J. gen. Microbiol. (1967), **49**, 443–449
Printed in Great Britain

Changes in Sensitivity to Acriflavine of *Escherichia coli* Grown in Media of Different Glucose Contents

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(*Accepted for publication 16 June 1967*)

SUMMARY

Organisms of *Escherichia coli* K-12 strains sensitive and resistant to acriflavine were plated on media with and without acriflavine after growth in media containing different concentrations of glucose. Proportionally more organisms produced colonies, in the presence of acriflavine, after growth in media containing a high concentration of glucose than in media with lower glucose contents. The final pH value of the growth medium was low with the high glucose media. With the resistant strain, the number of bacteria which survived acriflavine increased as the final pH value of the medium from which bacteria were harvested was decreased, but the initial glucose concentration rather than the final pH value of the culture was more influential in increasing survival with the sensitive strain. The acriflavine-binding capacity of the bacteria was affected by the initial glucose concentration of culture medium probably indirectly through a change of pH. Acriflavine sensitivity of the bacteria varied with the amount of acriflavine bound. The acriflavine-binding capacity of bacteria modified by the pH value of culture medium was stabilized in the course of several doublings of bacteria in that medium. The glucose concentration of the medium affected the acriflavine sensitivity of the sensitive strain through some mechanism other than the change of pH.

INTRODUCTION

In the studies on genetic control of acriflavine sensitivity of *Escherichia coli* K-12 it was found that the binding capacity of the bacteria for acriflavine and other basic dyes seemed to be controlled by a gene which controlled the acriflavine sensitivity and which lay in the vicinity of the marker for lactose utilization (Nakamura, 1966). A long period was needed for the acriflavine resistance to be expressed following introduction of the resistance gene into acriflavine-sensitive bacteria by mating (Nakamura, 1965). During these investigations it was observed that the acriflavine sensitivity of the bacteria was markedly affected by the initial glucose content of the medium in which the organisms had been grown. The present paper shows that this effect of glucose concentration was partly due to the change of pH value of the culture during growth, which in turn affected the acriflavine binding of the bacteria, and also that the pH shift was not the sole factor involved in the glucose concentration effect.

METHODS

Organisms. Two strains of *Escherichia coli* K-12 were used. One, 18/1042, is an acriflavine (AF)-sensitive strain, isolated as a spontaneous mutant from a wild-type AF-resistant female strain. The other, N90, is an AF-resistant female strain obtained

by a cross of 18/1042 with an AF-resistant Hfr strain of *Escherichia coli* K-12, strain W1895. The characters of these strains were described previously (Nakamura, 1965).

Media. The strains were maintained in brain–heart infusion (Difco). For experiments, bacteria were grown overnight at 37° in a liquid medium composed of (g./l.): Difco nutrient broth (solid), 8; NaCl, 5; glucose, 1; initially at pH 7·4. This medium will be referred to as standard broth. A stock solution of acriflavine was sterilized at 100° for 20 min. and stored in a refrigerator, for not more than 10 days.

For solid media, powdered agar (1·5 %) was added. AF-broth agar was prepared by adding AF solution to the broth agar after it had cooled to 60–70°.

Determination of acriflavine content of bacteria. As negative-charge density of bacteria is reported to decline remarkably during the active growth phase (Abramson, Moper & Gorin, 1942), bacteria in the stationary phase of a culture in broth were sampled and suspended in AF-media for the determination of AF-binding. The amount of bacteria to be suspended was turbidimetrically adjusted to be equivalent to about 700 μg. dry wt bacteria/ml. Samples taken from AF-media, after a varying period of time specified later, were centrifuged (14,500*g*) for 5 min. at 5°, and the dye concentration in the supernatant fluid determined spectrophotometrically at 450 mμ. The amount of AF lost from the supernatant fluid was taken as the AF-content of the bacteria and used as an index of their dye-binding capacity. Although the medium itself was coloured yellowish, the absorption spectrum of AF could be differentiated when the medium was diluted a few times; medium without dye used as reference.

