Osmotic Fragility of Bacteria Deprived of Carbon Dioxide

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SUMMARY

An absolute requirement for external carbon dioxide can easily be demonstrated for *Escherichia coli* strains B, C and K-12, and for a *Serratia marcescens* strain under anaerobic conditions, even in the presence of a very rich complex medium. During carbon dioxide deprivation such bacteria (with the exception of *E. coli* B) become extremely sensitive to the challenge of a sudden decrease in the osmotic pressure of the medium. This challenge does not seem to disrupt the gross structure of the bacteria, but it impairs in an apparently irreversible manner their ability to multiply.

INTRODUCTION

In the course of some experiments on bacteriophage infection of anaerobically growing bacteria, we accidentally observed a phenomenon which to our knowledge has not been noted before, and which we briefly characterize in this paper. We show that in some bacteria growing under anaerobic conditions in a very rich, complex medium, CO₂-deprivation produces a state of extreme osmotic fragility.

METHODS

Organisms. The following bacteria were used. *Escherichia coli* strain C: derivatives C-1a (Sasaki & Bertani, 1965), C-1055 (which is F⁺, streptomycin resistant, and polyauxotrophic), and C-107 (like C-1a, but lysogenic for phage P2). *E. coli* strain K-12: derivatives C600 (Appleyard, 1954), C600 lysogenic for phage λ , and CA154 (Brenner & Beckwith, 1965) cured of phage λ by hetero-immune superinfection (by D. Walker). *Escherichia coli* strain B and its derivatives: B-1 (made lysogenic for P2 and simultaneously cured of the dismune proplage of Cohen, 1959), B-16 (made radiation resistant by the method of Witkin, 1947) and B-17 (BC of Cohen, 1959 also made radiation resistant). *Serratia marcescens* strain SA-18 (Bertani, Torheim & Laurent, 1967).

In addition, two biotin-requiring mutants of *Escherichia coli* (C-1201 and C-1202) were isolated after treatment with the mutagen *N*-methyl-*N'*-nitro-*N*-nitrosoguanidine (Mandell & Greenberg, 1960; method of Adelberg, Mandel & Chen, 1965) from *E. coli* strain C-3 (Bertani & Six, 1958). Both have low reversion frequencies. Biotin-starved cultures, following residual growth, maintain a constant viable count for periods of the order of 5 to 8 hr after which morphologically anomalous forms appear. C-1201 bacteria have a greater tendency than C-1202 to develop into filamentous forms when starved of biotin. Both mutations are transducible by phage λ (see Del Campillo-Campbell, Kayajanian, Campbell & Adhya, 1967).

Unless otherwise stated, the observations to be described refer to *Escherichia coli* strain C-1a.

Media. Unless otherwise specified, bacteria were grown in LB medium (1 % Sheffield Chemicals' N-Z-amine type B, 0.5 % yeast extract, 1 % NaCl, 0.1 % glucose; Bertani, 1951), which is nutritionally, a very rich medium. This medium was adjusted at about pH 7.2. Viable assays were made on LB agar with the soft agar overlay technique, as for phage work. The melted soft agar was held at 45° in small tubes until needed. In some experiments tryptone broth (Sasaki & Bertani, 1965) and DIL (0.1 % Difco nutrient broth, 0.5 % NaCl) were used.

A defined medium (Davis; in Sasaki & Bertani, 1965) with the appropriate supplements was used in work with the biotin-requiring mutants. A biotin concentration of 0.01 µg./ml. was more than sufficient for normal growth of the mutants. The effects of biotin deprivation were studied in Davis medium supplemented with half strength Biotin Assay Medium (Difco).

Cultural techniques. All incubation was at 37°. Anaerobic growth was obtained by bubbling a tube culture with a mixture of N₂ + CO₂ (95 + 5, %, v/v). CO₂-free N₂ was obtained by bubbling N₂ first through two gas-washing bottles, each containing half litre of 30 % NaOH solution, and then through a 1/10 saturated Ba(OH)₂ solution in a large test tube (to detect inefficient CO₂ removal).

