ENZYMATIC ANALYSIS OF MUTANT AND WILD TYPE LACTIC STREPTOCOCCUS ORGANISMS DEVELOPING ON A SELECTIVE MEDIUM

by

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INTRODUCTION

Streptococcus lactis and *Streptococcus cremoris* are morphologically similar bacteria often grown together in mixed cultures to provide a starter for the manufacture of cultured dairy products. Despite the frequency with which these starter mixtures are used, little is known concerning the interactions of the microorganisms which they contain. The relative difficulty encountered in the separation and identification of these two species prompted the search for an agar medium which would distinguish colonies of *S. lactis* from colonies of *S. cremoris*. Such a medium would lend itself to physiological and biochemical studies of these microorganisms and could eventually be useful in genetic studies as well. The medium also could be used to determine the change in relative numbers of the two species during growth of starter cultures and manufacture of dairy products. Studies on the effect of antibiotics produced by one species on the growth of the other also would be facilitated by such a differential medium.

The primary criterion for the separation of *S. lactis* from *S. cremoris* is the production of ammonia from the amino acid arginine; only *S. lactis* is able to carry out this reaction. It was therefore desirable to study this

biochemical characteristic more carefully. Mutations in S. lactis resulting in the loss of the ability to hydrolyze arginine were induced by irradiation with ultraviolet light; the mutant organisms were isolated by plating on the differential medium developed during the present investigation. Comparative resting cell and cell-free extract studies on arginine degradation by wild type and mutant S. lactis organisms were carried out. It was the purpose of this phase of the present study to develop techniques and provide information that would be useful in subsequent genetic studies on these organisms.

During the course of the investigation, it was noted that colonies of lactic streptococcus organisms developing on media incubated in a candle-oats jar were much larger than those developing on media incubated in a regular aerobic incubator. A study therefore was undertaken to determine the cause of this phenomenon.

HISTORICAL

Selective Plating Procedures for Bacteria

Methods to distinguish one microorganism from another, by virtue of identifying characteristics of colonies developing on agar media, have been used by bacteriologists for many years. In 1916, Holt-Harris and Teague (20, p. 596-600) described a medium which would differentiate colonies of organisms which possessed or lacked the ability to ferment lactose. It was called eosin-methylene blue (EMB) agar, and since that time it has become a widely used selective medium; it has been used extensively in genetic studies as well as in the identification of members of the coli-aerogenes group of bacteria. A number of selective media also have been described which are suitable for the identification of pathogenic bacteria. Bismuth sulfite agar (62, p. 392-398), for example, has been used in the differentiation of typhoid organisms. Salmonella typhosa produces a characteristic black colony, while gram positive organisms and members of the coli-aerogenes group are inhibited. Hemolysis on blood agar plates has been useful in the classification of pathogenic streptococci into three groups (57, p. 81-93): the β-hemolytic streptococci, which produce clear zones of hemolysis around the colony; the α-hemolytic streptococci, which produce a zone of greenish discoloration around the colony

and the anhemolytic or γ-streptococci, which do not cause hemolysis.

Many genetic studies have been facilitated by the use of selective media for identification of colonies of bacteria possessing or lacking the characteristics under investigation. Avery, et al (2, p. 137-158), in their classical demonstration of the nature of the transforming principle, routinely plated their transformed cultures on blood agar plates for confirmation. The colonies of transformed organisms, which had gained the ability to synthesize a type specific polysaccharide capsule as a result of exposure to deoxyribonucleic acid (DNA) from an appropriate donor, were large, glistening and mucoid. They were easily distinguished from rough colonies of the non-transformed organisms.

Davis and Mingioli (8, p. 17-28) selected methionine-requiring mutants of Escherichia coli by comparing growth on plates of minimal media with growth on plates of minimal media to which methionine had been added. The development of the replica plating technique by Lederberg and Lederberg (36, p. 399-406) greatly facilitated the expanded use of this sort of selective procedure. They pressed the surface of a master plate to a sterile piece of velvet. The nap of the velvet acted as many tiny inoculating needles. This velvet in turn was pressed onto the surface of agar plates of various differential media.

Fulde and Shank (12, p. 48-49) indicated that an agar medium containing V8 juice could be used to differentiate catalase positive, nitrate reducing bacteria from catalase negative lactic acid bacteria. The former organisms produced large, white, lenticular colonies, while the latter produced green-black colonies with yellow halos.

Several authors have suggested the use of citrated whey agar for the identification and enumeration of citrate fermenting, aroma bacteria (Streptococcus diacetilactis and Leuconostoc species). Kneteman (25, p. 275-290) described a medium which consisted of acid whey, potassium ferrocyanide, ferric citrate, sodium citrate, sterile milk, and agar. He found that citrate-fermenting organisms produced blue colonies in contrast to white colonies of non-citrate-fermenting organisms. The blue colonies were due to the formation of prussian blue. This color change was inhibited by citrate surrounding the colonies of bacteria unable to use this substrate.

The medium proposed by Lundstedt (41, p. 64-68) was composed of 2 per cent citrate in albumin and globulin-free cottage cheese whey to which 1.5 per cent agar was added. Colonies of citrate-fermenting organisms were surrounded by bubbles of carbon dioxide (CO_2) which could be detected by holding the plate against a light source. Lundstedt estimated that the medium was correct only 85 per cent of the time.

The whey agar media described by Galesloot, et al (13, p. 127-150) were made turbid by the addition of calcium citrate. Citric acid-fermenting organisms produced clear zones around their colonies. Lactic acid streptococci produced little acid on the described media and were thus incapable of dissolving the calcium citrate.

No simple medium has been reported to date for the separation of S. lactis from S. cremoris.

On several occasions 2,3,5-triphenyl tetrazolium chloride (TTC) has been employed in agar media to detect bacterial mutants. It is one of a group of oxidation-reduction dyes which has been used in recent years to indicate the presence of enzymatic activity. Histologists have employed a number of tetrazolium dyes to indicate dehydrogenase activity in various plant and animal tissues (15, p. 389-396; 16, p. 271-278; 22, p. 65-66; 56, p. 317-320). In 1952, Laxminarayana and Iya (32, p. 124-125) suggested that the tetrazolium dyes might be applicable in several microbiological areas including taxonomy, nutritional studies, vitamin assay, quality control of milk, and vital staining. They went on to study the reduction of triphenyl tetrazolium bromide by lactic acid bacteria (34, p. 75-91) and found that lactic acid streptococci were generally more active in this respect than the lactobacilli. Among the streptococci, the enterococci were the most active, followed by S. lactis, S. pyogenes, and

S. cremoris in that order of reducing activity.

Weinberg (60, p. 240-242), in 1953, indicated that the incorporation of 0.05 per cent TTC into an agar medium would selectively inhibit gram positive and acid fast bacteria as well as actinomyces; gram negative bacteria, on the other hand, were not inhibited. They reduced the dye to the insoluble red formazan and developed intensely colored colonies which proved fully viable upon subculture. Less concentrated solutions of this dye (0.005 per cent) have been useful in detecting non-carbohydrate-fermenting mutants.

Newcombe and Whitehead (44, p. 243-251) used a mannitol-TTC agar to look for ultraviolet light induced mutants of E. coli. These authors noticed that 12 to 14 per cent of the colonies produced were sectored. However, they did not feel that the sectoring was related to mannitol fermentation since the number of sectored mutants was the same on mannitol-TTC, lactose-EMB, and xylose-TTC agar.

Lederberg (35, p. 695) also used TTC to detect non-carbohydrate-fermenting mutants. He suggested that the acid produced by the wild type colony was sufficient to inhibit dye reduction. Mutants did not produce acid and were thus capable of reducing the dye to the insoluble red formazan.

Zamenhof (64, p. 111-117) used TTC to detect lactose mutants of E. coli. Lactose fermenters produced colorless colonies; non-fermenters produced bright red colonies, and mutants with intermediate fermenting ability produced colonies of intermediate red colors.

Liska, et al (37, p. 1218-1223) studied the reduction of TTC by heterofermentative lactic acid bacteria and noticed a decrease in the amount of lactic acid produced upon the reduction of the dye. They suggested that the reduced nicotinamide adenosine dinucleotide ($NADH_2$) coenzyme, normally involved in the production of lactic acid from pyruvate, was instead oxidized by the TTC. Other investigators (4, p. 40-41; 10, p. 254-265) have indicated that a flavoprotein coenzyme also may be necessary for reduction of TTC to take place and that no reduction occurs through $NADH_2$ alone.

Effect of Carbon Dioxide on Lactic Acid Bacteria

Many organisms have been shown to require increased CO_2 tension to carry out various physiological activities at a maximum rate. Brucella abortus has been shown by Yaw and Kakavas (63, p. 392-398) to require CO_2 for protein synthesis. Lui (40, p. 282-288) noted that the colony sizes of certain hemolytic streptococci were markedly increased by incubation of plates in a CO_2 atmosphere. Bacillus anthracis requires CO_2 for the

synthesis of glutamyl polypeptide, but Thorne and Gomez (59, p. 129-130) have shown that this requirement can be replaced by the addition of 0.8 per cent sodium bicarbonate to the medium. The formation of slime by Streptococcus bovis requires the presence of CO_2, although "Tween 80" may be added to the medium with the same result, according to Dain, et al (7, p. 209-213).

Recent studies on the lactic acid streptococci indicate that CO_2 may be required for the growth of these organisms. Whitehead, et al (61, p. 24-31) reported that small amounts of CO_2 were required to reduce the lag period of cultures of S. lactis and S. cremoris in sterilized skim milk. They found that 0.2 to 2.3 per cent was necessary for optimal initial growth, with different strains requiring varying amounts of CO_2. Yeast extract (0.5 per cent) could substitute for CO_2 in the skim milk cultures. Garvie (14, p. 227-237) indicated that a strain of S. cremoris required CO_2 for good growth on milk agar; a mixture of hydrogen and 10 per cent CO_2 produced better results than CO_2 alone. An absolute CO_2 requirement was recently shown for S. lactis by Reiter and Oram (52, p. 175-176); carbon dioxide was removed from the growing culture by continuous sweeping of the medium with CO_2-free nitrogen gas. Sufficient CO_2 was produced metabolically to initiate growth if the gas were not continuously removed by the sweeping process.

Carbon dioxide fixation has been studied rather extensively in the lactobacilli. Lardy, et al (32, p. 721-731) have shown that C^{14}-labeled CO_2 could be found in the aspartic acid of Lactobacillus arabinosus; the fixation did not occur in the absence of biotin. Broquist and Snell (5, p. 431-444) later indicated that the large biotin requirement of L. arabinosus for aspartate synthesis was not shared by Lactobacillus fermenti or Clostridium butyricum. These authors suggested that some other mechanism for CO_2 fixation must be operative in the latter organisms. They also showed that CO_2 stimulated the growth of L. arabinosus in the absence of biotin and that less biotin was required by the organism in the presence of CO_2. They therefore concluded that biotin played an important part in the production of metabolically essential CO_2. The "sparing effect" of CO_2 on the biotin requirement of S. lactis has recently been reported by Reiter and Oram (53, p. 63-77).

Degradation of Arginine by Bacteria

Hills noted in 1940 (19, p. 1057-1069) that certain pathogenic organisms had the ability to produce ammonia and ornithine from the amino acid arginine. He suggested that a single enzyme, distinct from the arginase enzyme, was responsible for the conversion, and he called it arginine dihydrolase. He indicated that citrulline could

not be an intermediate in the reaction. However, Schmidt, et al (55, p. 771-783) showed that citrulline was indeed formed during the degradation of arginine by Clostridium perfringens and further indicated that two enzymes were probably involved in the dihydrolase system. Slade and Slamp (58, p. 455-466), in 1952, found that S. faecalis contained a similar mechanism for the breakdown of arginine. The amount of dihydrolase activity was dependent upon the arginine concentration in the medium and was low in the absence of arginine. They suggested that the enzyme system was adaptive. While whole cells of S. faecalis were incapable of utilizing citrulline as a substrate, cell-free extracts of the organism were fully capable, producing ornithine, CO_2, and ammonia. Inorganic phosphate, magnesium ions, and adenosine triphosphate (ATP) were required for the reaction. Arginine desimidase and citrulline ureidase were proposed to name the respective enzymes involved in the two-step degradation of arginine through citrulline to ornithine.

Oginsky and Gehrig (46, p. 791-797) confirmed that citrulline was an intermediate by paper chromatography and went on to characterize arginine desimidase (47, p. 799-805). No cofactors were necessary for enzyme activity. They also indicated that L-canavinine, a structural analog of arginine, was inhibitory to enzyme action but was not degraded. Kihara and Snell

(24, p. 485-495), however, were able to partially purify an enzyme which would convert L-canavinine to O-ureido-L-homoserine and ammonia. The enzyme was even more active in the conversion of arginine to citrulline and ammonia and was believed to be arginine desimidase.