Determination of pH value in culture media. A sample of the culture was centrifuged (14,500*g*) for 5 min. at 5°, and pH value of the supernatant fluid determined electrometrically.

Thus the experiments done took the following form: organisms were grown in various liquid media; after a given time the organisms were harvested and tested for their degree of survival in AF-media (by plating on AF-nutrient agar) and for AF-binding capacity; the pH value of the culture fluids at the time of harvesting was measured.

RESULTS

Glucose content of medium and AF-sensitivity of bacteria

Strains N90 (AF-resistant) and 18/1042 (AF-sensitive) were inoculated in broth media which contained glucose in concentrations ranging from 0·05 to 6·4 g./l. After overnight incubation at 37°, a part of each culture was diluted by a factor of 6×10^6 and plated on broth agar containing AF, 250 μg./ml., for resistant strain N90 and 1 μg./ml. for sensitive strain 18/1042, and on broth agar alone, as a control. Colony counts on AF-agar are expressed as % of those on the control plates; this gave a measure of the sensitivity (% survival) to acriflavine of the harvested organisms.

The rest of each culture was centrifuged and the pH value of the supernatant fluid determined.

Figure 1 represents % survivals of the two strains on the AF-agar plates and the final pH values of the media from which the bacteria had been harvested. The % survival was high when culture medium originally contained high concentrations of glucose. The % survival on AF-agar of strain N90 was related to the pH value of the

culture at the time of harvesting, but with strain 18/1042 the initial glucose concentration rather than the final pH value of the culture showed a better correlation with % survival on AF-agar.

Effect of initial glucose concentration in buffered media

To examine the effect of initial glucose concentration in cultures kept at a relatively constant pH value, broth medium was supplemented with phosphate buffer (pH 7·05; M/15 final concentration). The initial glucose concentrations were from 0·05 to 3·2 g./l. After growing strains N90 and 18/1042 in the media overnight, samples from the cultures were diluted and plated on AF-broth agar containing 250 μg./ml. and 1 μg./ml. of AF, respectively. Figure 2 shows that the % survival of strain N90 in the presence of AF remained unchanged as long as the pH value of the culture did not change, namely with initial glucose concentrations up to 1·6 g./l. With strain 18/1042, in contrast, the % survival increased even when the pH value remained constant. Hence, the initial glucose concentration of the culture medium influenced the AF-sensitivity of bacteria through final pH value in the former case and not through pH value in the latter case.

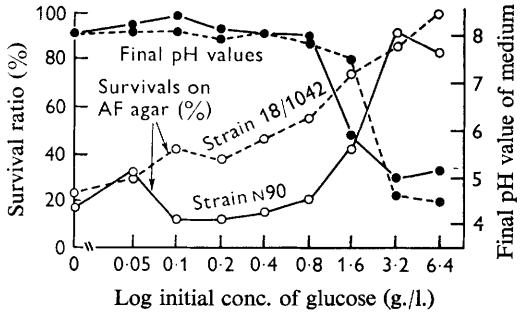

Fig. 1

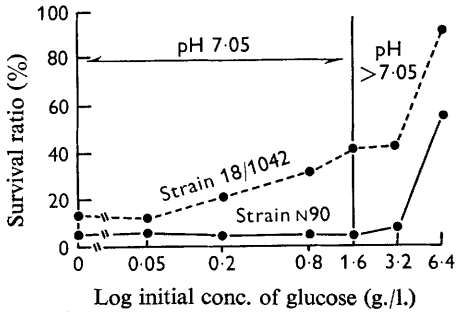

Fig. 2

Fig. 1. Effect of initial glucose concentration in culture medium on acriflavine (AF) sensitivity of *Escherichia coli* K12 AF-sensitive strain 18/1042 and AF-resistant strain N90. Bacteria were inoculated into broth media of graded glucose contents and, after overnight incubation at 37°, dilutions ($1/6 \times 10^6$) of cultures were plated on AF-containing broth agars. The pH values of the cultures at time of sampling were determined after centrifugation. – – –, Strain 18/1042; — strain N90; ○ survival ratio on broth agar containing 1 μg. AF/ml. for strain 18/1042 and 250 μg. AF/ml. for strain N90; ●, Final pH value of medium.

