RELATION OF BACTERIA TO VITAMINS AND OTHER GROWTH FACTORS

W. H. PETERSON AND MARY S. PETERSON

Department of Biochemistry, University of Wisconsin, Madison, Wisconsin

CONTENTS

Seven years ago the subject of growth factors was reviewed by Koser and Saunders (167) for this publication. Since that time, great progress has been made in the field. At that time only seven compounds could be listed as accessory growth substances for bacteria. Today the list of compounds has expanded to about twenty. Five of the additions are also new animal vitamins, but eight have not yet found a place in animal nutrition. The merging of the fields of animal and microbiological nutrition is well illustrated by the fact that three of these vitamins (biotin, pantothenic acid, and *para*-aminobenzoic acid) were discovered through microbiology, while the other two (pyridoxine and choline) appeared first in the field of animal nutrition. It is probably safe to predict that several of the compounds now known to be required by bacteria will some day become members of the vitamin family. A mutual exchange of ideas is bound to promote progress in both fields, and the benefits that accrue from such an exchange indicate the artificial nature of the boundaries that divide research work on cellular nutrition.

In contrast to the previous review, this paper will be limited to growth factors

for bacteria and the literature covered is that which appeared through 1944. A few references appearing in 1945 have been cited but no systematic effort has been made to review papers published during the present year. The developments in the field of bacteria alone have been so extensive during the last six years that it is difficult to cover the subject adequately in a review of reasonable length. Growth factors as used in this paper are defined as organic compounds that must be present in the medium in only minute quantities to promote the development of bacteria. The purpose of this limitation is of course to exclude the mineral elements and well-defined organic compounds such as amino acids. In most cases the limitation "minute amounts" is a sufficiently sharp criterion, but at times it becomes a narrow and almost vanishing boundary. For example, *Lactobacillus casei* requires only about twelve times as much tryptophane (352) as nicotinic acid (188). The nicotinic acid requirement, on the other hand, is a thousand fold that for biotin. The exclusion of the amino acids from the review logically required the omission of glutamine and asparagine in spite of the prominent place these two compounds occupy in growth-factor work. Whether this prominence rests on something other than amino-acid characteristics does not seem clear at the present time.

Substances that are not required preformed in the medium but if added increase the rate of growth are included in this review; as well as those that are required for the initiation and maintenance of growth. There are several reasons for not excluding the stimulatory substances. A compound that is indispensable in one medium may be unnecessary or only stimulatory in another (396, 449). Again, the microörganism that at first required the compound in the medium may be trained to grow without its addition (169, 361). It is probable that in such cases the microörganism has become able to synthesize as much of the compound as it needs for its metabolism. The success that has been attained in the adaptation of bacteria to dispense with various amino acids shows the remarkable latent powers of bacteria and suggests the possibility that similar success may be reached in training bacteria to dispense with growth substances.

RESPONSE OF BACTERIA TO GROWTH SUBSTANCES

Table 1 lists various compounds and unknown factors that have been reported to promote the growth of one or more species of bacteria. Numbers in the blocks refer to papers reporting a favorable effect. Where there is no entry, no report on the use of the compound has been found. If the compound has been tried and found to have no effect this result is indicated by the word "no." A statement regarding the manner in which this table has been compiled may be helpful to the reader.

No distinction is made between compounds that are required in the medium and those that are merely stimulatory to growth; because it is often impossible to distinguish between them. For example, Wood, Andersen and Werkman (447, 449) showed that a given strain of propionic acid bacteria required riboflavin in an ammonium sulfate medium but not in an amino acid medium. They also demonstrated that strains of propionics could be adapted by subculturing to dispense with riboflavin and thiamine.

Likewise, no attempt is made to distinguish between different strains of the same species with respect to their requirements. Some strains require a certain compound in a given medium and other strains do not. Difference of strains is one explanation for the seeming contradiction of an organism being listed as both requiring and synthesizing a growth factor.

Growth factors that have not been isolated are listed in the last column under the heading of unknown factors, if the reported factor appears to be distinct from the known compounds listed in the other columns. In case of papers published several years ago, it is difficult to decide whether or not a new factor was involved, as some of the compounds now known to promote the growth of bacteria were unknown or unavailable at the time. In connection with unknown factors the terms, Bios I, IIA, IIB, etc. are not used in this review as they have little meaning at the present time. In the field of the vitamins, the letters B_1, B_2, B_6, etc. are being dropped and the corresponding chemical terms thiamine, riboflavin, pyridoxine (pyridoxal, pyridoxamine), etc. are being substituted. Numbers or letters serve as suitable and temporary means of designation and also have the advantage that they can be readily abandoned when the pure compound has been isolated, identified, and properly named. On the other hand, to give chemical names to substances that have not been obtained in pure form appears also to be undesirable and unsound. Such terms are likely to be inappropriate and to carry a meaning which they do not legitimately possess. Their introduction is also unfair to other investigators in the same field who seek to avoid cluttering the literature with premature and inappropriate chemical nomenclature.

The bacteria as named by the authors cited are listed in alphabetical order. This procedure results in the listing of organisms that are regarded by some authorities as identical under two different names. Since there is disagreement among investigators as to the proper name to be applied, and since in some cases the name in general use is not that of systematic taxonomy, it would make the review confusing and less useful to list the bacteria under names other than those in current use.

Conclusions drawn from table 1

Approximately 130 bacteria have been found to need one or more of the compounds listed in the table. Organisms that are known to require the largest number of compounds are *Clostridium tetani*, *Lactobacillus arabinosus*, *Lactobacillus casei*, pneumococci, *Streptobacterium plantarum*, hemolytic streptococci and *Streptococcus lactis* with requirements of from 7 to 10 each. It is not implied, of course, that other bacteria do not utilize just as many growth factors as do the microörganisms listed. Probably all of these compounds are involved in bacterial metabolism, but when an organism is able to synthesize unsupplied factors their presence in the medium is not required. This aspect of bacterial nutrition will be dealt with in a later section.

Only a small proportion of the total number of heterotrophic bacteria have had their complete requirements for growth substances worked out. There is great need for systematic work to fill in the gaps in our knowledge, but with the

TABLE 1

Index of growth factor requirements of bacteria

Key to Table. The bacteria are listed in column one and the growth factors required by the bacteria are designated by numbers in the successive columns. The numbers refer to the bibliography and denote that the compound is reported as being required or stimulatory for the growth of the corresponding organism in column one. No attempt is made to distinguish between different forms of a growth factor, *e.g.* nicotinic acid *vs.* the amide, pyridoxine *vs.* pyridoxal.

