THE UTILIZATION OF CITRATE BY ESCHERICHIA COLI[1]

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The coliform bacteria—*Escherichia* and *Aerobacter* genera—are the subject of continued interest, particularly from the standpoint of differentiation and ecology, because they are so widely recognized as indicators of the sanitary quality of water and other foods, food-production plants, and eating places, as well as agents of spoilage of a wide variety of foods. Formerly the two genera were separated largely on the basis of the Voges-Proskauer (VP) and methyl red (MR) reactions, particularly after Levine (1916*a*,*b*) had shown that these tests correlated with differences in glucose metabolism and consequently with the origin of the bacteria. As a result, it was generally accepted that the species of *Aerobacter*, largely of nonfecal origin, were VP+ and MR−, whereas the species of *Escherichia*, commonly used as indicators of vertebrate fecal contamination, were VP− and MR+. The advent of Koser's citrate test, however, created a problem in differentiation which is perplexing even today.

Koser (1924*a*,*b*,*c*), as a result of an extensive study of the utilization of the salts of organic acids, found that sodium citrate was of special significance for the apparent separation of representative cultures of *Escherichia* and *Aerobacter*. *Escherichia coli* strains failed to develop visible turbidity, whereas the *Aerobacter aerogenes* cultures multiplied readily and produced luxuriant growth in the "synthetic" citrate medium. After further investigation Koser reported the correlation of citrate utilization with other differential characteristics and with habitat; claimed a better correlation of citrate utilization with the sanitary condition of water supplies than with other tests in common use at that time; and observed that the ability to utilize citrate as the sole source of carbon in an otherwise inorganic medium, as applied to the coliform bacteria, was a character not readily acquired or lost. Concurrently (1924*a*) and later (1926), Koser reported finding certain colilike bacteria that grew as luxuriantly in the citrate medium as did the cultures of *Aerobacter* although they resembled *E. coli* in other respects. Study of these citrate-utilizing, "intermediate" bacteria soon led to general confirmation of Koser's work and to the recognition of the wide distribution of such organisms in nature. (See Parr, 1939, and Vaughn and Levine, 1942, for a more detailed review.)

In contrast, generic classification has resulted in a near compromise allocation of these colilike, citrate-utilizing bacteria to the genus *Escherichia*, primarily be-

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cause their natural habitat has not been clearly demonstrated and because, unlike the normal "fecal" types of *E. coli*, they do utilize citrate in Koser's medium. Furthermore, the asserted vagaries of growth of *E. coli* in Koser's citrate broth or in Simmons' (1926) citrate agar have combined to delay any decisive conclusions. It was claimed even prior to the description of Koser's citrate test that *E. coli* could decompose citrate if grown in a suitable medium (Altobelli, 1914; Brown, 1921; and others). It has been reported by Ruchhoft *et al.* (1931), and adequately confirmed, that cultures of *E. coli* grow and multiply in Koser's citrate medium, although the maximum population levels reached remain below the limit of visibility to the naked eye. Parr (1938) and Parr and Simpson (1940) also have described the development of stable, citrate-utilizing "mutants" of *E. coli*, which they were able to cultivate on Simmons' citrate agar.

Great importance is attached to the test for *citrate utilization as a sole source of carbon* in the differentiation of the coliform bacteria. Nevertheless, there is no indisputable proof that *E. coli* does or does not decompose citrate under certain conditions in Koser's, Simmons', or other citrate media. Consequently, investigations were made in an attempt to determine under what conditions *E. coli* might grow and produce visible turbidity in Koser's citrate medium and actually might decompose the citrate.

EXPERIMENTAL RESULTS

As has already been stressed, the differentiation of *E. coli* from *E. freundii*, *E. intermedium*, and the species of *Aerobacter* is based upon the inability of the "coli" group to utilize citrate as a sole source of carbon (Yale, 1948). Thus, by definition at least, citrate should be the only carbon compound contained in the medium used for testing the ability of coliform bacteria to utilize citrate. In principle, therefore, Koser's liquid citrate medium is the only one that meets this requirement. Other citrate media in general use contain agar and organic pH indicators, either of which might contribute food for the growth of bacteria although actual utilization of citrate might not occur.

Growth of E. coli in citrate media. Growth rather than actual demonstration of the decomposition of citrate is commonly used as a criterion for a positive test of citrate utilization. It is to be expected, therefore, that under certain conditions growth (turbidity) alone might be a false criterion. The growth of *E. coli* in citrate media that contain agar is a case in point.

(*a*) *Growth in citrate agar media.* Some workers persist in using growth alone as a criterion of citrate utilization in citrate agar media, even though it is easily demonstrable that most, if not all, strains of *E. coli* will grow in such media. However, there is no evidence of actual utilization of citrate by the *E. coli* strains. It is quite obvious, then, if citrate agar media are used, growth alone cannot be used as a criterion for a positive citrate test. Simmons, who developed the first diagnostic citrate agar medium, was aware of the problem and overcame it by using change in pH value of the medium rather than growth as a criterion of citrate utilization.