Fig. 2. Effect of initial glucose concentration of broth medium buffered at pH 7·05 by phosphates on the AF-sensitivity of *Escherichia coli* K12. AF-sensitive strain 18/1042 and AF-resistant strain N90. Organisms were inoculated into broth medium of graded glucose concentrations buffered at pH 7·05 by phosphates. After overnight incubation at 37°, dilutions ($1/6 \times 10^6$) of cultures were plated on AF-containing broth agars. – – –, % survival of strain 18/1042 on 1 μg. AF/ml. broth agar; —, % survival of strain N90 on 250 μg. AF/ml. broth agar.

Acriflavine binding capacity and pH value of medium

It is known that bacteria of the AF-sensitive strain 18/1042 bind more AF than do bacteria of the AF-resistant strain N90 (Nakamura, 1966). In connexion with the effects of initial glucose concentration and the pH value of the medium at harvest, on the AF-sensitivity of the bacteria, the effects of these factors on the AF-binding capacity of bacteria are interesting. Bacteria of the two strains were grown overnight

in broth media adjusted initially to pH 5·4, 6·2, 7·2 and 8·0 by M/15 phosphate buffer. The bacteria were then harvested, washed 3 times with 0·85% (w/v) NaCl solution, and suspended in a broth medium containing 5 μg AF/ml. After incubation for 60 min. at 37°, the AF-binding capacity of these bacteria was determined. The binding capacity of either strain increased with increase of initial pH value of the culture medium, as shown in Fig. 3A. When bacteria grown in the standard broth without phosphate buffer were directly transferred into AF-containing broth media of various pH values, the AF-binding of the bacteria increased with increase of pH value (Fig. 3B). Thus, pH value of the preceding culture medium and pH value of the AF-medium were both effective on the AF-binding of the bacteria. Since the AF-sensitivity was lower at low pH values (Fig. 1, 2), the effect of pH value on sensitivity was the inverse of the effect on AF-binding in both strains.

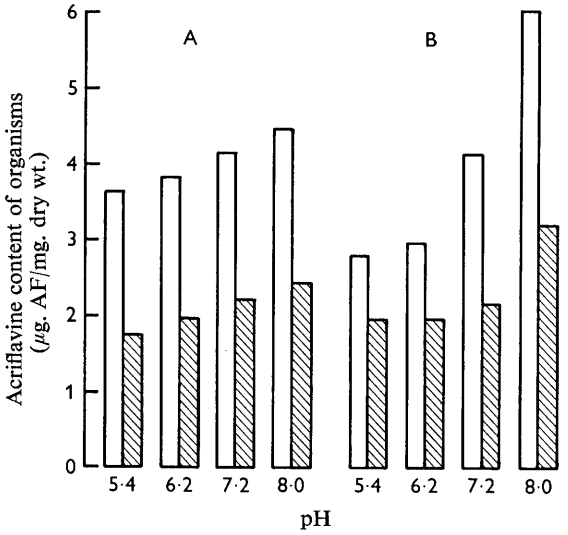

Fig. 3. Effect of pH value on AF-binding by *Escherichia coli* K 12 AF-sensitive strain 18/1042 and AF-resistant strain N90. A, strain N90 (shaded) and 18/1042 (unshaded) were grown overnight in broth media of initial pH values indicated, harvested, well washed and then incubated for 60 min. in the broth containing 5 μg. AF/ml. for measurement of AF-binding. B, Organisms of N90 (shaded) and 18/1042 (unshaded) grown in the standard broth were directly suspended in AF-broth (5 μg./ml.), buffered at the indicated pH values, and then incubated for 20 min. for determination of AF-binding.