In considering the decomposition of citrulline, Oginsky and Gehrig (48, p. 721-792) suggested that adenosine monophosphate (AMP), rather than the triphosphate, was actually involved. They proposed that a high energy bond occurred in the form of a phosphorylated intermediate compound. Knivett (27, p. 602-605) studied the breakdown of citrulline with cell-free extracts of S. faecalis and found that the system required inorganic phosphate, magnesium ions, and adenosine diphosphate (ADP). ATP was formed during the reaction. He showed (27, p. 606-610) that arsenate could replace phosphate in the reaction and that under these conditions no ADP or Mg^{++} was required. He therefore postulated that two enzymes were involved in the degradation of citrulline, one to bring about the formation of a phosphorylated intermediate and the other, a phosphokinase, to transfer the phosphate from the intermediate to ADP. He suggested that the arsenolated intermediate formed in the presence of arsenate was readily hydrolyzed, thus explaining the loss of the ADP requirement.

When ornithine was removed, Knivett (28, p. 480-485)

showed that citrulline disappeared from the reaction mixture in the absence of ADP or arsenate. Citrulline breakdown by cell-free extracts of S. faecalis was inhibited by L-ornithine. Citrulline formation occurred anaerobically in the presence of ornithine, ammonia, CO_2, and ATP.

Niven, et al (45, p. 651-660) demonstrated that S. lactis was able to produce ammonia from arginine. The conversion of citrulline to ornithine by cell-free extracts of S. lactis has been studied extensively by Korzenovsky and Werkman (29, p. 174-185). They established that stoichiometric quantities of CO_2, NH_3, ATP, and ornithine were formed from citrulline (30, p. 343-347). They also suggested that a phosphorylated intermediate was formed and that two enzymes were involved in the conversion.

Krebs, et al (31, p. 185-193) found that mammalian liver preparations were capable of arsenolysis and phosphorolysis of citrulline. They could not, however, demonstrate any ATP formation. Attempts to isolate a phosphocitrulline intermediate, through the hydroxylamine reaction or with radioactive phosphorous, were unsuccessful. They therefore postulated a mechanism for the reaction which, they felt, would also be applicable in bacterial systems.

Enzyme + Citrulline ⟶ Enzyme-citrulline

E-Citrulline + Phosphate ⟶ E(phosphate, citrulline)

E(phosphate, citrulline) ⟶ E(phosphate, ornithine) + CO_2 + NH_3

E(phosphate, ornithine) ⟶ E-phosphate + ornithine

They suggested that the E-phosphate bond in bacterial systems was a high energy bond which would participate in the formation of ATP.

In 1955, however, Jones, et al (23, p. 819-820) indicated that the phosphorylated intermediate which had been postulated by Knivett (26, p. 602-605) and Oginsky and Gehrig (46, p. 791-797) and Korzenovsky and Werkman (29, p. 174-185) was not a phospho-citrulline derivitive but was instead carbamyl phosphate. They also indicated that the citrulline biosynthetic pathway proceeded in the following manner:

Carbamate + ATP $\xrightarrow{Mg^{++}}$ Carbamyl phosphate + ADP

Carbamyl phosphate + ornithine ⟶ citrulline + Pi

Ravel, et al (49, p. 1452-1455) partially purified ornithine transcarbamylase, which catalyzes the synthesis of citrulline from ornithine and carbamyl phosphate, from S. lactis. They found that the reaction was completely

reversible and, in fact, proceeded in the reverse direction (that is, toward the formation of ornithine and carbamyl phosphate) in the presence of excess phosphate.

Carbamyl kinase, which catalyzes the synthesis of carbamyl phosphate from carbamate and ATP, also was partially purified from S. lactis by Ravel, et al (50, p. 525-531). They found that this reaction also was reversible and that the reverse and forward reactions proceeded at equal rates under identical conditions in the presence of a high concentration of ammonium carbamate. In the absence of ammonium carbamate, however, the formation of ATP proceeded faster than the formation of carbamyl phosphate.

Ravel and Shive (51, p. 413) indicated that biotin played a role in the production of active 4 carbon units for synthesis of ornithine transcarbamylase and is thus essential for enzymatic activity.

Arginine dihydrolase enzyme systems have been found in a variety of other microorganisms. Virulent strains of Pasteurella tularensis contain a citrulline ureidase enzyme while strains with low or no virulence do not (11, p. 345-349). Presence of the enzyme was not, however, directly related to virulence (43, p. 26-32). An arginine dihydrolase system has been reported in Staphylococcus aureus (38, p. xvii). The quantity of enzyme formed was found to depend on the concentration of arginine in the medium.

The arginine dihydrolase system has been implicated in the regulation of proteinase synthesis in S. faecalis var liquefaciens by regulating the amount of available arginine (18, p. 753-761). It was further indicated that some phase of the enzyme system was adaptive.

Bibb and Straugh (3, p. 79-80) have suggested that a specific permease system exists for citrulline in the Lactobacillus species which they studied. The permease system was induced by intracellular formation of citrulline from arginine or by extracellular citrulline.

Figure 1 shows the breakdown of arginine to ornithine, CO_2, and ammonia via the citrulline intermediate.

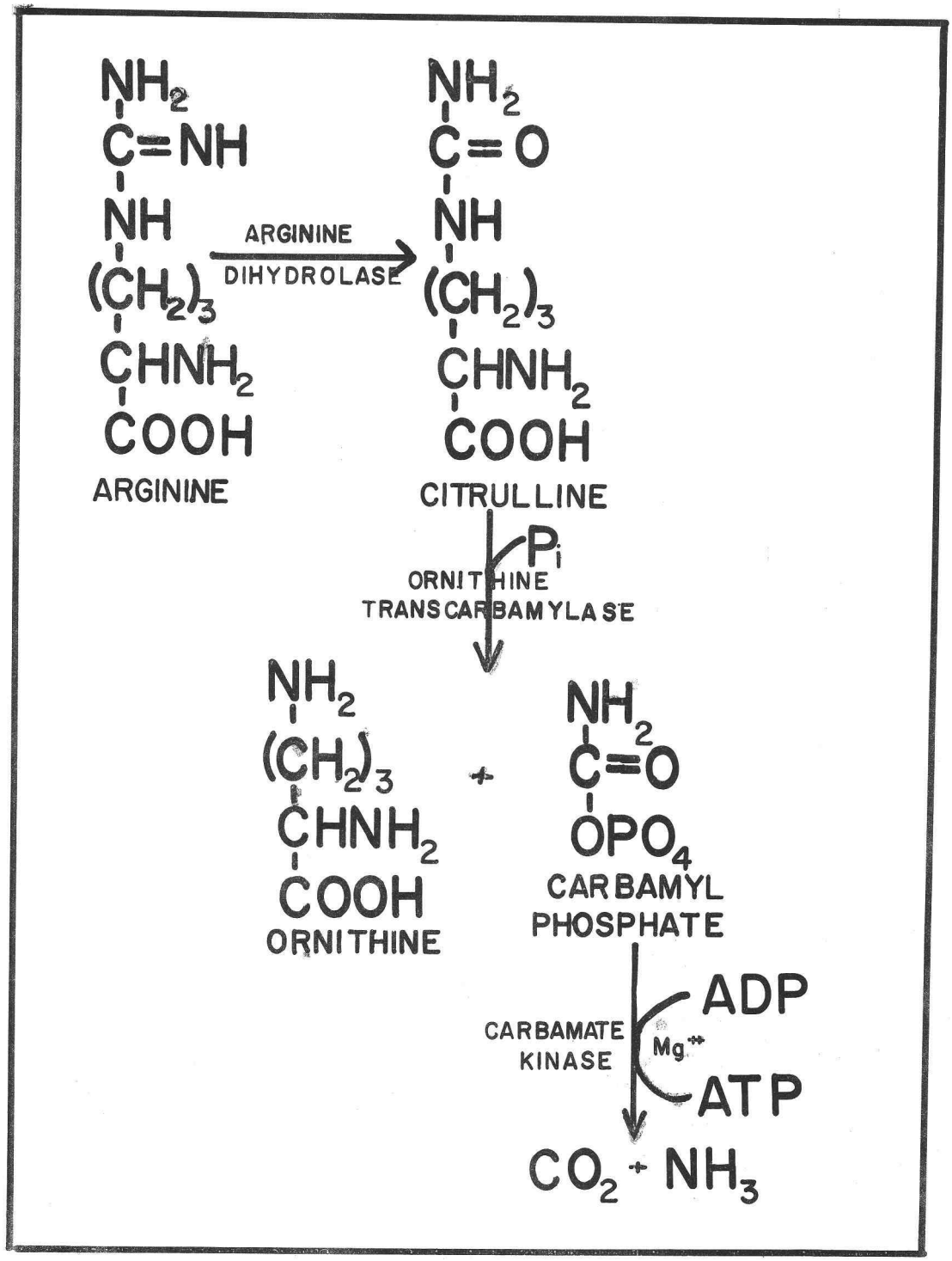

Figure 1. Pathway for arginine metabolism in S. lactis.

PART I

SELECTIVE PLATING PROCEDURES FOR IDENTIFICATION OF LACTIC STREPTOCOCCUS ORGANISMS

MATERIALS AND METHODS

Cultures Used

Strains of S. lactis, S. cremoris, and S. diacetilactis were obtained from the culture collection of the Oregon State University Department of Microbiology. Table 1 shows a list of the organisms used in the investigation. Cultures were maintained in lactic broth (9, p. 1611-1612) in the early phases of the investigation and were later carried in the medium of Korzenovsky and Werkman (K and W broth) (29, p. 174-185). The composition of these media may be seen in Tables 2 and 3, respectively. Cultures were transferred twice weekly, incubated at 30° C. for 24 hours (hrs.), and refrigerated between transfers at 4° C.

Test for Ability to Hydrolyze Arginine

The procedure of Niven, et al (45, p. 651-660) was used to test cultures for the ability to produce ammonia from arginine. The culture was grown in Niven's arginine broth for 24 hrs. A drop of the culture was then mixed with a drop of Nessler's reagent. The formation of a brown precipitate indicated the presence of ammonia.

TABLE 1

Strains of *S. lactis*, *S. cremoris*, and *S. diacetilactis* used in the present investigation

Strains of *S. lactis*	Strains of *S. cremoris*	Strains of *S. diacetilactis*
27	W	18-16
C2	KH	DRC-1
C10	C3	26-2
E	144-F	31-2
11955a	11602a	RM-1
7963	Da-1	6B-3
11454	1	11D-3
7962	E8	6B-1
	11603a	31-8
	9596	
	18-10	
	C13	
	9692	

TABLE 2

Composition of lactic broth*

Ingredient	Grams per liter
Tryptone	20.0
Yeast extract	5.0
Gelatin	2.5
Dextrose	5.0
Lactose	5.0
Sucrose	5.0
NaCl	4.0
Na acetate	1.5
Ascorbic acid	0.5
Tween 80	0.5 (ml.)
pH was 7.0	

*Lactic agar was made by adding 15.0 grams of agar per liter.

TABLE 3

Composition of Korzenovsky and Werkman medium

Ingredient	Grams per liter
Tryptone	10.0
Yeast extract	10.0
Glucose	2.2
K_2HPO_4	6.7
Tap water	33.0
L-arginine·HCl	2.5
pH was 6.8 to 7.0	

Basal Arginine Medium

This medium consisted of 0.5 per cent Tryptone, 0.5 per cent yeast extract, 0.2 per cent K_2HPO_4, 0.05 per cent glucose, 0.2 per cent L-arginine, and 1.5 per cent or 3.0 per cent agar in distilled water. The pH of the medium was adjusted to 6.0 with dilute hydrochloric acid (6N). It was then dispensed in 150-milliliter (ml.) quantities in 8-ounce prescription bottles and autoclaved at 121° C. (15 pounds of pressure) for 20 minutes. This basal medium was supplemented with one of two indicators as described below to provide an arginine-bromcresol purple agar and an arginine-TTC agar.

Indicators Used

A 2.2 ml. quantity of 0.4 per cent bromcresol purple in 0.008 N NaOH was added to the arginine medium containing 3.0 per cent agar before the medium was autoclaved.

The tetrazolium salts employed were filter-sterilized through a Seitz filter. They were added to the arginine medium containing 1.5 per cent agar immediately before agar plates of the medium were poured. Tetrazolium dyes are reduced by light and plates therefore were dried and stored in the dark. The 2,3,5-triphenyl tetrazolium chloride (TTC) was obtained from California Corporation for Biochemical Research, Los Angeles, California. All other tetrazolium salts were obtained from Nutritional

Biochemicals Corporation, Cleveland, Ohio.

Preparation of Agar Plates

Sterilized agar was poured from bottles into sterile petri plates. The plates were allowed to dry at room temperature for 24 hrs. Cultures were diluted in sterile, distilled water and 0.1 ml. aliquots of appropriate dilutions were distributed on the surface of the dried agar plates with a sterile glass spreader. The plates were then incubated in a candle-oats jar for 24 hrs. at 30° C. The candle-oats jar was prepared by placing moistened oats in the bottom of a large dessicator. A burning candle was placed in the dessicator at the time of incubation. The dessicator was sealed and the candle was allowed to burn out.

