JOURNAL OF BACTERIOLOGY
Vol. 87, No. 3, pp. 574-580 March, 1964
Copyright © 1964 by the American Society for Microbiology
Printed in U.S.A.

THE GENUS *VEILLONELLA*

II. NUTRITIONAL STUDIES

M. ROGOSA AND FERIAL S. BISHOP

National Institute of Dental Research, U.S. Public Health Service, U.S. Department of Health, Education, and Welfare, Bethesda, Maryland

Received for publication 11 October 1963

ABSTRACT

ROGOSA, M. (National Institute of Dental Research, U.S. Public Health Service, Bethesda, Md.), AND FERIAL S. BISHOP. The genus *Veillonella*. II. Nutritional studies. J. Bacteriol. **87**: 574-580. 1964.—A medium is described for the study of the vitamin, hypoxanthine, putrescine, or cadaverine requirements of 86 *Veillonella* isolates from man, rabbit, rat, and hamster. No organism required riboflavine or folic acid for growth. Niacin and calcium pantothenate were often stimulatory, but in nearly all cases were dispensable. Biotin and *p*-aminobenzoic acid were frequently stimulatory and sometimes indispensable for continued growth. *V. parvula* (antigenic group VI) required pyridoxal and thiamine and did not require putrescine or cadaverine. *V. alcalescens* (antigenic group IV) required pyridoxal, generally required thiamine, and also required putrescine or cadaverine. Of the isolates, 25 from the rat and 3 from the hamster (antigenic group II) generally behaved like *V. parvula*, except that a putrescine or cadaverine requirement was often observed. Spermine, spermidine, and agmatine could not replace putrescine or cadaverine. Although succinate is metabolized by resting cells, the organisms could not grow with succinate as an energy source.

Although the metabolism of certain *Veillonella* strains has been studied extensively and the organisms are easily grown anaerobically in lactate media containing yeast extract, casein digests, and similar materials, very little is known of their nutrition. Except for a preliminary note of a study with six human strains (Rogosa, 1955), there are no literature sources to be cited. With certain groups of organisms, requirements for essential metabolites, under carefully standardized optimal conditions, generally are characteristic for the species of a genus, even though sometimes an individual strain may behave exceptionally (Rogosa, Franklin, and Perry, 1961). Nutritional studies have been very helpful in the taxonomic study of diverse organisms (Rogosa et al., 1961*a*; Fildes, 1923, 1924; Knight and Proom, 1950; Proom and Knight, 1955), and the ability to grow cultures in a defined medium or a medium approaching that state may be invaluable in disclosing further problems for study as well as a clearer understanding of metabolic phenomena.

MATERIALS AND METHODS

Nutritional test medium. The composition of the basal medium used is shown in Table 1.

The solution of vitamin-free acid-hydrolyzed casein (Table 1) was prepared from the "vitamin-free, salt-free" casein hydrolysate (acid) powder of Nutritional Biochemicals Corp., Cleveland, Ohio. A 200-g amount was dissolved by heating in 1,600 ml of distilled water, cooled to room temperature, and adjusted to pH 4.0 with glacial acetic acid; 30 g of Norit A were added, and the mixture was stirred for 30 min. The solution was filtered through paper precoated with Hyflo Super-Cel (Johns-Manville, New York, N.Y.) on a Büchner funnel, and the pH was adjusted to 6.0 with 10 N NaOH. Norit A (20 g) was added to the filtrate, which was again stirred and filtered as previously. To the filtrate were added DL-tryptophan, 2 g; L-tyrosine, 800 mg; L-proline, 400 mg; DL-phenylalanine, 800 mg; and DL-histidine, 400 mg. Solution was effected by heating. The final volume was adjusted to 4 liters with distilled water, and the final concentration of solids was ca. 5%. Convenient samples were frozen in polypropylene screw-capped bottles, maintained at −20 C, and thawed in boiling water when required.

Salts A solution consisted of Na_2HPO_4, 15 g; KH_2PO_4, 5 g; and water to 500 ml. Salts B solution contained $FeSO_4 \cdot 7H_2O$, 0.312 g; $MgSO_4 \cdot 7H_2O$, 2.5 g; NaCl, 2.5 g; $MnSO_4 \cdot H_2O$, 0.190 g; and water to 500 ml.

