RECIPROCAL REPLACEMENT OF OLEIC ACID AND CO_2 IN THE NUTRITION OF THE "MINUTE" STREPTOCOCCI AND LACTOBACILLUS LEICHMANNII[1]

ROBERT H. DEIBEL[2] AND C. F. NIVEN, JR.

Division of Bacteriology, American Meat Institute Foundation, and the Department of Microbiology, University of Chicago, Chicago, Illinois

Received for publication January 10, 1955

In 1934, Long and Bliss observed and characterized a distinct group of streptococci known as the "minute hemolytic streptococci". These microorganisms were most frequently isolated from individuals suffering from typical streptococcal infections. Coinage of the term "minute" by Long and Bliss stems from two distinct characteristics of these organisms. The individual cells were described as being one-half to two-thirds the size of the ordinary *beta* hemolytic streptococci, and secondly, 48 to 96 hours of incubation were necessary before the colonies on blood agar developed sufficiently to be visible with the unaided eye. Typically, small areas of hemolysis first appeared on the blood agar plate before any colonies could be discerned.

Serologically, these streptococci fall into two of the Lancefield groups, namely, groups F and G (Bliss, 1937). All group F streptococci are minute, but only type 1, group G streptococci are minute streptococci. The type 1 antigens within the two serological groups are similar, if not identical, thus resulting in cross reactions among these strains.

While investigating the nutrition of the minute streptococci, Niven, Washburn, and Sherman (1946) observed that these microorganisms were unique in that all strains studied required folic acid. Other than an occasional group D strain, no streptococci that required this vitamin had been reported at that time.

Another unique characteristic noted by these investigators was that all strains required a high CO_2 tension for growth in a synthetic medium. Although these strains would grow in a yeast extract-tryptone-glucose medium, no growth occurred in a casein hydrolyzate medium unless the atmosphere above the tubes contained an appreciable concentration of CO_2. About 100 mm of CO_2 pressure appeared to be an optimal amount for growth.

In the present study it was observed that oleic acid or "tween 80", the polyoxyethylene derivative of sorbitan monooleate, would replace the CO_2 requirement for the minute streptococci. Also it was found that for two *Lactobacillus leichmannii* cultures CO_2 would replace their requirement for an unsaturated fatty acid.

MATERIALS AND METHODS

The cultures used in this study were maintained in APT broth (Evans and Niven, 1951). All cultures were transferred at least twice in this medium prior to the inoculation of experimental media. The composition of the casein hydrolyzate medium used to culture the minute streptococci is presented in table 1. This medium is based upon a synthetic medium devised for these microorganisms by Niven *et al.* (1946). The pH of the medium was adjusted to 7.2 prior to sterilization.

To culture the *L. leichmannii* strains the following additions per 100 ml of double strength medium were made to the streptococcal basal medium: vitamin B_{12}, 1 μg; *p*-aminobenzoic acid, 10 μg; sodium acetate, 2 g; L-asparagine, 40 mg; sodium thioglycolate, 20 mg; and enzyme hydrolyzed casein (GBI), 20 mg. These additions were based upon the medium used by Schweigert *et al.* (1950) and Scheid and Schweigert (1950) for the assay of vitamin B_{12} by this culture. The pH of the medium was adjusted to 6.4–6.6 prior to sterilization.

After autoclaving the media at 15 pounds pressure for 15 minutes, the tubes were inoculated with one drop of a washed, 24 hour culture diluted tenfold in saline. Serial transfers were made in critical experiments to confirm the nutritional adequacy of the test media.

The cultures were incubated in 8 liter desiccators containing approximately 550 mm air

[1] Journal paper no. 107, American Meat Institute Foundation.

[2] From M.S. thesis, University of Chicago.

