THIAMINE AND NICOTINIC ACID: ANAEROBIC GROWTH FACTORS FOR *MUCOR ROUXII*

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Received for publication, February 20, 1961

ABSTRACT

BARTNICKI-GARCIA, S. (Rutgers, the State University, New Brunswick, N. J.), AND WALTER J. NICKERSON. Thiamine and nicotinic acid: Anaerobic growth factors for *Mucor rouxii*. J. Bacteriol. 82:142–148. 1961.—*Mucor rouxii* requires preformed thiamine and nicotinic acid for anaerobic growth. Such requirements are not manifested during aerobic incubation. Aerobically, the fungus was shown to be able to synthesize both vitamins.

The yeastlike form and the filamentous form of anaerobically grown *M. rouxii* exhibit the same vitamin requirements.

Thiamine can be substituted by its thiazole moiety. Under certain conditions, nicotinic acid was partly substituted by tryptophan, kynurenine, 3-hydroxykynurenine, and 3-hydroxyanthranilic acid.

Anaerobically. the fungus (thiamine requiring) was about ten times more susceptible to pyrithiamine antagonism than the same organism grown aerobically (thiamine independent).

The vitamin requirements of microorganisms are frequently influenced by environmental conditions. Variations in temperature (Mitchell and Houlahan, 1946), pH (Lilly and Barnett, 1947), salinity (Robbins and Kavanagh, 1938), nitrogen source (Wood, Andersen, and Werkman, 1938), and the presence of other growth factors (Snell, 1951) have been reported as factors controlling the necessity or superfluity of a given vitamin. During the course of studies on the biochemistry of morphogenesis of the dimorphic phycomycete *Mucor rouxii* (Bartnicki-Garcia and Nickerson, 1959) we observed that nutritional requirements for growth became more complex in the absence of molecular oxygen. Abundant growth of *M. rouxii* was obtained aerobically in media containing glucose as the only source of preformed organic matter; under anaerobic conditions, growth did not occur unless complex media containing yeast extract or peptone were employed.

The requirements for anaerobic growth of this organism have been characterized. The loci sensitive to oxygen deprivation, the inactivation of which leads to the development of nutrilite requirements for anaerobic growth, have been explored. A preliminary report on this work has appeared (Bartnicki-Garcia and Nickerson, 1960).

MATERIALS AND METHODS

Microbiological procedures. M. rouxii strain IM 80, maintained in the culture collection of the Institute of Microbiology, was employed. Cultures were grown in 250-ml flasks containing 50 ml of basal medium of the following composition: glucose, 20 g; vitamin-free acid hydrolyzed casein (Difco vitamin-free casamino acids), 6.25 g; KH_2PO_4, 3.0 g; $MgSO_4 \cdot 7H_2O$, 0.5 g; $ZnSO_4 \cdot 7H_2O$, 1.8 mg; $FeSO_4 \cdot 7H_2O$, 1.0 mg; $MnSO_4 \cdot H_2O$, 0.3 mg; $CuSO_4 \cdot 5H_2O$, 0.4 mg; and distilled water to make 1 liter. The pH was adjusted to 4.5 with 2 N H_2SO_4. Glucose was autoclaved separately and added to the other components of the basal medium which had previously been sterilized by autoclaving at 121 C for 10 min. Pyrithiamine and the various growth factors were dissolved in water and sterilized by filtration through Millipore filters. Additions to the basal medium, including inoculum, did not exceed a volume of 1 ml. In one experiment, a medium I, with the following composition, was employed: peptone (Difco), 10 g; yeast extract (Difco), 3 g; glucose, 20 g; and distilled water to make 1 liter. The pH was adjusted to 4.5 with 2 N H_2SO_4 and the medium sterilized in the autoclave at 121 C for 10 min. Cultures were incubated on a reciprocating

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shaker at 28 C for 48 hr. The inoculum consisted of a thoroughly washed suspension of spores, incubated under 100% CO_2 for 8 to 12 hr; during the incubation period the spores germinated. Each flask was inoculated with a suspension that contained approximately 10^6 spores, as determined by direct count in a hemacytometer. For incubation under aerobic conditions, the flasks were covered with inverted 100-ml beakers. For incubation under anaerobic conditions, rubber stoppers with inlet and outlet glass tubing connections were adapted to the flasks. Carbon dioxide, bone dry grade, of 99.8% purity and prepurified nitrogen of 99.996% purity (both obtained from the Matheson Company, Inc., East Rutherford, N. J.) were flushed through the gas space of the flasks during the incubation period. Growth was estimated by the following procedure: cultures were filtered through pyrex sintered glass crucibles (porosity M), washed extensively with distilled water, dried at 80 to 90 C for 24 hr, and weighed.