Hypoxanthine stock solutions were made by dissolving 1 mg/ml of water with heating and cautious minimal addition of 10 N NaOH. Uracil was dissolved in water at 0.5 mg/ml by heating and cautious addition of concentrated NH_4OH.

Separate stock solutions of putrescine (5 mg/ml), pyridoxal HCl (1 mg/ml), thiamine HCl (1 mg/ml), calcium pantothenate (1 mg/ml), niacin (0.5 mg/ml), riboflavine (0.1 mg/ml), and *p*-aminobenzoic acid (PABA; 1 mg/ml) were dissolved in 20% aqueous ethanol, and 1 drop of concentrated HCl was added to each. Biotin and folic acid (1 mg/ml) were dissolved separately in 20% ethanolic 1% $NaHCO_3$ containing 1 drop of 10 N NaOH. These solutions were stored in the dark at 5 C, and desired dilutions were made only when final media were prepared. Any excess of a diluted solution was discarded.

The media were dispensed in 7-ml amounts into optically matched tubes (15 by 125 mm); the tubes were plugged with cotton, and autoclaved in racks at 121 C for 10 min. Only freshly constituted media were used, and first inoculations were generally made immediately on cooling and never into media more than 1 day old.

Inoculum and incubation conditions. The cultures were grown in medium V23A [10% (v/v) inoculum] consisting of Trypticase (BBL), 1%; yeast extract (BBL), 0.5%; 85% lactic acid, 1%; sodium thioglycolate, 0.075%; Tween 80, 0.01%; adjusted to pH 6.6 to 7.0 with solid K_2CO_3. The cells were washed three times in freshly sterilized water containing ca. 0.01% $Na_2S \cdot 9H_2O$ to assist in maintaining reduced conditions. Cultures were brought to original volume or a suitable volume in the same diluting fluid and to an optical density of approximately 1; inoculations of 1 drop were made. At least two serial passages were made in the nutritional test medium. Tubes were generally incubated 18 hr at 36 C, except for a small number of exceptional cases where growth was considered not plentiful enough. In these instances, incubation was continued for another 24 hr. Growth was expressed in terms of optical density as measured at 650 mμ in a Spectronic-20 colorimeter. Anaerobiosis in McIntosh and Fildes aluminum jars (Arthur H. Thomas Co., Philadelphia, Pa.) was either in an atmosphere of 95% N_2 plus 5% CO_2, or 95% H_2 plus 5% CO_2. Torbal jars (Torsion Balance Co., Clifton, N.J.) were also used, and contained a cold catalyst to react residual O_2 with 95% H_2 plus 5% CO_2. We found it absolutely necessary to check all jars for leaks. This was done manometrically under a positive pressure of 100 mm of Hg, and also with the liquid "Snoop" preparation (Nuclear Products Co., Cleveland, Ohio). Any necessary adjustments or alterations of the jars, particularly of the valves or indicator tubes, were done until the jars were assuredly leak-proof.

TABLE 1. *Composition of the basal nutritional test medium**

Component	Amt
Solution of vitamin-free acid-hydrolyzed casein (see text)	100 ml ~ 5 g
L-Cysteine-HCl	50 mg
Salts A and salts B, each	20 ml
Lactic acid (85%)	10 ml
Sodium thioglycolate	0.75 g
Hypoxanthine	30 mg
Uracil	2 mg
Putrescine dihydrochloride	4.5 mg
Pyridoxal HCl and thiamine HCl, each	2 mg
Calcium pantothenate and niacin, each	200 μg
Riboflavine	50 μg
p-Aminobenzoic acid and folic acid, each	1 μg
Biotin	10 μg

* The pH was adjusted to 6.8 with solid K_2CO_3. Final volume was brought to 1 liter with distilled water.