TABLE 1

Basal medium employed for the minute streptococci

Component	Quantity per 100 ml of Double Strength Medium
	mg
Acid hydrolyzed casein	1,000
K_2HPO_4	1,000
Glucose	2,000
Sodium citrate	1,000
Adenine sulfate	2
Guanine hydrochloride	2
Uracil	2
Thymine	2
Xanthine	2
L-Cystine	2
DL-Tryptophan	2
$MgSO_4 \cdot 7H_2O$	80
NaCl	4
$FeSO_4 \cdot 7H_2O$	4
$MnCl_2 \cdot 4H_2O$	14.4
	μg
Nicotinic acid	1,000
Riboflavin	200
Biotin	0.2
Folic acid	2
Thiamin hydrochloride	200
Pyridoxal hydrochloride	200
Calcium pantothenate	200

pressure and 100 mm CO_2 pressure. The desiccators were placed in a 37 C incubator. Growth intensity was estimated with the aid of a Coleman spectrophotometer.

EXPERIMENTAL RESULTS

Growth response to tween 80 and CO_2. While culturing the minute streptococci in a tryptone-yeast extract-glucose medium, it was observed that the addition of tween 80 greatly stimulated their rate of growth. However, this stimulatory effect of tween 80 could not be demonstrated in the casein hydrolyzate medium (table 1) when the cultures were incubated under a high CO_2 tension. In the casein hydrolyzate medium under CO_2 some of the cultures grew at a more rapid rate than in the artificial laboratory medium without CO_2 and tween 80.

These observations tended to relate tween 80 and CO_2 as being interchangeable in the nutrition of the minute streptococci. Therefore, the effect of tween 80 was tested in the casein hydrolyzate medium without an increased CO_2 tension. The results, as illustrated in table 2, indicate that the addition of tween 80 eliminates the need for the high CO_2 tension for successful growth in the simplified medium. Since biotin was added to the basal medium in excess of that required for growth, the replacement of CO_2 by tween 80 cannot be attributed to the ability of this fatty acid derivative to replace a biotin deficiency.

TABLE 2

Growth response (optical density × 100) of the minute streptococci to CO_2 and tween 80 in a casein hydrolyzate medium. Readings made after 48 hours at 37 C

Cultures	Additions to Basal Medium		
	CO_2 (100 mm)	Tween 80 (0.1%)	Control
Group F			
C628	55	54	0
H60R	75	54	0
C468	57	29	0
C41F	28	10	0
H127	13	45	0
Group G			
F68A	92	98	0
F163	98	99	0

As demonstrated in table 2, growth of the group G minute strains was superior to that achieved by the group F streptococci, and therefore the mutual exchange of tween 80 and CO_2 could be more readily demonstrated among the group G streptococci. As will be discussed in another publication, the group F streptococci require additional factors for satisfactory growth in the casein hydrolyzate medium. When these factors were added, the growth response to either tween 80 or CO_2 was of equal magnitude.

To determine specifically whether oleic acid was the active substance that replaced CO_2, the minute group G *Streptococcus* strain F163 was inoculated into the basal medium containing various concentrations of tween 80, sodium oleate, and sodium oleate plus "tween 40" (the polyoxyethylene derivative of sorbitan monopalmitate). As shown in table 3, oleic acid alone supported growth but only over a rather narrow range. When varying concentrations of oleate were detoxified by adding a constant amount of tween 40 (Williams, Broquist, and Snell, 1947), the growth response was directly

TABLE 3

Growth response (optical density × 100) of minute Streptococcus strain F163 to increasing concentrations of tween 80, sodium oleate, and sodium oleate plus tween 40 in a casein hydrolyzate medium. Readings made after 48 hours at 37 C

Tween 80 or Oleate Added (μg/10 ml)	Additions to Basal Medium		
	Tween 80	Sodium oleate	Sodium oleate plus tween 40*
0	0	0	0
1		4	2
3		34	6
5		40	
10	58	7	19
15		1	
20		0	50
30	72		
40		0	113
60			118
100	90	0	121
300	98		129
1,000	108		120
3,000	116		
10,000	116		

* Each tube contained tween 40 at a concentration of 1 mg per 10 ml.

proportional to the concentration of oleate in the medium. Thus, oleic acid, and not other chemical groups in the large tween molecule, appears to be the active principle involved in the replacement of CO_2.