Single strains of S. lactis, S. cremoris, and S. diacetilactis and mixtures of S. lactis and S. cremoris were tested by plating on the two arginine media containing either bromcresol purple or TTC.

RESULTS

The pH of the Media

In the use of either of the dyes, it was essential that the pH of the two media be 6.0 or lower. S. cremoris strains did not produce sufficient acid to turn the bromcresol purple medium yellow if the pH was above 6.0. In

the case of the arginine-TTC agar, all the colonies produced by S. cremoris were red if the pH was above 6.0.

Arginine-Bromcresol Purple Agar

The color of this medium at pH 6.0 was a light gray. Single strains of S. cremoris caused the agar to become light yellow. The colonies produced by this species were small with entire edges; strains KH, W, C3, and 144-F were tested. Typical results exemplified by colonies of S. cremoris 144-F developing on the arginine-bromcresol purple agar are shown in Figure 2.

Single strains of S. lactis caused the agar to become purple throughout. Colonial appearance was quite distinctive. The profuse growth in the center of the colony was surrounded by an area of thin growth. This was surrounded by an adjacent area of profuse growth. Strains 27, C2, C10, and E were tested. Colonies of S. lactis strain E were less distinctive than those produced by the other strains. Figure 3 shows colonies of S. lactis 27 developing on the arginine-bromcresol purple agar.

Agar plates of a mixture of S. lactis 27 and S. cremoris 144-F were entirely purple. Distinction between the two could be made, however, on the basis of the colonial morphology which was characteristic for each species.

Arginine-TTC Agar

The incorporation of 0.05 per cent TTC into the

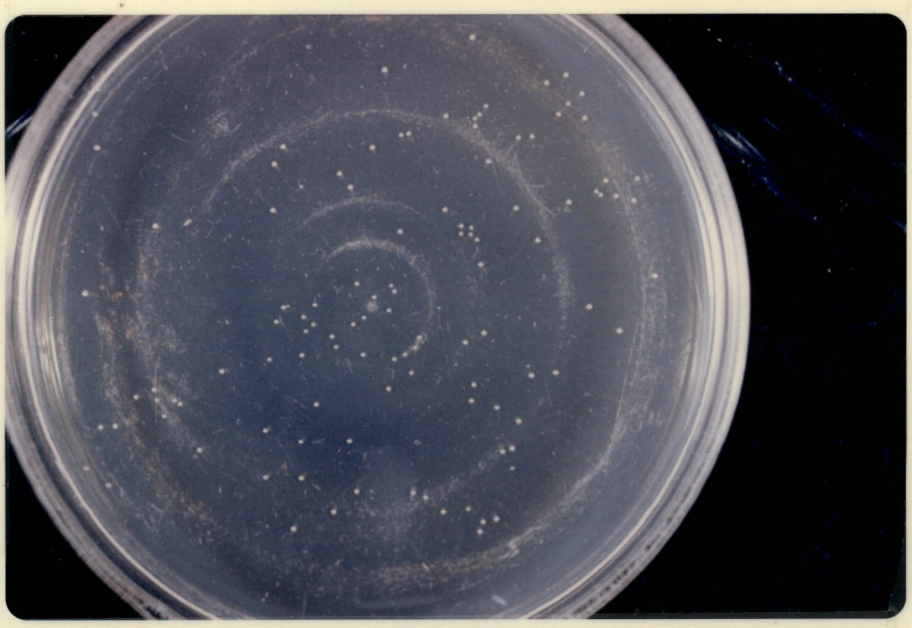

Figure 2. Colonies of S. cremoris 144-F developing on arginine-bromcresol purple agar upon incubation at 30° C. for 24 hrs. in a candle-oats jar.

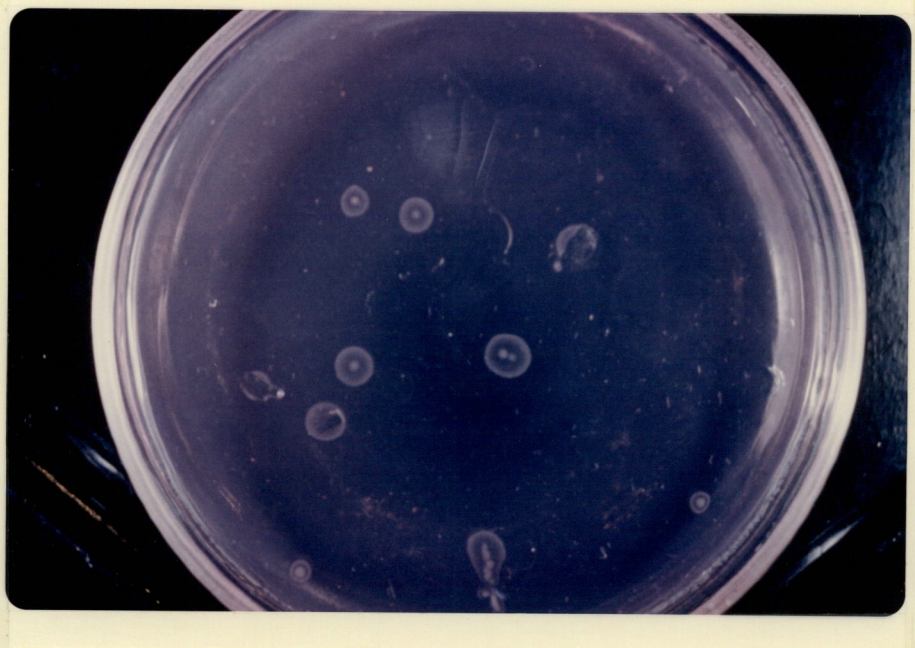

Figure 3. Colonies of S. lactis 27 developing on arginine-bromcresol purple agar upon incubation at 30° C. for 24 hrs. in a candle-oats jar.

arginine agar completely inhibited S. cremoris strains W, 144-F, C3, and KH. S. lactis E also was inhibited while the growth of S. lactis 27, C10, and C2 was markedly reduced. The incorporation of 0.005 per cent TTC, however, did not seem to have any influence on the growth of any of these organisms.

Table 4 summarizes the appearance of several strains of lactic acid streptococci on the arginine agar containing 0.005 per cent TTC. It may be seen that all except one of the S. cremoris strains produced white colonies. On the other hand, colonies of the S. lactis strains were red, indicating that these organisms were capable of reducing the dye to an insoluble red formazan. Colonies of S. lactis strain E did not appear within 27 hrs. of incubation but were easily recognized within 48 hrs. Figure 4 shows typical results when a mixture of S. lactis 27 and S. cremoris W were plated on this medium. Comparable results were obtained with mixtures of other S. lactis and S. cremoris organisms, and this was true whether or not the cultures were plated directly from broth or from nonfat milk cultures.

Most arginine-hydrolyzing strains of S. diacetilactis produced red colonies on arginine-TTC agar; strains which did not hydrolyze arginine (Rm-1, 6B-3, 11D-3 and 6B-1) produced white colonies. Three strains which gave a positive Nessler's test for ammonia (31-2, 18-16 and 31-8)

TABLE 4

Color of S. lactis, S. cremoris and S. diacetilactis colonies developing on arginine-TTC agar upon incubation at 30° C. for 24 hours in a candle-oats jar

Organism	Color of colony
Strain of S. lactis	
27	Red
C10	Red
E	Red
11955a	Red
7962	Tiny red center
7963	Red
11454	Red
Strain of S. cremoris	
W	White
KH	White
C3	White
144-F	White
11602a	White
Da-1	White
1	White
E8	White
9596	White
18-10	White
C13	Light pink
9225	White
Strain of S. diacetilactis	
DRC-1	Red
26-2	Red
31-2	Mixture of red and white
18-16	Mixture of red and white
31-8	Mixture of red and white
RM-1	White
6B-3	White
11D-3	White
6B-1	White

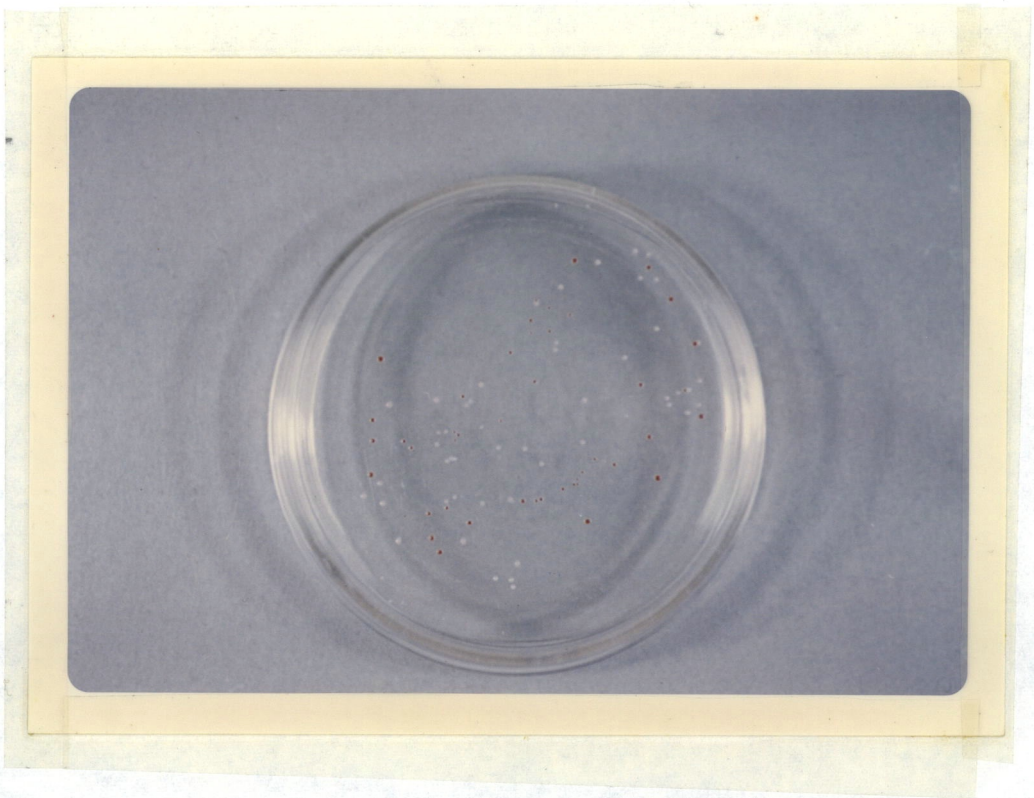

Figure 4. Colonies of S. lactis 27 and S. cremoris W developing on arginine-TTC agar upon incubation at 30° C. for 24 hrs. in a candle-oats jar.

produced a mixture of red and white colonies.

Other tetrazolium salts were tested in the basal arginine medium at a concentration of 0.005 per cent for possible use in differentiating S. lactis from S. cremoris. Table 5 indicates the results of these tests. Of the dyes tested, tetrazolium blue and tetrazolium red seemed to be as effective as TTC. This is substantiated in Figure 5, 6 and 7 which show colonies of S. lactis 27, S. cremoris W, and a mixture of these two, respectively, plated on arginine-tetrazolium blue agar. Figure 8 shows a mixture of these two organisms plated on arginine-tetrazolium red agar. In each case, colonies of S. cremoris were white, while those of S. lactis were colored.