RESULTS AND DISCUSSION

The composition of the basal medium (Table 1) was the result of numerous preliminary experiments in which the optimal balance of the various ingredients was determined. For instance, refrigerated L-cysteine·HCl (50 mg per liter) was weighed and added as media were prepared. Amounts greater than 75 mg per liter, particularly in a medium (not described here) containing known amino acids and other compounds, inhibited or prevented growth. The salts, lactate, and thioglycolate were titrated for optimal growth-promoting activity. A number of purines such as adenine, guanine, and xanthine were tried singly and in combinations with uracil, but those offered no advantage over hypoxanthine alone or hypoxanthine (30 μg/ml) plus uracil (2 μg/ml). Whiteley and Douglas (1951)

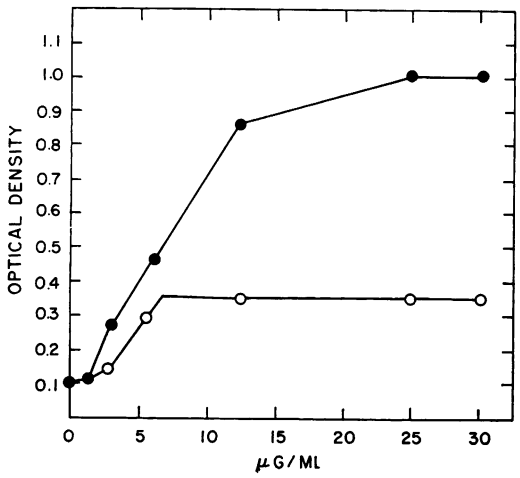

FIG. 1. *Response of Veillonella parvula strain PRI to increasing concentrations of hypoxanthine* (●) *and uracil* (○).

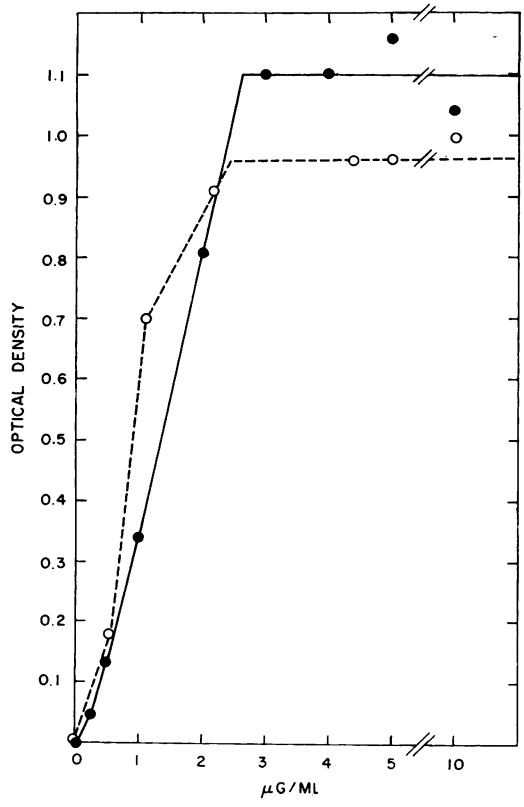

FIG. 2. *Growth response of Veillonella alcalescens strain BL-78 to increasing concentrations of putrescine* (●) *and cadaverine* (○).

had previously shown that some purines, including hypoxanthine were fermented. The results of one experiment illustrating the dependence of *V. parvula* strain PRI on hypoxanthine and to a markedly lesser extent on uracil are shown in Fig. 1. Hypoxanthine was highly stimulatory and induced maximal growth at concentrations >25 μg/ml, whereas uracil had a limited effect, and growth was only ca. one-third that in the presence of hypoxanthine. Most strains grew to some extent without any purine and uracil, but growth at best was only 50% maximal. If insufficient hypoxanthine (10 μg/ml) was present to support maximal growth, adenine, guanine, xanthine, or combinations of these, would substitute for hypoxanthine if enough were added (i.e., the equivalent of 30 μg/ml of hypoxanthine). *V. alcalescens* and certain other strains exhibited a total dependence on putrescine or cadaverine (Fig. 2). Concentrations >10 μg/ml caused inhibition or induced erratic growth. The amount of putrescine used (4.5 μg/ml; Table 1) was sufficient for maximal growth where it was required, and was without erratic effects. The concentrations of the vitamins were chosen in the same way. For instance, folic acid has often been found to inhibit these and other organisms when used at relatively high levels, particularly when it may be an artifact compound (Rogosa et al., 1961*a*). At 1 mμg/ml, folic acid has not been toxic. The pH of the medium in Table 1 was adjusted with K_2CO_3 because it was observed early that the residual bicarbonate often resulted in improved growth. Although the pH of the medium (Table 1) was 6.8 and all the experiments reported here were performed with such media, the *Veillonella* strains all grew well in the complete medium from pH 6.5 to 7.5 in an atmosphere of either 95% N_2 plus 5% CO_2 or 95% H_2 plus 5% CO_2; no significant difference in the results was observed in either atmosphere.