In an attempt to determine the extent of the reciprocity of CO_2 and oleic acid among other microorganisms, a collection of bacteria was obtained, each of which was recognized as having a requirement for either oleic acid or an increased CO_2 tension. Included in the collection was a recently isolated strain of *Brucella abortus* (strain 29726) obtained from Dr. H. J. Shaughnessy of the Illinois Department of Public Health. This organism was cultured in a trypticase soy broth (BBL). After two transfers in the medium (under an increased CO_2 tension), the culture was incubated under the following conditions: (1) 100 mm CO_2, (2) 0.1 per cent tween 80, (3) tween 80 plus CO_2, and (4) control incubated under atmospheric conditions. As shown in table 4, strain 27926 grew only when incubated under CO_2. Also noted was the failure of tween 80 to increase

TABLE 4

Growth of other bacterial species in media supplemented with tween 80 or CO_2

Culture	Tween 80 (0.1%)	CO_2 (100 mm)	Control
Brucella abortus, 27926	−*	+†	−
Lactobacillus acidophilus, 314	+	−	−
Lactobacillus acidophilus, 332	+	−	−
Lactobacillus acidophilus, 4355	+	−	−
Lactobacillus bulgaricus, 521	+	−	−
Lactobacillus helveticus, 8018	+	−	−
Lactobacillus leichmannii, 327	+	−	−
Lactobacillus leichmannii, 4797	+	+	−
Lactobacillus leichmannii, 313	+	+	−

* Indicates a negative growth response.
† Indicates a positive growth response.

the crop yield when the culture was incubated under an increased CO_2 tension.

Also in the collection were 12 *Lactobacillus* strains, each of which had been reported by other investigators to require oleic acid. Information concerning the basal media and additional growth factors required by these strains was obtained by consulting the literature (Kitay and Snell, 1950; Kitay *et al.*, 1950; Craig and Snell, 1951; Williams *et al.*, 1947). Attempts to culture four of these cultures (*L. acidophilus* ATCC 4356, *L. bulgaricus* ATCC 7993, *L. bulgaricus* ATCC 8001, *L. helveticus* ATCC 10386) under diverse conditions in simplified media met with failure, and they were therefore eliminated from the collection. Results concerning the reciprocal replacement of CO_2 and tween 80 among the remaining cultures are presented in table 4.

Two cultures of *L. leichmannii* strains 313 (ATCC 7830) and ATCC 4797 grew just as readily under CO_2 as they did in a medium supplemented with tween 80. Detailed results with these two cultures are given in table 5. As indicated in table 4, the remaining *Lactobacillus* cultures appeared to have a specific requirement for an unsaturated fatty acid. Therefore, it

TABLE 5

Growth response (optical density × 100) of Lactobacillus leichmannii strains 313 and 4797 to CO_2 and tween 80. Readings made after 48 hours at 37 C

Culture	Additions to the Basal Medium		
	CO_2 (100 mm)	Tween 80 (0.01%)	Control
Strain 313	100	110	0
Strain 4797	60	66	0

would seem that a reciprocal replacement between oleic acid and CO_2 does not exist for all bacteria that require one or the other of these substances.

Growth stimulation by yeast extract. The majority of the minute streptococci as well as the two *L. leichmannii* strains employed in this study grow quite readily in artificial laboratory media that contain yeast extract (Difco). This fact would imply that yeast extract contains a substance (perhaps lipoidal) that replaces the high CO_2 requirement of these microorganisms. Therefore, a cursory attempt was made to characterize the growth promoting substance(s) in yeast extract for minute *Streptococcus* strain F163.

When increasing increments of yeast extract were added to the basal casein hydrolyzate medium for the test strain, it was found that a level of approximately 0.1 per cent yielded a half-maximal growth response. Maximum growth was achieved with 0.5 per cent. Other medium constituents tested (Difco tryptone, tryptose; liver fraction "L" from General Biochemicals Corp.) showed comparatively weak activity.