The letter "S" means that the organism is reported to synthesize the growth factor. The reference to the synthesis is given in table 3.

The word "no" followed by a number signifies that the organism has been found not to require the growth factor preformed in the medium.

Where there is no entry in a block, it means that no report has been found regarding the need, synthesis, or dispensability of the compound for the corresponding organism.

ORGANISM	BIOTIN	NICOTINIC ACID	PANTOTHENIC ACID	PYRIDOXINE	PAB	RIBOFLAVIN	THIAMINE	MISC.	UNKNOWN
Acetobacter suboxydans	S	138, 185, 417	185, 417		185, 189, 417	S		Butyrolactone, 417; purines, 195	
Bacillus brevis	190								
B. butylicus	183								
B. dextrolacticus						6	6		Unnamed, 6
B. larvae	No, 204	No, 204	No, 204	No, 204		No, 204	204	Inositol, no, 204	
B. macerans	139						139		
B. mesentericus	48	S				S	S		
B. polymyxa	139								
B. proteus		93b, 148, 214, 215							
B. saccharobutyricus	183								
Bacterium bifidum						292, 294			

Bacterium brassicae			249, 384, 385			No, 381			
Bacterium radicicola (See *Rhizobium*)									
Bacterium tularense	407	407	407						Liver, etc., 407
Bacterium utile				253, 255					
Bacterium typhosus									"Sporogenes vitamin," 94
Betabacterium breve						292, 294			
Brucella abortus	163, 168, 218, 219, 220	141, 163, 168, 219, 220	163, 168, 219, 220		S		141, 163, 168, 218, 219, 220		
Brucella melitensis	220	163, 220	163, 220				163, 218, 220		
Brucella suis	220	163, 218, 220	163, 218, 220				163, 218, 220	β-Alanine and butyrolactone, 163	
Clostridium acetobutylicum	182, 183, 297a, 300, 432				123, 182, 183, 184, 300, 334, 335	S			"Acetone factor," 335
C. beijerinckii							No, 46a		Yeast, 46a
C. botulinum	190				S				"Sporogenes vitamin," 93a, 155; unnamed, 121b

TABLE 1—*Continued*

ORGANISM	BIOTIN	NICOTINIC ACID	PANTOTHENIC ACID	PYRIDOXINE	PAB	RIBOFLAVIN	THIAMINE	MISC.	UNKNOWN
C. butylicum	181, 183, 309a, 386	S	S	S	183	S	S		
C. chauvoei	190								
C. felsineum	183				183				
C. histolyticum	190								
C. parabotulinum									"Sporogenes vitamin," 86
C. pectinovorum							No, 46a		Yeast, 46a
C. septicum	29	29		29			29		Hydrolyzed casein, 29
C. sporogenes	183, 309a				S				"Sporogenes vitamin," 155, 298
C. thermosaccharolyticum	63	63	63		63		63		
C. tetani	90, 91, 183, 272, 273	91	90, 91, 272, 273	91	S	90, 91, 272, 273, 314	90, 91, 272, 273	Purines, 90, 91, 272, 273; oleic acid 90, 91; uracil, 90, 91	Eluate factor, 272; "folic acid," 90, 91, 273, 274
C. welchii	190			91				Uracil; oleic acid, 91	
Corynebacterium diphtheriae	420	42, 266, 267, 268, 269	271		S	S	S	β-Alanine or pantothenic acid, 166, 268, 270; oleic acid 65, 66; pimelic acid or biotin, 263, 264, 265, 420	Blood factor, 389; liver factor, 57, 59

C. diphtheriae (intermedius)		88, 89	88, 89				S	β-Alanine or pantothenic acid, 88, 89; pimelic acid, 88, 89	Liver factor, 53, 58
C. diphtheriae (mitis)		88, 89	88, 89			S	S	Pimelic acid, 88, 89; β-alanine or pantothenic acid, 88, 89	Liver factor, 55, 58
C. diphtheriae (gravis)		88, 89	88, 89		55	S	S	Pimelic acid, 88, 89; β-alanine or pantothenic acid, 88, 89; oleic acid, 66	Liver factor, 55, 58, blood factor, 66
Dysentery bacilli (See *Shigella*)									
Erysipelothrix rhusiopathiae						127		Oleic acid, 127	Unnamed, 127
Escherichia coli	S	S, 331*				S	S, 331*	Indole acetic acid, 13	
Gonococcus see *Neisseria gonorrhoeae*									
Hemophilus canis								Hematin, 208, 329a	
Hemophilus ducreyi								Hematin, 213a	

* X-ray treated strains are reported to require these factors.

TABLE 1—*Continued*

ORGANISM	BIOTIN	NICOTINIC ACID	PANTOTHENIC ACID	PYRIDOXINE	PAB	RIBOFLAVIN	THIAMINE	MISC.	UNKNOWN
H. influenzae								Hematin (factor X), 18, 71, 72, 93c, 103, 104, 208, 213, 291; coenzyme I (factor V), 18, 208; nicotinamide riboside, 104b	Unnamed, 18
H. parainfluenzae								Coenzyme I 18, 160a, 208–212, 261, 310; nicotinamide riboside, 104b, 343	Unnamed, 18
H. pertussis		121a							
Klebsiella pneumoniae		472							
Lactobacillus arabinosus	387, 466	384, 385, 387	249, 384, 385	S	131, 377	S, no, 381		Purines, 376, 377; pyrimidines, 376	Folic acid, 21 Tomato factor, 180a
L. acidophilus	190					51			
L. beijerinckii						51			
L. bulgaricus						51		Thymine, 394	Folic acid, 394

L. casei	188, 353, 354, 395, 416	188, 384, 385	188, 249, 308, 384, 385	37, 38, 188, 370, 371, 372, 379	54, 62	51, 188, 380, 381, 382, 383	21	Purines, 92, 399, thymine, 174, 394, 399; orotic acid, 52; fatty acids 20, 402	Eluate factor, 124, 378, 379; unnamed, 399, 400; folic acid 250, 252; vitamin B_6, 31, 309; *L. casei* factor, 125; misc., 54, 64, 76, 85, 92, 392
L. delbrückii			384, 385	37		51, 380, 381		Thymine, 394	Eluate factor, 124; folic acid, 394
L. gayonii						381			
L. helveticus						51			
L. jugurt						51			
L. lactis			249, 384, 385	37, 38, S		51, 175, 380, 381			
L. leichmanii						51			
L. lycopersici						447			
L. mannito-poeus						447, no, 381	358, 360		
L. pento-aceticus						No, 381, 447			
L. pentosus			384, 385		377	No, 381, S		Purines, 376, 377; pyrimidines, 376	
L. plantarum						No, 51			

