NUTRITION OF THE ENTEROCOCCI

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In connection with the isolation of pantothenic acid from natural materials, Woolley and Hutchings (1940) reported the development of a chemically defined medium for a strain of *Streptococcus zymogenes*, a hemolytic group D streptococcus. In addition to glucose, inorganic salts, buffer, and a reducing substance, the medium contained the three vitamins, riboflavin, pantothenic acid, and pyridoxine; and seven amino acids.

Schuman and Farrell (1941) extended this work and showed that a similar medium, differing somewhat in amino acid content, would support the growth of a culture of *Streptococcus fecalis*, a non-hemolytic group D streptococcus.

Later, Woolley (1941a) reported that a closely related *Streptococcus zymogenes* strain would not grow in the synthetic medium unless a larger variety of amino acids was used. Nicotinic acid also stimulated the growth of this strain.

In this laboratory, attempts to culture a number of enterococcus cultures in the above media have failed. A study was therefore undertaken to determine the additional substances required by these organisms, and also to determine whether or not the four major varieties or "species" within the enterococcus group have similar nutritive requirements.

CULTURES AND TECHNIQUE

The cultures used in this study consisted of five strains each of *Streptococcus zymogenes*, *Streptococcus durans*, and *Streptococcus fecalis*, and four strains of *Streptococcus liquefaciens*; all of which were selected at random from the laboratory stock culture collection. All had been shown previously to be enterococci and to be members of their respective "species" by a study of their physiological properties, and all belonged to the Lancefield serological group D. These nineteen strains were isolated from pasteurized milk, cheese, and the human intestine, no two strains having been obtained from the same sample.

The technique used throughout this study was essentially the same as previously reported (Smiley, Niven, and Sherman, 1943). The medium under test was inoculated with a fine wire needle from a 24-hour meat infusion broth culture. Care was taken, especially in all critical tests, to make several serial transfers in the same medium at 24-hour intervals whenever growth occurred.

GROWTH FACTOR REQUIREMENTS

In order to determine the growth factor requirements of these cultures, a basal medium was used which contained acid-hydrolyzed, vitamin-free casein, tryptophane, phosphate buffer, glucose, sodium thioglycolate, xanthine, adenine,

guanine, uracil, and salts in the concentrations listed in table 1. The salt mixture used by Woolley (1941b) was adopted (see table 1). By previous microbiological tests it was known that the casein hydrolysate was free from detectable quantities of riboflavin, pantothenic acid, nicotinic acid, pyridoxine, biotin, thiamine, and folic acid.

All the ingredients, including the test substances, were mixed together, the pH adjusted to 7.2–7.4, and 5 ml. quantities distributed into specially cleaned culture tubes and autoclaved at 15 pounds pressure for 15 minutes before inoculation. All cultures were incubated at 37°C. and the growth response measured with a photoelectric densitometer at different time intervals.

TABLE 1

A simplified medium for the enterococci

	micrograms
Riboflavin	10
Calcium pantothenate	10
Nicotinic acid	10
Pyridoxine	10
Biotin (crystalline, free acid)	0.01
Folic acid (concentrate)	0.1
Xanthine	50
Adenine	50
Guanine	50
Uracil	50
	milligrams
l-Tryptophane	0.3
Hydrolyzed casein	50
K_2HPO_4	40
Glucose	50
Sodium thioglycolate	1
Salts*	
	ml.
Water to make	10

* Salts composed of 20 mg. NaCl, 0.8 mg. $MgSO_4 \cdot 7H_2O$, 40 micrograms $FeSO_4 \cdot 7H_2O$, and 12 micrograms $MnCl_2$ per 10 ml. medium.

When only pantothenic acid, riboflavin, and pyridoxine were included in the basal medium, none of the nineteen cultures developed visible growth, even after an incubation period of five days. However, it was soon learned that when the basal medium contained the seven B vitamins listed in table 2, all cultures grew promptly and could be subcultured indefinitely in the same medium. Visible turbidity usually developed within 12 to 16 hours, with a maximum level reached within 24 hours.

Experiments were then designed to determine the vitamins that were necessary for each culture. Each of the 7 growth factors was omitted from the medium individually and the growth response determined. A summary of these experiments is shown in table 2.

No culture was found which was able to synthesize pantothenic acid, nicotinic

acid, biotin, or pyridoxine, whereas all grew equally well in the absence of added thiamine. Only two strains, a *Streptococcus fecalis* and a *Streptococcus durans* culture, were able to grow without added riboflavin.