DISCUSSION

Niven, et al (45, p. 651-660) noted that cultures of S. lactis were able to produce ammonia from arginine, while cultures of S. cremoris lacked this ability. This characteristic has since been the primary criterion for the separation of the two species. The procedure for isolating and identifying each of these organisms when grown in mixed culture is extremely tedious. It involves plating the mixture on some suitable agar medium, picking as many as 100 individual colonies and subculturing the isolates in Niven's arginine broth. A method of recognizing these organisms on an agar medium would be extremely

TABLE 5

Comparison of color of colonies of S. lactis and S. cremoris organisms developing on basal arginine agar medium supplemented with 0.005 per cent of various tetrazolium dyes

Dye	Color of colonies	
	S. lactis 27	S. cremoris W
Tetrazolium blue	Dark blue	Light blue
Tetrazolium red	Red	White
Neo-tetrazolium chloride	--[a]	--
Tetrazolium violet	Violet	Violet
DTDTBr[b]	--	--
INT[c]	--	--

[a] Indicates no growth due to inhibition by the dye

[b] 3(4,5 dimethyl thiazolyl 1-2)2,5 diphenyl tetrazolium bromide

[c] 2ρ Iodophenyl 3ρ nitrophenyl-5-phenyltetrazolium chloride

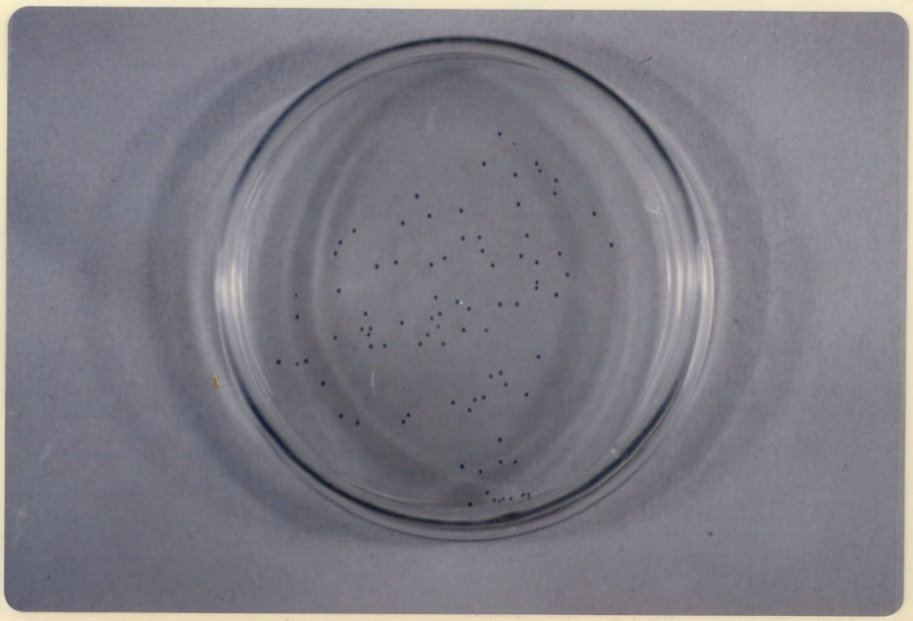

Figure 5. Colonies of S. lactis 27 developing on arginine-tetrazolium blue agar upon incubation at 30° C. for 24 hrs. in a candle-oats jar.

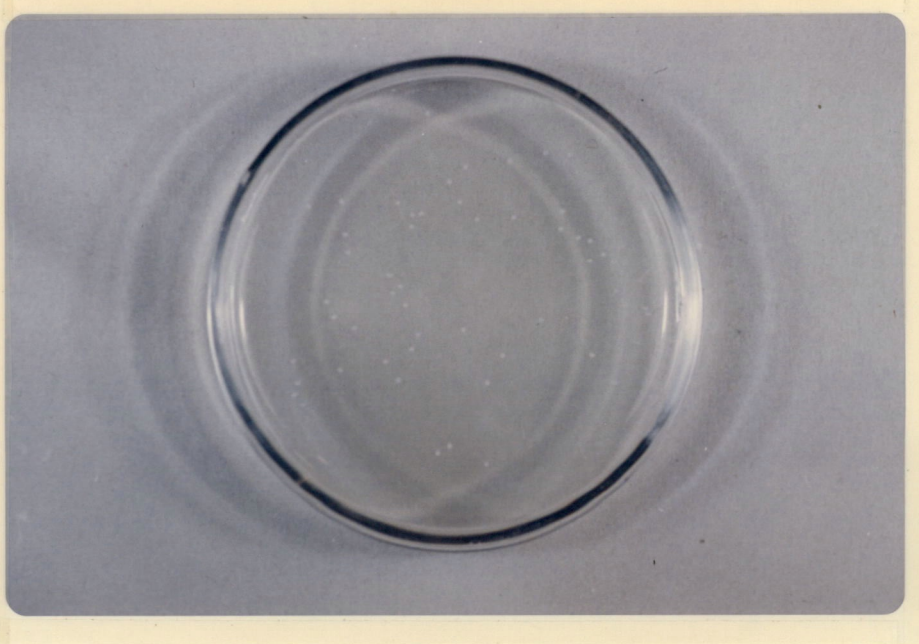

Figure 6. Colonies of S. cremoris W developing on arginine-tetrazolium blue agar upon incubation at 30° C. for 24 hrs. in a candle-oats jar.

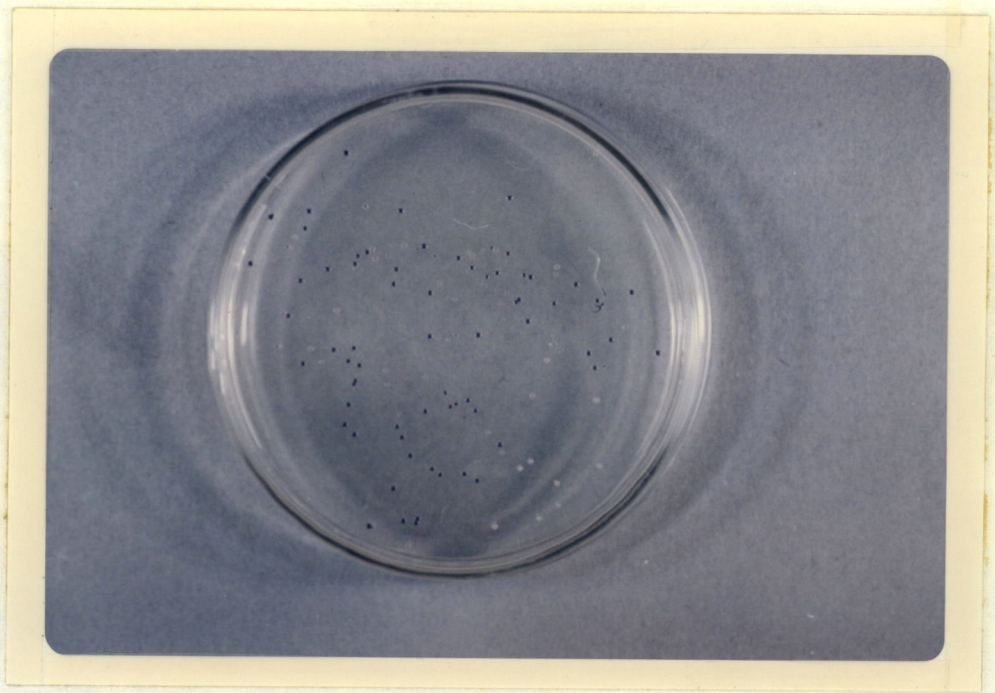

Figure 7. Colonies of S. lactis 27 and S. cremoris W developing on arginine-tetrazolium blue agar upon incubation at 30° C. for 24 hrs. in a candle-oats jar.

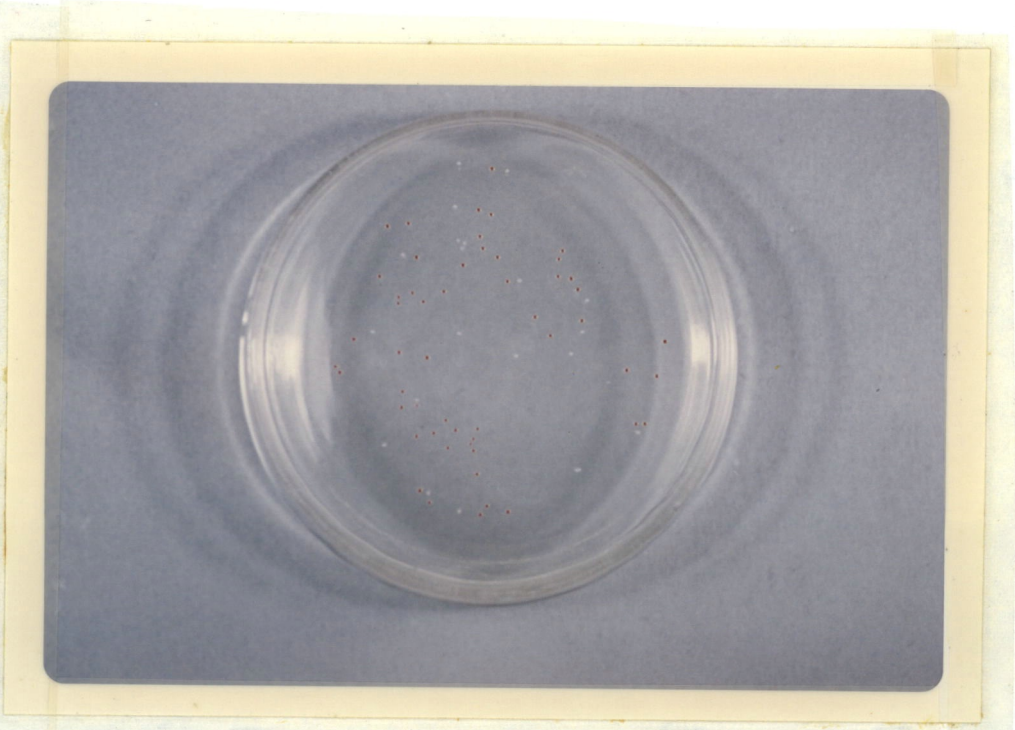

Figure 8. Colonies of S. lactis 27 and S. cremoris W developing on arginine-tetrazolium red agar upon incubation at 30° C. for 24 hrs. in a candle-oats jar.

desirable.

Since a significant difference in the pH was found to exist between cultures of S. lactis and S. cremoris when grown in Niven's arginine broth, it was hoped that the incorporation of a pH indicator, such as bromcresol purple, into an agar medium would result in differentiation of the two species. It was hypothesized that the acid produced by S. cremoris would diffuse into the agar immediately surrounding the colony and cause the bromcresol purple indicator to become yellow. The colony would then be surrounded by a yellow ring. The ammonia produced by arginine-hydrolyzing organisms would, in the same manner, cause the indicator to become purple and result in a purple ring around the S. lactis colonies. However, the ammonia diffused rapidly throughout the medium and, as a result, the entire plate became purple when the two organisms were mixed. Nevertheless, it was possible to distinguish most S. lactis colonies from colonies of S. cremoris strictly on the basis of morphology. However, the reason for the distinctive morphology of the S. lactis colonies is unclear. It is possible that arginine hydrolysis, which was presupposed in the design of the medium, is a factor in this phenomenon. Thus, while the arginine-bromcresol purple medium did not accomplish all that was originally intended, it was still useful in the differentiation of colonies of S. lactis and S. cremoris.

It became obvious, because of the rapid diffusion of ammonia through the agar, that a dye which would produce some change in the colony, rather than in the medium surrounding it, would be better suited to differentiate the organisms in question. Such dyes were available in the tetrazolium salt indicators. These dyes are reduced and absorbed by the bacterial cells, and no apparent change occurs in the surrounding medium.

The acid produced by S. cremoris was sufficient to inhibit the reduction of the tetrazolium dyes. The ammonia produced from the hydrolysis of arginine in the S. lactis colonies was sufficient to raise the pH of the medium so that reduction of the dye could proceed. It was found in this regard that the initial pH of the medium was extremely important; if it was 6.5 instead of 6.0, all of the colonies became colored. Thus it appeared that S. cremoris did not produce enough acid to inhibit dye reduction at pH 6.5. Clear-cut differentiation was possible, however, when the initial pH of the medium was 6.0 or less.

Arginine-TTC agar should be useful in the study of single strains of S. lactis and S. cremoris. It has already been used for the selection of non-arginine hydrolyzing mutants of S. lactis, a technique which will be described in Part III of this thesis. It could also be of some use in the separation and identification of mixtures of S. lactis and S. cremoris which are so often used in

the dairy industry. It should not, however, be regarded as a cure-all for problems encountered in the separation of lactic acid streptococci grown in mixed culture. The reduction of the dye is dependent upon pH, not upon the hydrolysis of arginine. It has, however, proved to be quite reliable in the case of S. lactis and S. cremoris. The reaction of S. diacetilactis is less consistant, for organisms from some of the white colonies produced by strains of this species seem to hydrolyze arginine.

The length of incubation may also be a factor to be considered. Most of the organisms tested produced detectable colonies within 24 hrs. S. lactis strain E was an exception and required a 48 hr. incubation period. Colonies of certain strains of S. cremoris changed from white to blue when incubated for 48 hrs. on arginine-tetrazolium blue agar. This did not occur on arginine-TTC agar.

Plates were incubated in these experiments in a candle-oats jar because larger colonies were produced under these conditions. The same results, with regard to dye reduction, could be obtained, however, by incubating the cultures aerobically.

PART II

THE EFFECT OF INCUBATION OF LACTIC STREPTOCOCCUS ORGANISMS IN ATMOSPHERES CONTAINING DIFFERENT AMOUNTS OF CO_2

Colonies of organisms developing on the surface of agar media incubated in a candle-oats jar were invariably larger than colonies developing on media incubated in a regular air incubator. Figure 9 shows typical results obtained by incubating lactic streptococcus organisms in a regular air incubator. Typical results obtained by incubating these organisms in a candle-oats jar may be seen in Figure 10. The candle-oats jar provided an atmosphere of CO_2 greater than that found in air and it therefore seemed advisable to test the effect of different amounts of CO_2 on the growth of the organisms in question.

MATERIALS AND METHODS

Cultures Used

S. lactis strain 27, S. cremoris strain W, and S. diacetilactis strain 18-16 were studied as representatives of each of the three species comprising the lactic group of streptococcus organisms. Cultures were transferred twice weekly into K and W broth (Table 3), incubated at 30° C. for 24 hrs., and refrigerated between transfers at 4° C.

