PROCEEDINGS
OF THE
NATIONAL ACADEMY OF SCIENCES
Volume 27 January 15, 1941 Number 1

PURINE AND PYRIMIDINE BASES AS GROWTH SUBSTANCES FOR LACTIC ACID BACTERIA

BY ESMOND E. SNELL AND HERSCHEL K. MITCHELL

FROM THE DEPARTMENT OF CHEMISTRY, UNIVERSITY OF TEXAS

Communicated November 25, 1940

In 1936 Richardson[1] showed that uracil was essential for the anaerobic growth of *Staphylococcus aureus*, but not for aerobic growth of the same organism. Of five strains tested three required uracil, while one required both guanine and uracil for growth. Thymine or cytosine did not replace uracil for this organism. These experiments suggested that hydrolytic products of nucleic acids might become factors limiting growth of various organisms under certain conditions. Bonner and Haagen-Smit[2] in 1939 showed that adenine greatly stimulated growth of leaves under defined conditions, while Möller[3] showed that adenine was required for growth of *Streptobacterium plantarum*. Pappenheimer and Hottle[4] recently showed that adenine was necessary for the growth of a strain of Group *A* hemolytic streptococci; it could be replaced by hypoxanthine, guanine, xanthine, guanylic acid or adenylic acid. They made the very interesting observation that adenine was unnecessary for growth of this organism if the carbon dioxide tension was maintained at a sufficiently high level.

Snell and Peterson[5] investigated the properties of an unidentified growth factor for *Lactobacillus casei* ϵ, and concluded that it showed some properties in common with purines, although it was not replaceable by any of the known naturally occurring purines. The failure of known purines to support growth does not indicate that they are not required, since they may be required in addition to other unidentified substances. In view of the above results we have investigated the rôle of purine and pyrimidine bases in the growth of lactic acid bacteria, utilizing in particular organisms which appear to be less fastidious in their growth requirements than is *L. casei* ϵ. Results of this investigation show that for various organisms of this group, each of the purine and pyrimidine bases which occur naturally in nucleic acids—guanine, adenine, uracil and thymine—may become the factor limiting growth.

Experimental.—The basal medium used had the following composition:

	AMOUNT PER 100 CC. OF MEDIUM
Acid hydrolyzed casein (*Labco*, vitamin-free)	0.5 g.
Sodium acetate	0.6 g.
Glucose	1.0 g.
Cystine hydrochloride	10 mg.
Tryptophane	10 mg.
Inorganic salts	
KH_2PO_4	50 mg.
K_2HPO_4	50 mg.
$MgSO_4 \cdot 7H_2O$	20 mg.
NaCl	1 mg.
$FeSO_4 \cdot 7H_2O$	1 mg.
$MnSO_4 \cdot 4H_2O$	1 mg.
Riboflavin	10 μg.
Calcium pantothenate	10 μg.
Vitamin B_6	10 μg.
Thiamin	10 μg.
Nicotinic acid	10 μg.
Biotin concentrate	equivalent to 0.01 μg. pure biotin

The biotin concentrate used was prepared essentially by following through the first few steps in Kögl's procedure.[6] It contained 0.2% biotin when assayed by the yeast method[7] against a sample of Kögl's pure biotin methyl ester. Biotin is essential for the growth of several of the lactic acid bacteria on media similar to that above; it was first reported essential for certain of these organisms by Möller;[3] all of the other vitamins listed with the exception of thiamin have been shown to be growth requirements of representative organisms of this group.[3, 5, 8, 9]

Supplements whose effects were to be tested were added in desired amounts to the culture tubes and the total volume adjusted to 5 cc. To these were then added 5 cc. of the above basal medium (prepared in double the given concentrations and adjusted to pH 6.8), the tubes were plugged with cotton, autoclaved at 15 pounds steam pressure for 15 minutes, cooled and inoculated. Inocula were grown for 24 hours at 30° in Medium B supplemented with 1 μg. of calcium pantothenate (Merck) per culture,[10] centrifuged out, resuspended in 0.9% sodium chloride solution and diluted to the desired density for use. Test organisms used were *Lactobacillus arabinosus* 17–5, *Lactobacillus pentosus* 124–2, *Leuconostoc mesenteroides* P-60 and *Streptococcus lactis* R. Extent of growth was measured by comparing turbidities quantitatively in the thermoelectric turbidimeter.[11] This was calibrated by use of a cell suspension of the organism in question.