Chemical analyses. Thiamine was assayed by the thiochrome method (Association of Vitamin Chemists, 1951) with an Aminco-Bowman spectrophotofluorometer. The activation and fluorescent spectra of the samples were compared with those of thiamine. Nicotinic acid was assayed by the cyanogen bromide method, with *p*-methylaminophenol sulfate as the color developing agent (Association of Vitamin Chemists, 1951).

Rcagents. The thiazole and pyrimidine moieties of thiamine were prepared according to the procedure of Williams et al. (1935) and were shown to be free from contaminating thiamine by the thiochrome method. Thiamine, nicotinic acid, L-cysteine, and pyrithiamine were purchased from the Nutritional Biochemicals Corporation, Cleveland, Ohio; DL-kynurenine and kynurenic acid from Mann Research Laboratories, Inc., New York, N. Y.; 3-hydroxyanthranilic acid from California Corporation for Biochemical Research, Los Angeles, Calif.; DL-tryptophan from Eastman Organic Chemicals, Rochester, N. Y.; 3-hydroxykynurenine was prepared by A. Butenandt, of the Max Planck Institut für Biochemie, Tubingen, and supplied to us by the courtesy of E. Katz. Glucose and inorganic salts were of reagent grade.

RESULTS

Anaerobic vitamin requirements. Abundant growth of *M. rouxii*, either aerobically or anaerobically, was obtained in the complex liquid medium I. The average yield per flask under aerobic conditions was 350 mg, whereas under anaerobic conditions it was 100 mg; thus the ratio of aerobic to anaerobic growth was 3.5. Good growth occurred aerobically in the defined basal medium (with 2.4 g/liter of ammonium sulfate as the sole nitrogen source), but no growth occurred in this medium if the cultures were incubated anaerobically (Table 1). The inclusion in the basal medium of a more complex source of nitrogen, such as vitamin-free casein hydrolyzate, did not suffice to meet the nutritional requirements of the fungus. The anaerobic growth factor requirements of the organism were then explored.

The basal medium was supplemented with a mixture of the following growth factors: riboflavin, thiamine, *p*-aminobenzoic acid, calcium pantothenate, nicotinic acid, and choline (all at a concentration of 0.5 mg/liter); folic acid and biotin (both 0.05 mg/liter); cobalamin, 0.007 mg/liter; inositol, 1.0 mg/liter; and adenine, guanine, cytosine, uracil, xanthine, and thymine (100 mg/liter). Anaerobically, this supplemented medium was found to support good growth of the fungus. A stepwise elimination of the several

TABLE 1. *Growth factor requirements of Mucor rouxii under aerobic and anaerobic incubation*

Additions to the basal medium*	Growth (mg dry weight/50 ml of medium)		
	Air	Nitrogen	CO_2
Casein hydrolyzate as nitrogen source:			
No addition	135	2	2
Thiamine	138	3	5
Nicotinic acid	133	2	2
Thiamine and nicotinic acid	142	43	46
Ammonium sulfate as nitrogen source:			
No addition	112	2	1
Thiamine	114	6	3
Nicotinic acid	116	5	2
Thiamine and nicotinic acid	115	36	30

* Thiamine and nicotinic acid supplied at a level of 1 mg/liter.

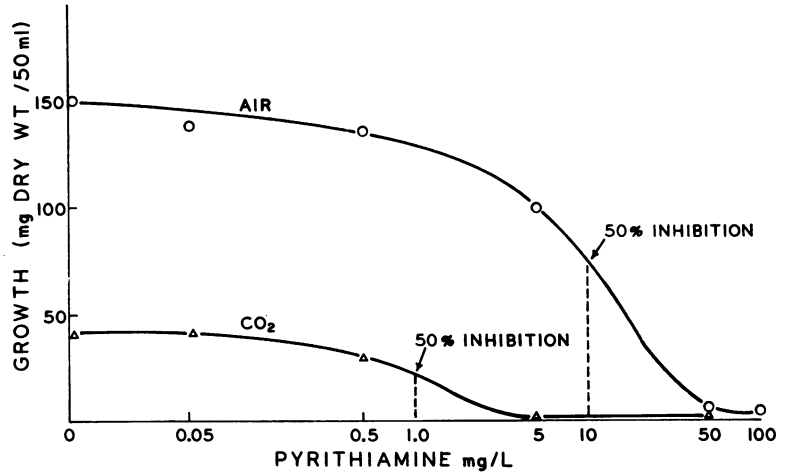

FIG. 1. *Effect of pyrithiamine on the growth of Mucor rouxii. Basal medium supplemented with 0.05 mg/liter of thiamine and 1.0 mg/liter of nicotinic acid. Cultures incubated either under air or CO_2.*