It is interesting to note that one *Streptococcus zymogenes*, three *Streptococcus fecalis*, and three *Streptococcus durans* cultures would not grow without added folic acid. This growth factor was added in the form of a concentrate (potency, 2200) which was obtained from Dr. E. E. Snell, University of Texas. As little as 0.001 microgram of this concentrate per ml. of medium would support half-maximum growth of a culture of *Streptococcus fecalis* tested, which indicates that the substance responsible for growth is folic acid and not a contaminating chemical in the material. A satisfactory curve was obtained in response to increasing quantities of the concentrate which, when calculated on the basis of pure folic acid added, corresponds approximately to that reported for "*Streptococcus lactis* R" by Mitchell and Snell (1941).

All cultures tested grew in the absence of the purine and pyrimidine bases but when these substances were included the rate of growth was greatly enhanced in

TABLE 2

Vitamin requirements of the enterococci

VITAMIN	NUMBER OF CULTURES TESTED	NUMBER OF CULTURES REQUIRING VITAMIN
Biotin	19	19
Nicotinic acid	19	19
Calcium pantothenate	19	19
Pyridoxine	19	19
Riboflavin	19	17
Folic acid	19	7
Thiamine HCl	19	0

most cases. The stimulatory effect of these substances upon various lactic acid bacteria has been reported by Snell and Mitchell (1941).

An interesting observation has been recently recorded by Snell and Guirard (1943) showing that alanine in relatively large amounts was able to replace pyridoxine for the culture of "*Streptococcus lactis* R" studied by them. Also, they found that glycine, and to a less extent threonine, serine, and beta-alanine are inhibitory. Inhibition by each of these substances could be quantitatively eliminated by adding pyridoxine or alanine to the medium.

These observations are confirmed in the present study. Using a pyridoxine-free casein hydrolysate medium containing 10 mg. of synthetic alanine per 10 ml. of medium, all of the 19 enterococci produced approximately maximum growth within 24 hours. The control medium containing neither pyridoxine nor added alanine would allow very little or no perceptible growth.

Likewise, glycine was found to be inhibitory in large amounts. Growth of the 19 enterococci in a casein hydrolysate medium containing 5 micrograms of pyridoxine and 20 mg. of added glycine per 10 ml. was compared to that occurring in a similar medium containing 5 micrograms of pyridoxine and no additional

glycine. The degree of inhibition varied with the individual strain, ranging from none (two cultures) to complete (five cultures).

Twenty-four other enterococci recently isolated from as many different samples of raw milk were tested for growth in the casein hydrolysate medium of table 1. All cultures grew promptly and could be subcultured indefinitely in the same medium. With the exception of folic acid, the specific vitamin requirements of these strains were not investigated. Only two of the 24 cultures were unable to synthesize folic acid.

AMINO ACID REQUIREMENTS

Woolley and Hutchings (1940) were able to culture their *Streptococcus zymogenes* strain in a medium containing the seven amino acids, isoleucine, lysine

TABLE 3

Amino acid requirements of Streptococcus zymogenes (26C1)

AMINO ACID OMITTED*	DENSITOMETER READING†
None	50
dl-Alanine	24
dl-Valine	0
dl-Leucine	0
dl-Isoleucine	0
dl-Serine	2
dl-Threonine	26
dl-Methionine	5
l-Tyrosine	16
d-Glutamic acid	0
d-Arginine 2HCl	0
dl-Lysine HCl	16
l-Histidine	2
l-Tryptophane	0

* Only the 13 amino acids listed in this table were included in the complete medium.
† Readings made after 43 hours' incubation; uninoculated medium reads zero.

tyrosine, cystine or methionine, arginine, tryptophane and glutamic acid, in the presence of the three vitamins mentioned previously. Schuman and Farrell (1941) reported a similar amino acid combination for one culture of *Streptococcus fecalis*, except that valine was included instead of isoleucine. Either of these amino acid combinations was found to be insufficient for any of the cultures in the present study. However, when a mixture of 20 amino acids was used to replace the casein hydrolysate, all cultures grew readily on serial transfer, except one strain of *Streptococcus liquefaciens*. Experiments were then designed to determine a minimum amino acid combination that would effectively replace casein hydrolysate for one culture of *Streptococcus zymogenes* (strain 26C1). All amino acids used were added in the concentration of one mg. per 10 ml. of medium.