TABLE 1—*Continued*

ORGANISM	BIOTIN	NICOTINIC ACID	PANTOTHENIC ACID	PYRIDOXINE	PAB	RIBOFLAVIN	THIAMINE	MISC.	UNKNOWN
Leptospira canicola		333a				333a	333a		In animal serum, 333a
L. hebdomadis icterohaemorrhagiae		472							
L. icterohaemorrhagiae		426, 472							
Leuconostoc mesenteroides	99, 100	99, 100	99, 100, 384, 385	37, 99, 100		No, 381	99, 100	Purines, 376, 377, no, 99; pyrimidines, 376, no, 99	
Listerella monocytogenes	128, 317					127, 317	128, 317	Hemin, 317	Casein factor, 127, 128
Mycobacterium paratuberculosis								Anti-hemorrhagic compounds, 460	
M. phlei		472						Ergosterol, 320	
M. tuberculosis								Ergosterol, 320	Liver, etc., 41
Neisseria gonorrhoeae		434?	434?	434?			434?	Cocarboxylase, 196; choline, 434	Liver, etc., 5, 197, 434
Pasteurella pestis	No, 84	328 No, 84	No, 84	No, 84	No, 84	No, 84	328 No, 84	Hematin, 328, no, 84	
P. suiseptica		25, 27, 28, 162	25, 27, 28						Unnamed, 25

P. tularense							25, 27		
Photobacterium phosphorescens						83			
Pneumococci (several types)	12, 39, 104a	12, 104a, 326, 327	12, 104a, 326, 327			326	12, 104a	Purines, 104a; choline, 12, 104a, 326, 327	Strepogenin, 455
Propionibacterium arabinosum			170			448	408, 448		Unnamed, 412
P. jensenii	412	No, 412	170, 412	No, 412	412	No, 412		Inositol, no, 412	
P. pentosaceum			170, 384, 385			449	356, 358, 360, 408, 448, 449		Eluate factor, 124; unnamed, 412
P. peterssonii	412	No, 412	412	No, 412	412	449, no, 412	356, 358, 412, 449	Inositol, no, 412	
P. rubrum	412		412	412		449	449		Unnamed, 412
P. shermanii			170						
P. technicum		No, 412	412	No, 412		No, 412		Inositol, no, 412	
P. thoenii	412	No, 412	170, 412	No, 412		449, no, 412	449	Inositol, no, 412	
P. zeae			170			S	408, 449		Unnamed, 412
Proteus morganii	No, 305	302, 303, 305	26, 78, 120, 302, 303, 305	No, 305	No, 305	No, 305	No, 305	Inositol, β-alanine, pimelic acid, purines and pyrimidines, no, 305	Unnamed, 305
P. vulgaris	S	93b, 214, 257, 260, 301, 336	S	S	S	S	S	Inositol, S	

TABLE 1—*Continued*

ORGANISM	BIOTIN	NICOTINIC ACID	PANTOTHENIC ACID	PYRIDOXINE	PAB	RIBOFLAVIN	THIAMINE	MISC.	UNKNOWN
Pseydomonas aeruginosae pyogenes	S	No, 341			S				
Rhizobium leguminosarum	277, 440, 446						No, 277		Unnamed, 61
R. lupinii	439, 440								
R. meliloti	440, 446								
R. phaseoli	440								Unnamed, 61
R. trifolii	281, 282, 283, 438, 439, 440, 446, S		440			437a, 439, S	33, 34, 279, 280, 281, 282, 283, 437a S	β-Alanine, 440	
Rhodospirillum rubrum	129							Pimelic acid, no, 129	
Salmonella gallinarum							135, 136, 137		
S. paratyphi		144, 145, 146, 147, no, 341			S				
S. pullorum	No, 136	135, 136, 137	No, 136	No, 136		No, 136	No, 136		
S. schottmuelleri		No, 341							

Sarcina flava						340	340		
Shigella dysenteriae		144 to 148			S				
S. paradysenteriae		80, 81, 82, 96, 162, 164, 165, 169, 336, 430, 472	430	No, 165	S			Uracil, 130	
Shigella sonnei		81, 164, 165		No, 165					
Spirillum serpens						S		Purines, 306	
Spironema gallinarum						No, 151	No, 151	Pimelic acid, no, 151	Chicken red cells, 151
Staphylococcus albus		166		422	S	S	166, 365		
S. aureus	315, 316, S	149, 152, 153, 154, 156, 160, 186, 315, 316, 435			S	S	119, 149, 153, 154, 156, 315, 316, 365,	Uracil, 329	Unnamed, 121b
S. pyogenes aureus	160, 176, no, 339	160, 176, 339, no, 341					160, 176, 339	Inositol, no, 339	
Streptobacterium casei						292, 293, 295			
S. plantarum	176, 254, 257, 259	176, 254, 257, 259	176, 254, 259	176, 253, 254, 255, 259	176, 259	259	176, 254, 255	Purines, 176, 254, 255	
Streptococci (hemolytic)		325	223, 224, 405	224	S	325		Betaine, 325; thiochrome, 325	

TABLE 1—*Continued*

ORGANISM	BIOTIN	NICOTINIC ACID	PANTOTHENIC ACID	PYRIDOXINE	PAB	RIBOFLAVIN	THIAMINE	MISC.	UNKNOWN
Streptococci (hemolytic) Group A	122, 299	299	30, 299	299		299	299	Purines, 299	Strepogenin, 392, 455; unnamed, 19, 110
Streptococci (hemolytic) Group B	284	284	284, 459	284, 459		284, 459	284		
Streptococci (hemolytic) Group C			458			458		Pyrimidines, 332	
Streptococci (hemolytic) Group D			453, 459	346, 459		459		Butyrolactone or pantothenic acid, 453	Strepogenin, 392
Streptococci (hemolytic) Group G			223						
Streptococcus bovis	287, 363	287, 363				S	287, 363		
S. cremoris	285	285	170, 285	285		285, 292, 295	285		
S. distendens			170						
S. durans	286	286	286	286		286	No, 286	Thymine, 394	Folic acid, 286, 394; SLR factor, 397
S. epidemicus			458			458			
S. faecalis	286	286	286, 346	286, 346		51, 286, 346	No, 286	Thymine, 394	Folic acid, 286, 394; unnamed, 464; SLR factor, 397

