspended and treated with EDTA. Dilution with medium was at 0 time on the graph. Portions were tested for actinomycin sensitivity at various times thereafter.

The effect of cold Tris presents an interesting parallel to the results of Strange and coworkers, who found that cold shock causes exponential cultures of Aerobacter aerogenes to lose intracellular constituents to the medium (Strange and Dark, 1962) and also results in RNA degradation when these chilled cells are resuspended at 37° (Strange and Postgate, 1964).

The above results suggest certain controls that are useful when EDTA-treated cells are used in metabolic studies. For instance, incorporation of tracers into RNA can be used to show that the cells synthesize RNA at a rate comparable to normally growing cells, and that all of this synthesis is sensitive to actinomycin. In addition, any investigation of systems not previously studied in EDTA-treated cells should include experiments indicating that these systems are functioning normally. For instance, to validate a recent study of β-galactosidase and thiogalactoside transacetylase induction in EDTA-treated E. coli, we felt it neeessary to show that the kinetics of induction of these two enzymes, and their differential rate of synthesis, was the same as in untreated cells (Leive and Kollin, 1967).

In summary, by employing the described conditions, and checking their effectiveness by appropriate controls, it is possible to prepare EDTA-treated cells suitable for use in many metabolic studies including studies of RNA and protein synthesis.

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