Standard experiment. An overnight static broth culture was diluted 200-fold into fresh broth and incubated anaerobically. When the bacterial count, as judged by counting with a microscope, had increased to between 5×10^7 and 10^8 bacteria/ml., the culture was again diluted (10- to 50-fold) in fresh broth (5 to 15 ml. in a large test tube) held at 37° and already equilibrated with the desired gas (either the N₂ + CO₂ mixture or CO₂-free N₂) by bubbling it for 15 to 30 min. Bubbling was continued at a rate of about 200 ml. gas/min. At various times throughout the experiment, samples of the culture were taken and colony counts made immediately following a dilution either in broth (control) or in distilled water (osmotic challenge) or in other media held at room temperature.

β-Galactosidase assay (Wallenfels, 1962). Bacteria resuspended in 0.1 M-sodium phosphate buffer (pH 7.4) were shaken with 1/10 volume of toluene at 42° for 15 min. A sample (0.1 ml.) of the suspension was then added to 3 ml. of a solution of o-nitrophenyl-β-D-galactopyranoside (Sigma Chemical Co., St Louis, Missouri) and incubated for 20 min. at 42°. The extinction was read in a colorimeter at 405 mµ. A suspension containing the same amount of carrier (see Table 4) bacteria of the CA154 derivative, which is unable to synthesize β-galactosidase was treated in exactly the same manner and used as a blank.

RESULTS

Escherichia coli strain C-1a, used in most of the experiments to be described, grows about equally well in broth (yield at saturation: 3 to 6×10^8 viable bacteria/ml.; maximum doubling rate: 20–22 min.) under aerobic as under anaerobic conditions. When, however, CO₂ is removed from an anaerobic, exponentially growing broth culture, by vigorously bubbling it with CO₂-free N₂, two phenomena can be observed (Fig. 1): (a) multiplication of the bacteria, as measured by viable count following appropriate dilution in broth, comes to a halt; (b) the bacteria become highly sensitive to a challenge consisting of a sudden dilution (usually 1/100) in distilled water.

To demonstrate these phenomena, the bacterial concentration during CO_2 -starvation must not be much above 10^7 bacteria/ml. The reason for this is, we believe, that the CO_2 released by the bacteria into the medium is efficiently re-utilized by other bacteria before it can be removed by the bubbling gas. The two phenomena are largely unaffected by minor pH changes in the medium (Fig. 1). They were also demonstrated in tryptone broth, in the medium used in the biotin-deprivation experiments (see below), and even in simple defined media, although in this case the osmotic fragility was erratic and less pronounced. They could not be demonstrated under aerobic conditions.

Fig. 1. Inhibition of multiplication and appearance of osmotic sensitivity in anaerobically growing *Escherichia coli* following removal of CO_2 . Standard experiment: at 2.5 hr the culture was diluted 1/50 to establish three parallel cultures differing in gas régime and in initial broth pH value. ●, viable counts following dilution in broth; ×, viable counts following dilution in distilled water. The first dilution step was 1/100 in all cases.

When the experiment is continued beyond the time shown in Fig. 1, the viable counts in the CO_2 -deprived cultures begin to decrease after 2 to 3 hr, even in the absence of challenge, and eventually reach the same low values observable after challenge.

The sensitivity to the challenge of dilution in water appears to be really an osmotic phenomenon. Simple defined media with sufficiently high solute concentrations are as good as broth as diluents, whereas half-strength broth behaves like water (Table 1).

The osmotic sensitivity does not develop immediately upon dilution into broth deprived of CO_2 , but takes at least 30 min. to reach its full expression. Chloram-

phenicol, an inhibitor of protein synthesis, added at the time when CO₂-deprivation begins, does not permit the development of the osmotic sensitivity (Table 2). No evidence of protection was found when the culture was exposed to chloramphenicol for a period of 2 hr just before CO₂-deprivation. These observations suggest that the development of osmotic sensitivity results from unbalanced synthesis.