S. lactis ATCC 8043*	207, 248, 376	207, 248, 376	207, 248, 249, 383, 384, 385	207, 248, 370, 371, 372		No, 381, S		Purines, 207, 376, 377; thymine, 174, 376, 394; alanine or pyridoxine, 369	Folic acid,† 207, 248, 250, 252; eluate factor,† 124; *L. casei* factor,† 125, 399, 400; vitamin B_c, 31, 174, 309, 410; factor SLR,† 142, 397
S. lactis	285	285, 324	285	285		292, 295, yes and no, 285, 324	Yes and no, 285	Purines, 324	Unnamed, 364, 464; strepogenin, 392
S. liquefaciens	286	286	286	286		286	No, 286		
S. mastitidis			170, 459	459		459, no, 51			
S. paracitrovorus						447			
S. pyogenes			458			458			
S. salivarius	287, 363	287, 363	287, 363			287, 363	287, 363	Uracil, 287, 363	
S. thermophilus						292, 295	418		
S. zymogenes	286	286	126, 286, 459	126, 224, 286, 459		51, 126, 286, 459	No, 286	Thymine, 394	Folic acid, 286, 394; factor SLR, 397
Thermobacterium bulgaricum						292, 295			
T. helveticum						2, 292, 295			Unnamed, 423
T. jugurt						295			
T. lactis						293, 294, 295			

* According to Niven and associates (116, 285, 286), this organism should be classified as *Streptococcus faecalis*.
† These factors are replaceable by thymine (171, 394).

accelerating rate of progress that is now being attained, another five years should close most of the gaps for the commonly used bacteria. Even though we know what compounds will promote growth for an indefinite number of transfers, it by no means follows that we have discovered the requirements for optimum growth and metabolism. Comparison of the defined medium with the best natural medium often shows that the former gives a slower rate of cell proliferation and formation of products than the latter. When equivalence has been reached, we may attempt to improve on nature. Today we are undoubtedly short of equivalence in most cases.

The compounds most frequently reported as promoting the growth of bacteria are: biotin, nicotinic acid, pantothenic acid, and riboflavin, each being required by about 50 organisms; thiamine 40; pyridoxine (pyridoxal, pyridoxamine) 25; *p*-aminobenzoic acid 15; about 15 each for several purines and pyrimidines; and 5 or less for more than a dozen other compounds. Some compounds, such as ascorbic acid and thioglycolic acid have been reported (9, 12, 49, 104a, 150, 216, 340, 458) as favoring the growth of certain bacteria, but it is probable that they do so by regulating the oxidation-reduction condition of the medium rather than as true growth substances. Because of this doubt regarding the function of these compounds, they have not been listed in the table.

Many of the above compounds are well-known vitamins for animals, but there are many compounds having potency for bacteria that have not yet found a place in the animal field. The opposite is of course also true; most of the fat-soluble vitamins are not known to have any potency for bacteria. A pair of compounds of great potency in the growth of higher plants is conspicuously absent from the lists of both animal and microbiological vitamins, *viz.*, auxins a and b. Their occurrence in plant and animal tissues, *e.g.*, seeds and liver, strongly suggests that they play an important role in the metabolism of such cells. It is not improbable that some day they will be found to serve as growth factors for bacteria.

Unidentified factors

The number of bacteria listed in table 1 as requiring unknown growth factors is about thirty. It seems unlikely that there is any such number of unknown factors. The requirements of these bacteria can probably be met in most cases by known compounds that were either not available or were not recognized as growth factors at the time the papers were published. In other cases, failure to grow may have been due to inadequate supplies of amino acids and mineral elements or to unsatisfactory buffer and oxidation-reduction conditions rather than to growth factor deficiencies. There remain, however, several instances in which the existence of an unidentified factor seems well-established, and other cases where its occurrence seems strongly indicated. These will be considered in order.

Sporogenes vitamin. This is the oldest unidentified factor and was first described by Knight and Fildes (155). Pappenheimer (298) made an extensive chemical investigation of its concentration and properties. His best preparation,

although not crystalline, was very potent: 0.04γ per ml of medium insured good growth of *Clostridium sporogenes*. The preparation was acidic in nature, very stable to heat and acids, and had an apparent molecular weight of about 200. In many respects, the data indicate a strong resemblance to biotin, but in other respects they show a marked unlikeness, notably in the absence of nitrogen and sulfur from the preparation. Further indication that biotin is probably involved in the action of the sporogenes vitamin comes from the reports of Peterson, McDaniel and McCoy (309a) and Lampen and Peterson (183) that certain strains of *C. sporogenes* require only biotin for growth on a synthetic medium although other strains fail to grow on this medium. Only biotin is required by several clostridia, but thiamine, riboflavin, pantothenic acid and *p*-aminobenzoic acid are required by others. On the basis of available data, it appears probable that "sporogenes vitamin" consists of several known factors but may also contain some as yet unidentified factor. A systematic study employing the known compounds would undoubtedly throw much light on the problem.

Norite eluate factor, Lactobacillus casei factor, vitamin B_c, folic acid, SLR factor. In 1939 Snell and Peterson (378) published an abstract and in 1940 a complete paper (379), concerning the occurrence, concentration, and properties of a growth factor required by *Lactobacillus casei*.[1] The factor was abundant in liver, malt products, and yeast, and occurred to a less extent in an extract of cereal grains. Adsorption on norite and elution therefrom was one of the most effective means of concentration, and because of this property, the factor was designated "norite eluate factor." In 1941, Hutchings *et al.* (124) published further details regarding the purification and properties of the factor and showed that it was required by several other lactic and propionic acid-forming bacteria, *e.g.*, *Streptococcus lactis* R, *Propionibacterium pentosaceum*, but could be synthesized by certain lactic types, *e.g.*, *Lactobacillus arabinosus*, *Leuconostoc mesenteroides*. In the same year, Stokstad (399) also reported the preparation of an active concentrate from liver which could be replaced in part by thymine.

In 1941 Mitchell, Snell, and Williams (250) published a brief note reporting the concentration from spinach of a factor required by *Streptococcus lactis* R (No. 8043 of the American Type Culture Collection)[2] but also potent for *L. casei*. Because it was considered to be "a nearly pure chemical entity" (later found to be about 30% pure) and because of its abundance in leaves, the factor was named "folic acid."