It is probable that the demonstrable growth of *E. coli* in citrate agar media

results from the presence of contaminating chemicals introduced with the inoculum, however small. The agar tends to keep the growth-promoting contaminants concentrated along the line of inoculation, and growth becomes apparent, whereas in Koser's liquid medium the contaminants diffuse and no concentration of cells can occur. On the other hand, since agar is a complex polysaccharide, the growth might result from its partial hydrolysis. Or, as has been demonstrated by Robbins (1939), the growth might originate from growth factors present in the agar.

(*b*) *Growth in Koser's liquid citrate medium.* Contaminants that could be introduced into citrate media with the inoculum would include traces of ingredients of the various media used for the preparation of the test cultures or, in the absence of chemical cleanliness, almost any compound used in the laboratory. Accordingly, tests were made to determine the least amounts of various sugars, alcohols, organic acids, amino acids, etc., which would support visible growth of *E. coli* and its varieties in Koser's liquid citrate medium.

Care was taken to avoid the introduction of chance chemical contaminants into the experiment. A fresh lot of Koser's citrate medium (Difco) was obtained and used throughout the experiment. All glassware was treated with cleaning solution and rinsed with distilled water that had been redistilled from pyrex. Redistilled water was used for the preparation of the basal medium and all solutions of the various chemicals to be tested. The basal citrate medium was sterilized in the autoclave. The chemical solutions were sterilized by filtration. The two were then combined under aseptic conditions.

The test chemicals were prepared in concentrations calculated to give 500 μg per ml of finished medium when 1 ml of the stock solution was diluted in 9 ml of Koser's medium. By further dilution the final concentrations tested included 50, 5, 0.5, 0.05, and 0.005 μg of chemical per ml of Koser's medium. Untreated Koser's medium was used as a control. No attempt was made entirely to eliminate chemical contaminants that originate from inoculation. Such contaminants were kept at a minimum by careful transfer of the liquid inoculum by a straight needle. The seeded needle was vigorously shaken and then immersed no more than 1 cm into the test medium.

Cultures of *E. coli*, *E. communior*, *E. neapolitana*, and *E. acidilactici*, represented by American Type Culture Collection strains 26, 207, 133, and 128, respectively, were used for these tests.[2] The cultures were grown in standard nutrient broth for 1 day at 30 C and then used as inocula in the manner already described.

Fifty-eight different compounds or mixtures were tested. However, only 24 of these, when present in concentrations of 50 μg or less per ml of medium, were found to support development of turbidity by the test cultures. Compounds that did not support growth under the conditions of this experiment included D-arginine·HCl, β-alanine, L-cystine, L-histidine·HCl, L-hydroxyproline, DL-leucine, DL-lysine·HCl, DL-methionine, DL-norleucine, DL-phenylalanine, DL-valine,

[2] These experiments were initiated during March, 1941, discontinued in December, 1941, as a result of the war, and not reinitiated until 1947. Consequently some different test cultures were used in the latter experiments.

DL-threonine, α-amino-*n*-valeric acid, urea, uric acid, hydantoin, uracil, adenine sulfate, calcium formate, sodium butyrate, sodium malonate, sodium L-malate, sodium D-tartrate, α-methyl glucoside, esculin, salicin, adonitol, glycerol, sucrose, calcium pantothenate, nicotinic acid, riboflavin, thiamine·HCl, and a mixture of mineral salts. It is probable that in concentrations greater than 50 μg per ml many of the compounds listed above, as well as many compounds not tested, would support growth of the test cultures.

The minimum additions of those compounds that enabled *E. coli* and related species to grow and produce turbidity in Koser's citrate medium are shown in table 1. It is clear that a variety of unrelated compounds (each independently tested in relatively small quantities) will enable *E. coli* and related varieties to grow and produce turbidity in Koser's citrate medium.

In all cases growth occurred without citrate utilization. It is probable therefore that, in the concentrations tested, the added compounds functioned only to provide food for the growth of the test cultures. It could be demonstrated by turbidity measurements (Evelyn colorimeter) and microscopic counts (Petroff-Hauser) that increased growth was roughly proportional to the amount of added chemical above that required for the production of discernible turbidity (visual and turbidimetric). Therefore, exhortations to use care in the preparation of the diagnostic citrate media as well as judgment in the execution of the test of citrate utilization as a sole source of carbon are well founded.

