cosis. It is interesting to note that the excess EDTA solution, which was capable of chelating calcium, had a more pronounced effect on shortening induction time and prolonging recovery time than the balanced EDTA solution. The binding of calcium ions during the induction of narcosis by either citrate or EDTA resulted in a prolongation of recovery time. Thus, it appears that ionized calcium is of importance in the rate of recovery from narcosis. The anomalous results with reference to the recovery times observed when narcosis was induced in calcium-free Ringer's solution and following a 4 hour pretreatment period in this solution remain unexplained at the present time.

Pretreatment of the fish in calcium-free Ringer's solution resulted in a loss of calcium ions as shown by quantitative analysis. This is in accord with observations of Krogh(8) who showed that various ions may be removed by diffusion, primarily across the gill capillaries, when fish are placed in ion-poor solutions. The inability of Ringer's solution containing excess calcium to modify the induction of narcosis even after the 4 hour pretreatment period may indicate either that excess intracellular calcium has no effect on narcosis or that calcium diffuses across cellular membranes and becomes incorporated into the cell with great difficulty.

Summary. 1. The induction time of pentobarbital narcosis was shortened by 50% when carried out in the presence of calcium binding substances. 2. Removal of calcium by binding with citrate ion or pretreatment of the fish in calcium-free Ringer's solution prior to induction of pentobarbital narcosis resulted in a prolongation of the induction time (90%). 3. Balanced calcium EDTA solution shortened the induction time (30%) and lengthened the recovery time (100%) in spite of its theoretical inability to chelate calcium, while excess EDTA caused a greater shortening in induction time (50%) and lengthening in the recovery time (200%). 4. Excess calcium exerted no effect on induction or recovery of pentobarbital narcosis.

1. Mazia, D., and Clark, J. M., *Biol. Bull.*, 1936, v71, 306.

2. Berwick, M. C., *J. Cell. and Comp. Physiol.*, 1951, v38, 95.

3. Heilbrunn, L. V., *An Outline of General Physiology, 3rd ed.*, Philadelphia, Saunders Co., 1952.

4. Clark, E. P., and Collip, J. B., *J. Biol. Chem.*, 1925, v63, 461.

5. Shelling, D. H., and Maslow, H. L., *ibid.*, 1928, v78, 661.

6. Technical Bulletin, *The Versenes*, Bersworth Chemical Co., Framingham, Mass., 1954.

7. Bersin, T., Mueller, A., and Schwartz, A., *Arzneimittel-Forsch.*, 1954, v4, 119.

8. Krogh, A., *Osmotic Regulation of Aquatic Animals*, Cambridge University Press, 1939, 130.

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Nutritional Requirements of *Micrococcus freudenreichii.** (23490)

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As a preliminary step in the investigation of certain enzyme systems in *Micrococcus freudenreichii,* it became desirable to grow the organism in a chemically defined medium which was capable of producing a high yield of cells. When a search of the literature failed to reveal the necessary information concerning the nutritional requirements of this organism, an investigation of these requirements was undertaken.

Materials and methods. Cultures and inocula. The culture employed in the studies was *Micrococcus freudenreichii* ATCC 8459. It was maintained on a stab in stock medium containing peptonized milk, 0.1%; tryptone, 0.1%; filtered tomato juice, 20%; and agar,

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TABLE I. Composition of Basal Medium.

Component	Amt/100 ml of double strength medium
Sodium acetate · $3H_2O$	2.4 g
Ammonium chloride	.3
Sucrose	3.0
Adenine sulfate	3 mg
Guanine · HCl	3
Xanthine	3
K_2HPO_4	100
KH_2PO_4	100
$MgSO_4 \cdot 7H_2O$	40
NaCl	2
$FeSO_4 \cdot 7H_2O$	2
$MnSO_4 \cdot H_2O$	1.5
DL-Alanine	80
L-Arginine · HCl	48.5
L-Asparagine*	80
DL-Aspartic acid	20
L-Cysteine · HCl	10
DL-Glutamic acid	60
Glycine	20
L-Histidine · HCl	12.5
L-Isoleucine	50
DL-Leucine	50
L-Lysine · HCl	50
DL-Methionine	20
DL-Phenylalanine	20
L-Proline	20
DL-Serine	10
DL-Threonine	40
DL-Tryptophan	8
L-Tyrosine	20
DL-Valine	50
Calcium pantothenate	.5
Thiamine · HCl	.5
Riboflavin	.5
Nicotinic acid	1
Pyridoxine · HCl	1
p-Aminobenzoic acid	.1
Biotin	1 μg
Folic acid	10

* *L*-Asparagine was included in basal medium only for those experiments which tested amino acid requirements of the organism. When it was found that slightly better growth was obtained in the absence of asparagine, this compound was omitted from subsequent experiments.