The two terms, "norite eluate factor" and "folic acid" obviously referred

[1] This organism is widely used in microbiological assays and at various times has been called *L. casei*, *L. casei* ϵ, and *L. helveticus*. On the basis of a recent bacteriological report (414) the most suitable name is *L. casei*. It is carried under the serial number 7469 by the American Type Culture Collection, Georgetown University Medical School, Washington, D. C. The discussion that follows applies only to this strain of *L. casei*. Other strains of *L. casei* may not have the same growth-factor requirements as Culture 7469.

[2] Attention is called to the recent reports of Niven and Sherman (286), Niven (285), and Gunsalus, Niven, and Sherman (116) stating that *S. lactis* R is really a strain of *Streptococcus faecalis*. The designation *S. lactis* R is retained in conformity with the terminology used in the original papers.

originally to the same factor or factors but with some special emphasis in each case on properties, occurrence, or test organism. Other investigators have shown that these factors are required by several additional bacteria.

In a series of four papers, Mitchell *et al.* (97, 245, 251, 252) reported in detail a procedure for the concentration of folic acid, and supplied much information regarding its chemical and physiological properties. On the basis of elementary analysis and molecular weight determinations (400 ± 50) a formula of approximately $C_{15}H_{15}O_8N_5$ is indicated.

Interest in these factors was greatly stimulated by the discovery that they were involved in animal nutrition. Hutchings *et al.* (123a) reported that the norite eluate factor was required for the growth of chicks. Since that time a large number of reports showing its importance for rats, chicks, and monkeys have appeared. A discussion of these reports is outside the scope of this paper, but they have been adequately covered in a recent review by Wieder (441).

The converging lines of investigation in microbiological and animal nutrition met in the isolation from liver of a crystalline compound by Pfiffner *et al.* (309) in 1943. This compound was potent in extremely small quantities for both the chick and *L. casei*. Because the initial research arose in pursuit of a chick antianemia factor which had been named vitamin B_c, this term was also used to designate the *L. casei* factor. The compound was obtained as yellow or orange colored crystals that in the most recently published analysis (Binkley *et al.*, 31) had the following percentage composition: C 52.45, H 4.29, N 19.7.

The announcement of Pfiffner *et al.* was soon followed by a note (Keresztesy *et al.*, 142) reporting the isolation[3] from an undisclosed source of a very potent factor for *S. lactis* R (later called SLR factor) but almost inactive for *L. casei*. In a later report, Stokes *et al.* (397) gave the interesting information that *S. lactis* R and other streptococci of the enterococcus group can convert the SLR factor into a form which is active for *L. casei* and other lactobacilli. Factor SLR is regarded by its discoverers as different from folic acid because of the lack of response of *L. casei* to it but, since folic acid is defined (Snell and Mitchell, 376; and Mitchell and Williams, 252) as the factor required by *S. lactis* R, there is obviously an overlapping of territory in these two terms.

A few months later in 1943, Stokstad (400) announced the isolation of crystalline compounds from both liver and yeast that had essentially the same composition and properties as vitamin B_c. Stokstad's compounds had equal potency for *L. casei*, but for *S. lactis* R the yeast product was only about one-half as potent as the liver isolate. A fourth crystalline compound derived from still another source was reported from the same laboratory by Hutchings *et al.* (125). This compound had about the same spectral absorption as the liver and yeast compounds but differed from that of highly purified folic acid. It was about 80 to 90 per cent as active for *L. casei* but only about 6 per cent as active for *S. lactis* R as the liver product.

A still further complication in the picture is brought about by the discovery

[3] The original report did not say whether or not the compound was crystalline but in a private communication Dr. Keresztesy states that it was obtained in crystalline form.

that, in yeast, vitamin B_c also occurs in the form of a conjugate that is highly active for the chick but has little potency for either *L. casei* or *S. lactis* R. However, on digestion with an enzyme found in kidney and other animal tissues but not in yeast, Bird *et al.* (32) found that the conjugate becomes highly active for both organisms. From the digestion material a crystalline compound was isolated identical in properties and potency with the crystals from liver. Another report on the possible existence of a "folic acid"-like material inactive for bacteria but having vitamin activity for the rat has been given by Welch and Wright (433). At the time this review is being written (January 1945), there appear to be: a pure compound that is very active for both organisms, a second that has the same activity as the first for *L. casei* but only one-half the activity

TABLE 2

Source and activity of crystalline or highly purified compounds required by Lactobacillus casei (A.T.C.C. 7469) and Streptococcus lactis R (A.T.C.C. 8043)

PREPARATION	NAME	SOURCE	TEST ORGANISM	AMOUNT FOR ½ MAX. ACTIVITY	INVESTIGATOR
				mγ/ml	
1	Vitamin B_c	Liver	*L. casei*	0·05	Pfiffner *et al.* (309)
	Vitamin B_c	Yeast	*L. casei*	·05	Binkley *et al.* (31)
	Vitamin B_c	Liver	*S. lactis*	·25	Hutchings *et al.* (125)
	Vitamin B_c	Yeast	*S. lactis*	·50	Stokstad (400)
2	Factor SLR	Undisclosed	*S. lactis*	·034	Keresztesy *et al.* (142)
	Factor SLR	Undisclosed	*L. casei*	20 had no effect	Keresztesy *et al.* (142)
3	*L. casei* factor	Undisclosed	*L. casei*	·061	Hutchings *et al.* (125)
	L. casei factor	Undisclosed	*S. lactis*	4·2	Hutchings *et al.* (125)
4	Folic acid	Spinach	*S. lactis*	·055*	Mitchell and Snell (248) Mitchell and Williams (252)
	Folic acid	Spinach	*L. casei*	·072*	Snell (369)

* Calculated for folic acid of 137,000 potency.

of the liver product for *S. lactis* R, a third that is active for *L. casei* and almost inactive for *S. lactis* R, a fourth that is just the reverse of the third, and a fifth, as yet not isolated in a pure state, that is inactive for both organisms.

The similarities and differences among the various compounds can perhaps be most readily seen from the brief tabulation set forth in table 2. Many other papers dealing with the testing and potency of these compounds have appeared, but so far no one group of workers has tested all of the pure compounds. Comparisons of potency are hence difficult to make, but vitamin B_c appears to be the most potent for *L. casei*, and factor SLR the most potent for *S. lactis* R. The potencies reported place these compounds among the extremely active growth factors, of which biotin is perhaps the best example.

