COLIFORM "MUTANTS," WITH RESPECT TO THE UTILIZATION OF CITRATE

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As has been shown (Parr, 1939), a test for the utilization of citrate as a sole carbon source is one of the three most widely used technical procedures for determining the identity and relationships of coliform bacteria. Most workers who have made use of it since its introduction by Koser (1923) consider the test satisfactory, whether they employ it as originally proposed (Koser, 1924a) or make use of the refinement of Simmons (1926). Since the citrate utilization test introduced new concepts into the bacteriology of coliform organisms, Koser (1924b) undertook experiments to determine if citrate utilization possessed those characteristics of stability essential to a diagnostic procedure. He showed that citrate utilization, or the lack of it, is "constant and reliable" and not readily acquired or lost. With these conclusions we are in complete accord. *Escherichia coli* does not satisfactorily utilize citrate as a sole source of carbon for growth purposes, and when sown in citrate broth (Koser test) or streaked on citrated agar (Simmons' test) fails to develop to the threshold of recognition. On the other hand, *Escherichia freundii*, *Aerobacter aerogenes*, and *Aerobacter cloacae* develop promptly, usually producing easily recognized growth within 24 hours.

Ruchhoft and co-workers (1931) have clearly shown that the colon bacillus does grow in Koser's citrate broth but that it only attains therein a population density of from one to five million cells per milliliter which is not enough to give a definitely recognizable turbidity. It is significant that these workers recovered typical *Escherichia coli* from such "negative" tubes weeks after

inoculation, in one case even after a year's time. They definitely state that such cultures did not exhibit any change in their biochemical features, although rough colonies were frequently encountered in platings from such aged broth sources.

Evidence exists that occasional coliform strains may be encountered which are neither absolutely negative nor completely positive with respect to the utilization in culture of citrate as a sole source of carbon. Parr (1938) reported as "mutants" certain coliform strains departing from the generally accepted rule. It remains to describe how these atypical strains behave, how "mutants" are handled to obtain pure breeding lines of descent, to give a preliminary statement as to the stability of derived lines, and to discuss the possible significance of such findings.

The senior author first observed the citrate "mutants" as early as 1934 (see Plate 1), and in his laboratory Evans (1935) induced their production by special cultural procedures. The clearest picture of what happens in nature with respect to citrate "mutants" can, however, best be obtained through a description of a culture, called H.23, which, with 64 other atypical coliform cultures, was sent to Parr by A. V. Hardy of the DeLamar Institute of Columbia University from his studies on *Shigella* from Indians carried out in New Mexico in the summer of 1937.

H.23, a slow lactose fermenter, when tested was indole positive, methyl red positive, and Voges-Proskauer negative. When inoculated[1] on a sector of Simmons' citrated agar it produced no immediate growth. H.23 was, therefore, apparently a slow lactose-fermenting *Escherichia coli* (Imvic $++--$). It was found, however, that on the fifth day after citrated agar had been inoculated, one colony appeared on the area sown. This "mutant" colony grew larger and by the seventh or eighth day was accompanied by a change in color of the surrounding medium from green to blue. No other colony developed on the area despite the holding of the plate for a total of fifteen days' observation.

[1] The citrated agar is inoculated by wiping a shaken loop of culture in broth (no visible film) on a sector of the plate the size of half a dollar.

The first impression from such results is that there has been a contamination of the medium. Subsequent work, involving the