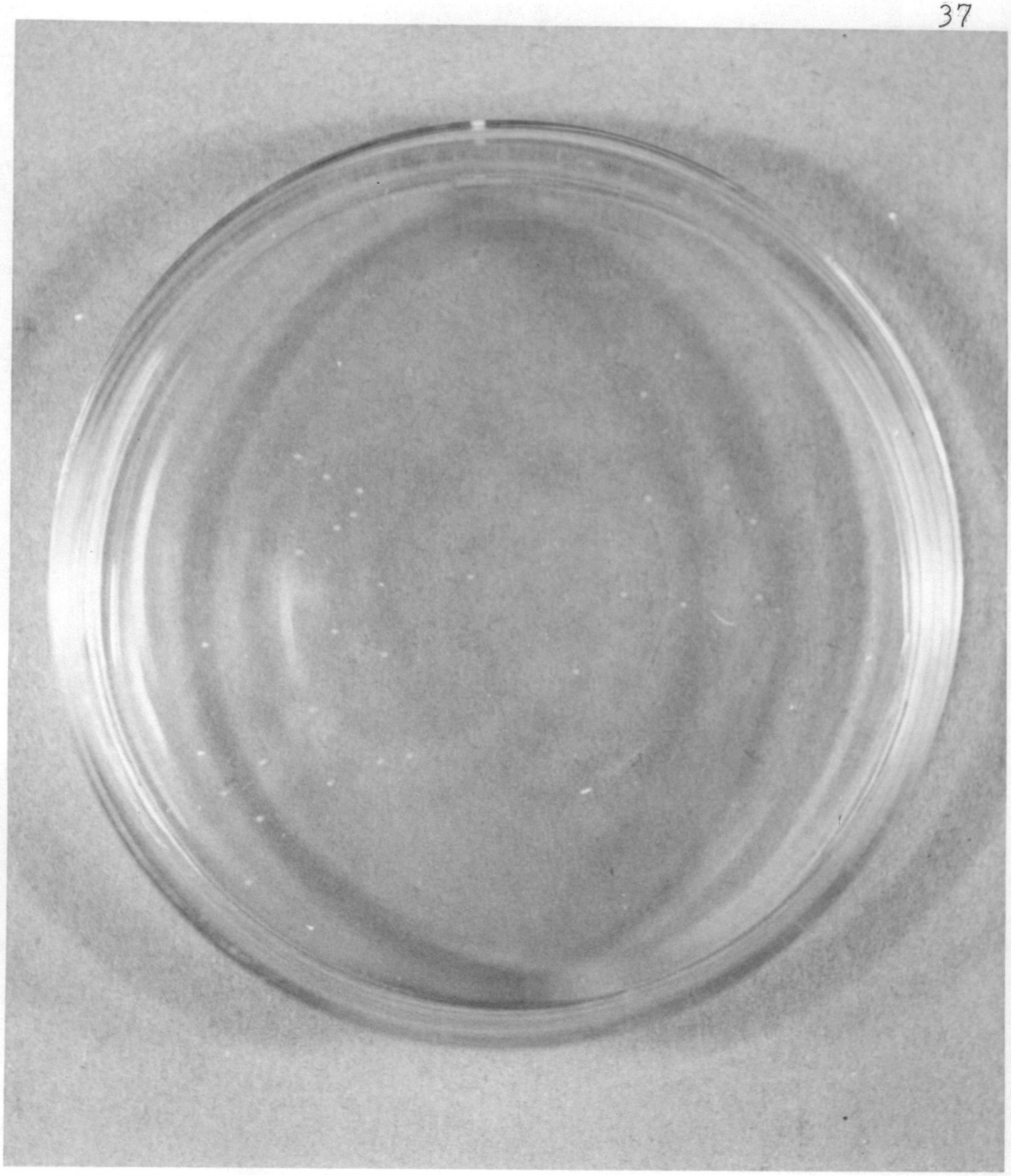

Figure 9. Colonies of lactic acid streptococci developing on lactic agar upon incubation at 30° C. for 24 hrs. in an aerobic incubator.

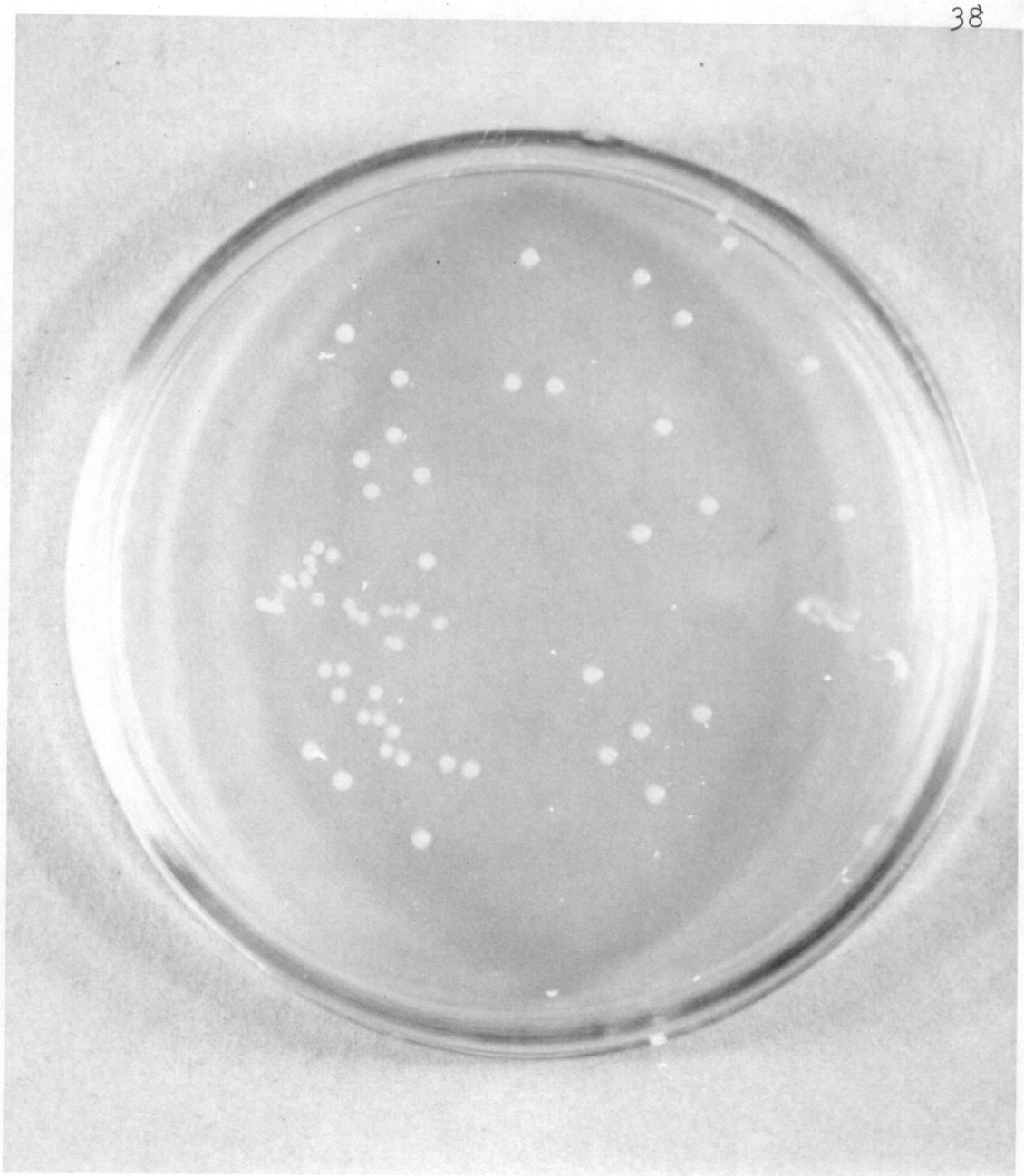

Figure 10. Colonies of lactic acid streptococci developing on lactic agar upon incubation at 30° C. for 24 hrs. in a candle-oats jar.

Preparation of Plates

Agar was poured into sterile petri plates from bottles of the sterile lactic agar described in Table 2 (Refer to page 20). The plates were allowed to dry at room temperature for 28 hrs. A 5-hr. culture of each of the above organisms was diluted in sterile distilled water, and four 0.1 ml. aliquots of an appropriate dilution (10^{-6}) of each culture was distributed over the surface of four dried agar plates with a sterile glass spreader. The inoculated plates were incubated under the conditions described below.

Incubation of Plates

All plates were incubated at a temperature of 30° C. for 24 hrs.; one plate of each organism was incubated aerobically in a regular air incubator; a second plate of each culture was incubated in a candle-oats jar, and a third plate of each organism was incubated in a CO_2 incubator (Model 3560, National Appliance Company, Portland, Oregon). The latter plates were exposed to levels of CO_2 varying from 2 per cent to 18 per cent. The desired CO_2 tension was established by evacuating a given percentage of air from the incubation chamber and replacing it with CO_2 delivered from a cylinder of this gas. About 600 ml. of water were placed in the bottom of the incubator to provide a high humidity (about 100 per cent).

The results of preliminary experiments suggested that a fourth plate of each organism should also be incubated aerobically but in an atmosphere of high humidity. This was accomplished by incubating these plates in a second CO_2 incubator, identicle to the one described in the preceeding paragraph. The incubation chamber was not evacuated, however, and a high humidity was achieved by adding about 600 ml. of water to a shallow pan in the bottom of the incubator.

Determination of Colony Size

The diameter of colonies was measured in millimeters by using a calibrated ocular micrometer in a Bausch and Lomb binocular microscope. Nine typical colonies on each plate were selected at random for measurement and the average colony size determined from these values.

RESULTS

The results of preliminary tests indicated that the degree to which the agar plates had been allowed to dry exerted a definite influence on the average size of the colonies produced by the organisms tested. For example, colonies of bacteria growing on plates dried for 48 hrs. were noticeably smaller than colonies of those growing on plates dried for 24 hrs. This suggested that the amount of moisture present in the medium was a significant

factor in colonial development. Thus the high humidity found in the candle-oats jar could play as important a role as increased CO_2 tension where colony size was concerned. Plates were therefore incubated aerobically in an atmosphere of increased humidity in an effort to measure this effect.

Table 6 summarizes the effect of the various conditions of incubation on colonies of S. diacetilactis strain 18-16. It may be seen that the average colony size was increased by growing the organisms in a candle-oats jar or at elevated CO_2 tension. Humidity did not seem to be a factor, however, since growth at high and low humidity was the same.

The effect of the various conditions of incubation on colonies of S. cremoris strain W is summarized in Table 7. An atmosphere of high humidity seemed to be as effective in increasing the average size of the colonies as was elevating the CO_2 tension or putting the plates in a candle-oats jar. The colonies produced by this strain of S. cremoris were larger under the three previously mentioned conditions than they were under conditions of low humidity.

The effect of the various incubation conditions on colonies of S. lactis strain 27 may be seen in Table 8. Colonies were larger in average size in the candle-oats jar and at high humidity than they were at low humidity.

TABLE 6

The relative* average size of colonies of S. diacetilactis 18-16 incubated under various conditions at 30° C. for 24 hrs.

Conditions	Per cent of size when grown in a candle-oats jar
CO_2 Incubation	
2 per cent	96
4 per cent	90
6 per cent	93
8 per cent	189
10 per cent	83
12 per cent	101
14 per cent	88
16 per cent	108
18 per cent	77
Candle-oats Jar	100
Aerobic Incubation	
Low humidity	70
High humidity	72

*The average size of colonies developing on plates incubated in the candle-oats jar was taken as 100 per cent.

TABLE 7

The relative* average size of colonies of *S. cremoris* W incubated under various conditions at 30° C. for 24 hrs.

Conditions	Per cent of size when grown in a candle-oats jar
CO_2 Incubation	
2 per cent	69
4 per cent	86
6 per cent	98
8 per cent	161
10 per cent	109
12 per cent	101
14 per cent	89
16 per cent	91
18 per cent	108
Candle-oats Jar	100
Aerobic Incubation	
Low humidity	65
High humidity	99

*See Table 6

TABLE 8

The relative* average size of colonies of S. lactis 27 incubated under various conditions at 30° C. for 24 hrs.

Conditions	Per cent of size when grown in a candle-oats jar
CO_2 Incubation	
2 per cent	115
4 per cent	104
6 per cent	110
8 per cent	177
10 per cent	113
12 per cent	111
14 per cent	159
16 per cent	125
18 per cent	104
Candle-oats Jar	100
Aerobic Incubation	
Low humidity	67
High humidity	105

*See Table 6

The greater increase, however, was seen when the plates were incubated at levels of CO_2 between 6 and 16 per cent.

The addition of "Tween 80" or bicarbonate to the medium was not effective in increasing colony size.

