EFFECT OF FLUOROACETIC ACID AND ALLIED FLUOROANALOGUES ON GROWTH OF *ESCHERICHIA COLI*

I. Pattern of Inhibition

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The pharmacological and biochemical aspects of fluoroacetate poisoning have been intensively studied in different animal species (Chenoweth, 1950). The work of Martius (1949) and the fundamental researches of Peters and his associates (1952*a*, 1952*b*) demonstrated that fluoroacetic acid is transformed *in vivo* into fluorocitric acid. The latter blocks the Krebs cycle at the citric acid stage by interfering with the action of aconitase.

Information concerning the action of fluoroacetate on microbial metabolism may be derived from the works of Kalnitsky and Barron (1947) and Black and Hutchens (1948) but to our knowledge no systematic study has been published on the effect of this substance on bacterial growth.

The present investigation was undertaken not only in order to gain better insight into the mode of action of fluoroacetic acid, but also in the hope that it might help to clarify some still highly controversial problems regarding the role and function of the tricarboxylic acid cycle in microorganisms (Ajl, 1951).

MATERIALS AND METHODS

All chemicals used were of reagent grade. The amino acids and vitamins were products of the National Biochemical Corp. (NBC) or of Hoffman La Roche Co.

Sodium pyruvate prepared from redistilled pyruvic acid according to the method of Robertson (1942) was twice recrystallized from aqueous ethanol. α-Ketoglutaric acid was prepared as described by Friedman and Kosower (1946) and recrystallized from *n*-chlorobutylacetate.

Sodium fluoroacetate was obtained by saponification, with saturated alcoholic NaOH solution, of ethyl fluoroacetate prepared according to a method of Bergmann and Blank (1953). The crude product was twice recrystallized from water-ethanol.

Diethyl sodio-oxalofluoroacetate (referred to in the text as oxalofluoroacetate) and fluoropyruvic acid were synthesized as described by Blank and Mager (1954) and Mager and Blank (1954).

Omega-fluorobutyric acid and omega-fluorohexanoic acids were furnished by Prof. T. L. M. Pattison, University of Western Ontario, London, Canada.

The minimal medium was of the following composition: $(NH_4)_2SO_4$, 0.5 per cent; KH_2PO_4, 0.2 per cent; $MgSO_4 \cdot 3H_2O$, 0.05 per cent. The pH was adjusted to 7.2 and the medium was autoclaved for 15 min at 121 C. Glucose (added aseptically), 0.5 per cent.

The various substances added to the minimal medium in the course of the experiments were sterilized usually by filtration through Seitz EK pads or through Corning sintered glass UF filters.

The strains of *Escherichia coli* employed in this study came from a local culture stock.

The inoculum, if not otherwise stated, was prepared by suspending in physiological saline a 6 to 7 hour culture from a 0.5 per cent peptone-beef extract (Difco) agar slant. The cells were washed twice in saline and the turbidity of the suspension was adjusted to an optical density of 0.06 at λ 540 mμ on the Coleman Junior spectrophotometer. This suspension, diluted 1:5 in saline, was added in amount of 0.1 ml to 5 ml medium dispensed in matched tubes. This inoculum contained about 500,000 cells as estimated by viable counts.

The tubes were incubated in vertical position at 37 C for varying periods of time. When more abundant growth was desired, the tubes were

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slanted and shaken in a reciprocating shaker at 100 strokes per minute. Anaerobic cultivation was carried out in Fildes-McIntosh jars evacuated and filled with a mixture of hydrogen and 2 per cent carbon dioxide.

Growth was usually measured in the Coleman photometer, at λ 540 mμ and expressed in terms of either per cent light transmission (tables) or optical density (figures).

All experiments were run in duplicate.

RESULTS

Course of growth inhibition by fluoroacetate. Figure 1 shows that different amounts of sodium fluoroacetate added to the minimal medium produced a marked delay in the onset of growth of *E. coli*. The delay was already appreciable at a concentration of 2.10^{-4} M (about 20 μg fluoroacetic acid per ml medium) and increased with mounting concentrations of the inhibitor. Permanent suppression of growth could not be achieved even by raising the concentration of fluoroacetate to 10^{-1} M. Once growth started, the eventual rate of proliferation, as well as the final amount of growth reached, was equal to that in the control culture.

With equal inocula the number of colonies obtained after 48 hours' incubation on minimal medium agar (minimal medium plus 2 per cent agar-agar) was not affected by the addition of fluoroacetate to the medium even at concentrations as high as 10^{-2} M. However, the colonies grown in the presence of fluoroacetate were of an appreciably smaller size than those on the control plates.