1.5%. Transfers were made at monthly intervals and were incubated at 26°C for approximately 24 hours. Inoculum medium was identical to the above with agar omitted. A transfer was made from stock culture approximately 8 hours before the inoculum was needed, and this was incubated at 26°C. The cells were centrifuged, washed twice in sterile 0.9% saline solution, then resuspended in the saline solution to give a reading of 70 to 80% transmittance in the Bausch and Lomb photoelectric colorimeter with a 660 mμ filter. Each assay tube was inoculated with a single drop of this suspension. *Basal medium.* Composition of the basal medium is shown in Table I. Final pH of the medium was adjusted to 7.0. *Procedure for growth tests.* In determining essentiality of various nutrients for growth, each compound was omitted individually from the basal medium. Six ml of single strength basal medium were added to each 18 x 150 mm culture tube, covered with aluminum caps, and autoclaved five minutes at 121°C. After the tubes had cooled, they were inoculated and incubated at 26°C until maximum growth. Growth was determined by turbidimetric reading in a Bausch and Lomb photoelectric colorimeter using a 660 mμ filter.

Results. Effect of CO_2. In early experiments, it was found that growth was often slow and light, and that results were difficult to duplicate. When CO_2 was supplied by the addition of a filter sterilized solution of $NaHCO_3$, growth was found to be much more rapid, heavier, and more reproducible from one experiment to another. In view of these effects, 0.2 mM of $NaHCO_3$ was routinely included in each 6 ml culture by addition of a filter sterilized solution to the autoclaved medium.

The stimulatory effect of CO_2 on the growth of *M. freudenreichii* is similar to that observed by previous workers with various other microorganisms(1,2) and to results obtained in previous work in our laboratory with *Streptococcus bovis*(3,4). In the latter instance, however, CO_2 was implicated in the synthesis of amino acids, both by the finding that $C^{14}O_2$ was rapidly incorporated into the carbon skeleton of amino acids, and by the fact that CO_2 was not required when the medium contained a complete mixture of amino acids. It is unlikely that CO_2 functions in the synthesis of amino acids in *M. freudenreichii* when a complete mixture is supplied. The likelihood exists that CO_2 functions in other synthetic pathways, however.

Amino acid requirements. In repeated experiments, the omission of any one of the following amino acids resulted in growth failures: arginine, glutamic acid, histidine, leucine, methionine, threonine, tryptophan, and

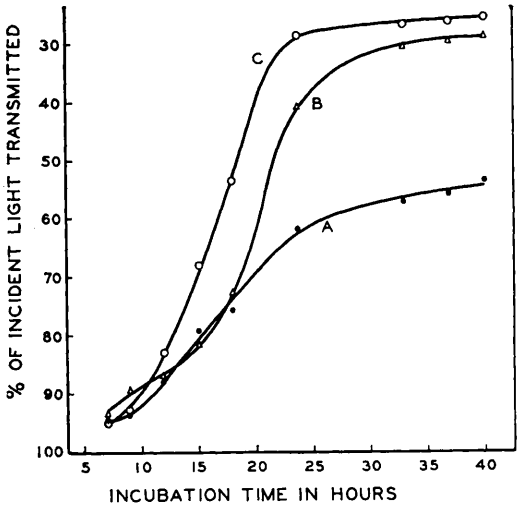

FIG. 1. Growth rates of *M. freudenreichii* on 3 complete amino acid mixtures. A, mixture in basal medium. B, mixture of Henderson and Snell(6). C, mixture shown in Table II.

valine. If, however, $NaHCO_3$ were omitted from the medium, the organism required aspartic acid in addition to the amino acids listed above. Asparagine was incapable of replacing aspartic acid in the absence of CO_2. It is well known that commercial samples of leucine, isoleucine, and valine are sometimes cross-contaminated with each other. The leucine, isoleucine, and valine used in these studies were free of such contamination as shown by assay with *Lactobacillus plantarum* (*Lactobacillus arabinosus* 17-5).

Attempts were made to grow *M. freudenreichii* in a medium identical to the basal medium except that the complete amino acid mixture was replaced by a mixture containing only the essential amino acids in the same ratio in which they occur in the complete mixture. These efforts were unsuccessful, but moderate to heavy growth could be obtained from the essential amino acids after prolonged incubation by altering the ratios of histidine to arginine and of leucine to valine to values of 3:1 or 4:1. The addition of alanine, glycine, isoleucine, phenylalanine, and serine to the 8 essential amino acids produced more rapid growth, but the lag phase was still longer than when the complete amino acid mixture was supplied.