DISCUSSION

When this investigation was undertaken, it was hoped that a definitive statement could be made regarding the reason for increased colony size produced by the lactic acid streptococci when grown in a candle-oats jar. The results of these experiments, however, cannot be regarded as conclusive, and additional studies should be initiated in an attempt to claify the picture presented here. It would appear that each of the organisms tested responded to the candle-oats jar atmosphere for different reasons. S. lactis strain 27, for example, seemed to require increased CO_2 tension as well as high humidity. S. cremoris strain W seemed to require only high humidity, while S. diacetilactis strain 18-16 responded to increased CO_2 tension but not to high humidity.

Colonies developing on an agar medium are limited to growth in the thin film of water which is present on the surface of the medium. If the agar becomes too dry, nutrients cannot diffuse to the organisms, and growth will be severely limited. The effect of high humidity on the strain of S. lactis and the strain of S. cremoris tested

could be simply to prevent the agar from drying out too rapidly. Since S. diacetilactis strain 18-16 did not respond to increased humidity, it must be assumed that the colony size of this organism is limited by some factor other than diffusion of nutrients through the agar. The fact that the S. diacetilactis strain responded to increased CO_2 tension would seem to indicate that CO_2 is required for maximum growth by this organism. S. lactis strain 27 also responded significantly to the increased CO_2 tension, suggesting that this strain of S. lactis, as well as the ML3 strain investigated by Reiter and Oram (52, p. 175-176), possesses a CO_2 requirement.

The function of the candle-oats jar, in the case of the organisms tested, could be to provide conditions of high humidity and/or increased CO_2 tension. More extensive experiments should be performed and more strains of each species should be tested before any positive statement regarding the effect of the candle-oats jar, increased CO_2 tension and high humidity is made.

PART III

ARGININE METABOLISM BY WILD TYPE AND MUTANT STRAINS OF *STREPTOCOCCUS LACTIS*

MATERIALS AND METHODS

Cultures Used

S. lactis strain 27 was used throughout this phase of the study. *S. cremoris* strain 144-F was also examined to see if enzymes for arginine degradation were present. Mutant and wild type organisms were transferred twice a week in K and W broth (Table 3), incubated at 30° C. for 24 hours, and refrigerated at 4° C. between transfers.

Preparation of Mutants

One-tenth ml. of a 5-hr. culture of *S. lactis* 27 was spread on the surface of arginine-TTC agar plates. The plates were irradiated with ultraviolet light for varying lengths of time by placing them 6 inches from a 30 watt General Electric germicidal bulb emitting 12.37 ergs per second per square centimeter. They were then wrapped in towels to prevent photoreversal of any mutation which may have occurred, and incubated in a candle-oats jar at 30° C. for 24 hrs. Mutant organisms which did not hydrolyze arginine produced white colonies and were readily distinguished from the red colonies produced by the wild type organism. Isolates were subcultured in Niven's arginine broth and tested with Nessler's reagent for the

production of ammonia. They were also respread on arginine-TTC agar to confirm that white colonies would be produced by the mutant cultures.

Mutants were not obtained by single irradiation exposures, even though the cultures were irradiated as long as 200 seconds. If colonies were isolated from a plate which had been irradiated, cultured in K and W broth, respread on arginine-TTC agar, and irradiated a second time, mutants which lacked the ability to hydrolyze arginine could be found. The two mutants studied were isolated in this manner and were designated 1601 and i501.

Preparation of Resting Cells for Manometric Experiments

Two-liter volumes of K and W broth in flasks were inoculated with 10 ml. of a 5-hr. broth culture of the organism to be studied. The flasks were incubated at 30° C. for 6 hrs. Cells were harvested in the cold by centrifugation at 7,000 revolutions per minute (r.p.m.) in a Servall centrifuge (Model SS-3). They were then washed twice in 0.05 molar (M) potassium phosphate buffer, pH 6.5, and resuspended in 30 ml. of the buffer. Protein was estimated in the following manner. Wet weight of the cells was determined to the nearest mg. by weighing on a torsion balance. Dry weight of the cells was estimated to be about 10 per cent of the wet weight. Of this 10 per cent, 14 per cent was estimated to be nitrogen. The

estimated per cent nitrogen multiplied by 6.25 gave a rough estimate of the amount of protein present in the sample.

Preparation of Cell-Free Extracts for Manometric Experiments

A nine-liter volume of K and W broth was inoculated with 200 ml. of a 5-hr. broth culture of the organism to be tested. The flask was incubated at 30° C. for 5-6 hrs. The cells were harvested in a Sharpless centrifuge, washed twice in 0.05 M potassium phosphate buffer, pH 6.5, and resuspended to 30 per cent suspension (wet weight) in the buffer. The suspension was subjected to sonic disintegration in a 10 kilocycle, Raytheon oscillator for 30 min. Cell debris was removed by centrifugation at 14,000 r.p.m. for 20 min. in a Servall centrifuge (Model SS-1). The cell-free preparation was distributed in 5 ml. quantities into screw cap tubes and stored at -4° C. Total protein was determined colorimetrically by the method of Lowery, et al (39, p. 265-275).

Manometric Determination of Enzyme Activity

The breakdown of both L-arginine (Nutritional Biochemicals Corporation, Cleveland, Ohio) and L-citrulline (California Corporation for Biochemical Research, Los Angeles, California) was followed manometrically. The reaction was conducted in double side arm Warburg flasks

of 3.2 ml. capacity. Reports of other workers suggested the advisability of testing the effect of various cofactors on the reaction. Figure 11 shows the effect of various conditions on the amount of CO_2 produced from L-citrulline by cell-free extracts of S. lactis 27 (wild type). It may be seen that the greatest activity occurred in the presence of phosphate, Mg^{++}, and ADP. It was therefore decided that the complete reaction mixture should consist of 5 µmoles of ADP (Sigma Chemical Company, St. Louis, Missouri), 10 µmoles of $MgCl_2$, 0.03 M phosphate buffer (pH 6.5), 10 µmoles of substrate (citrulline or arginine), and enzyme in the form of whole cell suspension or cell-free extract. The reaction was started by the addition of substrate from one side arm. The reaction was allowed to proceed until the production of CO_2 began to level off (usually 50 min. to 1 hr.). Acid (3N H_2SO_4) was then tipped into the main compartment from one of the side arms to stop the reaction. The reaction mixture was assayed colorimetrically for citrulline by the method of Archibald (1, p. 121-142) and for ornithine by the method of Chinard (6, p. 91-95). All manometric experiments were conducted in an atmosphere of nitrogen at 30.2° C.

RESULTS

Studies of cell-free extracts of S. cremoris strain

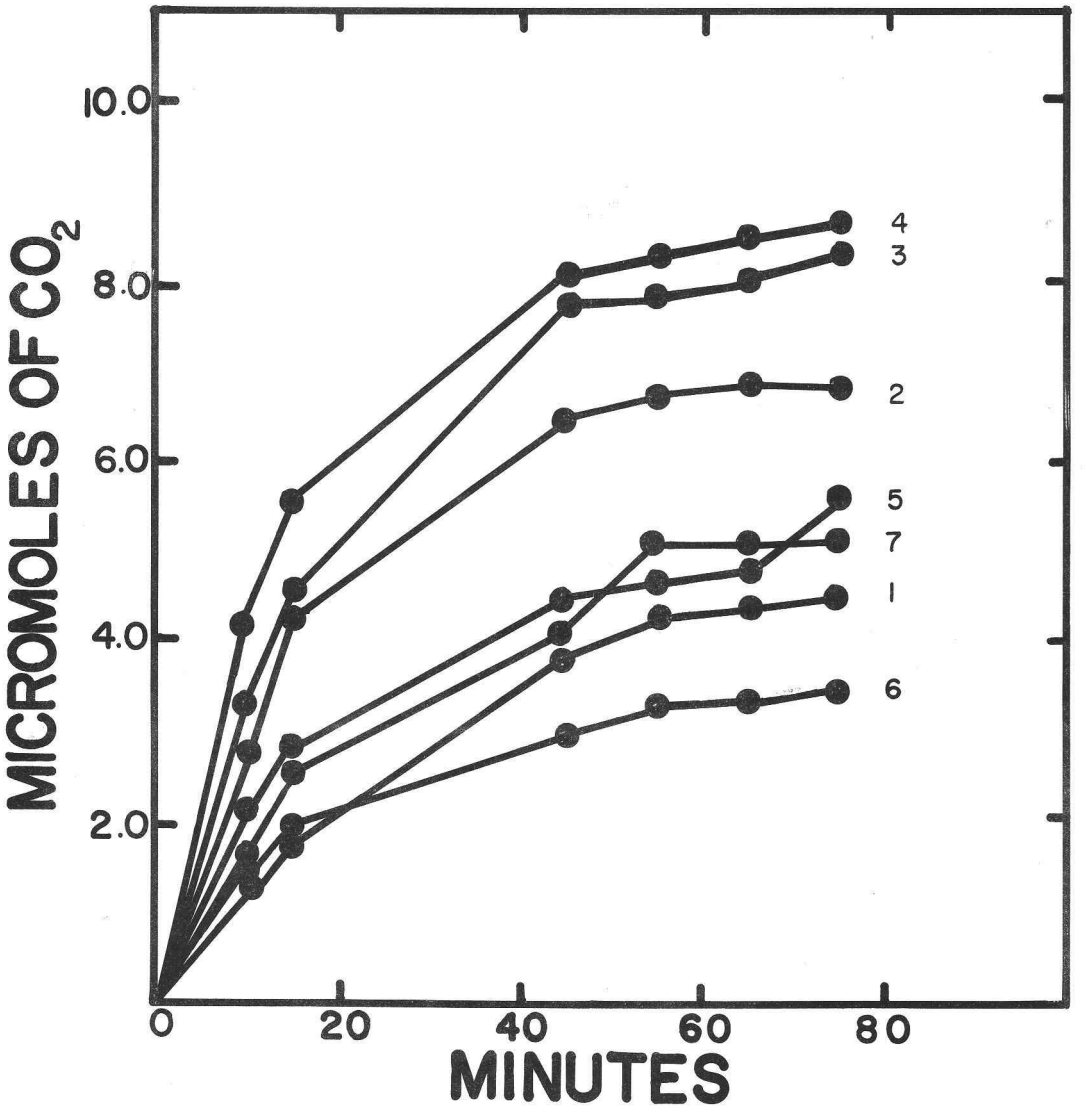

Figure 11. Effect of various conditions on the number of μmoles of CO_2 produced from 10 μmoles of citrulline by cell-free extracts of S. lactis 27 at 30° C.

Cell-free extract (1), cell-free extract+phosphate (2), cell-free extract+phosphate+Mg^{++} (3), cell-free extract+phosphate+Mg^{++}+ADP (4), cell-free extract+Mg^{++}+ADP (5), cell-free extract +Mg^{++} (6), cell-free extract +ADP (7).

144-F showed that no CO_2 was produced when either citrulline or arginine was supplied as a substrate.

Figure 12 compares the amount of CO_2 produced from the substrates arginine and citrulline by cell-free extracts of the wild type organism and of mutant 1601. It may be seen that the wild type organism produced nearly 9 µmoles of CO_2 from 10 µmoles of either substrate. The mutant 1601, on the other hand, produced only 6 µmoles of CO_2 from either substrate. The flasks containing arginine as the substrate were assayed to see if any accumulation of citrulline had occurred. It was found that flasks containing cell-free extracts of mutant 1601 contained 2.8 µmoles of citrulline. Two and one half µmoles of ornithine could be determined by the Chinard assay procedure.

Figure 13 compares the amount of CO_2 produced from arginine and citrulline by whole cells of the wild type organisms and by whole cells of mutant 1601. The wild type organism produced slightly less than 10 µmoles of CO_2 from 10 µmoles of arginine. However, only 3 µmoles of CO_2 were produced when citrulline was the substrate. Whole cells of mutant 1601 produced less than 1 µmole of CO_2 from either substrate.

