The Role of Amine Buffers in EDTA Toxicity and Their Effect on Osmotic Shock

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SUMMARY

The lysis of *Escherichia coli* following exposure to EDTA in tris buffer is similar for most F⁺, F⁻ and Hfr strains. Survival and release of acid soluble nucleotide material was identical in male and female strains. Following osmotic shock by the EDTA + tris method, regrowth patterns of F⁻, F⁺ and Hfr strains showed no significant differences. Physiological buffers—Tes, Hepes, Bicine—cannot replace tris in EDTA + lysozyme lysis of *E. coli* or in osmotic shock to release surface enzymes. Tes, Hepes and Bicine can replace tris in causing lysis of *Pseudomonas aeruginosa* either with EDTA alone or with lysozyme.

INTRODUCTION

The role of ethylenediaminetetra-acetic acid (EDTA) and amine buffers such as tris (hydroxymethyl) aminomethane (tris) in altering the surface of Gram-negative bacteria and hence permeability has received increasing attention during the last few years. Repaske (1958) demonstrated that some Gram-negative bacteria could be lysed by use of EDTA and lysozyme in a tris buffer. Neu & Heppel (1964) showed that exposure of *Escherichia coli* to tris and EDTA followed by a sudden osmotic shift released a number of degradative enzymes from the bacteria which remained viable. Subsequently, Neu & Chou (1967) showed that an osmotic shock following EDTA + tris treatment resulted in the release of degradative enzymes from most Enterobacteriaceae. It was shown (Leive, 1965) that EDTA caused a non-specific increase in permeability in *E. coli*. Neu, Ashman & Price (1966, 1967) showed that exposure to EDTA could result in breakdown of RNA, and at 4° tris alone caused the nucleotide pool to be lost from various Enterobacteriaceae.

Recently, Goldschmidt, Goldschmidt & Wyss (1967) noted that EDTA and tris toxicity were perhaps related to the fact that male bacteria possess exposed pili. Since we had not noted such striking differences in toxicity between male, female and Hfr strains in our studies on osmotic shock we thought this should be explored. Voss (1967) has shown the role of various compounds in affecting bactericidal activity of EDTA. It seemed of value to determine the role that other physiological hydrogen ion buffers of amine type (Good *et al.* 1966) might play in the role of EDTA toxicity.

METHODS

Organisms. *Escherichia coli* strains were those previously used in our laboratory and strains that were a generous gift of Dr R. Rudner.

Media and growth conditions. Stock cultures were maintained on Penassay slopes (Difco). Organisms were grown in Penassay broth (Difco) or the high phosphate or low phosphate media of Neu & Chou (1967). Organisms were incubated at 35° on a rapid rotary shaker, and growth was followed by extinction at 600 m μ in a DU spectrophotometer (Beckman). Viability was determined on serial dilutions in Penassay broth, which were plated on either Penassay agar or nutrient agar containing 0.5 % NaCl.

Osmotic shock. Stationary-phase organisms were harvested at 16 hr (1 %, v/v, inoculum). Exponential organisms were harvested at 5×10^8 bacteria/ml.; they were washed twice with room temperature 0.01 M-tris+HCl (pH 7.5)+0.03 M-NaCl or 0.85 % NaCl as noted. Washed organisms were suspended at a ratio of 5×10^8 bacteria/ml. to 80 ml. of 0.5 M-sucrose+0.03 M-tris (pH 7.5). EDTA was added to a concentration of 1 mM for stationary organisms, and after 5 min. of mixing the organisms were removed by centrifugation. The pellet was resuspended at 3° in an equal volume of water for 5 min. The organisms were again removed by centrifugation. Exponential organisms were treated with 0.1 mM-EDTA and resuspended in 0.5 mM-MgCl₂ at 3°.

Chemicals. EDTA was used as the disodium salt. Tris was purchased from Sigma and pH adjusted with HCl. Tes, *N*-tris (hydroxymethyl) methyl-2-aminoethanesulfonic acid; Hepes, *N*-tris (hydroxymethyl) methyl-2-aminoethanesulfonic acid; Bicine, *N,N*-bis (2-hydroxyethyl) glycine, were purchased from Calbiochem.