Ten grams of yeast extract were saponified according to the method of Hilditch (1947) and then extracted with ether under alkaline conditions (nonsaponifiable fraction), and then again after being acidified (saponified fraction). These two fractions, as well as the residue, were tested for growth promoting activity. Little activity resided in the ether extractable fractions. On the other hand, the activity remaining in the residue closely paralleled that of the unfractionated yeast extract. These results would indicate that the active principle from yeast extract for the growth of strain F163 in the absence of oleic acid or an increased CO_2 tension is an ether-insoluble, nonlipoidal substance. The substance was found to be readily dialyzable.

Similar experiments conducted with *L. leichmannii* strain 313 as the test organism revealed that the saponified fraction and the residue fraction showed approximately equal activities, thus indicating that at least part of the growth promoting properties of yeast extract was due to the presence of lipoidal substances.

Growth stimulation with pyruvate. Regardless of the ultimate fate of the fixed CO_2 in the cells, some primary fixation product (of perhaps simple chemical nature) is undoubtedly formed by the cells. It would be expected that this substance, if added to the medium, would allow growth to occur in the absence of CO_2 or oleic acid. In this connection it was observed that pyruvic acid would partially replace the CO_2 requirement for the minute streptococci. In comparison to the effects of tween 80 or CO_2, however, growth in the presence of pyruvate was much slower and less intense. Typical growth responses to these substances are presented in table 6.

Although growth occurred among the minute streptococci in the presence of 0.5 per cent pyruvate, this concentration appeared to be insufficient for *L. leichmannii*, strain 313. When the concentration was increased to 1.0 per cent, strain 313 was able to grow, but the minute strains appeared to be somewhat inhibited at this level (table 6).

These results indicate that pyruvate is not a primary fixation product by these microorganisms but that it is more likely serving indirectly as a

TABLE 6

Effect of pyruvate upon the growth of representative CO_2 requiring cultures

Additions	Minute Streptococcus				*Lactobacillus leichmannii* Strain 313	
	F163 (group G)		H60R (group F)			
	24 hr	48 hr	24 hr	48 hr	24 hr	48 hr
None	0*	0	0	0	0	0
Pyruvate (0.5%)	0	76	0	22	0	0
Pyruvate (1.0%)	0	59	0	12	0	36
Tween 80 (0.2%)	95	98	0	54	110	117
CO_2 (100 mm)	98	104	61	62	110	125

* Figures denote optical density × 100.

source of CO_2. This conclusion is based upon the supposition that such high concentrations would not be required if it were a fixation product. Other lactic acid bacteria are known to metabolize pyruvate by a dismutation reaction with the production of lactate, acetate, and CO_2; or by another mechanism in which acetoin and CO_2 are produced.

It should also be pointed out that 0.3 per cent yeast extract that had been saponified and ether extracted (the residue fraction) afforded superior growth of the minute *Streptococcus* strain F163 than did 0.5 per cent pyruvate. This presents further evidence that pyruvate is not a direct fixation product of this organism.

Other substances tested for CO_2 replacement. In an attempt to find a direct CO_2 fixation product or an intermediate metabolite that would substitute for CO_2 or oleic acid in the nutrition of these microorganisms, a large number of substances were tested for their ability to support growth in the casein hydrolyzate basal media. Minute *Streptococcus* strain F163 and *L. leichmannii* strain 313 were employed as test organisms. In this discussion it would perhaps contribute little to name all the chemicals tested for their activity. Suffice it to say that special attention was paid to the fatty acid series, the dicarboxylic acids, certain D-amino acids, and a large number of other organic acids, aldehydes, and alcohols that are known to be of biological significance.

Each substance was tested at both 0.1 per cent and 0.5 per cent levels under atmospheric conditions. Another tube with 0.5 per cent of the substance was inoculated and incubated under CO_2 to determine the possible toxicity of the compound. If the substance was toxic at this level, lower dilutions were employed. Besides pyruvate, every substance tested in this manner failed to support growth of the test microorganisms in the absence of tween 80 or CO_2.