Table 1. *Effect of diluent composition on the survival of anaerobically growing Escherichia coli C-1a deprived of CO₂*

Standard experiment: results of viable count (colony count) assays made 1 hr after the beginning of CO₂ deprivation, with various diluents (held at room temperature). The first dilution step was in all cases 1/10. The 100 % value is set equal to the titre of the culture at the beginning of CO₂ deprivation. Values greater than 100 % result from the residual multiplication that could occur under these conditions (see, for example, Fig. 1). (a), (b), and (c) are different experiments. LB or DIL, preceded by a multiplication factor, indicates a medium with the same qualitative composition as LB or DIL, but differing in total solute concentration, the normal concentration being taken as 1.0.

Composition of the diluent used	Percentage original colony count		
	(a)	(b)	(c)
LB	42	170	110
1.5 LB	150	—	90
2.0 LB	110	—	90
0.5 LB, or 0.25 LB, or 0.1 LB	ca. 0.5	—	ca. 0.01
Distilled water	—	0.1	—
DIL	—	0.2	—
4 % sucrose in 0.83 DIL	—	135	—
8 % sucrose in 0.67 DIL	—	115	—
12 % sucrose in 0.5 DIL	—	130	—
1 % NaCl+0.01 M-MgSO ₄	—	140	—
2 % NaCl+0.01 M-MgSO ₄	—	160	—

Table 2. *Escherichia coli* strain C-1a. *Effect of chloramphenicol on the development of osmotic fragility*

Standard experiment. At time 0 the exponentially growing anaerobic culture was diluted into broth with various gas régimes. Chloramphenicol was added at time 0 in Expt. a, 10 min. before dilution (i.e. at time -10 min.) in Expt. b. The viable titre at time 0 (8×10^8 bacteria/ml. in Expt. a, and 10^8 bacteria/ml. in Expt. b) is set equal to 100 %.

Expt.	Gas régime	Chloramphenicol present (μ g./ml.)	Viable count in % of original titre					
			At 30 min. with dilution in		At 60 min. with dilution in		At 120 min. with dilution in	
			Broth	H ₂ O	Broth	H ₂ O	Broth	H ₂ O
a	N ₂ only	None	190	0.1	—	—	63	<0.1
	N ₂ only	25	120	16	—	—	150	19
	N ₂ only	50	105	60	—	—	93	73
	N ₂ +CO ₂	50	190	55	—	—	87	66
b	N ₂ only	None	210	2.8	115	1.6	84	1.2
	N ₂ only	100	150	170	190	110	180	155

Death of the osmotically sensitive bacteria is not a result of the handling involved in the colony-count procedure. In fact, their inviability following the challenge was also shown by end-point titration in broth. The effect of dilution in distilled water could be

demonstrated immediately: once the dilution in water was made the survival did not vary significantly upon keeping the tube (at room temperature) for as long as 30 min.

Microscopical observation (phase contrast) of bacteria in the osmotically sensitive state did not reveal any gross morphological abnormality. This applied also to such bacteria after dilution in distilled water; in other words, the killing was not due to visible rupture or distortion of the cell envelopes and loss of cytoplasm. Quantitatively, these experiments presented some difficulties, since the bacterial concentration after dilution in distilled water was very low when conditions that best produced the effect were chosen. In some experiments therefore, at various times following CO₂ removal, the bacteria, after dilution in either broth or distilled water, were centrifuged down and concentrated more than 100-fold, before being examined and counted in a microscope (Table 3).