Enzyme assays. 5'-Nucleotidase, cyclic phosphodiesterase and inorganic pyrophosphatase assays are those previously published (Neu, 1967).

RESULTS

Previous studies of osmotic shock of Enterobacteriaceae had not shown differences in F⁺ and F⁻ strains in our laboratory (Neu & Chou, 1967). Table 1 shows that when overnight cultures of various *Escherichia coli* strains were exposed to 1 mM-EDTA at

Table. 1. *Effect of buffers on lysis of various male and female Escherichia coli strains*

Strain	Buffer		
	Tris	Hepes	Tes
	Per cent A ₆₀₀ change		
Hfr Hayes	4.9	1.4	0
CR 34 (F ⁻)	3.2	3.1	2.2
CR 161 (F ⁺)	18	0	0
W 1192 (F ⁺)	16	13	26
Hfr 3000	7	+4	+3
W 1485 (F ⁻)	14	0	0
W 1117 (F ⁻)	5	3	—

Overnight cultures were washed with 0.03 M-NaCl+0.01 M-tris-HCl and resuspended at 21° in 0.03 M-buffer at 10^{10} bacteria/ml. Change in extinction was followed for 15 min. at 600 m μ .

21° there seemed to be increased lysis of some F⁺ strains as compared with some F⁻ strains. Hfr strains seemed as stable as the F⁻ strains, to the effect of EDTA in tris buffer. Two other buffers, Hepes and Tes, showed much less lysis than did tris.

A comparison of the survival of eight strains of *Escherichia coli* after osmotic shock was made. No differences were noted in release of acid soluble material, release

Table 2. *Effect of buffer on EDTA + lysozyme lysis of Escherichia coli*

Buffer (pH)	Change in extinction		Nucleo- tidase release		Change in extinction		Nucleo- tidase release		Change in extinction		Nucleo- tidase release		Change in extinction		Nucleo- tidase release	
	(%)	A_{260}	CR 34 (F ⁻), Survival (%)	A_{260}	(%)	CR 4 (F ⁻), Survival (%)	A_{260}	(%)	CR 161 (F ⁺), Survival (%)	A_{260}	(%)	CR 161 (F ⁺), Survival (%)	A_{260}	(%)	CR 161 (F ⁺), Survival (%)	A_{260}
Tris (7.4)	93	0.329	2	100	90	0.309	10	98	73	0.374	22	100	73	0.374	22	100
Hepes (7.1)	19	0.137	100	28	5	0.245	73	20	19	0.161	78	32	19	0.161	78	32
Tes (7.6)	50	0.184	85	66	10	0.206	98	34	28	0.240	75	48	28	0.240	75	48
Bicine (7.4)	31	0.148	73	42	13	0.319	71	50	18	0.192	60	29	18	0.192	60	29

Organisms were grown to early stationary phase in tris medium. They were washed twice with 0.85% NaCl at 21 °C. 10^{10} bacteria were suspended in 0.03 M-buffer. EDTA concentration was 1 mM and lysozyme 10 μ g./ml. Change in extinction was at 600 m μ . A_{260} release was determined on the supernatant fluid of 10 min. treated bacteria.

of surface enzymes or survival of W1485 (-), CR161 (+), K10 (-), W1117 (-), AB1192 (+), AB282 (+), P4 × 6 (Hfr) and Hfr. Hayes. Growth of bacteria in either the high phosphate or low phosphate media did not alter the results. Growth patterns after osmotic shock of F⁻, F⁺ and Hfr strains were identical.

Comparison of male and female strains in relation to time of exposure and type of buffer demonstrated that F⁺ and Hfr strains had a decreased survival after exposure to 0.03 M-Tes + 0.25 mM-EDTA for 60 min. Tris and Bicine buffers did not show increased toxicity as time of exposure to EDTA and the buffer was increased.