It is unnecessary to describe in detail the various combinations of amino acids

that were attempted. Table 3 shows a mixture of 13 amino acids, the minimum number found which would allow prompt and satisfactory growth of this strain.

It can be seen that six of the 13 amino acids were essential, no growth occurring within 43 hours when each was omitted individually. Apparently, other amino acids can not replace any of these six amino acids since no growth resulted when they were omitted singly from a medium containing 20 amino acids. Woolley and Hutchings, and Schuman and Farrell reported for their respective cultures that tryptophane and glutamic acid were the only essential amino acids which could not be replaced by others.

Of the 19 enterococcus cultures studied, only three *Streptococcus durans* and one *Streptococcus liquefaciens* culture failed to grow upon continued subculture in the simplified medium containing the 13 amino acids listed in table 3. Thus, it would appear that the amino acid requirements of the enterococci vary somewhat among individual strains.

No attempt was made to determine the specific amino acid requirements for all enterococcus cultures studied. However, it should be pointed out that of the 19 cultures tested only one *Streptococcus fecalis* and one *Streptococcus durans* culture were able to grow in a casein hydrolysate medium which contained no added tryptophane.

HEAT STERILIZATION OF THE MEDIUM AS AFFECTING GROWTH

Using a number of different lactic acid bacteria, S. Orla-Jensen (1931) and A. D. Orla-Jensen (1933) observed that no growth (or greatly delayed growth) would occur in a glucose, yeast autolysate medium when the sugar was added aseptically after heat sterilization of the medium. All cultures behaved in this manner, with the exception of *Streptococcus liquefaciens*, cultures of which grew equally well when glucose was added either before or after autoclaving the medium.

Using a synthetic medium, a similar observation was reported recently for members of the species *Streptococcus salivarius* (Smiley, Niven and Sherman, 1943). No growth would develop when sterile glucose was added aseptically unless the medium contained small amounts of pyruvate or acetaldehyde. It was concluded that when glucose was heated in the presence of the other constituents of the medium, a small amount was decomposed to yield certain substances which could serve as hydrogen acceptors in the initial carbohydrate metabolism of the growing culture.

In the present study, 10 enterococcus cultures, representing the four "species" of this group, were inoculated with a fine wire needle into tubes of casein hydrolysate medium to which glucose had been added, before and after heat sterilization. All cultures grew satisfactorily irrespective of whether glucose was added before or after autoclaving. All cultures developed maximum turbidity within 24 hours, no difference being noted in the rate of growth in the two media.

Attempts were then made to determine the substance or substances in the medium which allowed the initiation of growth under these conditions. A *Streptococcus fecalis* and a *Streptococcus zymogenes* culture were able to grow

with equal rapidity in a casein hydrolysate medium under strict anaerobic conditions regardless of whether glucose had been added before or after autoclaving. Likewise, similar results were obtained when the medium contained only the 13 amino acids of table 3, instead of casein hydrolysate as an amino acid source. Equal rates of growth also resulted when glycerol and mannitol were substituted for glucose in similar experiments. It would therefore appear that the enterococci have a mechanism for the initial carbohydrate fermentation which is somewhat different from that of *Streptococcus salivarius*.

In this laboratory, group B streptococci have also been found to be able to initiate growth in casein hydrolysate media which did not contain glucose during heat sterilization.

DISCUSSION

With respect to their growth factor requirements the enterococci seem to be a homogeneous group. All cultures tested required pantothenic acid, nicotinic acid, pyridoxine and biotin. All strains were able to synthesize thiamine. Variations in requirements were found only for two vitamins, riboflavin and folic acid.

It should be recalled that Woolley and Hutchings, and Schuman and Farrell reported that only riboflavin, pantothenic acid and pyridoxine were required by the enterococcus cultures studied by them. Therefore, it should be expected that occasional enterococcus strains might be found which would be able to synthesize biotin and nicotinic acid. However, it is possible that the basal media employed by these pioneer investigators were contaminated with sufficient quantities of these two factors to allow growth. Aside from the above reports, no other streptococcus has been found which is able to synthesize either of these two vitamins. Pantothenic acid also seems to be an essential vitamin for all streptococci thus far studied. Variations have been found only among riboflavin, pyridoxine, thiamine, and folic acid.