It is probable that these compounds possess some unit structure in common. Those compounds for which most information is available, *viz.*, vitamin B_c, *L. casei* factor, and folic acid, contain a carboxyl group. The methyl ester is inactive but much of the potency of the free acid can be recovered after saponification of the ester. The *L. casei* factor appears to contain a free amino group as it becomes inactive on treatment with nitrous acid, on acetylation, or on benzoylation. *L. casei* factor and folic acid have been related to xanthopterin because of a similarity in ultraviolet absorption spectra (Stokstad 400, Hutchings *et al.*, 125, Mitchell, 245, and Bloom *et al.*, 36) and hence it seems probable that the several factors contain a unit structure similar to that of xanthopterin. Additional evidence of a relationship to xanthopterin is found in certain common physiological properties: cure of fish anemia, relation to one another in synthesis or destruction by rat liver, and inhibition of synthesis by *Aerobacter aerogenes* (Wright *et al.*, 465, 467–469).

Although *L. casei* can be grown in continued subculture in a medium containing only crystalline compounds (174, 416), unidentified factors that stimulate initial growth have been reported by Feeney and Strong (92, 93), Pollack and Lindner (313), Light and Clarke (202), Dolby *et al.* (76), and Sprince and Woolley (392), but it is not clear how much of the effect obtained is beyond that resulting from the addition of increased amounts of known compounds, *e.g.*, amino acids and vitamins. Thus Feeney and Strong could replace their yeast extract with a mixture of compounds of which asparagine and glutamine were the most effective. Pollack and Lindner found glutamine was ten times as potent as peptone, the source of their factor, and Chu and Williams (62) report that when glutamine, pyridoxal, and *p*-aminobenzoic acid were added to the basal medium, peptone had no effect. Snell (369) found that 0.5% hydrolyzed casein, the amount usually used in the basal media, does not supply sufficient alanine; and Dolby and Waters (77) obtained increased growth on the addition of leucine, isoleucine, and threonine. Lowry and Bessey (206) discovered that making conditions more anaerobic, *e.g.*, by addition of cysteine to the medium and replacement of air with CO_2, markedly improved acid production and presumably growth of *L. casei*. The size of the inoculum and the way it was prepared also appear to be of importance in determining the effect of stimulating substances. Besides a factor that stimulates initial growth, Dolby, Happold, and Sanford postulate two other unknown factors for *L. casei*, but they do not appear to have excluded the several compounds recently isolated in crystalline form by American investigators.

The situation that exists with respect to *L. casei* appears to be repeated with *S. lactis*. Smith (364), Sprince and Woolley (392), and Wright and Skeggs (464) report growth-promoting effects with unidentified factors obtained from yeast, liver and casein. Niven (285), on the other hand, tested 21 strains of *S. lactis* and all grew in 24 hours in a defined medium provided sufficient unheated filter-sterilized glutamine and asparagine were added to the autoclaved medium. When these compounds were autoclaved in the medium, the response was erratic. Pollack and Lindner (312) and Wright and Skeggs (464) suggest that

asparagine or glutamine may be involved in the structure of proteins or in the synthesis of compounds which are more active for the bacteria than the original compounds.

Factors for other lactic acid bacteria. Orla-Jensen *et al.* (294, 295) have reported that unidentified growth factors contained in milk are required for various lactic acid bacteria. Some of the effects they obtained from "milk bios" were probably due to *p*-aminobenzoic acid and to the recently isolated compounds that promote the growth of *L. casei* and *S. lactis* R.

Möller (254, 255) reported that three unidentified factors, G, H′, and J, were required by *Streptobacterium plantarum*, but apparently these have been replaced by known compounds, for in later papers Möller and Schwarz (259) and Kuhn and Schwarz (178) state that H′ is *p*-aminobenzoic acid, and that the organism can be grown in media containing only known crystalline compounds. In a later paper, Kuhn *et al.* (176) give the composition of the synthetic medium; this consists of 26 very carefully purified compounds (glucose, amino acids, salts and growth factors). An acid-stable factor found in tomato juice that stimulates the growth of *Lactobacillus arabinosus* has been reported by Kuiken *et al.* (180a).

Corynebacterium diphtheriae factors. A group of English investigators (Chattaway *et al.*, 53, 55, 57, 58, 59) have published several notes and papers regarding unknown factors required by *intermedius*, *mitis*, and *gravis* strains of the diphtheria bacteria. The factors appear to be closely related to one or another of the recently isolated *L. casei* and *S. lactis* R factors. Differences or identities can be established only when pure compounds have been tested.

Factors for Erysipelothrix and *Listerella.* Hutner (127) reported that *Erysipelothrix rhusiopathiae* required an unidentified factor found in yeast and peptone, that was partly adsorbed by fuller's earth and completely adsorbed by charcoal. In the same paper Hutner dealt with the requirements of *Listerella monocytogenes* and found that it required an unknown factor also found in yeast and peptone and similar in properties to the erysipelothrix factor. In a later abstract, Hutner (128) states that this factor is not folic acid, xanthopterin, oleate, or acid-hydrolyzed yeast nucleic acid. The factor is found in so-called "vitamin-free" casein and withstands acid hydrolysis.

Gonococcus factors. A thermolabile factor required by certain fastidious strains of *Neisseria gonorrhoeae* has been identified by Lankford and Snell (198) as glutamine, but all strains tested by Lankford *et al.* (197) required one or more thermostable factors found in liver and other tissues. Certain other exacting strains required cocarboxylase in addition to the unidentified factors (Lankford and Skaggs, 196). This requirement for cocarboxylase recalls the relationship between nicotinic acid and coenzymes I and II with respect to the requirements of the hemophilus bacteria. Gould (105) found that glutathione is essential for certain strains of gonococci after they have been cultured in the laboratory for a few weeks. When freshly isolated, they did not require glutathione. This is an example of an induced requirement, a phenomenon that is much less common than a relinquished requirement. Gould also reported that, by suitable treatment, the organisms could be adapted to dispense with glutathione.

Starch has been emphasized by several investigators as having a striking effect on the growth of gonococci, but Gould *et al.* (106) found that it acts only as a protection against the inhibitory effect of certain samples of agar and can be replaced by charcoal. The same authors reported that meat infusion contains an unidentified factor that greatly stimulated the growth of their strains of *N. gonorrhoeae*.

Factors for hemolytic streptococci. Woolley (455), and Sprince and Woolley (392) reported on a factor from liver which promoted the growth of four strains of hemolytic streptococci and one strain of pneumococcus. It is insoluble in most organic solvents, adsorbed with difficulty by charcoal but readily by $BaSO_4$, and is stable to acid and alkali. The most potent concentrates were about 100 times as active as the starting material; about 10γ per ml of medium gave a maximal effect.