Table 3. *Escherichia coli* strain C-1a. Recovery of morphologically normal bacteria following inactivation by osmotic challenge

In a standard experiment, 1 ml. samples were diluted 1/40 either in broth or in distilled water, assayed by viable count, centrifuged, and resuspended in a small volume of broth to which formalin was added later (Expt. B) or directly in formalin (0.3 % final formaldehyde concentration) (Expt. A). The titres of these suspensions were determined under a microscope with a Petroff-Hausser counting chamber (also by viable count in Expt. B).

Expt.	Time (min.) after beginning of CO ₂ deprivation	Percentage survival to osmotic challenge	Percentage effi- ciency of the concentration by centrifugation (unchallenged bacteria)	Percentage relative recovery of challenged bacteria by microscopic count
		(1)*	(2a)†	(3)§
A	30	1.0	39	27
	60	2.8	57	77
	90	3.0	49	68
	120	8.7	46	80
			(2b)‡	
B	30	1.4	54	100
	90	4.9	70	78
	120	6.7	36	76

* (1) = $100 \times (\text{viable count following dilution in H}_2\text{O}) / (\text{viable count following dilution in broth})$.

† (2a) = $100 \times (\text{total count in concentrated suspension, corrected for the volume change}) / (\text{viable count before concentration})$.

‡ (2b) $100 \times (\text{viable count in concentrated suspension, corrected for the volume change}) / (\text{viable count before concentration})$.

§ (3) = $100 \times (\text{total count in concentrated, challenged suspension}) / (\text{total count in concentrated, unchallenged suspension})$.

The efficiency of the concentration process performed thus was low and variable (possibly due to bacteria sticking to the walls of the centrifuge tube, partially washing off the pellet, and the large error in estimating pellet volume). Nevertheless, the apparent morphological integrity of the majority of the bacteria inactivated by the osmotic challenge was clearly indicated by the data.

Another experiment (Table 4) also indicated that killing of osmotically sensitive bacteria by dilution in distilled water was not accompanied by a mass leakage of cell contents. The β -galactosidase activity of bacteria first challenged by dilution in water

and then concentrated by centrifugation (with removal of the supernatant) was essentially identical with that of unchallenged bacteria.

In other experiments, all the *Escherichia coli* C derivatives listed in Methods, the two C600 derivatives of *E. coli* K-12, and the *Serratia marcescens* strain were tested for osmotic fragility following anaerobic CO₂ deprivation; they all gave the effect like strain C-1a. But the effect could not be demonstrated with any of the four lines of *E. coli* B tested, even though the absolute requirement for CO₂ under anaerobic conditions could be demonstrated even with *E. coli* B. Attempts to demonstrate a similar osmotic fragility with the two biotin-requiring mutants when starved of biotin under aerobic conditions (in the presence of CO₂) were completely unsuccessful.

Table 4. *Escherichia coli* Strain C-1a. Recovery of β -galactosidase activity in CO₂-deprived, osmotically challenged bacteria

Standard experiment. Bacteria were grown however in modified LB (glucose replaced by 0.25% lactose) to obtain full induction of β -galactosidase. The transfer to N₂-only bubbled broth involved a six-fold dilution. The challenge consisted of a fifty-fold dilution into H₂O. A similar dilution in ordinary LB served as control. Following these dilutions, and the viable count assays, the samples were re-concentrated by centrifugation and re-suspended in buffer, before assaying for enzyme activity. To assure a good recovery in the concentration step, a small volume of a concentrated suspension of mutant bacteria unable to synthesize β -galactosidase was added to each sample as carrier. An equal volume of double strength LB was added to the CO₂-starved sample in H₂O before centrifugation to make the physical environment (which might affect pellet formation, etc.) identical with that of the control in broth. Comparable suspensions of carrier bacteria alone were used as blanks. The concentration of carrier bacteria in the final enzyme assay mixture was of the order of 10⁷/ml.