These studies have not eliminated the possibility that a growth factor exists whose presence would obviate the high CO_2 requirement for the respective organisms under study. Indeed, the quantities of oleic acid sufficient to replace CO_2 are of such low magnitude as to suggest this possibility. Lytle *et al.* (1951) noted that the "pyruvate oxidation factor" could be replaced with CO_2 in the nutrition of *Streptococcus faecalis*. Through the courtesy of Dr. I. C. Gunsalus, Department of Bacteriology, University of Illinois, Urbana, a sample of lipoic acid was obtained, and its activity tested for minute *Streptococcus* strain F163 and *L. leichmannii* strain 313. After 72 hours of incubation, 2.4 units of this factor per 10 ml of basal medium showed no activity for either strain in the absence of CO_2 and tween 80. In the presence of CO_2, tween 80, or pyruvate, no additional growth response was observed with this factor. Also, the addition of 9.4 units of coenzyme A per 10 ml of basal medium yielded no growth response when similarly tested.

DISCUSSION

In light of the well known ability of oleic acid to replace biotin partially or completely in the nutrition of many microorganisms, and the possible role of biotin in CO_2 fixation, the question naturally arises as to the possible role of this vitamin in the results herein presented. No evidence was found to indicate that biotin is concerned with the reciprocal replacement of CO_2 and oleic acid in the nutrition of the minute streptococci and *L. leichmannii*. This possibility, however, has not been eliminated. These bacteria belong to the general group of microorganisms that require an unsaturated fatty acid in spite of the presence of biotin. In the basal media employed, oleic acid eliminates the need for biotin, however; and the requirement for this vitamin could be demonstrated only when the minute streptococci and *L. leichmannii* were cultured under CO_2.

An interesting relationship between oleic acid, CO_2, and biotin has been observed by Broquist and Snell (1951). If the hydrolyzed casein in the basal medium of *L. arabinosus* was replaced by a mixture of amino acids (including aspartic acid), it was noted that oleic acid would not produce a growth response comparable to that of biotin. However, if oleic acid was added to the biotin-free medium, and the organism was cultured under increased CO_2 tension, it was found that a growth response comparable to that of the addition of biotin was achieved. In view of the evidence obtained from their study, Broquist and Snell concluded that biotin apparently is concerned (1) with the production of metabolically essential amounts of CO_2, and (2) in the synthesis of oleic and aspartic acids.

In view of the diverse chemical nature of

CO_2 and oleic acid, it is difficult to conceive that one is directly substituting for the other in the metabolism of the microorganisms employed in this study. Since oleic acid is required in such small quantities, it is conceivable that this fatty acid functions as a coenzyme in some metabolic activity of the microrganisms which obviates the necessity for a high CO_2 tension.

The possibility that oleic acid may act as a biological catalyst is suggested by the observations of Lichstein and Boyd (1951). These investigators found that oleic acid was necessary for the optimal activity of the formic hydrogenase and hydrogenlyase enzyme systems of *Escherichia coli*. However, the mechanism by which this fatty acid manifests its activity was not determined specifically. The evidence obtained by these investigators suggested that the action of oleic acid may reside in its functioning as a cofactor in the hydrogenlyase enzyme system of *E. coli*.

The observation that yeast extract contains a nonlipoidal substance capable of supporting growth of the minute streptococci in the absence of increased CO_2 tension or oleic acid suggests further studies. It is possible that this substance is closely related to the direct fixation product of CO_2 by these microorganisms.

A substance in yeast autolyzate that is capable of obviating the requirement for increased CO_2 tension for certain meningococcus strains has been reported by Tuttle and Scherp (1950). Later work by these authors (Larson and Scherp, 1954) indicates that this substance is not identical to that required by the microorganisms employed in this study.

In view of the evidence presented, it seems likely that the major path of CO_2 fixation by the minute streptococci and *L. leichmannii* involves a unique pathway not hitherto recognized. None of the substances reported to replace the CO_2 requirements of various microorganisms possessed any observable growth promoting activity under the conditions tested.

Little is known concerning the quantity of CO_2 that is fixed by these microorganisms during growth. Therefore, a quantitative comparison between the CO_2 and oleic acid requirements cannot be made at this time. One preliminary experiment with *L. leichmannii* strain 313 in which radioactive CO_2 was employed indicated that relatively large quantities of CO_2 are fixed during growth in the absence of added oleic acid. Although the results were not conclusive, the experiment indicated that little or no fixed CO_2 resided in the lipoidal fraction of the cells.