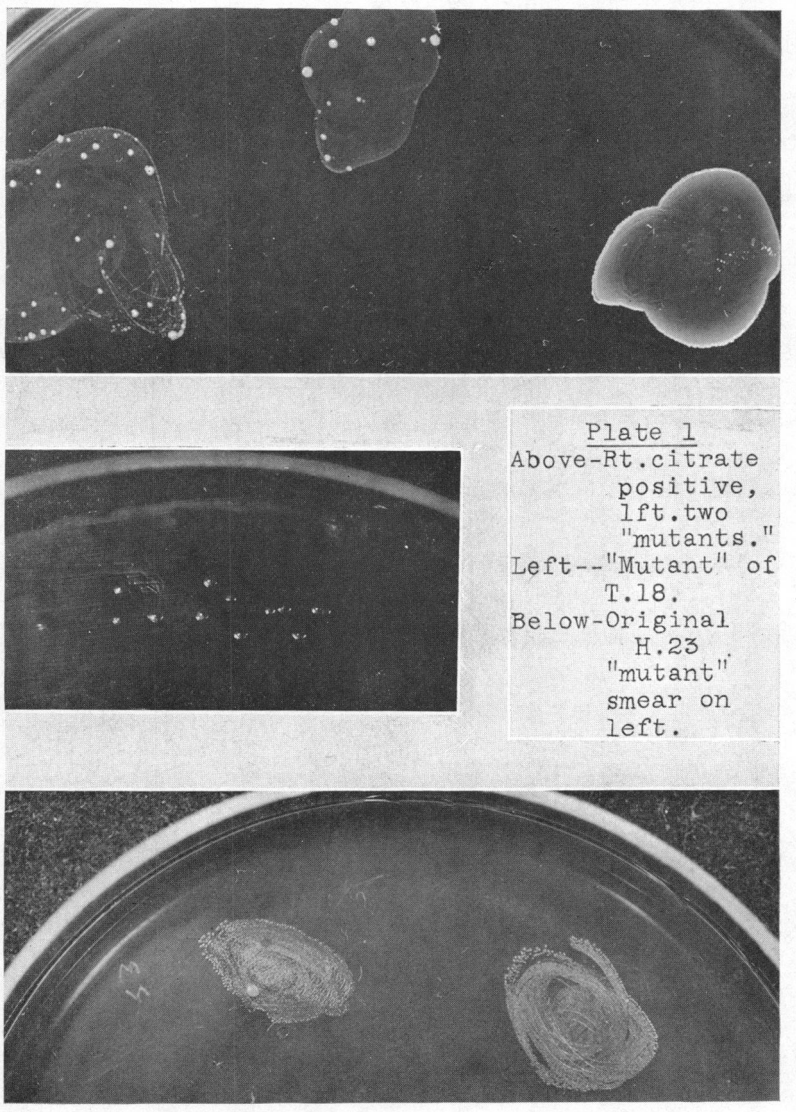


Plate 1
Above-Rt.citrate positive, lft.two "mutants."
Left--"Mutant" of T.18.
Below-Original H.23 "mutant" smear on left.

handling of many "mutant" strains and their derivatives, has eliminated this point as a proper explanation. "Mutants" appear

regularly and constantly on citrated agar from strains known to produce them; such colonies do not appear on citrated agar when strains are under study which do not produce them; the contamination rate for citrated agar is very low and is mostly confined to molds; and, most satisfactorily, the colonies which appear as "mutants" can be shown to have biochemical and serological identity with the culture under study from which they arise.

Nor was the appearance of these "mutant" colonies on citrated agar on an otherwise negative smear the manifestation of a culture mixture. We have, for instance, carried another strain, L.W.P.6-2, from its original isolation from fresh feces through 66 "platings out" and in testing each subculture have found that colonies transferred to citrated agar gave "mutants."

Neither do we believe that this phenomenon is one common to all *Escherichia coli*, merely missed in the past because tubes and plates in most laboratories have been discarded too soon for the "mutants" to be seen. We have tested numerous strains of *Escherichia coli* on citrated agar, holding the plates for as long as four weeks for observation (one day at 37°C., thereafter at room temperature), and we do not find that this "mutation" appears on any but a very small fraction of the total. It should be noted that in this work Koser's citrate broth cannot be used. A solid plating medium is necessary to fix and isolate the colony which may develop from a cell which manifests the ability to metabolize citrate. Difco products have been used throughout.