growth factors indicated that anaerobic growth did not occur unless thiamine and nicotinic acid were present in the medium. The omission of any other factor did not significantly affect the anaerobic growth of the organism.

When the basal medium was supplemented with thiamine and nicotinic acid, substantial growth was achieved anaerobically. Table 1 summarizes the results obtained with the basal medium containing either ammonium sulfate or vitamin-free casein hydrolyzate as the nitrogen source. The addition of vitamins to the cultures incubated aerobically was without significant effect on the total growth produced after incubation for 48 hr. In the presence of thiamine and nicotinic acid, the ratio of aerobic to anaerobic growth varied from 3.2 to 3.8, values comparable to the ratio obtained in medium I. Thus, the addition of thiamine and nicotinic acid fully satisfied the anaerobic growth factor requirements *M. rouxii*.

The response of the fungus to various concentrations of thiamine and nicotinic acid revealed that 0.1 mg/liter of thiamine and 0.5 to 1.0 mg/liter of nicotinic acid represented the minimal concentrations for optimal growth.

In most experiments, vitamin-free casein hydrolyzate was employed as the nitrogen source because it supported better growth, and greater constancy of morphological development was obtained under the different gas atmospheres employed.

Effect on morphology. The anaerobic atmosphere, whether nitrogen or CO_2, had little effect on the dry weight of mycelium obtained. But, as reported previously (Bartnicki-Garcia and Nickerson, 1959), the presence of high tensions of CO_2 induced a dramatic morphological change. Growth under CO_2 consisted of budding yeast-like structures, whereas in cultures incubated under nitrogen a typical filamentous mycelium developed. This dual morphological potential was clearly evident in the casein hydrolyzate medium. In the ammonium sulfate medium, cultures incubated under CO_2 contained a small proportion of filamentous forms, whereas cultures incubated under nitrogen possessed a small proportion of spherical structures.

Effect of pyrithiamine. Growth of *M. rouxii*, either aerobically or anaerobically, was not inhibited by the presence of 100 mg/liter of pyrithiamine in a medium containing 5 mg/liter of thiamine (Bartnicki-Garcia and Nickerson, 1960). However, when the ratio of pyrithiamine was further increased, total inhibition of growth resulted. The effect of different concentrations of pyrithiamine on growth in a basal medium containing 0.05 mg/liter of thiamine is illustrated in Fig. 1. The antagonistic effect (inhibitory index) of pyrithiamine was calculated according to Woolley and White (1943). The inhibitory indices for the aerobically and anaerobically grown cultures were 400 and 40, respectively. On this basis, the anaerobically grown yeast cells were ten times more susceptible to pyri-

TABLE 2. *Aerobic synthesis of thiamine and nicotinic acid by Mucor rouxii*

Fraction analyzed	Vitamin per gram of mycelium	
	Thiamine	Nicotinic acid
	μg	μg
Mycelium	7–8	3280
Filtrate	<1	2970
Total	7–8	6250

TABLE 3. *Replacement of thiamine and nicotinic acid requirements of Mucor rouxii for anaerobic growth*

Additions to the basal medium*	Growth (mg dry weight/50 ml medium)	
	Nitrogen	CO_2
No addition	2	2
Thiamine		
+ Nicotinic acid	40	42
+ DL-Tryptophan	4	2
+ DL-Kynurenine	5	2
+ 3-Hydroxykynurenine	10	2
+ 3-Hydroxyanthranilic acid	15	5
+ Anthranilic acid	2	2
+ Kynurenic acid	3	2
Nicotinic acid		
+ L-Cysteine	2	2
+ Thiazole moiety	37	42
+ Pyrimidine moiety	4	3

* Thiamine and nicotinic acid supplied at the level of 1 mg/liter; all other additions at 10 mg/liter.

thiamine than the aerobically grown filamentous cells.