The amount of CO_2 produced from arginine and citrulline by cell-free extracts of mutant i501 and of the wild type organism is compared in Figure 14. Only 3.5 µmoles of CO_2 were produced by the mutant from 10 µmoles of

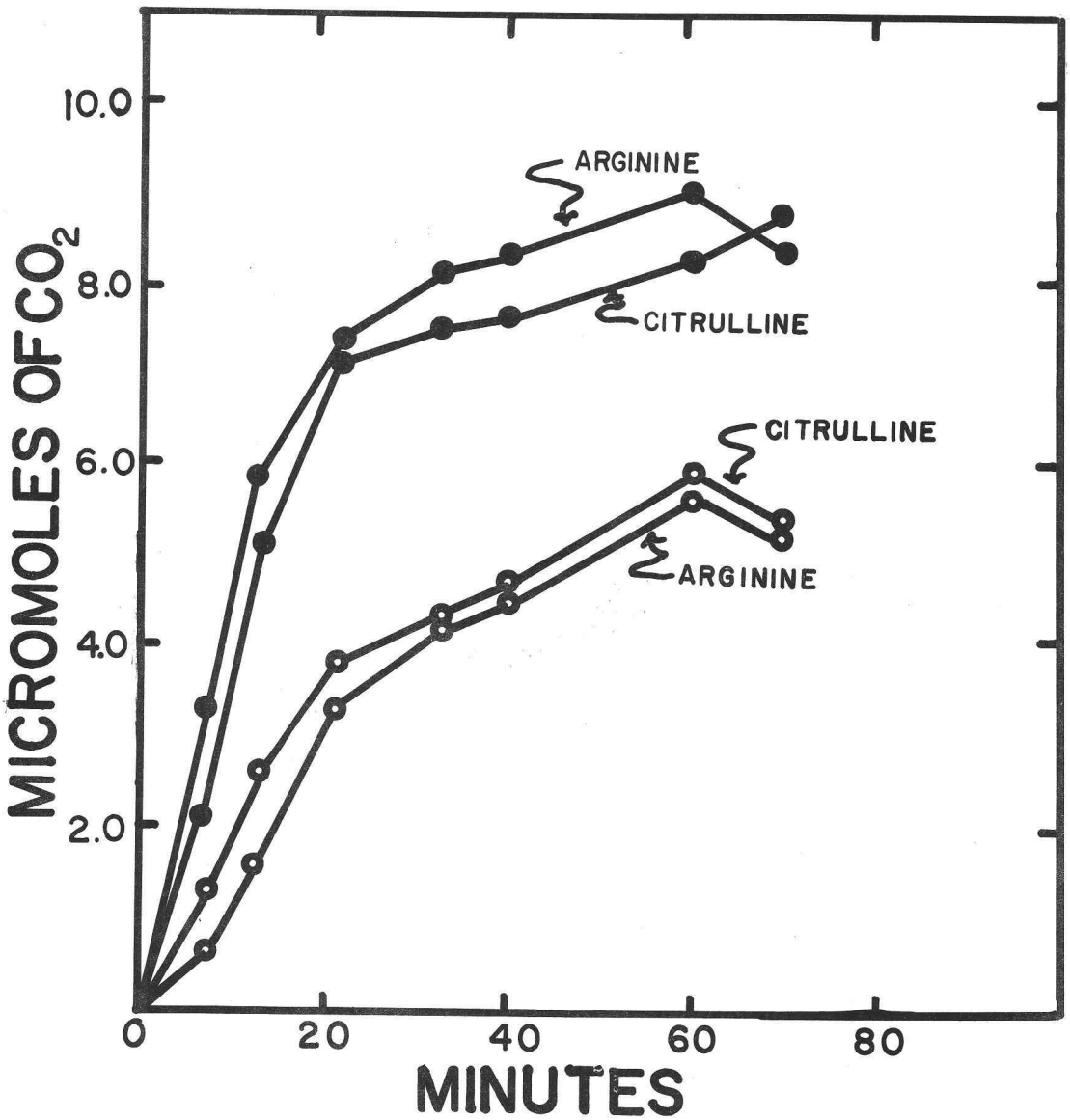

Figure 12. Comparison of μmoles of CO_2 produced by cell-free extracts of wild type (closed circles) and mutant 1601 cells (open circles) of S. lactis 27 incubated at 30° C. in the presence of either arginine or citrulline.

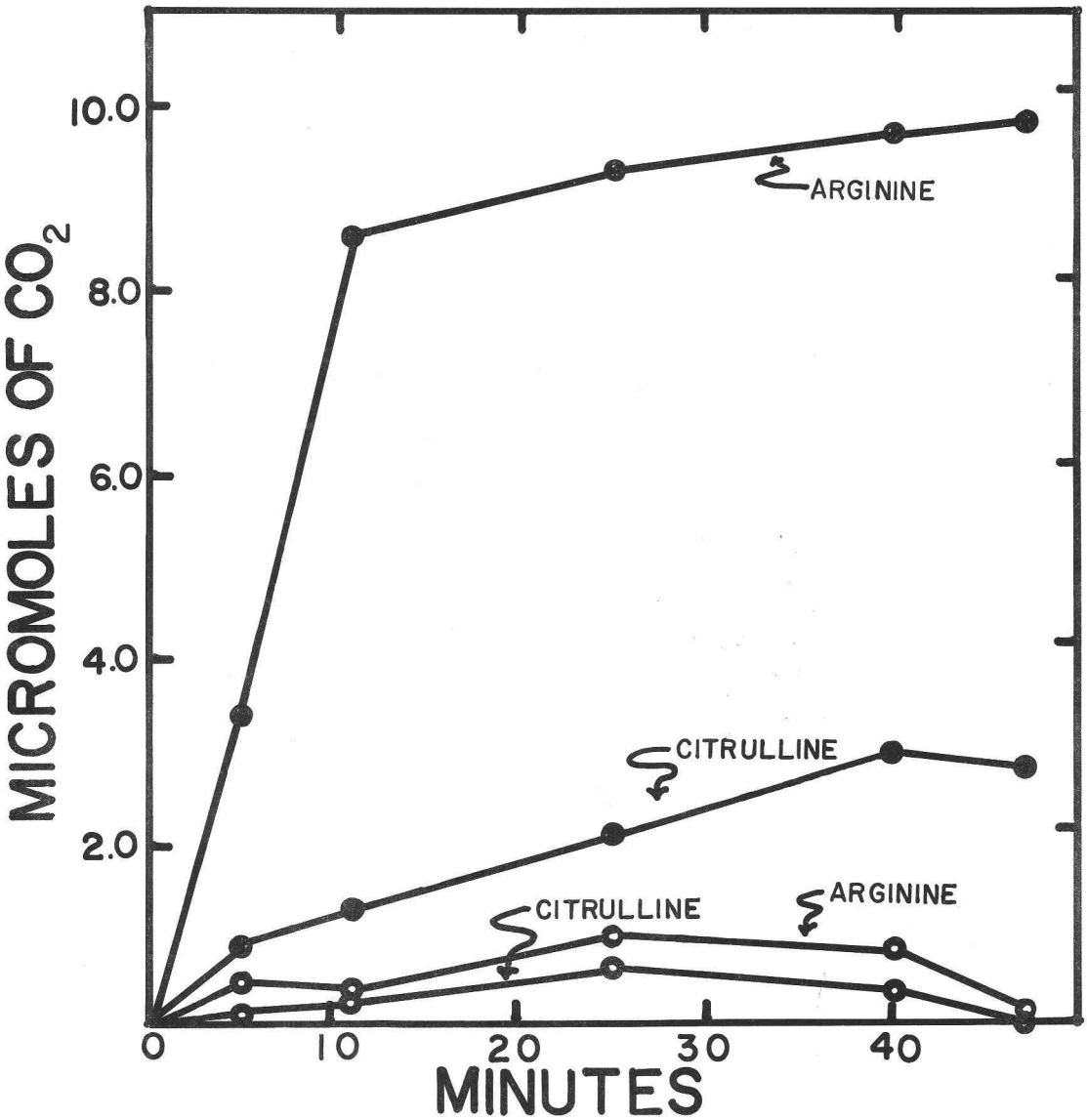

Figure 13. Comparison of μmoles of CO_2 produced by wild type resting cells (closed circles) and mutant 1601 cells (open circles) of S. lactis 27 incubated at 30° C. in the presence of either arginine or citrulline.

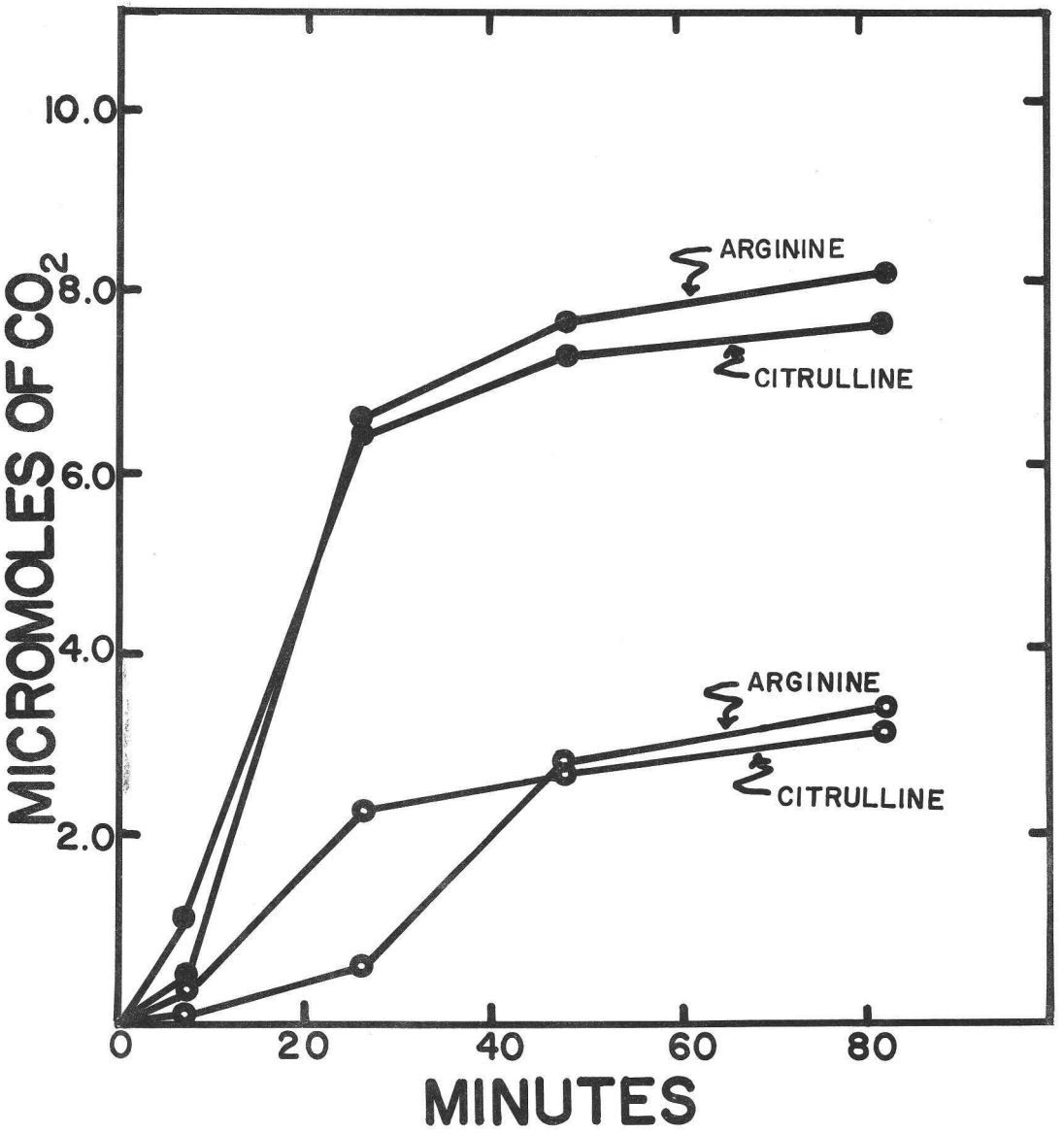

Figure 14. Comparison of μmoles of CO_2 produced by cell-free extracts of wild type (closed circles) and mutant i501 cells (open circles) of S. lactis 27 incubated at 30° C. in the presence of either arginine or citrulline.

citrulline. A pronounced lag in CO_2 production was noticed when arginine was the substrate. The total amount of CO_2 produced from arginine during the reaction time by the mutant was about the same as the amount of CO_2 produced from citrulline. When the arginine flasks were assayed for citrulline, it was found that about 7 μmoles of citrulline had accumulated in the flasks during the reaction time. Two μmoles of ornithine could be measured.

Figure 15 compares CO_2 production from the two substrates by whole cells of the wild type and of mutant i501. It may be seen that less than 1 μmole of CO_2 was produced from either substrate by whole cells of mutant i501.