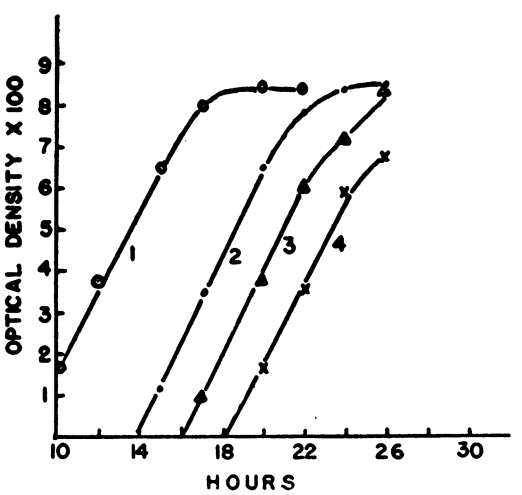

Figure 1. Effect of sodium fluoroacetate on growth of *Escherichia coli* in the minimal medium. Curve 1, control; curve 2, fluoroacetate 4×10^{-4} M; curve 3, fluoroacetate 2×10^{-3} M; curve 4, fluoroacetate 4×10^{-3} M.

TABLE 1

Inhibition of growth of Escherichia coli in the presence of various sources of carbon

Source of Carbon	Amount of Fluoroacetate Added	Growth after Various Periods of Incubation		
		15 hr	24 hr	48 hr
	μg/ml			
Glucose	0	63	45	45
	100	100	98	46
	400	100	97	46
Sodium pyruvate	0	79	74	74
	100	100	98	76
	400	100	98	78
Sodium α-ketoglutarate	0	84	65	40
	100	100	86	55
	400	100	98	75
Sodium malate	0	65	56	56
	100	100	64	58
	400	100	72	55
Sodium succinate	0	57	47	47
	100	90	51	48
	400	100	65	51
Sodium fumarate	0	68	58	59
	100	100	98	58
	400	100	100	58
Sodium acetate	0	96	51	45
	100	100	83	41
	400	100	94	48

In the minimal medium, glucose was replaced by 0.3 per cent of the respective source of carbon. Succinate was used in 0.1 per cent concentrations, as larger amounts proved inhibitory. Incubation was carried out in a shaking apparatus.

When glucose in the minimal medium was replaced by another utilizable source of carbon such as pyruvate or intermediates of the Krebs cycle, essentially the same type of inhibition was obtained. However, the magnitude of the effect varied with the different substances (table 1).

Similarly when asparagine or alanine were used instead of ammonium sulfate as a source of nitrogen, the pattern of growth inhibition remained unaltered, although growth was much more rapid and profuse than in the standard medium.

The bacteriostatic effect of fluoroacetate was largely independent of the age of the inoculum. Inocula taken from 30 and 54 hour cultures did

TABLE 2

Growth inhibition of Escherichia coli by fluoroacetate in aerobic and anaerobic cultures

Amount of Fluoroacetate Added	Aerobic Cultures		Anaerobic Cultures	
	15 hr	24 hr	15 hr	24 hr
$\mu g/ml$				
0	53	25	67	67
80	100	56	84	67
200	100	98	99	66
400	100	100	100	91

not differ in their susceptibility to fluoroacetate from the usually employed 6 to 7 hour inoculum.

The size of the inoculum was also of little influence, since the degree of growth inhibition produced by a given concentration of fluoroacetate remained practically constant while the inoculum varied over a thousandfold range from 2.5×10^4 to 2.5×10^7 cells. However, with inocula ranging from 200 to 1000 cells, the period of growth inhibition tended to be more prolonged.

Varying the initial pH of the medium from 6.2 to 7.8 did not in any way modify the results, nor did incubation at 30 C instead of 37 C (usually employed) alter the response to fluoroacetate. Forced aeration tended to intensify the inhibitory effect of fluoroacetate, while anaerobic conditions of cultivation resulted in a decrease of the effect (table 2).

Cause of spontaneous release of inhibition. The characteristic growth curve obtained in the presence of fluoroacetate (figure 1) indicated that at a certain stage of incubation a spontaneous reversal of inhibition took place.

The possibility first considered was that the reversal might be due to a detoxifying modification of the molecule of fluoroacetic acid induced by the bacteria. Such an assumption appeared *a priori* untenable in view of the known chemical stability of fluoroacetic acid (Swarts, 1896) and in view of the relatively enormous amounts of the inhibitor used in certain experiments in comparison with the small number of bacterial cells. In addition a bioassay performed on rats showed that the amount of fluoroacetic acid added to the culture remained unchanged even after maximum growth had been attained.