Among the species which have been *thus far studied*, certain strains of enterococci appear to be the only streptococci which are unable to synthesize folic acid. Of the 43 enterococcus cultures studied by us, only nine required this vitamin for growth.

As an aid in concentrating folic acid from natural materials, Mitchell, Snell and Williams (1941) used as a test organism a streptococcus termed "*Streptococcus lactis* R." This culture, which is used for the microbiological assay of folic acid (Mitchell and Snell, 1941), has been shown to be physiologically and serologically identical with *Streptococcus fecalis*.

Further studies on the vitamin requirements of this organism have confirmed the above findings. In this laboratory, three cultures of "*Streptococcus lactis R*" obtained from different laboratories (but from the same original source) have been found to agree closely with the enterococci in their vitamin requirements. These cultures grew promptly in the medium of table 1. Riboflavin is not required, which is in harmony with the findings of Snell, Guirard and Williams

(1942). Two of the 19 enterococcus cultures investigated in this study did not require riboflavin.

The amino acid requirements of "*Streptococcus lactis* R", as reported by Snell and Guirard (1943), do not correspond exactly to those found for the strain of *Streptococcus zymogenes*. However, the fact that four other enterococci did not grow in the minimum amino acid combination for this strain indicates that significant differences in the amino acid needs occur between individual cultures. As shown in another paper (Niven), the amino acid and vitamin requirements of *Streptococcus lactis* differ widely from those of the enterococci and "*Streptococcus lactis* R."

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SUMMARY

The vitamin and amino acid requirements of 19 enterococci, representing the four major varieties or "species" within this streptococcus group, are reported.

All cultures studied required pantothenic acid, nicotinic acid, pyridoxine, and biotin. Seventeen strains required riboflavin, whereas only seven required folic acid.

Fourteen of the 19 cultures were able to grow in a medium containing 13 amino acids, the minimum combination found for one strain of *Streptococcus zymogenes*.

No significant difference in nutritive requirements was found among strains of the four enterococcus "species."

REFERENCES

MITCHELL, HERSCHEL K., AND SNELL, ESMOND E. 1941 Assay method for "folic acid." Univ. Texas Publ., No. 4137, 36–37.

MITCHELL, H. K., SNELL, E. E., AND WILLIAMS, R. J. 1941 The concentration of "folic acid." J. Am. Chem. Soc., **63**, 2284.

ORLA-JENSEN, A. D. 1933 Hitherto unknown activators for the growth of lactic acid bacteria. J. Soc. Chem. Ind., **52**, 374–379.

ORLA-JENSEN, S. 1931 Die Abhängigkeit de Milchsäuregärung von der Art und Weise, in welcher die Sterilisierung der Nährböden ausgeführt wird. IX^{e} Congr. intern. Laiterie, Compt. rend., Copenhagen.

SCHUMAN, ROSLYN L., AND FARRELL, MICHAEL A. 1941 A synthetic medium for the cultivation of *Streptococcus fecalis*. J. Infectious Diseases, **69**, 81–86.

SMILEY, K. L., NIVEN, C. F., JR., AND SHERMAN, J. M. 1943 The nutrition of *Streptococcus salivarius*. J. Bact., **45**, 445–454.

SNELL, ESMOND E., AND GUIRARD, BEVERLY M. 1943 Some interrelationships of pyridoxine, alanine and glycine in their effect on certain lactic acid bacteria. Proc. Nat. Acad. Sci. U. S., **29**, 66–73.

SNELL, ESMOND E., GUIRARD, BEVERLY M., AND WILLIAMS, ROGER J. 1942 Occurrence in natural products of a physiologically active metabolite of pyridoxine. J. Biol. Chem., **143**, 519–530.

SNELL, E. E., AND MITCHELL, H. K. 1941 Purine and pyrimidine bases as growth substances for lactic acid bacteria. Proc. Nat. Acad. Sci. U. S., **27**, 1–7.

WOOLLEY, D. W. 1941a Studies on the nutritive requirements of bacteria. J. Bact., **42**, 155–163.

WOOLLEY, D. W. 1941b A new growth factor for certain hemolytic streptococci. J. Exptl. Med., **73**, 487–492.

WOOLLEY, D. W., AND HUTCHINGS, BRIAN L. 1940 Synthetic media for culture of certain hemolytic streptococci. J. Bact., **39**, 287–296.