Some general observations may be noted. Although 86 strains from individual humans or animals were described here, 107 strains have been cultured repeatedly through at least three passages in the medium of Table 1. Very rarely (<2%), individual strains failed to grow, but this was attributable to poor growth in source media. When the vigor of the culture was reestablished by repeated passages in medium V23A, growth ensued. All strains grew well and

TABLE 2. *Growth responses of 21 strains of Veillonella parvula (antigenic group VI) to vitamins and putrescine*

Optical density	Deficiency										
	None	Pyridoxal	Thiamine	Calcium pantothenate	Riboflavine	Niacin	PABA	Folic acid	PABA and folic acid	Biotin	Putrescine
Mean*	0.85	0.06	0.01	0.54	0.82	0.80	0.20	0.74	0.13	0.31	0.82
Range*											
0–0.25	0†	21	21	0	0	1	16	0	17	7	0
0.26–0.75	3	0	0	20	4	3	4	7	4	13	3
0.76–>1.00	18	0	0	1	17	17	1	13	0	1	18

* Figures are optical densities at 650 mμ measured with a Spectronic-20 colorimeter.
† Number of strains in range.

TABLE 3. *Growth responses of 12 strains of Veillonella alcalescens (antigenic group IV) to vitamins and putrescine*

Optical density	Deficiency										
	None	Pyridoxal	Thiamine	Calcium pantothenate	Riboflavine	Niacin	PABA	Folic acid	PABA and folic acid	Biotin	Putrescine
Mean*	0.68	0.07	0.25	0.46	0.62	0.62	0.57	0.68	0.27	0.23	0
Range*											
0–0.25	0†	12	8	0	0	0	2	0	4	8	12
0.26–0.75	8	0	2	12	6	8	6	4	8	4	0
0.76–>1.00	4	0	2	0	6	4	4	8	0	0	0

* Figures are optical densities at 650 mμ measured with a Spectronic-20 colorimeter.
† Number of strains in range.

were not significantly limited by the absence of exogenous riboflavine. The same was true of folic acid, which appeared to be inert even in those cases where PABA was required for growth (Tables 2 to 6). Preliminary results with pteroic acid, dihydropteroic acid, folinic acid, dihydrofolic acid, tetrahydrofolic acid, *p*-aminobenzoyl glutamate, and pteroyltriglutamate indicate the need for continued detailed study to determine the biologically active compound or compounds. In general, a niacin deficiency was not severely limiting, and only two strains grew poorly enough to exhibit an optical density <0.26. However, some stimulation of growth by niacin was occasionally observed, even though continued growth occurred in its absence. There were only four instances in which calcium pantothenate was required but here also stimulation was often observed, as reflected by means and the ranges of optical densities in Tables 2, 3, 4, and 6, but not Table 5 (data for antigenic group I, catalase-positive, hamster strains). Biotin was often highly stimulatory and occasionally was indispensable for continued growth (Tables 2 to 6).