Work with the H.23 culture proceeded along two lines, each accomplishing an end which complemented and completed the findings obtained by the other procedure.

On the one hand the tryptone broth culture of H.23, from which the citrated agar had been stroked (see above), was plated on Endo's agar October 27, 1927. Twenty of the colonies which appeared on the plates were tested on citrated agar. The technic used was to touch one of the colonies with a straight needle. A tiny bit of the inoculum so obtained was placed on a sector of a Simmons' citrated agar plate and the rest was inoculated into a tube of tryptone broth numbered to correspond to the sector on citrated agar inoculated. Then, with a loop needle, the

trace of colony placed on the citrated agar was carefully spread out over an area about the size of a quarter. The size of the spread has depended upon whether three, five or six colonies are to be tested on a single plate of citrated agar. The inoculated citrated agar plates and tryptone broth tubes have been incubated over-night at 37°C. and thereafter held at room temperature.

In the case of the 20 colonies so inoculated, deriving from an Endo's agar plate made from the H.23 broth culture, no growth was evident on the citrated agar for three days. On the fourth day a single "mutant" colony appeared on each of three of the twenty test areas. In five days a fourth sector showed one colony and in seven days a fifth sector revealed one colony. By ten days 12 of the 20 citrated agar sectors showed one or more colonies (1, 1, 1, 2, 2, 2, 4, 6, 6, 8, 9, 14). From one of the twenty broth tubes, that corresponding to a sector showing six "mutant" colonies, a plating on Endo's agar was made and 18 of the colonies developing were picked to citrated agar and to broth. This time no one of the 18 smears showed a "mutant" colony until the sixth day, when on one sector one such colony appeared. By eleven days eight of the 18 smears showed "mutant" colonies (1, 1, 1, 1, 2, 2, 2, 2).

We were able to continue this process indefinitely, always being able to show among the colonies sampled in any given subculture on Endo's agar some which when smeared on citrated agar gave rise to "mutant" colonies.

Not all of the smears made gave rise to "mutant" colonies (12 of 20; 8 of 18; 14 of 20; 5 of 20; 4 of 20; 8 of 10, and so on). In other words, the number of cells capable of "mutating" in such a culture as H.23 is not large. One may stroke out thousands of cells on an area of citrated agar without obtaining a "mutant" colony, even though there are capacities for "mutation" in the culture under test. Early in the work we satisfied ourselves that whenever, as in the work just described, a smear on citrated agar showed no "mutant," it was nevertheless possible to obtain "mutants" from the colony in question on Endo's agar. This was done by plating the broth culture corresponding to the

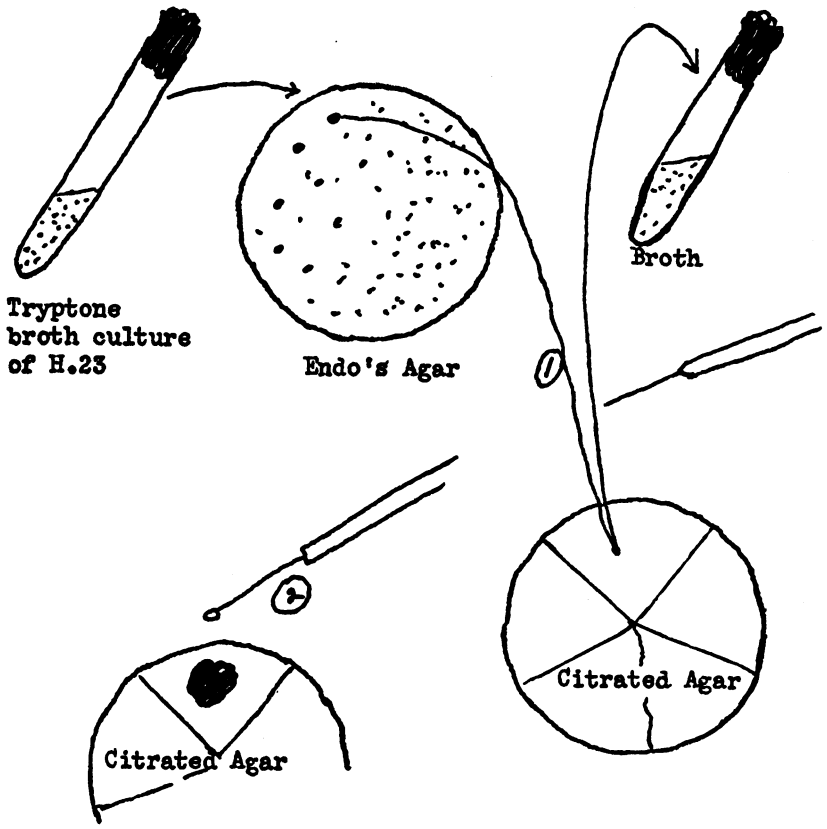
negative citrated agar sector on Endo's agar and testing colonies so obtained. We never failed to obtain in any such check one or more "mutants" to demonstrate that the capacity for "mutation" was not absent.