The effect of supplementing the basal medium with various purine and pyrimidine bases on the growth of *L. arabinosus* is shown in table 1, columns 1–4.

TABLE 1

EFFECT OF ADENINE, GUANINE AND URACIL ON GROWTH OF *Lactobacillus arabinosus*

μG. SUPPLEMENT PER 10 CC. MEDIUM	MG. MOIST CELLS PRODUCED PER CULTURE (10 CC.)				
	ADENINE SULFATE	GUANINE HYDROCHLORIDE	URACIL	URACIL + 100 μG.* ADENINE PER CULTURE	URACIL + 100 μG.† ADENINE PER CULTURE
0	1.2	1.2	1.2	9.0	4.9
0	1.2	1.2	1.2	9.0	4.8
0.3	...	...	...	..	5.4
1.0	2.0	1.9	...	10.0	7.3
3.0	2.3	2.2	1.4	11.0	9.0
10.0	3.7	2.4	1.5	9.7	8.0
30.0	5.2	4.1	1.4	11.0	8.0
100.0	9.0	4.9	1.7	..	...
300.0	9.0	4.8	...	..	...

* Columns 2–5: 24-hr. incubation at 30°. Inoculum 30 μg. moist cells per culture tube.

† Column 6: 18-hr. incubation at 30°. Inoculum 10 μg. moist cells per culture tube.

Adenine has a striking effect on growth; guanine replaces it effectively at low doses, but does not permit growth to proceed to such a high level. Uracil is ineffective when tested alone.

The inoculum in the above experiment was about 30 μg. moist cells per 10 cc. of medium; at the end of 24 hours the control tube (without adenine) contained 1.2 mg. of cells, while the tube with adequate adenine contained 9.0 mg. of cells. Evidently extensive multiplication has occurred even in the absence of added adenine; its rate, however, is limited by the amount of adenine present. Separate experiments showed that the organism could be serially subcultured indefinitely in the base medium without adenine; if growth were allowed to continue for several days, density of growth in the cultures without adenine approached that in cultures containing adenine.

Thus adenine should be considered as a substance limiting the *rate* of growth (stimulatory substance) rather than as a substance limiting the *extent* of growth (essential growth substance) for this particular organism. It is evident, however, that such distinctions are of little importance in so far as the biochemical importance of a compound is concerned. Thus an external supply of biotin greatly increases rate of growth of yeast, but is not essential for growth;[6, 12] it is, however, essential for the growth of certain clostridia[13] and lactic acid bacteria.

The rate at which multiplication must occur to produce visible growth will be increased either by decreasing the amount of inoculum or the incubation period, or both. Under these conditions uracil, though completely ineffective in growth stimulation in the absence of adenine, proved stimulatory to growth in its presence (table 1, columns 4–6). Separate experiments showed that uracil was completely replaced by cytosine* and guanine by xanthine in the growth of *L. arabinosus*. Addition of both

guanine and adenine resulted in only a very slight growth increase over that secured with adenine alone.

Exactly the same relationships between the presence of adenine and uracil and growth proved to hold with *Lactobacillus pentosus* as with *L. arabinosus*. Our results (table 2) showed that increasing the carbon dioxide tension had no effect on the growth of these organisms or on the growth-stimulating properties of adenine, in contrast to its effect on certain hemolytic streptococci.[4] Adenylic acid (yeast) proved to be almost unavailable, but had slight activity at high levels.

TABLE 2

EFFECT OF CARBON DIOXIDE TENSION ON GROWTH REQUIREMENT FOR ADENINE; AVAILABILITY OF YEAST ADENYLIC ACID

	L. arabinosus					*L. pentosus*				
μg. supplement per culture*	0	3.0	10.0	30.0	100.0	0	3.0	10.0	30.0	100.0
Mg. moist cells per culture										
Adenine sulfate (CO_2-free air)	2.3	2.7	3.3	4.8	5.8	1.3	2.0	2.4	3.8	4.2
Adenine sulfate (10 mm. CO_2 tension)	2.0	2.3	2.8	4.2	5.6	1.1	1.8	2.4	3.9	4.9
Adenylic acid (CO_2-free air)	2.3	2.1	2.3	2.4	2.7	1.4	1.4	1.6	1.9	2.2
Adenylic acid (10 mm. CO_2 tension)	2.0	2.0	2.0	2.2	2.7	1.3	1.3	1.4	1.8	2.2

* All tubes were supplemented with 100 μg. uracil. Inoculum 30 μg. moist cells per culture.