The 21 strains of *V. parvula* (Table 2) were judged to be members of this species because they were identical with a reference strain of *V. parvula*, No. Te3, received from Prévot. These organisms were all of human origin, shared the same group antigen (VI), and were all catalase-negative. In addition to the characteristics already mentioned, all strains required pyridoxal and thiamine and did not require putrescine. Typical dose-response curves of *V. parvula* strain PRI to pyridoxal and pyridoxamine are shown in Fig. 3, and the quantitative response to

TABLE 4. *Growth responses of 25 strains of Veillonella (antigenic group II) from the rat and 3 strains from the hamster*

Optical density	Deficiency										
	None	Pyridoxal	Thiamine	Calcium pantothenate	Riboflavine	Niacin	PABA	Folic acid	PABA and folic acid	Biotin	Putrescine
Mean*	0.98	0.11	0.05	0.53	0.96	0.77	0.64	1.00	0.29	0.41	0.36
Range*											
0–0.25	0†	26	28	4	0	1	2	0	14	4	10
0.26–0.75	1	2	0	12	1	6	12	2	10	23	11
0.76–>1.00	27	0	0	12	27	21	14	26	4	1	7

* Figures are optical densities at 650 mμ measured with a Spectronic-20 colorimeter.
† Number of strains in range.

TABLE 5. *Growth responses of 19 strains of Veillonella (antigenic group I) from the hamster*

Optical density	Deficiency										
	None	Pyridoxal	Thiamine	Calcium pantothenate	Riboflavine	Niacin	PABA	Folic acid	PABA and folic acid	Biotin	Putrescine
Mean*	1.03	0.38	0.14	1.10	1.03	0.59	0.80	1.05	0.47	0.47	0.17
Range*											
0–0.25	0†	9	15	0	0	0	0	0	6	1	14
0.25–0.75	0	0	0	0	0	14	5	0	10	13	2
0.76–>1.00	19	10	4	19	19	5	14	19	3	5	3

* Figures are optical densities at 650 mμ measured with a Spectronic-20 colorimeter.
† Number of strains in range.

TABLE 6. *Growth responses of two strains of Veillonella (antigenic group III) from the rat*

Determination	Deficiency										
	None	Pyridoxal	Thiamine	Calcium pantothenate	Riboflavine	Niacin	PABA	Folic acid	PABA and folic acid	Biotin	Putrescine
Mean	0.80	0.08	0.06	0.66	0.89	0.82	1.00	1.00	1.00	0.31	0.89
Total range*	0.74–1.00	0.05–0.11	0.04–0.08	0.47–1.00	0.80–1.05	0.64–1.10	1.00	0.92–1.10	0.96–1.05	0.27–0.48	0.85–0.96
No. of strains in the range	2	2	2	2	2	2	2	2	2	2	2

* Figures are optical densities at 650 mμ measured with a Spectronic-20 colorimeter.

thiamine is seen in Fig. 4. Two additional members of a *Veillonella* sp., KON (human) and 8/59 (from the rat), resembled *V. parvula* nutritionally, in lack of catalase, and in other respects, but possessed a different antigen (group V).

The isolates of *V. alcalescens* (Table 3) were identical with *V. alcalescens* No. 259 from Prévot. Ten of these isolates were of human origin; six were isolated from the human mouth and four from human bacteremias after dental operations (*see* Rogosa et al., 1960). Two additional strains,

one from the rat and one from the rabbit, are also included in Table 3. There were no organisms such as these from either the hamster or the guinea pig. *V. alcalescens* required pyridoxal, but varied in the response to thiamine. Most strains required thiamine, but four of them grew through successive transfers without it. *V. alcalescens* required putrescine, whereas *V. parvula* did not. Also, *V. alcalescens* differed from *V. parvula* in decomposing H_2O_2. The catalase of *V. alcalescens* does not appear to have an Fe heme grouping, since this species (like all the veillonellae) has been consistently benzidine-negative as tested by the procedure by Deibel and Evans (1960), and cytochrome compounds have not been demonstrated. Two strains of a *Veillonella* sp., ERN and FIS, behaved nutritionally and otherwise like *V. alcalescens* except that they possessed an additional antigen (group VII).

The 25 strains from the rat and 3 from the hamster (Table 4) required pyridoxal and thiamine for good growth and nutritionally behaved like *V. parvula* (all of human origin), except that a deficiency of putrescine frequently either prevented or severely limited growth of the animal strains. The organisms from the rodent also contained a distinctive antigen (group II).

The antigenic groups mentioned are based on the work previously reported by Rogosa, Hampp, and MacKintosh (1961*b*), and this aspect of the study of the genus will be reported more fully elsewhere.

