um. 1952, U. of Minn. Press, Minneapolis, p. 217.
2. McMaster, P. D., and Kruse, H., *Fed. Proc.*, 1951, v10, 564.
3. Latta, H., *J. Immunol.*, 1951, v66, 635.
4. Crampton, C. F., and Haurowitz, F., *J. Immunol.*, 1952, v69, 457.
5. Fischel, E. E., and Kabat, E. A., *J. Immunol.*, 1947, v55, 337.
6. Kekwick, R. A., and Cannan, R. K., *Biochem. J.*, 1936, v30, 227.
7. Coons, A. H., and Kaplan, M. H., *J. Exp. Med.*, 1950, v91, 1.
8. Kaplan, M. H., Coons, A. H., and Deane, H. W., *J. Exp. Med.*, 1950, v91, 15.
9. Coons, A. H., Leduc, E. H., and Kaplan, M. H., *J. Exp. Med.*, 1951, v93, 173.
10. Robertson, J. S., *Austral. J. Exp. Biol. Med. Sc.*, 1952, v30, 59.
11. Dixon, F. J., Bukantz, S. C., Dammin, G. J., and Talmage, D. W., *Fed. Proc.*, 1951, v10, 553.
12. Lurie, M. B., *J. Exp. Med.*, 1942, v75, 247.

Received January 30, 1953. P.S.E.B.M., 1953, v82.

Clotting of Citrated Plasma by Bacteria which Destroy the Anticoagulant: Effect of Sodium Fluoroacetate. (20233)

ABRAHAM I. BRAUDE, JAY P. SANFORD, JANE BARTLETT, AND JOYCE FELTES.
(Introduced by Reuben Kahn.)
From the Department of Internal Medicine, University of Michigan, Ann Arbor

When certain bacteria are incubated in citrated plasma, they frequently remove the anticoagulant, citrate, and the blood clots (1,2). Clotting is a dependable end point of utilization of citrate and could offer a simple means of studying bacterial metabolism of citrate. The present study was conducted to clarify how the rate of bacterial utilization of citrate in plasma is related to bacterial growth and to clotting. In addition, sodium monofluoroacetate and potassium cyanide, substances known to affect metabolism of citrate in experimental animals, were tested for their action on the clotting of citrated plasma by bacteria.

Method. In all experiments, organisms were tested for clotting by inoculating 1.0 ml of citrated human plasma with 0.1 ml of a culture grown for 24 hours in tryptose phosphate broth. Inoculated plasmas were incubated at 37°C and examined daily to 8 days for clotting. Clotted plasmas were completely jelled. Unclotted plasmas remained entirely fluid. One ml of ACD solution (1.32 g sodium citrate, 0.48 g citric acid, and 1.47 g dextrose per 100 ml) was used for 4 ml of blood. Results were recorded only if heavy bacterial growth had occurred. Quantitative determinations of citrate were made on whole clotted plasmas, including fluid within the clot, by the method of Lardy(3). The *rate of clotting* of citrated plasma containing increasing concentrations of sodium monofluoroacetate or potassium cyanide was determined after inoculation with various strains of *Aerobacter aerogenes* or enterococci. The rate at which *bacteria destroyed citrate* was determined in mixtures containing 3 parts of citrated plasma

TABLE I. Clotting by Bacterial Consumption of Citrate. Results obtained by growth of 116 strains in citrated blood.