H.23 was continued in this way for 15 sub-cultures in the preliminary work, during which 188 colonies on Endo's agar were tested, and there was no break in the constant yield of "mutant" colonies in each sub-culture. In no case was a typically positive, prompt citrate reaction obtained. As noted above, certain smeared sectors failed to reveal "mutant" colonies, but in each case it was possible to show that the Endo's agar colony smeared contained cells which could "mutate." Since it was found that few if any "mutant" colonies developed on a citrated agar sector after ten days, this time interval was adopted as the minimum time for holding the citrated agar plates. We have observed the appearance of "mutants" in much the same way in each of the seven other coliform cultures studied in detail,—T.16, T.18, T.20, P.C.14, L.W.P.6-2, and L.W.P.11-3. Were no other data available we should be inclined to think of the phenomenon as merely another instance of the "unstable variant" described by Deskowitz, (1937). See diagram 1.

However, when we gave attention to the "mutant" colonies which appeared on the citrated agar, the findings could not be so readily explained. Thus, to illustrate our second approach to the problem, the "mutant" colony which appeared, October, 1937, on a sector of citrated agar was touched lightly with a needle and inoculated into broth which, after incubation, was plated on lactose indicator agar. Twenty of the resulting colonies were fished, as above, to sectors of citrated agar and to broth. This time, however, 19 of the 20 sectors showed on over-night incubation the typically positive citrate reaction, i.e., growth uniformly over the entire area smeared, and a color change in the medium from green to blue. One sector of the 20 was negative and remained so during the fourteen days of observation. On it no "mutant" colony appeared at any time.

The broth corresponding to this negative sector (C.162) was plated on Endo's agar and 20 colonies were picked to citrated

agar and broth. All 20 sectors were entirely negative during a period of observation of 14 days. Again, a broth culture from



This perpetuates the "mutant" in series.

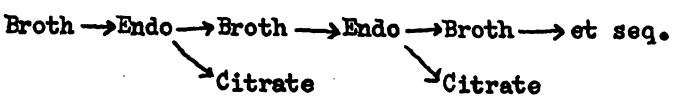


DIAGRAM 1

this series (C.245) was plated and another lot of 20 colonies picked to citrated agar and broth. As before no growth of any kind was observed on the citrated agar sectors inoculated. This

procedure was repeated until 19 subcultures involving a test of 194 colonies from Endo's agar had been made. No one of the 194 grew on citrated agar or gave rise thereon to "mutant" colonies.

On the other hand, when a broth culture corresponding to one of the 19 positive sectors was plated (C.163) and 20 colonies fished to citrated agar and broth, growth occurred on all 20 sectors promptly, completely, and typically. From this subculture, broth C.265 was plated on Endo's agar and 20 colonies picked. Again on citrated agar all sectors streaked were positive. This procedure was repeated until 22 sub-cultures, involving 187 colonies, had been tested without a single negative appearing among the 187 sectors inoculated on citrated agar (see diagram 2).