Aerobic synthesis of thiamine and nicotinic acid. As a working hypothesis it was assumed that the anaerobic requirement for growth factors originated from the inability of the fungus to synthesize these substances when deprived of molecular oxygen. To demonstrate that thiamine and nicotinic acid were synthesized by aerobically grown cells, assays for thiamine and nicotinic acid were performed on the mycelia and culture filtrates of aerobically grown cultures. The results (Table 2) showed that thiamine was present in the mycelium in small quantities. Thiamine was not detected in culture filtrates. Nicotinic acid was synthesized in comparatively greater amount, and about half of it was excreted into the medium. The presence of thiamine and nicotinic acid was confirmed by a biological assay, using *M. rouxii*, itself, as the test organism. Aerobically grown mycelium (100 mg dry weight) was hydrolyzed in 0.1 N H_2SO_4 at 121 C for 30 min. The hydrolyzate was sterilized by filtration through a Millipore filter, and added to 50 ml of basal medium. The medium was inoculated; and after incubation under CO_2 for 48 hr, 64 mg of yeast-like growth was obtained. Thus, the presence of the requisite growth factors in the aerobically grown mycelium was verified.

Replacement of thiamine and nicotinic acid. To locate the possible biosynthetic step(s) at which molecular oxygen is apparently necessary for the syntheses of thiamine and of nicotinic acid, known precursors or related metabolites of these vitamins were added to the basal medium. The results, summarized in Table 3, indicated that thiamine was fully replaceable by its thiazole moiety under either nitrogen or CO_2. Cysteine, which was postulated to be an intermediate in thiazole biosynthesis (Shimomura et al., 1957), was ineffective in this system.

Nicotinic acid was partly substituted, in increasing order of activity, by tryptophan, kynurenine, 3-hydroxykynurenine, and 3-hydroxyanthranilic acid. On the other hand, kynurenic acid and anthranilic acid were without significant effect. When the atmosphere was CO_2, only 3-hydroxyanthranilic acid exhibited nicotinic acid-replacing ability; this effect was slight, but probably significant.

3-Hydroxyanthranilic acid oxidase activity. This oxygen-requiring enzyme has been shown to participate in the formation of nicotinic acid in mammalian liver (Bokman and Schweigert, 1951). Demonstration of the operation of this oxygenase in *M. rouxii* would provide a plausible explanation for the inability of this fungus to synthesize nicotinic acid under anaerobiosis. 3-Hydroxyanthranilic acid oxidase was assayed according to the spectrophotometric and fluorometric methods described by Bokman and Schweigert (1951). Acetone powders and Hughes press extracts of aerobically grown *M. rouxii* were employed, but oxidation of 3-hydroxyanthranilic acid was not detected.

DISCUSSION

The results reported suggest that the absence of molecular oxygen limited the syntheses of nicotinic acid and of the thiazole moiety of thiamine. Conceivably, in the absence of oxygen, some of the enzymatic systems responsible for the synthesis of thiazole and nicotinic acid are inoperative. Although no report of the intervention of oxygen in the biosynthesis of thiamine has been found, our data suggest that molecular oxygen is required for the synthesis of the thiazole moiety.

An extensive literature on the biosynthesis of nicotinic acid has appeared in recent years. In *Neurospora crassa*, and in mammalian liver, nicotinic acid is synthesized via tryptophan and 3-hydroxyanthranilic acid; the mechanism of biosynthesis in mammalian liver has been studied most closely. There are three known steps that require molecular oxygen: (i) the tryptophan peroxidase-oxidase system which converts tryptophan into formylkynurenine (Hayaishi et al., 1957), (ii) the oxidation of L-kynurenine to 3-hydroxykynurenine (De Castro, Price, and Brown, 1956) and (iii) a 3-hydroxyanthranilic oxidase (Bokman and Schweigert, 1951) which cleaves the aromatic ring of 3-hydroxyanthranilic acid to 1-amino-4-formyl-1,3-butadiene-1,2-decarboxylic acid (Wiss, Simmer, and Peters, 1956). Presumably, this biosynthetic pathway is highly vulnerable to oxygen depletion. The fact that nicotinic acid can be partly replaced, in order of increasing activity, by L-tryptophan, DL-kynurenine, 3-hydroxykynurenine, and 3-hydroxyanthranilic acid, would tend to support the role of these intermediates as precursors of nicotinic acid in *M. rouxii*. The enzymatic transformations of these intermediates could take place, although at a much reduced rate, under the anaerobic conditions employed (traces of oxygen may have been present). In fact, Bokman and Schweigert (1951) reported that 3-hydroxyanthranilic acid was slowly oxidized in vitro under anaerobic conditions. On the other hand, our failure to detect 3-hydroxyanthranilic acid oxidase activity in *M. rouxii* does not support the presence of the above indicated biosynthetic pathway in this organism.