Attempts to extend these results to one of the heterofermentative lactic acid bacteria, *Leuconostoc mesenteroides*, showed that in the presence of adenine and uracil only slight growth occurred in the first culture; this failed on subculture. Further experiments (table 3) showed that with this organism, guanine rather than adenine was the principal growth substance involved, while adenine had only a slight effect. Guanine is apparently essential for the growth of this organism; in its presence the organism responds further to additions of uracil, cytosine or thymine, which are interchangeable in their growth effects. This response appears partly in reducing the concentration of guanine at which a certain level of growth is achieved. Xanthine replaces guanine to a considerable extent, but does not bring growth to the same high level.

The fact that thymine and uracil are interchangeable in their growth effect on *L. mesenteroides* suggested that for certain organisms, thymine might prove to be an essential growth substance. This proved to be the case with *Streptococcus lactis*. This organism grew only slightly on the base medium supplemented with guanine, adenine and uracil and growth

TABLE 3

PURINE AND PYRIMIDINE BASES AS GROWTH FACTORS FOR *Leuconostoc mesenteroides*

SUBSTANCE ADDED	μG. PER CULTURE	MG. CELLS PER CULTURE*	SUBSTANCE ADDED	μG. PER CULTURE		MG. CELLS PER CULTURE
None	...	0.05	Adenine	100		3.5
			Guanine	100		
Uracil	100	0.15	Uracil	100		
			Guanine		30	3.9
					100	4.7
Cytosine	100	0.15	Cytosine	100		
			Guanine		30	3.8
					100	4.5
Thymine	100	0.15	Thymine	100		
			Guanine		30	3.7
					100	4.7
Adenine	10	0.05				
	30	0.20				
	100	1.20	Adenine	100		
			Uracil	100		
Guanine	10	1.8	Guanine		30	3.9
	30	2.5			100	4.5
	100	3.4				
Xanthine	10	2.0				
	30	2.7				
	100	2.6				

* 24-Hr. incubation at 30°. Inoculum 30 μg. moist cells per culture. Adenine and guanine were added as the sulfate and hydrochloride, respectively.

failed on subculture; much heavier growth was obtained which could be serially subcultured for several transfers when thymine was added. The experiments are summarized in table 4. Adenine is also required by this organism; a mixture of adenine and thymine produces growth as heavy as that produced by a mixture of adenine, guanine, thymine and uracil. Guanine can partially replace adenine, but growth is not as heavy. Uracil does not replace thymine.

Discussion.—From the above results it is evident that each of the purine and pyrimidine bases of nucleic acids may under certain conditions become the factor limiting growth of certain of the lactic acid bacteria. Thus adenine greatly stimulates growth of *L. arabinosus* and *L. pentosus*, and is essential for growth of *S. lactis*. Uracil greatly stimulates growth of *L. arabinosus*, and is helpful to *L. mesenteroides*. Guanine is essential for growth of the latter organism. Thymine is essential for growth of *S. lactis*.

In general, the naturally occurring amino derivatives of the purine or pyrimidine bases are replaceable by the corresponding oxy-derivative. Thus cytosine and uracil are interchangeable, as are guanine and xanthine. In some cases (but not all) guanine and adenine are interchangeable; such

TABLE 4

EFFECT OF THYMINE, URACIL, ADENINE AND GUANINE ON THE GROWTH OF *Streptococcus lactis*

SUBSTANCE ADDED	μG. PER CULTURE	MG. CELLS PER CULTURE*	SUBSTANCE ADDED	μG. PER CULTURE		MG. CELLS PER CULTURE
None	...	0.1	Guanine	100		1.0
			Uracil	100		
Uracil	100	0.05				
Thymine	100	0.1	Guanine	100		2.0
			Thymine	100		
Adenine	100	1.5	Guanine	100		2.7
			Adenine	100		
Guanine	100	1.1	Thymine	100		
			Uracil	100		
Adenine	100	1.1				
Guanine	100		Adenine†	100		
			Thymine		0	0.05
Adenine	100	1.0			0.3	0.15
Uracil	100				1.0	0.50
					3.0	1.70
Adenine	100	2.7			10.0	2.70
Thymine	100				30.0	3.1
					100.0	3.1

* Incubation time: 16 hrs. at 30°. Inoculum 20 μg. moist cells per culture.