Thus, from one single parent strain, H.23, we were able to develop three true-breeding lines differing with respect to their utilization of citrate as a sole carbon source. There was, first, the citrate-unstable form which when streaked on citrated agar shows no visible growth for several days, after which growth occurs, but only as one or more isolated, "mutant" colonies, and not as a uniform film of growth covering the entire area inoculated. If sub-cultures on Endo's agar are made from the broth corresponding to such a streaking on citrated agar, colonies picked from the plate will reproduce the picture. If, on the other hand, further work is carried out from one of the "mutant" colonies instead of from the broth from which is was derived, one may obtain a positive citrate-utilizing line which develops quite as promptly and completely as would a culture of *Escherichia freundii*. And, thirdly, also starting with a "mutant" colony, one may obtain a line which refuses citrate completely, behaving exactly as would *Escherichia coli*.

This is the typical picture which we have obtained for all strains we have studied in detail, eight in all, with one exception. P.C.14 has not as yet yielded all three of the lines, but may do so with further manipulation. It is obvious from a consideration of the factors involved that one may not on first trial obtain these three lines, and that our success in doing so was in part good luck. We have almost always found that when we studied a

"mutant" colony yielding pure positives and complete negatives, the positives greatly outnumber the negatives. Chance selection of twenty colonies from an Endo's agar plate may well fail to include a negative. To date we have made detailed study of 107 colonies appearing as "mutants" on otherwise negative citrated agar sectors. Forty-eight of these when put into broth and plated on lactose indicator agar yielded, as far as tested, only positive progeny. (Obviously all colonies appearing on a plate could not be tested.)

Again it must be remembered that citrated agar is not toxic to citrate-negative organisms of the coliform group. Such forms probably persist, even multiply below the recognition threshold for some time. A "mutant" colony lies over, even contains, some of these negative cells. Furthermore, it is not improbable that when the "mutant" colony starts to develop, its metabolism of the citrate molecule presents the immediate environment with food materials which citrate-negative coliform cells can use. Thus, when such a "mutant" colony is propagated in broth and plated out, it may yield progeny negative to citrate, and some which "mutate" on citrate, as well as the derived positive forms. Thus, among the 107 "mutant" colonies studied there have been some like "Colony on C.6734," which was planted in broth and after incubation plated on Endo's agar. When 30 colonies were picked from the plates there were 19 positive sectors on citrated agar, three negative smears, and eight areas on which "mutants" appeared. If, however, the worker is careful to touch a "mutant" colony at its highest point of elevation, and deals with such colonies as soon as they can be handled safely instead of waiting until they have remained on the plate a week or longer, he is likely to obtain progeny which are either mostly positive with a few negatives, or entirely positive.

If this phenomenon is merely another instance of the "unstable variant," so well described by Deskowitz (1937), it is hard to understand how absolutely negative lines may arise, as they undoubtedly do. According to the theory of the unstable variant, each single cell possesses the capacity to develop the variant. *Bacterium coli-mutabile* is probably an "unstable variant."

Every cell in such a culture possesses the potentiality for fermentation of lactose when cultured on a substrate containing that sugar. But lines entirely negative to lactose are not encountered. In the work here reported we have had not the slightest difficulty in isolating negative lines which breed true and persist as negative through many sub-cultures. One may postulate that when a "mutant" colony develops, it contains many cells which have taken on the capacity for the prompt utilization of citrate but it also contains some cells which have no such capacity at all, and it may contain, from the underlying film of inoculum out of which it grows, cells which have not as yet "mutated," but which do so when the "mutant" colony is fished to citrated agar for study. The theory advanced by Reed (1933), to explain bacterial "mutation" on the basis of unequal cell division with respect to some gene-like determinant would explain such a phenomenon.