Putrescine and cadaverine are the decarboxylation products of L(+)-ornithine and L(+)-lysine, respectively. Herbst and Snell (1948, 1949*a*, *b*) found that *Hemophilus parainfluenzae* required putrescine for growth, and that spermine, spermidine, and agmatine could replace putrescine. Martin, Pelczar, and Hansen (1952) showed that putrescine was required for the growth of *Neisseria perflava*, and that spermidine, agmatine, and perhaps cadaverine could substitute. For the apposite *Veillonella* strains, however, putrescine and cadaverine were the only amines promoting growth. The corresponding carboxylic acids were ineffective. Agmatine, the amine product from L(+)-arginine, and arginine itself were inactive. In addition, an array of compounds tested at 27.94×10^{-6} to 55.98×10^{-6} M were without effect; these included histamine, tyramine, γ-aminobutyric acid, β-alanine, glutamine, spermine, spermidine, asparagine, citrulline, creatinine, guanidine, urea, and thiourea. These

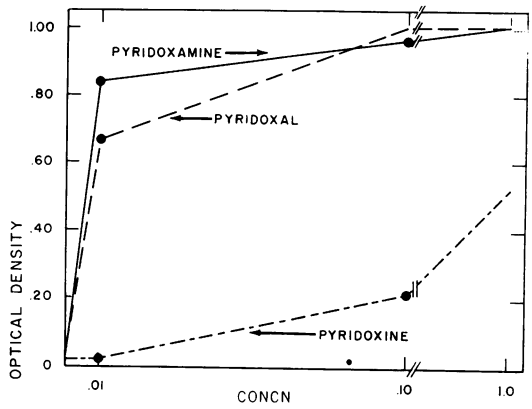

FIG. 3. *Growth response of Veillonella parvula strain PRI to pyridoxamine, pyridoxal, and pyridoxine. Concentrations shown are in μg/ml.*

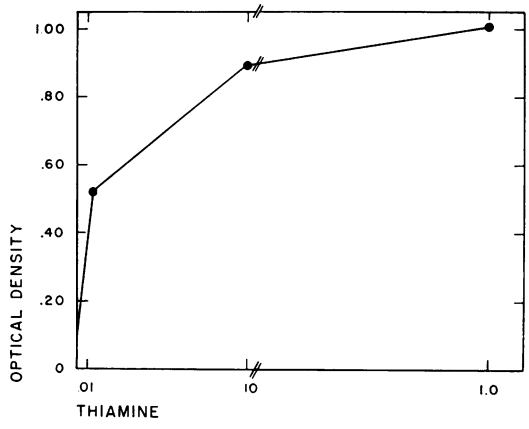

FIG. 4. *Growth response of Veillonella parvula strain PRI to varying concentrations (μg/ml) of thiamine.*

TABLE 7. *Growth of Veillonella alcalescens strain BL-78 in nutritional test medium containing various energy sources*

Substrate (0.1 M)	Growth period	
	1 day	2 days
Lactate	0.68*	0.62
Fumarate	0.19	0.82
Malate	0.37	0.91
Oxaloacetate	0.37	0.78
Pyruvate	1.10	1.10
Succinate	0.04	0.06

* Figures are optical densities at 650 mμ measured with a Spectronic-20 colorimeter.

compounds were filter-sterilized, and separate portions were also sterilized in the medium. The activity of putrescine and cadaverine was unaffected by autoclaving.

The filter-sterilized sodium salts of fumaric, malic, oxaloacetic, pyruvic, and succinic acids were substituted for lactate as energy sources. Relative growth obtained with *V. alcalescens* BL-78 is illustrated in Table 7. Except for erratic results with lactate, all these compounds are metabolized by resting cells of *Veillonella* (Johns, 1951; Rogosa, 1964), and succinate is fermented to propionic acid and CO_2 very probably by the transcarboxylation mechanism of Swick and Wood (1960), Wood and Stjernholm (1961), and Stjernholm and Wood (1961). However, the organism could not grow with succinate as a substrate. Also, the putrescine or cadaverine growth requirement was not spared for *V. alcalescens* by the substitution of fumarate, malate, oxaloacetate, or pyruvate for lactate.