The fact that the inhibition was not affected by the size of the inoculum seemed to argue against an alternative hypothesis that the delayed growth in the presence of fluoroacetate might be due to the emergence of a resistant mutant. Since growth occurred invariably with inocula as small as 500 cells per culture tube, an improbably high mutation rate would have to be postulated. Finally this hypothesis could be dismissed by showing that, after five serial passages of *E. coli* in a medium containing fluoroacetate (2.10^{-3} M), the strain retained its original susceptibility to the inhibitor as compared with the parent culture.

In order to account for the fact that the fluoroacetate induced inhibition affects specifically the lag period, it was assumed that fluoroacetate slows down the rate of synthesis of metabolites essential for initiation of growth. Accordingly, it was reasoned that normal growth would start as soon as these metabolites have been produced in an adequate amount by the cells introduced with the inoculum. This assumption seems to be borne out by the results of the following experiments.

A series of tubes, each containing 5 ml of the minimal medium, was inoculated by the usual procedure and incubation was started. At different intervals, fluoroacetate was added to a pair of tubes to a final concentration of 4.10^{-3}M.

The results summarized in table 3 show that preincubation for 3 hours before addition of fluoroacetate resulted in a marked shortening of the time lag for appearance of visible growth. Over this period plate counts revealed less than a twofold increase in cell number. Obviously the effect of preincubation could not be ascribed merely to an increase of the population since, as

TABLE 3

Effect of fluoroacetate added to cultures of Escherichia coli after various periods of preincubation

Time of Incubation Before Addition of Fluoroacetate* (hr)	Growth after Various Periods of Incubation			
	15 hr	19 hr	21 hr	36 hr
0	100	100	100	82
3	95	86	82	82
5	90	83	83	83
Control (without fluoroacetate added)	84	82	82	82

* 200 μg per ml.

pointed out before, even a hundredfold increase of the inoculum did not significantly affect the extent of inhibition induced by fluoroacetate.

In a further experiment 25 ml medium were inoculated as usual and incubated for 7 hours at 37 C. After this period the organisms were centrifuged down (20 min at 5000 rpm) and the supernatant, sterilized by heating for 10 min in a boiling water bath, was transferred in 5 ml portions to four sterile tubes. One pair of tubes served as control, while to the other one fluoroacetate (2.10^{-4} M) was added, and all the tubes were reinoculated. Fluoroacetate produced no growth inhibition in the treated medium.

A Seitz sterilized filtrate of a 48 hour culture of *E. coli* in the minimal medium, when added in a proportion of 1:10 to a fresh culture, completely abolished the inhibitory effect of fluoroacetate. The same result was obtained when a filtrate of *E. coli* grown in the presence of fluoroacetate was similarly tested.

These results demonstrate that *E. coli*, in the course of its growth, produces and causes the accumulation in the medium of a substance(s) capable of nullifying the inhibition produced by fluoroacetate.

Effect of other fluoroanalogues on the growth of E. coli. ω-Fluorobutyric acid was found to inhibit growth of *E. coli* in a manner both qualitatively and quantitatively identical with that observed with fluoroacetic acid while ω-fluorohexanoate

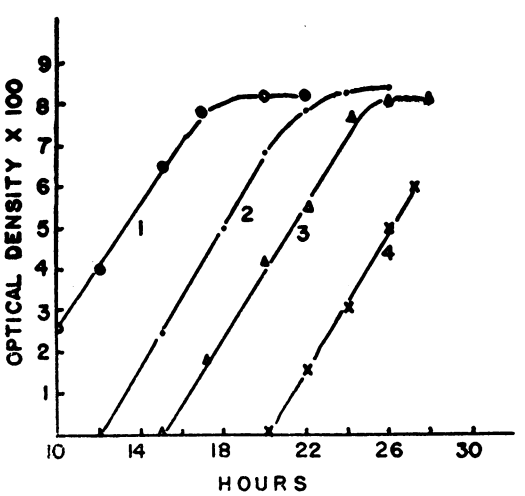

Figure 2. Effect of diethyl sodio-oxaloacetate and sodium fluoropyruvate on growth of *Escherichia coli*. Curve 1, control; curve 2, fluoropyruvate 2×10^{-5} M; curve 3, oxalofluoroacetate 2×10^{-4} M; curve 4, fluoropyruvate 10^{-4} M.

was completely inert. As illustrated by figure 2, oxalofluoroacetate and fluoropyruvate exhibited essentially the same pattern of inhibition as fluoroacetate. However, the dose capable of inducing a still discernible delay (3–4 hours) in the onset of growth was about 25 to 50 times, respectively, lower with these compounds than with fluoroacetate.

Fluoropyruvate, at concentrations above 2.5×10^{-2} M, effected a permanent suppression of growth which finally resulted in sterilization of the culture.