The essential identity of the three lines derived from a single parent strain has been established by comparisons of their morphology and biochemistry and by serological tests using sera prepared with the parent strains as antigens. For H.23, 13 strains were used—the original, four other "mutating" strains, four negative strains, and four positive strains. For the culture T.16[2] nine strains were used—the original, one other "mutating" strain, three negative strains and four positive strains. These strains were simply well defined and stabilized cultures picked out from the complex family trees of H.23 and T.16 at wide inter-

[2] T.16 was isolated by the senior author from long-stored feces and placed in his type collection as an atypical coliform intermediate. When first isolated and stabilized it developed promptly and typically on citrate and had the Imvic formula ++−+. When tested on citrate, June 30, 1937, it was at first negative, but by July 5, it had developed "mutant" colonies and when again tested on citrate December 6, 1937, it was again negative on citrated agar for several days following which one colony appeared on the otherwise negative smear. T.16 was probably originally an *Escherichia coli* which in long stored feces (in the cold room) acquired the character of citrate utilization, but not so perfectly or completely but that years later in culture it reverted to its original reaction, *Escherichia coli*, with a potentiality for citrate mutation. We suspect that many ++−+ (Imvic) strains are of this type. Such strains well illustrate Adami's (1892) concept of the development of bacterial races, an old paper which should be read by all bacteriologists.

vals in the study so as to clearly test the essential relationships and identities.

All 13 of the H.23 strains were culturally alike except for citrate utilization and the same was true for the nine strains of T.16. With antisera prepared with the original parent strain as antigen, in each of the two cases, all 22 derived strains agglutinated either to titer, one tube below titer or one tube above titer, which seems quite satisfactory in view of the alleged serological heterogeneity of coliform bacteria. Absorption tests were not carried out and as yet no serological tests have been done with strains other than the 22 H.23 and T.16 cultures.

The study of the citrate variants has been by no means confined to the mere derivation of the 22 strains mentioned above. Many other cultures have been given considerable manipulation in order to arrive at some notion of their stability. A "mutant" must not only present a new character believed to be significant, but it must exhibit the new character with reasonable constancy. For example, the nine strains of T.16 were cultivated in series through 13 passages of lithium chloride broth (6/2/38 to 6/23/38). At the end of the series the nine were tested for their action on citrated agar and seven of the nine bred true; two, both considered negative, gave "mutant" colonies (1, 2) upon prolonged cultivation on citrated agar. Again, these nine strains were cultured in series through 17 passages of colchicine alkaloid broth (0.1 per cent in Tryptone broth) and after four weeks of constant exposure to the influence of this chemical, were sown on citrated agar. In this series eight of the nine strains bred true, the ninth, a negative, giving one "mutant" colony on the citrated agar sector on the fifth day. The negative derivative, T.16 no. 5, both "mutants" and all four positives, bred absolutely true to type in all of these experiments. These facts seem to emphasize that there are differences in the degree of fixity of the characters of a new race, which is exactly what Adami postulated more than forty years ago. At the time the preliminary work with H.23 and T.16 was done in 1937 and 1938 we were inclined to think that the stability of the derived positive was much greater than that of the derived negative, for during that period all of

our derived positive strains bred true. More recently we have encountered some which yield a few negatives as well as many positives when cultured, but right along with them we have strains like H.23 no. 12 which has been subcultured (up to March 11, 1940) 50 times in the past year, following a period during which it was held in the ice box in the desiccated form. In this past year's work with H.23 no. 12, 714 colonies on Endo's agar have been tested and each one of them has been completely, promptly and typically positive on citrated agar. Study is in progress on the stability of derived citrate variants. We already know that many of them are stable, quite within the limits of what might be expected from any normal strain of bacteria; but it must be admitted that we encounter other strains in the same family (H.23 or T.16, for example) for which the stabilization is less perfect. If there is any mechanism for inheritance among bacteria which may eventually be made out it will have to explain imperfect stabilization of "mutants" as well as provide an understanding of how the new forms arise.