Utilization of citrate by E. coli. Although it has been reported that cultures of *E. coli* could decompose citrate if grown in a suitable medium (Altobelli, 1914; Brown, 1921; Grey, 1924; and others), careful scrutiny of the results of these investigations did not dispel all reasonable doubt as to the authenticity of the cultures of *E. coli* used. Therefore it was desirable to determine what cultural conditions would be required for *E. coli* and related varieties to decompose citrate, on the assumption that these bacteria actually might decompose citrate when grown in suitable media.

Exploratory tests using qualitative criteria of utilization (increases in carbon dioxide production and pH value of the test medium as compared with controls) showed that cultures of *E. coli* and related varieties did decompose citrate if grown in the presence of 0.25 to 0.5 per cent peptone or glucose contained in Koser's citrate medium. Quantitative confirmation was then sought to determine the extent of utilization.

To determine actual citrate decomposition the accessory chemicals were added in concentrations of 0.01, 0.1, 0.25, and 0.5 per cent to Koser's citrate medium. The test media were then prepared in 30-ml amounts in 60-ml bottles, sterilized in the usual manner, inoculated with a 4-mm loopful of 24-hour lactose broth culture of the test organism, incubated at 30 C for 4 days, and then analyzed for citrate content as compared with uninoculated controls.

The procedure used for citrate determination was based on the Hartmann (1943) modification of the Hartmann and Hillig gravimetric pentabromacetone method as outlined in Winton and Winton (1945). This method was further modified in that a molar potassium bromide solution was substituted for crystal-

TABLE 1

Minimum additions of organic compounds that enabled E. coli and related species to grow and produce turbidity in Koser's citrate medium

COMPOUND	BACTERIA			
	E. coli	*E. acidilactici*	*E. neapolitana*	*E. communior*
	Minimum concentrations,* μg/ml, supporting visible growth, 4 days at 30 C.			
Peptone (Difco)	50	50	—†	50
Tryptone (Difco)	50	50	50	5
Proteose peptone (Difco)	50	50	50	5
L-Asparagine	0.5	0.5	0.5	0.5
L-Cysteine·HCl	50	50	50	50
Glycine	0.5	0.5	0.5	0.5
L-Proline	50	50	50	5
DL-Serine	50	50	—	50
L-Tryptophan	50	50	50	5
L-Tyrosine	50	50	50	50
L-Aspartic acid	50	50	50	5
Glutamic acid	0.5	0.5	5	0.5
Nucleic acid from yeast	50	50	50	50
Allantoin	50	50	—	50
Glutathione	—	—	—	50
D-Xylose	5	5	5	0.5
L-Arabinose	50	50	50	50
Glucose	0.5	5	5	0.5
Fructose	0.5	0.5	50	0.5
Maltose	50	50	50	50
Lactose	5	5	5	0.5
Potassium acetate	50	50	50	50
Sodium propionate	50	50	50	50
Sodium lactate	50	50	50	50

* Maximum addition 50 μg per ml of medium; 5, 0.5, and 0.05 μg per ml also tested. Each compound represents a separate test. Each figure represents approximately the minimum quantity of chemical required for the development of turbidity by the test culture in Koser's medium. No utilization of citrate was detected. Turbidity was measured with the Evelyn colorimeter to confirm visual inspection.

† (—) = no visible growth with 50 μg per ml. Higher concentrations enabled cultures to grow.

line potassium bromide. The bromination time also was extended to 30 minutes. These changes simplified the method for this specific use and recoveries were just as uniform.

Four cultures of *E. coli* were used for this experiment. Culture 26 was from the

TABLE 2

Utilization of citrate in Koser's medium by E. coli

CULTURE	ACCESSORY COMPOUND	FINAL CONCENTRATION OF COMPOUND			
		0.01%	0.1%	0.25%	0.5%
		Per cent of citrate utilized, 4 days, 30 C			
J V R	Peptone (Difco)	—*	—	—	65.2
	Glucose	6.2	99.7	84.1	93.7
	Fructose	4.0	91.7	98.5	89.9
	Na-acetate	0	0	30.8	71.9
	Ca-formate	0	0	0	0
	L-Asparagine	0	6.2	11.8	13.2
	L-Aspartic acid	0	12.5	5.0	0
26	Peptone (Difco)	—	—	—	30.2
	Glucose	0	99.3	75.1	58.2
	Fructose	3.2	93.0	97.1	67.9
	Na-acetate	0	4.9	22.6	22.9
	Ca-formate	0	0	0	0
	L-Asparagine	0	4.8	2.9	9.7
	L-Aspartic acid	0	0	0	0
S 5-101	Peptone (Difco)	—	—	—	93.1
	Glucose	7.6	99.4	54.1	66.1
	Fructose	4.2	88.0	99.8	92.7
	Na-acetate	0	13.0	25.6	56.9
	Ca-formate	0	0	0	0
	L-Asparagine	0	1.1	31.2	8.8
	L-Aspartic acid	0	0	0	0
S 5-102	Peptone (Difco)	—	—	—	86.3
	Glucose	6.2	99.4	65.1	60.3
	Fructose	12.9	89.2	98.8	92.7
	Na-acetate	0	20.8	42.0	96.1
	Ca-formate	0	0	0	0
	L-Asparagine	0	7.2	7.5	12.5
	L-Aspartic acid	0	0	0	0
Aerobacter control 182†	Peptone (Difco)	—	—	—	—
	Glucose	9.8	99.1	—	—
	Fructose	13.9	89.2	70.0	61.7
	Na-acetate	0	0	0	0
	Ca-formate	0	0	0	0
	L-Asparagine	0	7.6	7.6	9.5
	L-Aspartic acid	1.3	4.0	34.4	—

* — indicates no test was made.