Because one of the objectives of the investigation was to find a medium which would support vigorous growth of *M. freudenreichii*, different complete mixtures of amino acids were tested for ability to produce heavy cell yields. Fig. 1 shows the results obtained when different amino acid mixtures were compared in the presence of $NaHCO_3$. Mixture A was contained in the basal medium and is similar to that of Sauberlich and Baumann (5). Mixture B is that of Henderson and Snell(6), and Mixture C has the composition shown in Table II. All 3 mixtures are very similar qualitatively, but show considerable variations in the ratios of individual amino acids. It is obvious that growth of *M. freudenreichii* is affected to a considerable extent by the relative amounts of various amino acids in the medium.

Vitamin requirements. When all vitamins were omitted, little if any growth occurred. Upon the individual omission of each vitamin, repeated tests showed that absence of no single vitamin caused complete growth failures. The absence of pantothenic acid, folic acid, and riboflavin reduced the growth drastically, however, and niacin and pyridoxine both appeared to possess a slight stimulatory effect, although not nearly so marked as that of the first 3 compounds. Thiamine, biotin, and *p*-amino-benzoic acid could be left out without any effect on growth.

TABLE II. Composition of Amino Acid Mixture C.

Component	mg/100 ml of double strength medium
DL-Alanine	68.7
L-Arginine • HCl	83.1
L-Aspartic acid	34.4
L-Cysteine • HCl	17.2
L-Glutamic acid	103.0
Glycine	34.4
L-Histidine • HCl	21.3
DL-Isoleucine	85.9
DL-Leucine	85.9
L-Lysine • HCl	85.9
DL-Methionine	34.4
DL-Phenylalanine	34.4
L-Proline	34.4
DL-Serine	17.2
DL-Threonine	68.7
DL-Tryptophan	13.7
L-Tyrosine	34.4
DL-Valine	85.9

The possibility existed that the 3 most stimulatory vitamins were, in reality, essential to growth of the organism, and that small amounts were being supplied as contaminants from other constituents of the medium. In order to test the possibility that the vitamins used were cross-contaminated, a complete lot of new vitamins was opened and the tests were repeated several times, with the same results as above. If contamination were involved, it seems likely that it came from some constituent of the medium besides the other vitamins. Alternatively, the organism may be capable of synthesizing pantothenic acid, folic acid, and riboflavin at sub-optimal rates which permit limited growth.

Purine and pyrimidine requirements. The omission of all purines and pyrimidines from the otherwise complete medium resulted in growth failures. It was found that individual addition of adenine, guanine, xanthine, hypoxanthine, adenosine, guanosine, or xanthosine supported heavy growth of *M. freudenreichii*. Thymine, uracil, cytosine or thymidine were incapable of supporting growth. It appears, then, that the requirement of the organism can be met by any purine or its riboside, but not by pyrimidines or their pentosides.

Carbohydrate requirements. Out of 21 carbohydrates tested, heavy growth was supported by glucose, maltose, trehalose, fructose, mannose, sucrose, lactose, starch, and galactose. It thus appears that the organism can use a wide variety of carbohydrates as its energy source.

Summary. The nutritional requirements of *Micrococcus freudenreichii* 8459 for amino acids, vitamins, purines, pyrimidines, and carbohydrates have been studied. Three complete mixtures of amino acids gave considerable variation in growth, indicating that the organism is sensitive to the ratios of the various amino acids present. Eight amino acids were indispensable for growth. No single vitamin was shown to be essential, but growth was light in the absence of pantothenic acid, riboflavin, or folic acid. The single addition of any of the purines or purine ribosides tested supported heavy growth, but none of the pyrimidines or their pentose derivatives tested permitted growth in the absence of a purine. Of 21 carbohydrates tested, 9 were capable of serving as an energy source for *M. freudenreichii*. Growth was greatly improved when CO_2 was supplied to the medium as $NaHCO_3$.

1. Lyman, C. M., Moseley, O., Wood, S., Butler, B., and Hale, F., *J. Biol. Chem.*, 1947, v167, 177.

2. Huhtanen, C. N., Carleton, F. J., and Roberts, H. R., *J. Bact.*, 1954, v68, 749.

3. Prescott, J. M., and Stutts, A. L., *ibid.*, 1955, v70, 285.

4. Prescott, J. M., Ragland, R. S., and Stutts, A. L., *ibid.*, 1957, v73, 133.

5. Sauberlich, H. E., and Baumann, C. A., *J. Biol. Chem.*, 1948, v176, 165.

6. Henderson, L. M., and Snell, E. E., *ibid.*, 1948, v176, 15.

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Effects of High Fat and Carbohydrate Diets on Rat Liver Phosphatase.* (23491)

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The fact that adaptation of rats to a high fat diet increases the rate of utilization of fat and decreases that of carbohydrate during a subsequent fasting period(1,2) suggested the possibility that with the change in metabolic pattern, the activity of some enzymes involved in carbohydrate metabolism may also be altered. We considered particularly alkaline phosphatase because several authors had shown that the phosphatase activity of some tissues underwent changes due to starvation,

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