Grossowicz (110) has also reported an unknown factor found in tomato juice that is active for hemolytic streptococci. It was fairly resistant to heating under neutral or alkaline conditions but was destroyed by peroxide and other oxidizing reagents.

Miscellaneous factors. Thompson reported (412) that three of nine strains of propionic acid bacteria would not grow in a medium containing the known growth factors but grew satisfactorily when yeast extract was added.

All of the indispensable factors for the continued subculture of *Proteus morganii* are known, but Pelczar and Porter (305) found that meat infusion broth contains a stimulatory substance that about doubles the growth in the chemically-defined medium.

The same situation exists for *Rhizobium trifolii* according to West and Wilson (437a). Various tissue and microbial extracts contain a heat-stable substance that stimulates growth but is not essential for successful continued transfer of the organism on a synthetic medium.

Unidentified factors have been reported for *Bacterium tularense* (407), *Clostridium acetobutylicum* (70) and *Thermobacterium helveticum* (423) but, since not all of the known growth substances were tested, the effects noted may have been due to known rather than to unknown factors.

Interchangeability of growth factors

Large quantities of one or more factors may substitute for small quantities of another factor. Kögl and van Wagtendonk (160) obtained the same growth of *Staphylococcus pyogenes aureus* with large quantities (5γ per ml) of thiamine and nicotinic acid and no biotin as they did with small quantities (0.05γ) of the two factors and 0.005γ of biotin. In other words, increasing the thiamine and nicotinic acid 100-fold dispensed with the need for biotin in the medium. Möller (254) reported that a 1000 times larger dosage of *i*-inositol replaced nicotinic acid for *Streptobacterium plantarum*, and in a later paper (256) he stated that large amounts of pure *l*-tyrosine (including the synthetic compound) could meet the thiamine requirements of this organism. Snell and Mitchell (376) showed that *Streptococcus lactis* R could be cultured in the absence of unknown

factors (later shown to be folic acid) if thymine was added to the medium. Stokes (394) reported that thymine gave complete replacement of folic acid for *S. lactis* R, *Streptococcus durans*, three strains of *Streptococcus faecalis*, and partial replacement for *Streptococcus zymogenes* when used in concentrations about 5,000 times that of folic acid. Stokstad (399), Stokes (394), and Krueger and Peterson (174) have shown that fair though not optimal growth of *Lactobacillus casei* can be obtained in the absence of the various casei factors if liberal quantities of thymine are contained in the medium. Another example of replacement was observed by Snell and Guirard (374) for *S. lactis* R, with purified alanine taking the place of pyridoxine. About 500γ of alanine were equivalent to 1γ of pyridoxine. The authors suggest that alanine may be one of the constituents utilized in the synthesis of pyridoxine. Snell and Mitchell (377) found that methionine, adenine, guanine, xanthine, or hypoxanthine could replace *p*-aminobenzoic acid in the nutrition of *Lactobacillus arabinosus* and *Lactobacillus pentosus*.[4] Snyder and Broh-Kahn (390) report that cysteine can take the place of hemin for *Hemophilus influenzae* but this report was not confirmed by Bass *et al.* (18). The growth of this organism on a hemin-free medium has been a subject of controversy for a long time. Ghon and Preyss (103, 104) maintained that such growth was due to small amounts of hematin in the other constituents of the medium. Besides the ever-present danger of contaminants, the effect of heat sterilization on the constituents of the medium must not be overlooked. Bovarnick (43–46) found that the moderate growth of dysentery bacteria in a medium in which nicotinamide had been replaced by asparagine or glutamic acid was due to the formation of the amide from these two compounds by heat sterilization. Many examples of interchangeability among purines and pyrimidines are noted in table 1.

Structural specificity of compounds

Many papers dealing with the response of bacteria to large numbers of analogs of the vitamins or parts of their molecules have appeared during the past five years. In many cases the analog not only possesses no growth-promoting property but acts as an inhibitor toward the growth factor. This inhibition may usually be reversed by addition of larger quantities of the growth factor to the medium. The promotion of growth by one compound and inhibition of growth by another is interpreted as indicating that the two compounds play competing roles in some enzyme system. As Koser and Saunders (167) listed the papers that dealt with derivatives of thiamine, riboflavin, and nicotinic acid and parts of the molecule of thiamine and pantothenic acid, in general only papers that have been published since the preceding review will be mentioned here.

Thiamine. Knight (154) found that the pyrimidine and thiazole parts of

[4] In recent personal correspondence Dr. Snell says that their medium apparently contained small amounts of *p*-aminobenzoic acid which in the presence of methionine and the purines were adequate for the needs of the bacteria. Much larger amounts of *p*-aminobenzoic acid were required for growth when these substances were omitted. Landy and Streightoff (195) have also noted that purines markedly increased the sensitivity of *Acetobacter suboxydans* to *p*-aminobenzoic acid.

thiamine together were as active on a molar basis as the intact molecule for *Staphylococcus aureus*. Sarett and Cheldelin (337), on the other hand, obtained no response to the mixture of pyrimidine and thiazole halves of the thiamine molecule with *Lactobacillus fermentum*.

The pyridine analog of thiamine, 2-methyl-4-amino-5-pyrimidyl-methyl-(2-methyl-3-hydroxyethyl)-pyridinium bromide first called "heterovitamin B_1" by Baumgarten and Dornow (22) and Schopfer (345a) but more appropriately named pyrithiamine by Woolley and White (461) has slight growth-promoting properties but is more noteworthy because of its antagonistic action toward thiamine. *S. aureus* which requires either thiamine or the two parts of thiamine in the medium was rather susceptible to pyrithiamine. The inhibition index (ratio of pyrithiamine to thiamine) was 2000. Bacteria that can synthesize thiamine were generally insensitive to pyrithiamine (inhibition index greater than 2,000,000) but the quantity of thiamine synthesized (*e.g.* 0.02γ/ml in the cells of *E. coli*) appeared insufficient to neutralize the quantity of pyrithiamine in the medium (500γ/ml). Wyss (470) tested pyrithiamine as a therapeutic agent against *S. aureus* but since non-toxic levels were not antibacterial in the blood, he concluded that it had little chemotherapeutic value. Sarett and Cheldelin (338) found pyrithiamine was more inhibitory to the utilization of diphosphothiamine (cocarboxylase) than of thiamine for the growth of *Lactobacillus fermentum*.