ACKNOWLEDGMENT

The authors wish to thank Dr. Rebecca C. Lancefield, Rockefeller Institute for Medical Research, and Dr. Harry W. Seeley, Cornell University, for the minute streptococcus cultures employed in this study.

SUMMARY

A reciprocal replacement of CO_2 and oleic acid has been found to exist in the nutrition of the minute streptococci and two strains of *Lactobacillus leichmannii*. This mutual exchange of CO_2 and oleic acid could not be demonstrated among other bacteria known to require one or the other of these substances.

High concentrations of pyruvate partially eliminated the need for either CO_2 or oleic acid by the test microorganisms. Yeast extract contains a nonlipoidal, dialyzable substance which also replaces the CO_2 or oleic requirements of the test microorganisms. This substance was not found to occur in any appreciable quantity in other common, commercial medium constituents.

REFERENCES

Bliss, E. A. 1937 Studies upon minute hemolytic streptococci. III. Serological differentiation. J. Bacteriol., **33**, 625–642.

Broquist, H. P., and Snell, E. E. 1951 Biotin and bacterial growth. J. Biol. Chem., **188**, 431–444.

Craig, J. A., and Snell, E. E. 1951 The comparative activities of pantethine, pantothenic acid, and coenzyme A for various microorganisms. J. Bacteriol., **61**, 283–291.

Evans, J. B., and Niven, C. F., Jr. 1951 Nutrition of the heterofermentative lactobacilli that cause greening of cured meat products. J. Bacteriol., **62**, 599–603.

Hilditch, T. P. 1947 *The chemical constitution of natural fats*. 2nd ed. John Wiley and Sons Inc., New York

Kitay, E., and Snell, E. E. 1950 Some additional nutritional requirements of certain lactic acid bacteria. J. Bacteriol., **60**, 49–56.

Kitay, E., McNutt, W. S., and Snell, E. E. 1950 Desoxyribosides and vitamin B_{12} as growth factors for lactic acid bacteria. J. Bacteriol., **59**, 727–738.

LARSON, A. D., AND SCHERP, H. W. 1954 Amino acids as substitutes for carbon dioxide in the growth of *Neisseria meningitidis*. Bacteriol. Proc., **1954**, 101–102.

LICHSTEIN, H. C., AND BOYD, R. B. 1951 The effect of oleic acid and of biotin on the formic hydrogenlyase and formic dehydrogenase enzyme systems. J. Bacteriol., **62**, 415–423.

LONG, P. H., AND BLISS, E. A. 1934 Studies upon minute hemolytic streptococci. I. The isolation and cultural characteristics of minute beta hemolytic streptococci. J. Exptl. Med., **60**, 619–631.

LYTLE, V. L., ZULICK, S. M., AND O'KANE, D. J. 1951 Replacement of the pyruvate oxidation factor by carbon dioxide. J. Biol. Chem., **189**, 551–555.

NIVEN, C. F., JR., WASHBURN, M. R., AND SHERMAN, J. M. 1946 Folic acid requirements of the minute streptococci. J. Bacteriol., **51**, 128.

SCHEID, H. E., AND SCHWEIGERT, B. S. 1950 Some factors affecting the potencies of vitamin B_{12} and *Leuconostoc citrovorum* factor of certain natural products. J. Biol. Chem., **185**, 1–8.

SCHWEIGERT, B. S., GUTHNECK, B. T., AND SCHEID, H. E. 1950 Amino acid requirements of *Lactobacillus leichmannii*. J. Biol. Chem., **186**, 229–234.

TUTTLE, D. M., AND SCHERP, H. W. 1950 Studies on the carbon dioxide requirements of *Neisseria meningitidis*. Bacteriol. Proc., **1950**, 39–40.

WILLIAMS, W. L., BROQUIST, H. P., AND SNELL, E. E. 1947 Oleic acid and related compounds as growth factors for lactic acid bacteria. J. Biol. Chem., **170**, 619–630.