† This bacterium conforms to the species *Aerobacter aerogenes* except for its inability to utilize citrate as a sole source of carbon.

American Type Culture Collection; strain JVR was isolated from spoiled calcium tartrate; cultures S5105 and S5102 were isolated from human feces especially for this study.

The data shown in table 2 amply illustrate that cultures of *E. coli* actually do decompose appreciable amounts of citrate if Koser's citrate medium is supplied with sufficient other compounds as food materials. Adaptation apparently does not explain the utilization of citrate by *E. coli*. Although the added compounds supported visible growth of the test cultures when present in quantities of 50 μg or less per ml of Koser's medium, citrate utilization did not occur. The citrate was decomposed only when the concentration of the other compound was increased to the range of 0.1 to 0.5 per cent.

Glucose and fructose stimulated the greatest decomposition of citrate. However, it is apparent from table 2 that different optimum concentrations of these two compounds are required. Almost complete decomposition of the citrate resulted when Koser's medium contained 0.1 per cent glucose or 0.25 per cent fructose. In contrast, the effect of sodium acetate was noticeably increased as more acetate was added. The results with L-asparagine and L-aspartic acid were erratic. Calcium formate had no effect on citrate utilization under the conditions of this experiment.

The exact function of these additional compounds is not known. However, despite the claim of Lominski *et al.* (1947), it is not possible to accept the idea that these compounds function as true hydrogen acceptors without more substantial evidence. It is hoped that more experimentation will clarify their function.

DISCUSSION

The results of these experiments stress again the need for care and judgment in the preparation of the medium and the execution of the test for the utilization of citrate as a sole source of carbon. It is more important, however, that the results explain, in part at least, a fundamental difference between *E. coli* and other coliform bacteria.

In reality, Koser's citrate test is but a measure of the ability or inability of different coliform bacteria to initiate and at least partially complete the decomposition of citrate in the absence of other compounds. *E. coli* and related varieties do not decompose citrate unless such other compounds are available in appreciable quantities. On the other hand, most of the cultures which, by definition, would be recognized as *A. aerogenes* or *A. cloacae* as well as *all* isolates of *E. freundii* and *E. intermedium* do not require these additional compounds in order to decompose citrate. This interpretation conforms to the presently accepted conception of the test for citrate utilization. However, isolates of *Aerobacter* are known that do not utilize citrate as a sole source of carbon but, as shown in table 2, require the presence of other compounds before they can decompose it. No adequate provision has been made for the classification of such isolates of *Aerobacter*, although such types have been known for almost as long as Koser's citrate test has been used (Ruchhoft *et al.*, 1931). Furthermore, such unusual non-citrate-utilizing strains cannot exist among the isolates of *E. freundii* and *E. intermedium*, for, by definition, the two latter species *must* utilize citrate as a sole source of carbon; otherwise they must be classified with *E. coli* and related varieties. Such a compromise situation with regard to classification is unwarranted.

Furthermore, because of the importance of the genus *Escherichia* as an index of sanitation for foods, food products, food production plants, etc., such a situation is also untenable. It is therefore obvious that steps should be taken to establish the ecology of the species now known as *E. freundii* and *E. intermedium* but formerly classified with the "fecal" coli group and given different specific designations.

SUMMARY

The growth and development of turbidity in Koser's citrate medium by *E. coli* and related species (*E. acidilactici*, *E. communior*, and *E. neapolitana*) was found to be dependent upon the addition of supplementary compounds. When such compounds as acetate, lactate, propionate, glucose, fructose, L-asparagine, glutamic acid, etc., were added to Koser's citrate medium in concentrations ranging from 0.5 to 50 μg per ml of medium, the test cultures produced turbidity but did not utilize the citrate.

The utilization of citrate in Koser's medium by *E. coli* was dependent upon the addition of these supplementary compounds in concentrations ranging from 0.1 to 0.5 per cent. The function of these supplementary compounds in the decomposition of citrate by *E. coli* is not known.

The experiments specifically show the reasons for the exercise of care and judgment in the preparation of the medium and the execution of the test for the utilization (decomposition) of citrate as a sole source of carbon.

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