It has been indicated above that the citrate "mutant" does not occur so frequently as to endanger the value of the citrate utilization test in coliform studies. We have studied 9924 cultures of colon-typhoid organisms including numerous strains from fresh and from stored feces, atypical coliform cultures from A. V. Hardy, strains from infectious diarrhea of the newborn, *Salmonella* types, *Eberthella* and *Shigella* types, and paracolon types. In all, we have encountered 83 citrate "mutants" from 21 specimens or sources. These 21 include one fresh fecal specimen from which 54 of 60 colonies studied in detail were citrate "mutants." They also include Evans' derived coliform "mutant" which he produced in our laboratory in 1935 from culture T.24. This organism was originally *Escherichia freundii* (Imvic $-+-+$) isolated more than fifteen years earlier by Koser from soil. It was one of twelve coliform organisms with which Evans was working in a study on the effect of holding cultures of coliform bacteria for long periods of time in Stearn's gentian violet broth (1923).

Of the 12 organisms Evans had under study only T.24 was

appreciably altered. How it lost its ability to utilize citrate is described by Evans (1935): "This change occurred gradually, there being first delayed citrate utilization, then little by little citrate-negative colonies began to predominate over citrate-positive ones, until when the experiment was suspended, 6/5/35, with one exception all colonies picked from Endo's plates produced only, for a substantial amount of inoculum rubbed on citrate plates, from one to thirty positive colonies, these colonies appearing four to five days after inoculum was rubbed on the plates. The exception noted was that the inoculum from one colony on Endo's medium, transferred to nutrient agar and

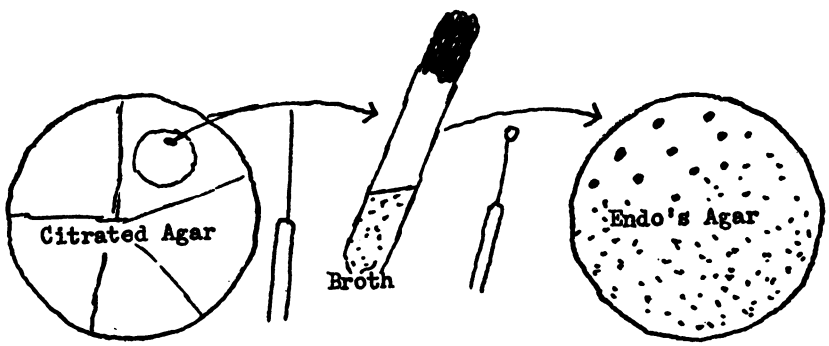


DIAGRAM 2. NOW PICK FROM THE COLONIES ON THE ENDO'S AGAR PLATE TO BROTH AND CITRATED AGAR AS OUTLINED IN DIAGRAM 1

For instance, H.23 of November 2, 1937 on Endo's 20 colonies fished, of which 19 positive on citrated agar, promptly, completely and typically. 1 negative on citrated agar, two weeks incubation, no "mutants" appearing.

repeatedly tested out on citrate never produced any citrate-positive colonies up to the time the experiment was interrupted." Evans' data are significant particularly because they relate to a much-tested strain considered as a typical *Escherichia freundii.* Significant also, as we see it, is the fact that the treatment given by Evans to all the strains with which he worked produced results in only one culture. Exceptions like this to the general outcome of an experiment, which have generally been disregarded in the past by many workers as something to be thrown out or excused, may in our opinion be the best part of the experiment. We are firmly convinced that bacteriologists must recognize differ-

ences in stability of bacterial strains, and where different laboratories are using different strains, or even identical strains, some times the same procedure may not yield the same results.