DISCUSSION

Cell-free extracts of the wild type organism, S. lactis strain 27, have been shown to contain the necessary enzymes for degradation of arginine and citrulline. The data indicate that arginine readily enters the cell, but that citrulline enters with difficulty. It is possible that the cell does not possess a specific concentrating mechanism for citrulline and that entrance of this substrate occurs by diffusion only. On the other hand, it also is possible that a permease enzyme exists which is induced by the presence of intracellular or extracellular citrulline. Evidence that such a mechanism exists in the

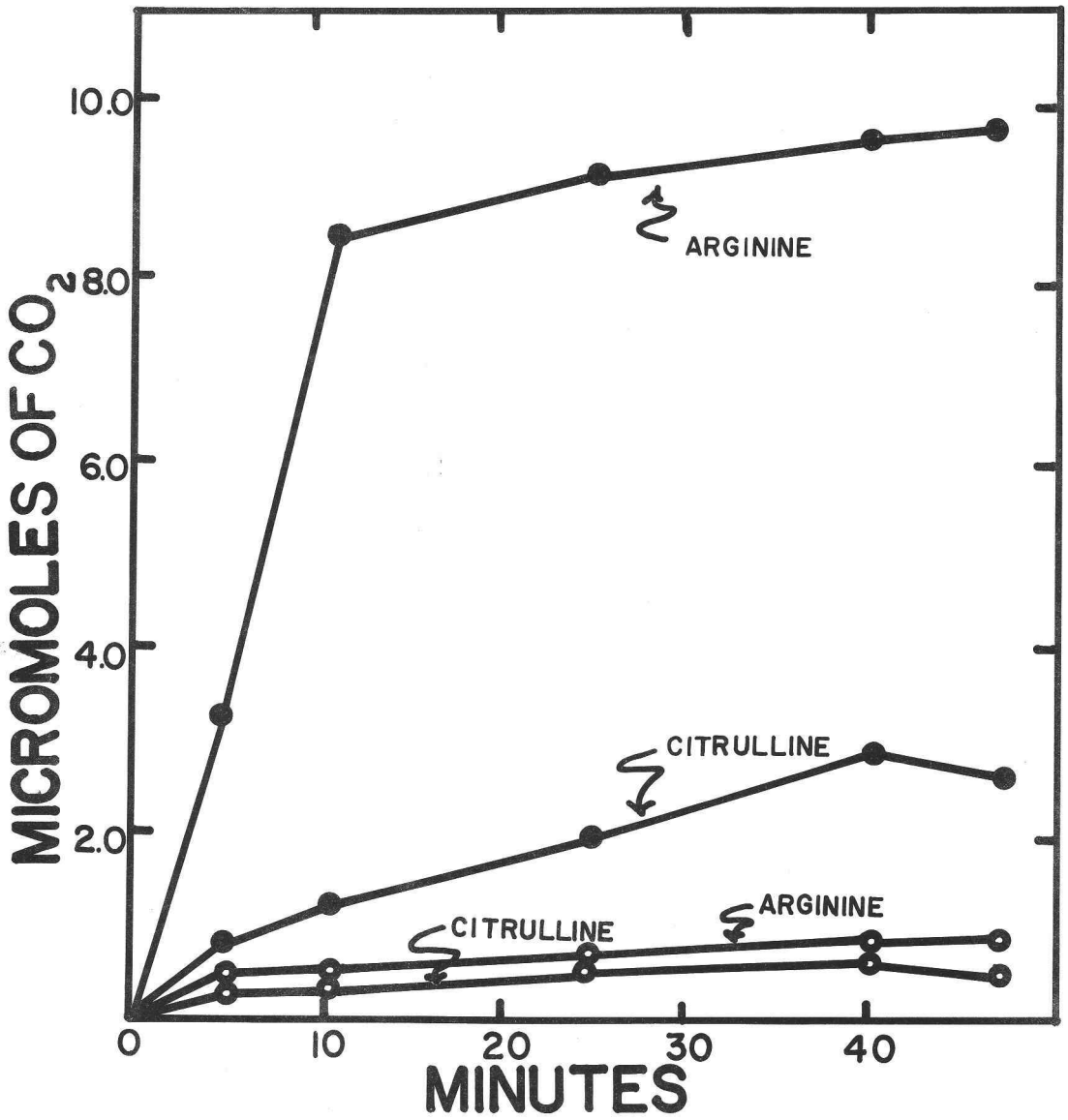

Figure 15. Comparison of μmoles of CO_2 produced by resting wild type cells (closed circles) and mutant i501 cells (open circles) of S. lactis 27 incubated at 30° C. in the presence of either arginine or citrulline.

lactobacilli has been presented by Bibb and Straugh (3, p. 79-80). It is not inconceivable that the same mechanism could exist in the lactic streptococci. It certainly is apparant from Figure 13 (Refer to page 54) that the cell is not completely impermeable to citrulline, since some CO_2 was formed from that substrate by resting, whole cells of the wild type organism.

An examination of the data concerning mutant 1601 would lead one to the conclusion that the conversion of arginine to citrulline was unimpared, since citrulline accumulated when arginine was the substrate for cell-free extracts of this organism.

It is somewhat difficult to explain the discrepancy between the amount of CO_2 formed (6 µmoles) and the amount of ornithine measured colorimetrically (2.5 µmoles) with mutant 1601, since no CO_2 should be formed without the concomitant production of ornithine. This may have been due to the inadequacy of the assay in the presence of accumulated citrulline. Chinard (6, p. 91-95) reported that interference of the assay would occur if 10 times as much citrulline as ornithine were present in the assay mixture. The data with mutant i501 in this regard, however, are more reasonable; 3.5 µmoles of CO_2 and 2.0 µmoles of ornithine were found.

If the breakdown of citrulline had been impared, one of two enzymes could be involved. If the ornithine

transcarbamylase enzyme were damaged, citrulline would not be broken down into ornithine and carbamyl phosphate. Thus, little CO_2 would be formed, even if the carbamate kinase enzyme were functioning properly, since CO_2 can be formed only from the breakdown of carbamyl phosphate. This would account for the accumulation of citrulline.

On the other hand, if the carbamate kinase enzyme had been damaged, very little CO_2 would be formed. In this case, an accumulation of ornithine, greater than the amount of CO_2 produced, might be expected to occur, provided that no damage to the ornithine transcarbamylase enzyme had been incurred. One would not expect the reversal of the citrulline to ornithine reaction, since Ravel, et al (49, p. 1452-1455) have reported that the formation of citrulline by this enzyme is inhibited in the presence of excess inorganic phosphate. All the present studies were carried out in phosphate buffer. It could therefore be postulated that some damage had occurred to the ornithine transcarbamylase enzyme in mutant 1601.

No CO_2 was produced from either arginine or citrulline by whole cells of mutant 1601. This might be expected in the case of citrulline, since the wild type organism, which readily converted this substrate to ornithine, CO_2 and ammonia once it was inside the cell, apparently had difficulty concentrating this substrate.

However, this was somewhat unexpected in the case of arginine. From studies of the wild type (Figure 13), it may be seen that CO_2 was produced from arginine almost as soon as it entered the cell. It must be assumed, therefore, that arginine was immediately converted to citrulline, which then was broken down. It therefore seems reasonable to expect that, if arginine were capable of entering the mutant cell, some CO_2 would be produced, even if it were a small amount.

It is possible that the arginine to citrulline reaction was readily reversed by the whole cells as soon as citrulline began to accumulate. Thus the limited action of the damaged ornithine transcarbamylase (or carbamate kinase) enzyme would go unnoticed. It is also possible that a specific permease existed for arginine which was destroyed by the ultraviolet treatment. This is an attractive hypothesis, since the arginine dihydrolase system has been reported by a number of workers (3, p. 79-80; 26, p. 602-605; 29, p. 174-185; 58, p. 455-466) to be adaptive. The formation of arginine desimidase would not necessarily be impared in the case of a permease mutation, since sufficient excess arginine would probably be present in the medium to enter the cell non-enzymatically and cause induction of that enzyme.

If a permease mutation had occurred, this would mean that mutant 1601 was a double mutant. Such a situation

would not be too unlikely, since both mutants studied were obtained by irradiating the culture twice. If damage had first occurred to the citrulline to ornithine enzyme, it is possible that sufficient ammonia would have been produced from the breakdown of arginine to allow colonies of the organism to appear red on the arginine-TTC agar medium. A mutant of this type would therefore have gone unnoticed.

The reason for the lag in the production of CO_2 from arginine by cell-free extracts of mutant i501 (Figure 14) is not clear. It is possible that the carbamate kinase enzyme was damaged by irradiation and that a certain level of carbamyl phosphate was necessary before any breakdown of that compound to CO_2 and ammonia could occur. Again, one would expect, if such were the case, to find an accumulation of ornithine greater than the amount of CO_2 produced. However, this was not found to be true.

The accumulation of citrulline by mutant i501 when arginine was the substrate again suggested that the arginine desimidase enzyme was not damaged. Also, the reduced CO_2 production (Figure 14 as compared to Figure 12) suggested that which ever enzyme was damaged was impared more severely in i501 than in mutant 1601. At the present time, it is not possible to say whether or not the same enzyme was damaged in both cases, nor is it possible to say with certainty which enzyme was affected. Since

methods for partial purification of both ornithine transcarbamylase (49, p. 1452-1455) and carbamate kinase (50, p. 525-531) from S. lactis have been reported, the answer to determining which enzyme or enzymes in the mutant organisms have been affected may be approached by using purified systems.

Ornithine transcarbamylase and carbamate kinase have been studied extensively as biosynthetic enzymes. The biosynthesis of arginine in E. coli was one of the first repressible enzyme systems to be studied (42, p. 215). In a repressible system, the product of the biosynthesis is capable of repressing (stopping) the synthesis of one of the enzymes involved in its production. It is usually an all or none phenomenon. In E. coli, arginine has been shown to repress the formation of ornithine transcarbamylase, which is the first enzyme peculiar to the arginine biosynthetic pathway from ornithine. If biosynthesis and degradation of arginine proceed by the same pathway in S. lactis, it seems likely that such a repression mechanism could not exist in that organism.

Recent reports have been made concerning a nonrepressible strain of E. coli (17, p. 961-971). The enzymes involved in arginine synthesis in this strain seem to be induced, rather than repressed, by the presence of arginine. Such a situation could be analogous to the synthesis of enzymes involved in arginine metabolism in

S. lactis. Some part of the degradative system in S. lactis has been reported to be inducible. Thus, if biosynthesis proceeds by reversal of the enzymes involved in degradation, the induction in this case is entirely analogous to induction of arginine enzymes in the nonrepressible strain of E. coli.

The reactions involved in the synthesis of citrulline in S. lactis have been shown to be readily reversible, and it has thus been assumed that arginine degradation and biosynthesis proceed through the action of the same enzymes. If this is true, it would be interesting to see if mutants i501 and 1601, whose enzymatic degradation of arginine has been impared, now require arginine for growth. If they did not possess some requirement for that amino acid, this would suggest that synthesis proceeds via some other pathway, or that enzymatic activity had been damaged in one direction only. The latter does not seem very probable.

It has recently been shown by Reiter and Oram (53, p. 63-77) that many strains of S. cremoris do not require arginine for growth. This is interesting in view of the fact that these organisms are unable to hydrolyze arginine with the production of CO_2 and ammonia. Does this imply that a different mechanism exists in S. cremoris for the synthesis of arginine than exists in S. lactis? Studies of cell-free extracts of S. cremoris 144-F indicated that

one or more enzymes involved in the production of CO_2 from arginine did not exist in this organism. Further studies would be necessary to determine whether one or all of the enzymes involved in arginine degradation are missing in this organism. If different mechanisms for arginine synthesis exist in these two organisms, then S. cremoris, which once was believed to be a variety of S. lactis, is unrelated to this latter organism in at least one more characteristic.

Is it possible that the same enzymes for arginine metabolism exist in S. cremoris as in S. lactis, but that their action is not reversible? Further studies of these organisms would help to clarify this question.

Reiter and Oram also have shown that at least two strains of S. diacetilactis (DRC-1 and DRC-2) require arginine, although both have been shown by Sandine, et al (54, p. 161-174) to possess the ability to hydrolyze it. This would certainly imply that synthesis and degradation of arginine proceed by different pathways in S. diacetilactis.

The block in arginine synthesis in DRC-1 and DRC-2, according to Reiter and Oram, occurs in the synthesis of arginine from citrulline. If this is the case, it is possible that arginine desimidase is involved in the degradation, but not in the synthesis, of arginine in S. diacetilactis.

It would be extremely interesting to extend studies of the mutant organisms isolated in this investigation and to study more extensively the mechanism of arginine synthesis and degradation in S. lactis, S. cremoris, and S. diacetilactis.

SUMMARY

A medium containing arginine and 2,3,5-triphenyl tetrazolium chloride (TTC) has been described which may be used to distinguish S. lactis from S. cremoris. Acid produced by strains of S. cremoris tested inhibited reduction of the dye to an insoluble red formazan and the organisms produced white colonies. The ammonia produced as a result of hydrolysis of arginine by the strains of S. lactis tested was sufficient to allow dye reduction to occur and red colonies were produced. Some strains of S. diacetilactis produced both red and white colonies, regardless of their arginine-hydrolyzing abilities.

The effect of carbon dioxide on the size of colonies produced by the lactic acid streptococci also was studied. S. lactis strain 27 seemed to respond to high humidity as well as increased CO_2 tension. S. diacetilactis strain 18-16 appeared to respond to increased CO_2 tension but not to high humidity. S. cremoris strain W apparently required only high humidity.

Mutants of S. lactis strain 27, which lacked the ability to produce ammonia from arginine, were obtained by ultraviolet light irradiation and selection on arginine-TTC agar. Enzymes involved in arginine degradation were studied manometrically in mutant and wild type S. lactis 27 organisms and in S. cremoris strain 144-F.

Enzymes for arginine degradation were found to be present in mutant organisms, but in a reduced amount. No enzymes for arginine degradation were found in S. cremoris 144-F.

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