	Anaerobic culture			
	Exponentially growing in the presence of CO ₂		Diluted 1/6 and main- tained for 1 hr in the absence of CO ₂	
	Diluted in broth	Diluted in H ₂ O	Diluted in broth	Diluted in H ₂ O
Viable count/ml. culture	2.6×10^8	3.0×10^8	4.3×10^7	2.5×10^6
No. of bacteria expected to be present in 0.1 ml. of the re-concentrated samples (assuming full recovery and neglecting carrier bacteria)	5.2×10^6	6.0×10^6	5.4×10^6	(5.4×10^6)
Extinction reading in the enzyme assay (for a layer thickness of 1.0 cm.)	0.34	0.33	0.72	0.65

DISCUSSION

The necessity of an external supply of CO₂ for the growth of many bacteria under certain conditions was shown long ago (see Gladstone, Fildes & Richardson, 1935, for early references). Later work (Lwoff & Monod, 1947; Ajl & Werkman, 1949; McLean & Purdie, 1952; Wiame & Bourgeois, 1955) showed that the addition of certain compounds to simple defined media decreased drastically the CO₂ requirement and its incorporation. Several CO₂-dependent, metabolic reactions could thus be identified. Not all essential CO₂-requiring reactions were detected in these experiments, since in several cases a residual CO₂-dependence could not be eliminated. A technical difficulty arises from the fact that both CO₂-fixing and CO₂-releasing reactions

occur in a cell, so that the extent of the requirement for an external CO₂ supply depends on the balance of the two sets of reactions under the cultural conditions used, and on the efficiency with which intracellular CO₂ diffuses and is exchanged with extracellular CO₂. To establish experimentally conditions of absolute CO₂ deprivation it is thus necessary to maintain a sufficiently high rate of removal of the CO₂ produced by the cells.

In our experiments with a nutritionally rich broth as culture medium, anaerobic conditions, and sufficiently low concentrations of bacteria, we succeeded in showing an absolute requirement for external CO₂ with all the bacterial strains examined. All the tests were done with exponentially growing cultures, thereby excluding the complexities of growth initiation by resting bacteria. We did not succeed in showing the requirement in similar cultures growing aerobically, presumably because of a higher rate of CO₂ production under such conditions.

The broth we used was expected to contain all amino acids and nucleic acid bases that the bacteria would need if the corresponding synthetic pathways were blocked. Under these conditions the factor, or factors, which cannot be synthesized in the absence of CO₂ must either be absent from the medium or unavailable to the bacteria because of difficulties in uptake. In recent years reactions which require CO₂ have been demonstrated in the synthesis (and degradation) of long-chain fatty acids. Since lipids are an important element in the structure of cell envelopes, it seems reasonable to suggest that the osmotic fragility observed by us might result from limitation or defects in the synthesis of fatty acids, leading to an abnormal composition of the cell envelopes, and consequently to extreme sensitivity to osmotic challenge. Since biotin is the coenzyme involved in the carboxylation reactions recalled above, we thought that it might be possible to produce a similar osmotic fragility, even in the presence of CO₂, by starving bacteria of biotin. In fact, there are indications in the literature (see Gavin & Umbreit, 1965) that deprivation of biotin affects the permeability of the bacteria. Our experiments in this direction were, however, unsuccessful.

Many effects of abrupt changes in the osmotic pressure of the suspending medium have been described in the bacteriological literature (see, for example, among the latest, Bayer (1967), and Heppel, (1967)). In terms of viability, the effect produced in our experiments can be very drastic, even following relatively small changes in solute concentration of the medium. The effect is not necessarily connected with that described by Meynell (1958), in which bacteria were challenged by dilution in ice-cold water, then stored cold for several hours before being assayed. We were able to reproduce Meynell's results with *Escherichia coli* c. Unlike the osmotic fragility following CO₂ deprivation, Meynell's effect was not immediately demonstrable following dilution in cold water; storage was essential for its manifestation. We have no explanation for the absence of osmotic fragility following CO₂ deprivation in *E. coli* strain B. It might be rewarding to study the inheritance of this difference in crosses between strains B and C, or B and K-12.

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