The citrate "mutant" has been of peculiar interest to us because it represents a phenomenon occurring in nature. With the exception of Evans' work, which we regret has never been extended, all "mutations" dealt with have been encountered in the regular line of cultural procedure. The forms described and the lines derived have not arisen through the use of X-rays, radium or special chemicals. Citrated agar has been used as far as possible only as an indicator, and the general propagation and plating out of strains has been done with broth and on lactose indicator agar on which all types concerned grow equally well.

Our data are not offered as an unfavorable criticism of the citrate utilization test. In fact, the results attest, in general, to the stability of coliform organisms with respect to their utilization of citrate as a sole source of carbon. We suspect that if other tests, such as the Voges-Proskauer or indole production tests were equally adaptable to "mutation" study, such experiment would reveal fully as much change of character in the strains studied.

What the results do show is that bacterial taxonomy should be conservative. In dealing with strains exhibiting slight differences in character, habitat, or action on a host, effort should be made to ensure that the characteristics utilized to define new species not only show stability combined with facility and exactness of elucidation, but also correlation with other known descriptive facts.

It could be argued that in this work new species have been evolved. Michelson and Dulaney (1936) reported the transformation of *Bacterium coli-mutabile* into *Bacterium aerogenes* and Minkewitsch, Rabinowitsch, and Joffe (1936) placed cultures of *Bacterium coli* (*communis*) in soil, free from coliform bacteria, and eventually recovered citrate-utilizing, sucrose-fermenting forms. In our laboratory Evans would seem to have changed *Escherichia freundii* into *Escherichia coli* with intermediate citrate "mutating" forms appearing along the route. As species are now recognized, our own "transformations" have been only as between *Escherichia coli* and *Escherichia freundii* of atypical

nature. We do not believe that the significance of this work lies in any transformations made *per se*, but in the demonstration that "mutations" may occur from which pure lines may be derived.

The citrate "mutant" has, moreover, provided us with an apparently new mechanism for the ready study of "mutation." In dealing with slow-fermenting forms it is easy to obtain the prompt fermenter and to preserve the slow-fermenting *mutabile* type, but the absolute negative does not appear. The citrate "mutant" yields three lines, the variant, the positive, and the negative. Further study with it may lead to a better understanding of bacterial "mutation."

One of the puzzling factors about the citrate "mutant" and about the slow-lactose-fermenting forms is the delay in appearance of the "mutant" after the inoculation of suitable material has been made. The whole theory behind the purification of a bacterial culture by plating it on a solid medium from a growth or suspension in fluid medium is that the inoculum is spread so diffusely that at many points single cells are deposited, from which distinct colonies grow. Certainly if a culture of coliform organisms which utilizes citrate be spread on a plate of citrated agar, distinct growth will be visible the next day. In the case of the citrate "mutant," however, a smear on citrated agar usually shows no sign of a colony for at least three days and frequently this time interval may be four, five, or six days. Why the delay? Secondary colonies on *Bacterium coli-mutabile* appear on lactose indicator agar only after a lapse of days. Typical cultures of this organism sown in lactose broth may show no visible acidity for more than a week. We feel that we shall never have the exact answers to what actually does go on in strains like these until methods better than those now in use have been developed.

CONCLUSIONS

A description has been presented of a coliform citrate "mutant" which can be so handled as to yield true breeding lines of the unstable variant, of a stable positive citrate-utilizer, and of a stable negative citrate-utilizer. The method used in obtaining

these three lines is described. The probability that such forms represent contaminations or mixtures is ruled out. It is felt that new material, valuable for the study of bacterial genetics has been presented, and that the evidence that bacterial taxonomy should be conservative has been added to. The need for improved methods of study for this "mutant" and for others longer known to bacteriologists is emphasized. It is suggested that differences in stability may exist in different strains of certain bacterial species, with the result that in occasional studies the results obtained will depend upon the nature of the strains utilized, making it possible for good workers in different places to obtain conflicting results.

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