Since the AF-sensitivity of 18/1042 organisms decreased as initial glucose concentration of the medium increased even at the same pH value (Fig. 1, 2), the effect of initial glucose concentration on AF-binding capacity was examined by using buffered culture media. Organisms of strains N90 and 18/1042 were grown in broth media containing different concentrations of glucose and adjusted to pH 7·4 by phosphate buffer. After overnight incubation, the bacteria were harvested and washed and their AF-binding capacities determined by suspension in AF-containing broth. Table 1 shows that for both strains the AF-binding varied with the pH value rather than by the initial glucose concentration.

Table 1. *Effect of glucose concentration of buffered medium on acriflavine(AF)-binding capacity of Escherichia coli K-12 AF-sensitive strain 18/1042 and AF-resistant strain N90*

Organisms were inoculated in media buffered at pH 7·4 and containing graded initial concentrations of glucose and incubated overnight. The organisms were then harvested, well washed, and then incubated for 60 min. in broth containing 5 μg AF/ml.

	Initial glucose concentration in culture medium (g./l.)						
Strain	0	0·05	0·20	0·80	1·60	3·20	6·40
	Final pH value of culture						
18/1042	7·31	7·32	7·29	7·19	7·18	6·91	5·80
N90	7·41	7·38	7·36	7·33	7·20	7·05	6·17
	AF-content of organisms after treatment (μg AF/mg. dry wt)						
18/1042	4·31	4·12	3·88	4·05	3·88	3·53	3·16
N90	2·44	2·44	2·53	2·60	2·55	2·38	1·90

Table 2. *The need for multiplication for modification of acriflavine(AF)-binding capacity by the pH value of the culture medium*

Strains 18/1042 and N90 were inoculated with the indicated inoculum sizes into broth media adjusted to pH 6·0 and pH 8·0 and, after overnight incubation at 37°, organisms were well washed and suspended for 60 min. in broth at pH 7·4 containing 5 μg. AF/ml.

Strain	pH of culture medium	Inoculum size (cells/ml.)	Doublings	AF-content (μg. AF/ mg. DW)
18/1042				
a	6·0	$9·1 \times 10^8$	2·2	3·44
	8·0	$9·0 \times 10^8$	1·2	3·48
b	6·0	$9·6 \times 10^7$	5·7	3·47
	8·0	$8·7 \times 10^7$	4·2	3·88
c	6·0	$9·3 \times 10^6$	8·6	3·44
	8·0	$8·1 \times 10^6$	7·6	3·80
d	6·0	$8·9 \times 10^5$	11·9	3·44
	8·0	$9·3 \times 10^5$	11·0	3·85
e	6·0	$9·8 \times 10^4$	15·0	3·44
	8·0	$9·8 \times 10^4$	14·2	3·85
f	6·0	$9·5 \times 10^3$	18·5	3·44
	8·0	$9·5 \times 10^3$	17·8	3·90
N90				
a	6·0	$9·9 \times 10^8$	1·4	1·81
	8·0	$1·1 \times 10^9$	1·0	1·73
b	6·0	$1·1 \times 10^8$	5·0	1·81
	8·0	$7·8 \times 10^7$	4·8	2·08
c	6·0	$9·6 \times 10^6$	8·2	1·78
	8·0	$1·1 \times 10^7$	7·5	2·08
d	6·0	$9·6 \times 10^5$	11·5	1·81
	8·0	$8·6 \times 10^5$	11·1	2·13
e	6·0	$1·0 \times 10^5$	14·7	1·81
	8·0	$9·9 \times 10^4$	14·1	2·12
f	6·0	$1·1 \times 10^4$	17·9	1·82
	8·0	$1·0 \times 10^4$	17·3	2·12

Modification of acriflavine-binding capacity and multiplication of the organisms

The AF-binding capacity of the bacteria was controlled not only by pH value of the AF-containing solution (Fig. 3B), but also by the pH of the medium in which they had been grown (Fig. 3A). The following experiment was designed to see whether growth in the medium was necessary for pH value of the medium to modify AF-binding capacity of the organisms.