There have been a few reports on the influence of molecular oxygen on the growth factor requirements of microorganisms. Richardson (1936) found that a strain of *Staphyloccocus aureus* required uracil for its anaerobic growth but could dispense with the requirement under aerobic conditions. Kluyver (1940) pointed out that *Torulopsis utilis* required "bios" factors only when oxygen was available in low concentrations; the requirement was no longer apparent when higher oxygen tensions were present. Andreasen and Stier (1953, 1954) found that both ergosterol (or certain other sterols) and oleic acid (or certain other unsaturated fatty acids) were indispensable requirements for anaerobic growth of *Saccharomyces cerevisiae* in a defined medium. Shockman (1956) found that 3 strains of *Streptococcus faecalis* had an absolute requirement for thiamine (plus lipoic acid) when grown under aerobic conditions, but there was no such requirement when the organism was grown anaerobically. This is the converse of the situation in *M. rouxii*. Since experimental evidence was not provided for the presence or absence of thiamine in the anerobically grown *S. faecalis*, it is not known whether the anaerobic dispensability for thiamine originated from the fact that it was synthesized anaerobically or whether the anaerobic metabolism of *S. faecalis* does not demand thiamine. The latter hypothesis seems somewhat more plausible; *S. faecalis*, a homofermentative lactic acid organism, could possibly operate anaerobically without cocarboxylase. In *M. rouxii*, such would not be the case; approximately half of the glucose metabolized anaerobically is converted to ethanol, thus necessitating the presence of thiamine.

The simultaneity of the thiamine and nicotinic acid requirements may not merely be incidental, but of more profound biochemical significance. Watanabe (1960) recently isolated auxotrophic mutants of *Salmonella typhimurium* resistant to streptomycin. All of the 15 mutants tested were slow growing and required both thiamine and nicotinic acid. Transductional studies suggested that the four characters derived from mutation in a single locus. It is conceivable, in light of our findings with *M. rouxii*, that the two vitamin requirements in these salmonella mutants originated from a block in the utilization of molecular oxygen. Similarly, the other two characters may be associated with limited oxygen utilization; thus, streptomycin resistance has already been correlated with limited oxygen uptake of certain bacteria (Umbreit, Smith,

and Oginsky, 1951); and a slower growth rate is usually an inherent characteristic of a facultative aerobe with restricted access to oxygen.

Woolley and White (1943) found that thiamine-independent organisms showed a greater tolerance to pyrithiamine than thiamine-dependent ones. Our results indicate that the relationship is also valid when thiamine dependence and thiamine independence can be phenotypically established in a single organism. It is noteworthy, however, that the inhibitory indices reported by Woolley and White are considerably greater than the corresponding indices in *M. rouxii*.

Dimorphic fungi may show different vitamin requirements in their mycelial and in their yeast forms. Salvin (1949) found a requirement for biotin exhibited by the yeast form, but not by the mycelial form of *Histoplasma capsulatum*. In *M. rouxii*, different nutritional requirements are not associated with form development; both the anaerobic filamentous form (N_2) and the anaerobic yeastlike form (CO_2) require thiamine and nicotinic acid.

No systematic survey of anaerobic growth factor requirements of microorganisms appears to have been made. It is conceivable that they are of more frequent occurrence than is indicated above. Recognition of the anaerobic limitation in the growth factor synthesizing capacity of microorganisms may be of importance in the study of biosynthetic mechanisms and of microbial ecology.

ACKNOWLEDGMENTS

Supported in part by grants (2E-43 and E-251) from the National Institutes of Health, U. S. Public Health Service.

LITERATURE CITED

ANDREASEN, A. A., AND T. J. B. STIER. 1953. Anaerobic nutrition of *Saccharomyces cerevisiae*. I. Ergosterol requirement for growth in a defined medium. J. Cellular Comp. Physiol. **41**:23–36.

ANDREASEN, A. A., AND T. J. B. STIER. 1954. Anaerobic nutrition of *Saccharomyces cerevisiae*. II. Unsaturated fatty acid requirement for growth in a defined medium. J. Cellular Comp. Physiol. **43**:271–281.