† From a separate experiment to determine the quantitative requirement for thymine. Inoculated from a 24-hr. culture of the organism in a tube of the basal medium supplemented with adenine alone.

an interchange, however, never results in as good growth as does use of the preferred purine.

Thymine has not been previously recognized as essential for growth of any living organism, although Hammet and Lavine[14] reported that this compound accelerated proliferation of *Obelia geniculata*. That it should be required by certain bacteria is not surprising, since thymonucleic acid is a recognized constituent of the nuclei of plant cells[15] while thymine and 5-methyl cytosine have been isolated from the nucleic acid of tubercle bacilli.[16]

Summary.—For growth on media of known composition certain lactic acid bacteria require one or more of the following: adenine, guanine, thymine or uracil. In several cases where continued growth occurs in the absence of an added supply of these compounds, its rate is greatly increased by their presence.

We wish to thank the Rockefeller Foundation for grants in support of this investigation, and Dr. R. J. Williams for his suggestions during the course of this work.

* We wish to thank Professor T. B. Johnson for kindly sending us samples of thymine and cytosine.

[1] Richardson, G. M., *Biochem. Jour.*, **30**, 2185 (1936).

[2] Bonner, D. M., and Haagen-Smit, A. J., *Proc. Nat. Acad. Sciences*, **25**, 184 (1939).
[3] Möller, E. F., *Zeit. physiol. Chem.*, **260**, 246 (1939).
[4] Pappenheimer, A. M., Jr., and Hottle, G. A., *Proc. Soc. Exptl. Biol. Med.*, **44**, 645 (1940).
[5] Snell, E. E., and Peterson, W. H., *Jour. Bact.*, **39**, 273 (1940).
[6] Kögl, F., and Tonnis, B., *Zeit. physiol. Chem.*, **242**, 43 (1936).
[7] Snell, E. E., Eakin, R. E., and Williams, R. J., *Jour. Am. Chem. Soc.*, **62**, 175 (1940).
[8] Snell, E. E., Strong, F. M., and Peterson, W. H., *Jour. Bact.*, **38**, 293 (1939).
[9] Snell, E. E., and Strong, F. M., *Enzymologia*, **6**, 186 (1939).
[10] Snell, E. E., Strong, F. M., and Peterson, W. H., *Biochem. Jour.*, **31**, 1789 (1937).
[11] Williams, R. J., McAlister, E. D., and Roehm, R. R., *Jour. Biol. Chem.*, **83**, 315 (1929).
[12] Williams, R. J., Eakin, R. E., and Snell, E. E., *Jour. Am. Chem. Soc.*, **62**, 1204 (1940).
[13] Snell, E. E., and Williams, R. J., *Jour. Am. Chem. Soc.*, **61**, 3594 (1939).
[14] Hammet, F. S., and Lavine, T. F., *Growth*, **1**, 135 (1937).
[15] Feulgen, R., Behrens, M., and Mahdihassan, S., *Zeit. physiol. Chem.*, **246**, 203 (1937).
[16] Johnson, T. B., and Coghill, D., *Jour. Am. Chem. Soc.*, **47**, 2838 (1925).

CONTACT WITH MEASLES

BY EDWIN B. WILSON AND JANE WORCESTER

HARVARD SCHOOL OF PUBLIC HEALTH

Communicated, November 27, 1940

Our colleague Dr. W. L. Aycock has raised the question as to the frequency with which an individual comes in contact with the virus of measles. Although the question seems impossible to answer with definiteness because of inadequacy of data, a discussion of it is not without interest and importance. One reason for its importance is the widespread belief that, at least for some diseases, immunity develops progressively from repeated contacts with the agent rather than from a single contact. In dealing with the kind of statistical material available to us for the case of measles we have no method of attack which will enable us to test the hypothesis of the progressive development of immunity from repeated contacts.

It will therefore be assumed that when a person comes first in contact with the virus he acquires the disease and thereby becomes immune. The average age at which one has measles according to reports of cases is:

For Massachusetts,[1]	1932–1937, inclusive	7.08 years
For Massachusetts,[1]	1932–1937, "under 20,"	6.72 years
For Providence, R. I.,[2]	1919–1935, "under 22,"	5.81 years

As infants are immune for about half a year, we may deduct 0.5 from these figures and assume 6.5 for all ages or 6.2 for ages under 20, in Massachusetts,