ACKNOWLEDGMENTS

The authors acknowledge the technical assistance of Alfred J. Beaman, and are indebted to Ronald Green for the preparation of the pure samples of spermine and spermidine, and to Tetsuo Shiota for the folic acid derivatives and related compounds.

LITERATURE CITED

DEIBEL, R. H., AND J. B. EVANS. 1960. Modified benzidine test for the detection of cytochrome-containing respiratory systems in microorganisms. J. Bacteriol. **79**:356–360.

FILDES, P. 1923. The classification of haemoglobinophilic bacteria, based upon their relation to blood-pigment and to the 'vitamine' factor. Brit. J. Exptl. Pathol. **4**:265–271.

FILDES, P. 1924. The growth requirements of haemolytic influenza bacilli, and the bearing of these upon the classification of related organisms. Brit. J. Exptl. Pathol. **5**:69–74.

HERBST, E. J., AND E. E. SNELL. 1948. Putrescine as a growth factor for *Hemophilus parainfluenzae*. J. Biol. Chem. **176**:989–990.

HERBST, E. J., AND E. E. SNELL. 1949*a*. The identification of putrescine as a growth factor for *Hemophilus parainfluenzae*. J. Biol. Chem. **181**:47–54.

HERBST, E. J., AND E. E. SNELL. 1949*b*. The nutritional requirements of Hemophilus parainfluenzae 7901. J. Bacteriol. **58**:379–386.

JOHNS, A. T. 1951. The mechanism of propionic acid formation by *Veillonella gazogenes*. J. Gen. Microbiol. **5**:326–336.

KNIGHT, B. C. J. G., AND H. PROOM. 1950. A comparative survey of the nutrition and physiology of mesophilic species in the genus *Bacillus*. J. Gen. Microbiol. **4**:508–538.

MARTIN, W. H., M. J. PELCZAR, JR., AND P. A. HANSEN. 1952. Putrescine as a growth factor requirement for *Neisseria*. Science **116**:483–484.

PROOM, H., AND B. C. J. G. KNIGHT. 1955. The minimal nutritional requirements of some species in the genus *Bacillus*. J. Gen. Microbiol. **13**:474–480.

ROGOSA, M. 1955. Nutrition of the *Veillonella*. J. Dental Res. **34**:721–722.

ROGOSA, M. 1964. The genus *Veillonella*. I. General cultural, ecological, and biochemical considerations. J. Bacteriol. **87**:162–170.

ROGOSA, M., J. G. FRANKLIN, AND K. D. PERRY. 1961*a*. Correlation of the vitamin requirements with cultural and biochemical characteristics of *Lactobacillus* spp. J. Gen. Microbiol. **25**:473–482.

ROGOSA, M., E. G. HAMPP, AND M. E. MACKINTOSH. 1961*b*. Serological studies on the genus *Veillonella*. Bacteriol. Proc., p. 127.

ROGOSA, M., E. G. HAMPP, T. A. NEVIN, H. N. WAGNER, JR., E. J. DRISCOLL, AND P. N. BAER. 1960. Blood sampling and cultural studies in the detection of postoperative bacteremias. J. Am. Dental Assoc. **60**:171–180.

STJERNHOLM, R., AND H. G. WOOD. 1961. Methylmalonyl isomerase. II. Purification and properties of the enzyme from propionibacteria. Proc. Natl. Acad. Sci. U.S. **47**:303–313.

SWICK, R. W., AND H. G. WOOD. 1960. The role of transcarboxylation in propionic acid fermentation. Proc. Natl. Acad. Sci. U.S. **46**: 28–41.

WHITELEY, H. R., AND H. C. DOUGLAS. 1951. The fermentation of purines by Micrococcus lactilyticus. J. Bacteriol. **61**:605–616.

WOOD, H. G., AND R. STJERNHOLM. 1961. Transcarboxylase. II. Purification and properties of methylmalonyl-oxaloacetic transcarboxylase. Proc. Natl. Acad. Sci. U.S. **47**:289–313.