Bacteria	Strains tested, No.	Strains which clotted citrated human blood, No.
A. aerogenes	18	16
A. cloacae	2	2
K. pneumoniae	8	6
Salmonella species	5	4
Diphtheroid	2	2
Ps. aeruginosa	15	7
B. alcaligenes	1	1
Paracolon species	4	2
E. freundii	8	3
E. coli	20	12
Shigella species	2	1
Staphylococcus coagulase—	15	2
Enterococcus	13	4
Str. viridans	3	0
Totals	116	62

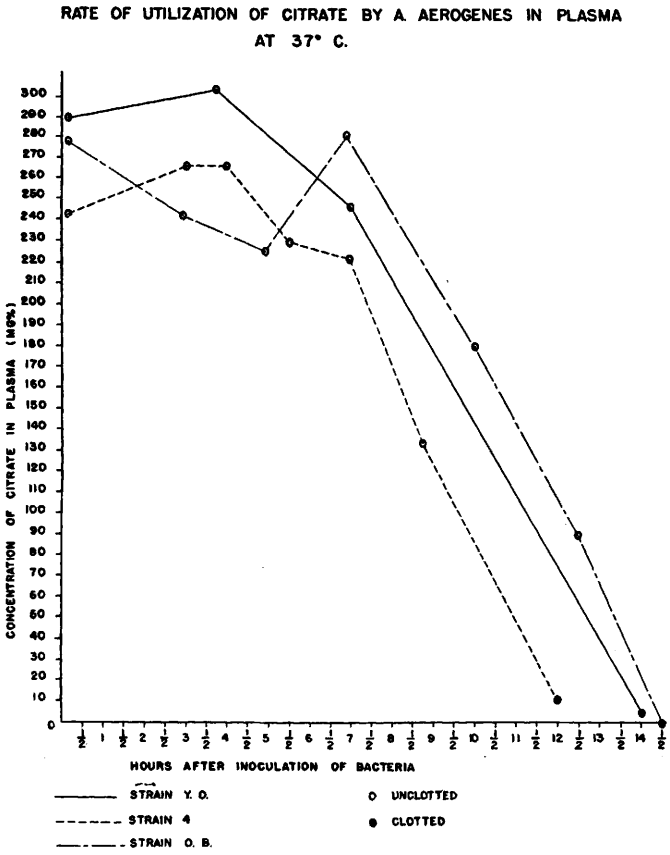

FIG. 1. Rate of utilization of citrate in plasma by *A. aerogenes* and relationship to clotting.

and 1 part of a 24 hour broth culture of *A. aerogenes*. These inoculated plasmas were divided into aliquots which were incubated simultaneously at 37°C. Individual aliquots were removed at intervals from the incubator and rapidly frozen. The aliquots were later thawed and examined for citrate content. Bacterial destruction of citrate was also determined in aliquots containing 10 mg of fluoroacetate per ml of clear plasma which had been examined turbidimetrically in the Coleman spectrophotometer for bacterial growth. *Anaerobic* studies were conducted in jars whose air had been evacuated and replaced by helium.

Results. The occurrence of clotting in plasma inoculated with any of 116 strains of bacteria is shown in Table I. Clotting was characterized by sudden jelling of the entire sample of plasma. The clot did not retract for 24 hours. Results with coagulase-positive staphylococci are omitted because the coagulase reaction masked clotting resulting from removal of citrate. The concentration of citrate after plasmas were clotted by strains of enterococci and *A. aerogenes* was frequently zero and usually less than 30 mg %. These bacteria did not clot heparinized plasma, oxalated plasma, serum or fibrinogen solution.

The rate of utilization of citrate with 3 strains of *A. aerogenes* is shown in Fig. 1. Citrate concentration falls abruptly after a stationary period when relatively little change is observed. Bacterial growth curves in Fig. 2 illustrate that the sharp fall in citrate concentration takes place after the bacteria have been in the stationary growth phase for about 4 hours. A secondary increase in bacterial density (A-B in Fig. 2) appears during the period of rapid destruction of citrate. This secondary rise in bacterial density, presumably due to bacterial growth, was sharply reduced