Freshly grown organisms of strains N90 and 18/1042 were washed with saline and inoculated into broth media adjusted to pH 6·0 and 8·0, the inoculum sizes being graded as shown in Table 2. After overnight incubation at 37°, the bacteria were washed with saline and suspended in broth containing 5 μg. AF/ml. The AF-content of the bacteria after incubation for 60 min. is represented in Table 2. The pH value of the incubation did not affect the AF-binding when bacteria underwent only one or two doublings in that medium.

DISCUSSION

It is known that the surface of bacteria bears a negative charge under physiological conditions, mainly because of the Donnan equilibrium and the ionization of surface components. Albert (1951) showed that the positive ionization of acridine dyes is an important factor for their antibacterial action, and that an equilibrium between dye and bacteria is rapidly established. Hence, it may be considered that acriflavine (and other basic dyes) binds primarily with negatively charged sites on and near the cytoplasmic membrane, where acriflavine-sensitive metabolic machinery may be located.

It was shown by the experiments described above that the initial glucose concentration of the medium markedly affected the AF-sensitivity of the organisms grown in it, even after they had been well washed. And in the AF-resistant strain N90 a positive correlation was observed between the AF-sensitivity of organisms and the pH value of the medium from which they had been harvested. Three metabolic processes can operate to shift the pH value of culture medium used: (1) unbalanced uptake and output of anions and cations; (2) formation of basic substances from nitrogenous compounds; (3) formation of acids from neutral metabolites. The pH decrease observed with the high glucose media was probably due to the third factor.

The AF-binding capacity of organisms was markedly affected by pH value of the medium in which they had been grown, much acriflavine being bound by organisms from high pH media (Fig. 3A). This suggests a possible explanation for the observation that the organisms from high pH cultures were more sensitive to acriflavine than were those from low pH cultures. Thus, in the case of the AF-resistant strain (N90), and partly in the case of the AF-sensitive strain (18/1042), the effect of initial glucose concentration on the AF-sensitivity of the grown organisms might be accounted for by modification of the AF-binding capacity of organisms because of the pH shift of the culture to more acid values.

The AF-binding increased when the organisms were simply treated with acriflavine at an increased pH value (Fig. 3B) and thus it might appear that, in the experiments illustrated in Fig. 3A, growth at a given pH value might not have been necessary to produce the effect of pH on subsequent AF-binding after washing of the organisms. However, this effect of pH, prior to washing of the organisms and exposure to acriflavine, on the binding of acriflavine was lost when the organisms were not permitted

to multiply at all (unpublished data) or to undergo more than two doublings at a given pH (Table 2). When the organisms were allowed to multiply by a factor more than two or so in a buffered broth medium, the AF-binding capacity characteristic of the pH value of the buffered broth medium in which the organisms were grown was retained even after washing. It is assumed that, when the organisms grew in media of different pH values, irreversible modification corresponding to the pH value occurred in the nature and amount of AF-binding sites.

The present results also show that a large initial glucose concentration increased the AF-tolerance of the AF-sensitive strain (18/1042) even when the pH value of the medium did not change. In the buffered medium the AF-binding capacity of the organisms did not change according to the initial glucose concentration (Table 1). Hence the initial glucose concentration of the culture medium influenced the AF-tolerance of the AF-sensitive strain not through changes in the AF-binding sites. There may possibly be a second process connecting glucose concentration and modification of AF-sensitivity in the AF-sensitive strain. In both of the strains the degree of binding of acriflavine by cells is affected by the pH value of medium but not by the glucose concentration directly.

The author thanks Professor J. Ashida of Kyoto University for his interest in this work.

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ADDENDUM

After submission of this paper the author received a preprint from Dr S. Silver. They include the same experimental results as in this paper with respect to the effect of pH of acriflavine solution on the acriflavine uptake of *Escherichia coli.* SILVER, S., LEVINE, E., & SPIELMAN, P. M. Acridine binding by *Escherichia coli*: pH dependency and strain differences. Submitted to *J. Bact.*