The inhibition of oxalofluoroacetate and fluoropyruvate could be overcome by addition of sterile culture filtrates. However, the reversal was less complete than in the case of fluoroacetate.

Isolation of drug tolerant strains and a study of their cross-resistance to various fluoroanalogues. The striking similarity in the pattern of inhibition exhibited by these fluorinated antimetabolites suggested the possibility that all these compounds might act via fluoroacetic acid arising from their degradation by the bacterial cells. Such a mechanism, while consistent with the behavior of fluorobutyric acid (McCombie and Saunders, 1946), could not account for the observation that oxalofluoroacetate and fluoropyruvate are much more powerful inhibitors of growth than fluoroacetate. Further evidence against this hypothesis was provided by the results of the experiments with resistant strains.

In the isolation of resistant strains the technique of Szybalski (1952) was employed, while the relative size of the colonies served as a guide for their presumed resistance. The largest colonies were picked out and again streaked on the "gradient plate", so as to obtain discrete colonies. By repeating this procedure several times eventually two stable mutants were isolated. The mutant designated *FR* grew unhampered in the presence of fluoroacetate in concentrations as high as 2.5×10^{-2} M. It showed equal resistance to fluorobutyrate and diminished susceptibility to oxalofluoroacetate. A second mutant labeled *OFR* was partially resistant to both oxalofluoroacetate and fluoropyruvate. With this strain the minimal inhibitory doses of the last mentioned compounds were about 10 times higher than the corresponding doses for the parent strains. On the other hand, strain *OFR* remained fully sensitive to fluoroacetate.

Unless differences in permeability are invoked, these results, although indicating some kind of interrelationship in the mode of action of these substanes, appear to argue against the hypothesis of a common active intermediate. At least in the case of the mutant *FR*, lack of permeability to fluoroacetate appears to be ruled out by the finding that fluoroacetic acid strongly stimulated the oxidation of acetate by this strain (Mager, unpublished results). It would be, of course, difficult to visualize that such an effect might have been brought about by a substance which fails to penetrate to the site of the enzyme action.

DISCUSSION

The basic feature of the growth inhibition produced by fluoroacetate and the other fluorinated compounds studied in this work is the spontaneous and complete reversal following a more or less prolonged lag time.

A similar pattern of activity has been observed with some basic dyes (Dubos, 1929; Ingraham, 1933), with furacin (Cramer and Dodd, 1946) and more recently with *p*-fluorophenylalanine (Atkinson, Melvin and Fox, 1951) and pyridine-3-sulfonic acid (Moore and Boylen, 1952).

In the case of furacin the occurrence of delayed but normal cell multiplication after a period of growth suppression was correlated with reduction of the compound by the inoculated bacteria, leading to its detoxification (Cramer, 1947). On the other hand, the spontaneous reversal of the *p*-fluorophenylalanine induced inhibition has been attributed by Atkinson *et al.* (1951) to selection of resistant mutants.

In our case it could be demonstrated that reversal of growth inhibition is brought about by some product(s) of the bacterial cells elaborated even in the absence of significant cell proliferation. It appears likely that these products are metabolites required for initiation of growth and that the fluoroanalogues cause prolongation of the lag period by decreasing the rate of their formation.

Data from the literature (Kalnitsky and Barron, 1947; Black and Hutchens, 1948) and our own experimental results to be reported at a later date suggest that the fluoroanalogues block primarily some steps of the tricarboxylic acid cycle. From differences in the degree of the inhibitory effect and from the lack of cross-resistance among the drug tolerant strains to heterologous compounds it may be deduced that the various fluorinated antimetabolites interfere with *different* component reactions of the Krebs cycle. However, no matter at what stage the cycle is interrupted, the final result is a derangement of the synthesis of certain metabolites essential for early reproduction. This interpretation may also explain the remarkable uniformity of the inhibition pattern exhibited by the various fluoroanalogues studied.

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SUMMARY

Sodium fluoroacetate, sodium fluoropyruvate and diethyl sodio-oxalofluoroacetate cause a temporary inhibition of growth of *Escherichia coli* in a minimal culture medium. The period of inhibition is followed by normal and unimpaired proliferation.

The inhibitory power of fluoropyruvate and oxalofluoroacetate is 50 to 25 times, respectively, higher than that of equivalent amount of fluoroacetate.

The isolation of two drug resistant strains, one tolerant to fluoroacetate and the other one to oxalofluoroacetate, is described. The resistant strains show a rather strict specificity for the compound with little cross-resistance to heterologous compounds.

The factors responsible for the spontaneous reversal of inhibition are analyzed and the possible mechanism involved is discussed.

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