Association of Vitamin Chemists, Inc., [ed]. 1951. Methods of vitamin assay. Interscience Publishers, Inc., New York.

BARTNICKI-GARCIA, S., AND W. J. NICKERSON. 1959. The yeast-like form of *Mucor rouxii*. Abst. IX Intern. Botan. Congr. (Montreal), v. 2, p. 22.

BARTNICKI-GARCIA, S., AND W. J. NICKERSON. 1960. Thiamin and nicotinic acid: anaerobic growth factors for *Mucor rouxii*. Bacteriol. Proc. **1960**:163.

BOKMAN, A. H., AND B. S. SCHWEIGERT. 1951. 3-Hydroxyanthranilic acid metabolism. IV. Spectrophotometric evidence for the formation of an intermediate. Arch. Biochem. Biophys. **33**:270–276.

DE CASTRO, F. T., J. M. PRICE, AND R. R. BROWN. 1956. Reduced triphosphopyridine nucleotide requirement for the enzymatic formation of 3-hydroxykynurenine from L-kynurenine. J. Am. Chem. Soc. **78**:2904–2905.

HAYAISHI, O., S. ROTHBERG, A. H. MEHLER, AND Y. SAITO. 1957. Studies on oxygenases. Enzymatic formation of kynurenine from tryptophan. J. Biol. Chem. **229**:889–896.

KLUYVER, A. J. 1940. Microbial metabolism and its significance to the microbiologist. Proc. Intern. Congr. Microbiol., 3rd. Congr. (New York), p. 73.

LILLY, V. G., AND H. L. BARNETT. 1947. The influence of pH and certain growth factors on mycelial growth and perithecial formation by *Sordaria fimicola*. Am. J. Botany **34**:131–138.

MITCHELL, H. K., AND M. B. HOULAHAN. 1946. Neurospora. IV. A temperature-sensitive riboflavinless mutant. Am. J. Botany **33**: 31–35.

RICHARDSON, G. M. 1936. The nutrition of *Staphylococcus aureus*. Necessity for uracil in anaerobic growth. Biochem. J. **30**:2184–2190.

ROBBINS, W. J., AND F. KAVANAGH. 1938. Thiamin and growth of *Pythium butleri*. Bull. Torrey Botan. Club **65**:453–461.

SALVIN, S. B. 1949. Cysteine and related compounds in the growth of the yeastlike phase of *Histoplasma capsulatum*. J. Infectious Diseases **84**:275–283.

SHIMOMURA, F., J. OGATA, H. NAKAYAMA, AND R. HAYASHI. 1957. Thiamine biosynthesis in bacteria. J. Vitaminol. (Osaka) **3**:96–105.

SHOCKMAN, G. D. 1956. The acetate requirement of *Streptococcus faecalis*. J. Bacteriol. **72**: 101–104.

SNELL, E. E. 1951. Growth factors in bacterial nutrition. *In* F. Skoog, [ed], Plant growth substances. University of Wisconsin Press, Madison, 476 p.

UMBREIT, W. W., P. H. SMITH, AND E. L. OGINSKY. 1951. The action of streptomycin. V. The formation of citrate. J. Bacteriol. **61**:595–604.

WATANABE, T. 1960. Transductional studies of thiamin and nicotinic acid requiring streptomycin resistant mutants of *Salmonella typhimurium*. J. Gen. Microbiol. **22**:102–112.

WILLIAMS, R. R., R. E. WATERMAN, J. C. KERESZTESY, AND E. R. BUCHMAN. 1935. Studies of crystalline vitamin B_1. III. Cleavage of vitamin with sulfite. J. Am. Chem. Soc. **57**:536–537.

WISS, O., H. SIMMER, AND H. PETERS. 1956. Über die Umwandlung der 3-Hydroxyanthranilsäure in Chinolinsäure und Nicotinsäure im tierischen Organismus. I. Die enzymatische Oxydation der 3-Hydroxy-anthranilsäure. Z. physiol. Chem. Hoppe Seyler's, **304**:221–231.

WOOD, H. G., A. A. ANDERSEN, AND C. H. WERKMAN. 1938. Nutrition of the propionic bacteria. J. Bacteriol. **36**:201–214.

WOOLLEY, D. W., AND A. G. C. WHITE. 1943. Selective reversible inhibition of microbial growth with pyrithiamine. J. Exptl. Med. **78**:489–497.