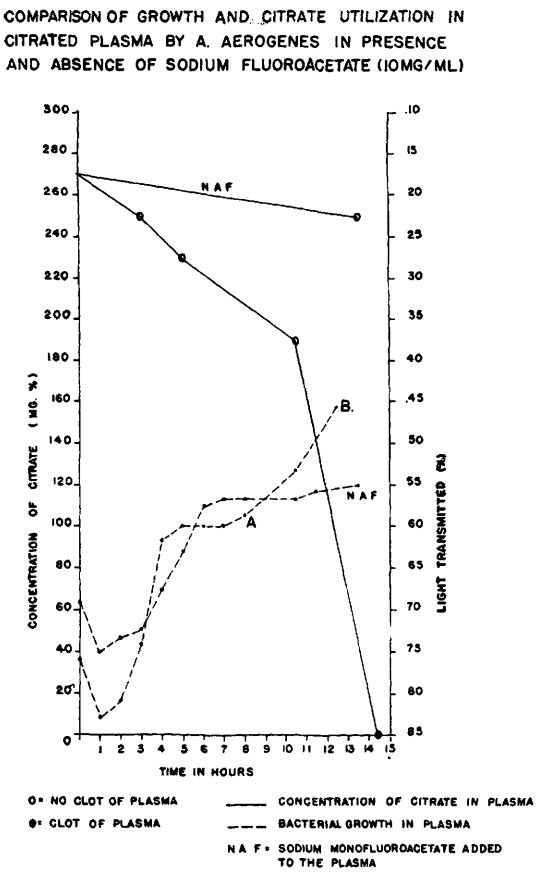

FIG. 2. Comparison of growth and citrate utilization in citrated plasma by *A. aerogenes* in presence and absence of sodium fluoroacetate (10 mg/ml).

by fluoroacetate at the same time that clotting and citrate utilization were prevented.

Fluoroacetate inhibited the clotting produced by many strains of both *A. aerogenes* and enterococci. As shown in Fig. 3, delay of bacterial clotting bore almost a linear relation to the concentration of fluoroacetate. Fluoroacetate itself was no anticoagulant and did not change pH. Complete anaerobiosis did not delay clotting produced by 13 strains of *A. aerogenes*, but repeatedly abolished or reduced the inhibitory action of fluoroacetate (5.0 g per ml) on bacterial clotting. Whenever clotting was prevented by fluoroacetate, the utilization of citrate was found to be inhibited.

The presence of 0.1 M KCN inhibited clotting of citrated plasma by 8 strains of *A. aerogenes*, but did not inhibit clotting by 11 strains of enterococci. Surprisingly, KCN actually hastened clotting by 6 strains of enterococci, although KCN has no effect of its own on clotting.

Discussion. The evidence presented here demonstrates that clotting of citrated plasma may be used as a dependable and simple end point of citrate utilization in studying bacterial metabolism. Under controlled conditions, a given strain can be depended upon to clot citrated plasma after uniform periods of incubation.

Data obtained in this study by observing bacterial clotting, and supported by measurement of citrate, indicate that sodium monofluoroacetate inhibits citrate utilization by *A. aerogenes* and enterococci. Fluoroacetate was examined for its effect on clotting of citrated plasma by bacteria because it has been found to prevent citrate utilization in animals. It has been postulated that fluoroacetate prevents oxidation of citrate in the tricarboxylic acid cycle(4,5). It is possible therefore that in preventing utilization of citrate by *A. aerogenes*, fluoroacetate acts in an oxidative cycle resembling the citric acid cycle. The loss by fluoroacetate of its inhibitory effect on clotting under anaerobic conditions further indicates that fluoroacetate activity was related to oxidative bacterial metabolism.

In the case of enterococci, however, it would be unlikely that an oxidative cycle of the tricarboxylic type is involved in utilization of citrate. Enterococci possess no iron-enzymes, and it was not surprising to discover that KCN did not prevent or delay clotting by these bacteria. The marked inhibitory effect of fluoroacetate on clotting by enterococci suggests that the antagonistic action of fluoroacetate to citrate utilization is not limited to oxidative systems resembling the conventional citric acid cycle.