Table 3. *Effect of buffer upon release of enzymes from Escherichia coli by osmotic shock*

Buffer (pH)	Per cent released				A ₂₆₀	Survival (%)
	5'-Nucleotidase	Acid phosphatase	3'-Nucleotidase	Inorganic pyrophosphatase		
Tris (7.4)	90	58	75	2	0.435	84
Tes (7.6)	20	30	34	3	0.435	92
Hepes (7.1)	10	25	33	7	0.423	100
Bicine (7.4)	21	26	38	10	0.448	97

An overnight culture of K37 was subjected to osmotic shock as outlined in Methods. Inorganic pyrophosphatase is used as an internal enzyme to detect cell damage.

Table 4. *Effect of buffer on EDTA toxicity for Pseudomonas aeruginosa*

Buffer	Change in extinction (%)			Survival (%)	
	EDTA	EDTA + Lysozyme	A ₂₆₀ EDTA	EDTA	EDTA + Lysozyme
Tris	25	81	0.796	34	10
Tes	18	24	0.715	62	9
Bicine	15	24	0.923	46	12

An early stationary-phase culture was harvested at 21° and washed twice with 0.85% NaCl. Washed organisms at a concentration of 10¹⁰ bacteria/ml. were exposed to 0.1 M-EDTA in the buffers at 0.03 M at a pH of 7.5 at 21°. Change in extinction was read at 10 min. at 600 mμ. A₂₆₀ release was determined on supernatant of treated cells. Bacteria treated with lysozyme at 20 μg/ml.

The effect of various buffers on the EDTA + lysozyme lysis of *Escherichia coli* (Table 2) show that lysis was significantly less with all the other physiological buffers at a similar pH for both male and female *E. coli*. Survival correlated with the lower release of acid soluble material and release of surface enzymes as represented by 5'-nucleotidase. An identical response was seen in the comparison of the same buffers used in osmotic shock to release surface enzymes. The other buffers—Tes, Hepes, Bicine—did not adequately replace tris (Table 3).

Washing of bacteria with either 0.85% NaCl or 0.01 tris-HCl (pH 7.4) did not affect the EDTA + lysozyme lysis with tris as the buffer. Similarly, bacteria washed with tris and then suspended in Tes, Bicine or Hepes and EDTA did not show more lysis than NaCl-washed bacteria.

Treatment of *Aerobacter* showed that Hepes and Bicine were as ineffective as tris in promoting EDTA + lysozyme lysis of *Aerobacter* strains. *Aerobacter* did however release surface enzymes with osmotic shock (Neu & Chou, 1967).

Although Tes and Bicine were not effective in replacing tris in EDTA lysis of *Escherichia coli*, they were as effective in causing lysis of *Pseudomonas aeruginosa* either with EDTA alone or with lysozyme (Table 4).

DISCUSSION

Although the results of Goldschmidt *et al.* (1967) suggested that all male strains of *Escherichia coli* were more sensitive to the toxic effects of EDTA + tris, we have been unable to confirm this. Some strains did show increased toxicity as compared with most female strains, but in general toxicity was similar for F⁺, F⁻ and Hfr strains. Occasional male strains that did show increased toxicity may have had defects of transport as indicated by their delayed regrowth following osmotic shock.

It is clear that tris has an effect on the bacterial cell wall unlike similar buffers such as Tes, Bes, Bicine and Hepes. Although these buffers can replace tris in other systems, they cannot be used to cause EDTA + lysozyme lysis or in the osmotic shock treatment to cause release of surface enzymes and transport factors from Enterobacteriaceae.

Gray & Wilkinson (1965) have shown that *Pseudomonas aeruginosa* is sensitive to EDTA in a variety of buffers. All of the buffers used—Tes, Hepes, Bicine—were effective in causing EDTA lysis.

These studies illustrate that tris cannot be replaced by other buffers in the systems using EDTA to alter permeability of bacteria except in very sensitive organisms. It is possible either that these other buffers form weaker homologues with EDTA than tris or that they have less ready access to the cations which stabilize the cell. The mechanisms of the different responses to buffers other than EDTA in *Escherichia coli* are under study.

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