Summary and conclusions. The relationship of bacterial growth to utilization of citrate and clotting of plasma has been determined. Clotting of citrated plasma may be used as a dependable and simple end point of citrate utilization by certain strains of bacteria. Fluoroacetate was found to prevent clotting by interfering with utilization of citrate by *A. aerogenes* and the enterococci.

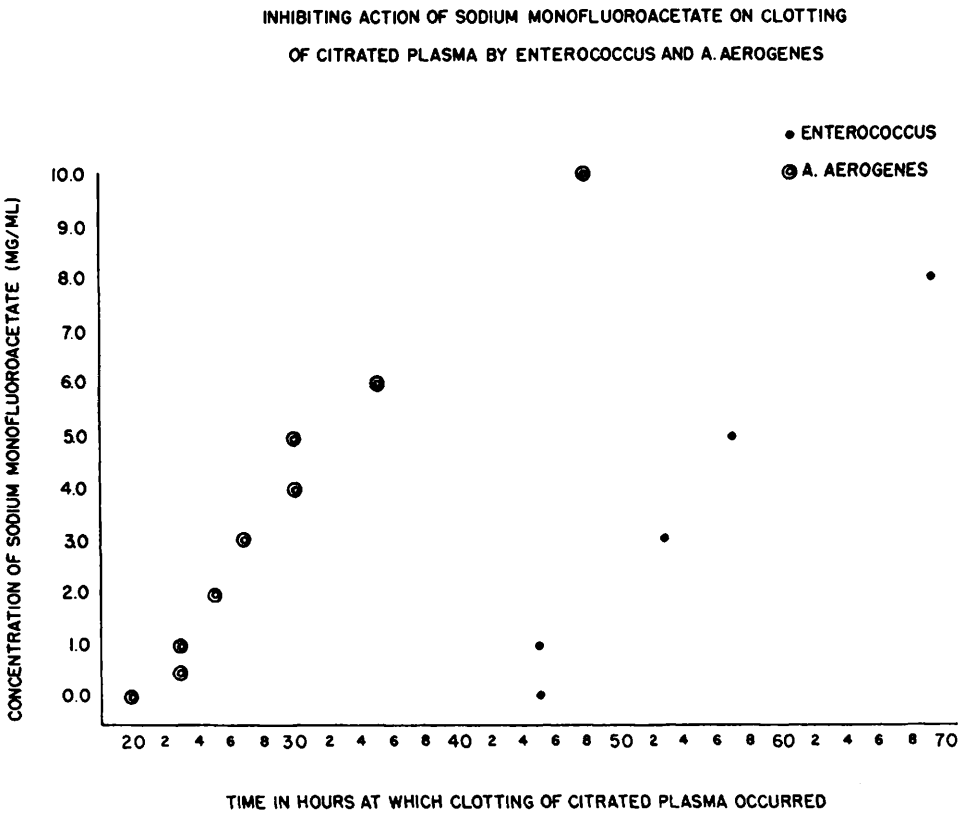

FIG. 3. Inhibiting action of sodium monofluoroacetate on clotting of citrated plasma by enterococcus and *A. aerogenes*.

Cyanide had no effect on clotting by enterococci.

The sodium fluoroacetate was generously provided by Dr. Maynard Chenoweth.

1. Braude, A. I., Sanford, J., Bartlett, J., and Mallery, O. T., *J. Lab. Clin. Med.*, 1952, v39, 902.

2. Harper, E. M., and Conway, N. S., *J. Path. Bact.*, 1948, v60, 247.

3. Lardy, H. A., In Umbreit, W. W., Burris, R. H., and Stauffer, J. F., *Manometric Techniques and Tissue Metabolism*. Burgess Publishing Co., Minneapolis, Minn., 1949, 163.

4. Buffa, P., and Peters, R. A., *J. Physiol.*, 1950, v110, 488.

5. Elliott, W. B., and Kalnitsky, G., *J. Biol. Chem.*, 1950, v186, 487.

Received February 27, 1953. P.S.E.B.M., 1953, v82.