Riboflavin. Derivatives of riboflavin have been investigated by Snell and Strong (381) for potency toward *Lactobacillus casei* and for *Streptobacterium plantarum* and *Bacterium lactis acidi* by Kuhn (175) and Möller (255). Few modifications were equal to the natural compound, and many were inactive. The same compounds were tested on rats and the results in general were in good agreement with the bacteriological data. Several examples of the competitive action between riboflavin and structurally related compounds, *e.g.*, 6,7-dichlorriboflavin (Kuhn *et al.*, 179), a phenazine analog (Woolley, 457), propamidine and other antimalarial drugs (Madinaveitia, 235) with respect to lactic acid bacteria and hemolytic streptococci have been reported. Kuhn *et al.* made the interesting observation that the inhibition ratio of analog to riboflavin increased with time of incubation. About six times as much inhibitor was required for a six-day incubation of *S. plantarum* as for a two-day period. The authors interpreted the results as indicating that the bacteria slowly synthesized riboflavin and eventually overcame the action of the dichlor derivative.

Nicotinic acid. Pelczar and Porter (301) tested thirteen compounds related to nicotinic acid for activity toward 189 strains of *Proteus vulgaris* and related species. Only compounds closely related to nicotinic acid, *e.g.*, salts, amide, and ester, were active. Coramine (diethyl nicotinamide) was found to be active for *P. vulgaris* as is also the case for *Streptobacterium plantarum* (Möller and Birkofer, 257), but was found inactive for *Lactobacillus arabinosus* (Teply and Elvehjem, 409). Naturally occurring bound forms of nicotinic acid have been reported and their properties studied by Oser *et al.* (297), Andrews *et al.* (8), and Krehl *et al.* (171, 172).

Thiazole 5-carboxylic acid (isosteric with nicotinic acid) had about 0.1% of the activity of nicotinic acid for dysentery bacilli (Schmelkes, 345). Pyridine-3-sulfonic acid, the sulfur analog of nicotinic acid, was practically without effect on the growth of *Proteus vulgaris* but strongly inhibited the development of *Staphylococcus aureus* and *Streptobacterium plantarum* (McIlwain, 225; Möller and Birkofer, 257). The inhibition was counteracted by more nicotinic acid or amide and in case of *S. plantarum* by heavy metals, especially iron (257).

Picolinic acid also inhibited *S. plantarum*. Nicotinic acid failed to reverse this inhibition but heavy metals (zinc was the most effective) counteracted the picolinic acid completely (Möller and Birkofer, 258). Wood and Austrian (451) also showed that nicotinamide and cozymase block the action of chemically unrelated compounds. Structural similarity appears thus not to be the only basis for antagonism between compounds.

The requirement of *Hemophilus parainfluenzae* for cozymase has been shown by Schlenk and Gingrich (343) and Gingrich and Schlenk (104b) to reside in the inability of the organism to link the nicotinamide with the ribose. Nicotinamide nucleoside but not other parts of the nucleotide can replace the cozymase molecule.

Pantothenic acid. Additional reports showing the replacement of pantothenic acid by β-alanine for subspecies of *Corynebacterium diphtheriae* (Evans *et al.*, 88, 89), and *Rhizobium trifolii* (West and Wilson, 440) have been published. The butyrolactone part of the pantothenic acid molecule is all that is needed by a strain of hemolytic streptococci (Woolley, 453) and by a culture of *Acetobacter suboxydans* (Underkofler *et al.*, 417). Either of the two components as well as the pantothenic acid itself stimulates the growth of *Brucella suis* (Koser *et al.*, 163). Numerous combinations of analogs of the β-alanine part of pantothenic acid (Weinstock *et al.*, 431; Kuhn *et al.*, 180; Snell, 366, 367; Barnett and Robinson, 15; McIlwain, 226–233; Pollack, 311; Madinaveitia *et al.*, 236) and of the butyrolactone part (Woolley and Hutchings, 458, 459; Subbarow and Rane, 405; Mitchell *et al.*, 249) have been tested with various bacteria but the potency of every compound has been either negative or much less than that of the natural component. Compounds containing the sulfur analogs of β-alanine, *e.g.*, pantoyltaurine, are especially interesting since like the sulfa drugs they possess inhibiting properties and their effect can be reversed by pantothenic acid. The reversibility suggests that pantothenic acid and pantoyltaurine probably play competing roles in some enzyme system. Strains of bacteria that are resistant to pantoyltaurine usually have the ability to synthesize pantothenic acid. Resistance is possessed by some strains that occur in nature but can be induced in others.

Pyridoxine. The specificity of various derivatives of pyridoxine has been determined for *Streptobacterium plantarum* (Möller, 255) and *Lactobacillus casei* (Bohonos *et al.*, 38) and comparison made with the response of the rat to the same compounds. A good parallelism was found between microbiological and rat potency, but no derivatives were as active as pyridoxine itself.

In contrast to the generally lesser activity of analogs of growth factors, Snell

et al. (375) have discovered a naturally occurring derivative of pyridoxine, provisionally called pseudopyridoxine, that is several thousand times more active for *Streptococcus lactis* R than the laboratory compound. Other lactic acid bacteria showed a similar response to pseudopyridoxine. In a later paper, Snell (368) showed that the activity of pyridoxine could be increased many fold by autoclaving the pyridoxine with the medium. Autoclaving with amino acids, ammonia, and other constituents of the medium also brought about the conversion. As a result of a cooperative effort to solve the problem, Snell (370–372) and Harris *et al.* (118) have discovered that the activity of pseudopyridoxine can be accounted for by two synthetic compounds, "pyridoxal" and "pyridoxamine." In these compounds the 4-hydroxymethyl group of pyridoxine is replaced by a formyl (—CHO) or an aminomethyl ($—CH_2NH_2$) group, respectively. These compounds are from 5000 to 9000 times as active as pyridoxine for *S. lactis* R, but all three compounds have the same activity for *Saccharomyces carlsbergensis*. This difference in response permits a differential assay for pyridoxine and pseudopyridoxine. The latter comprises a considerable portion (from less than 5 to more than 50%) of the total vitamin B_6 present in food, body tissues and urine In a personal communication, Dr. Snell states that "pyridoxal and pyridoxamine are active for all organisms so far tested which need pyridoxine as well as for some that do not use pyridoxine." It is probable that the functional forms of pyridoxine are compounds identical with or closely related to the aldehyde or amine. Some organisms, *e.g.*, yeast, readily bring about the transformation from the relatively inactive to the very active form. The inability of an organism to bring about this transformation might explain failure to grow on a synthetic medium. Obviously much work done with pyridoxine should be repeated with the two new compounds. Another outgrowth of this work is to reduce the apparent requirement of certain organisms for pyridoxine. The quantity of pyridoxine required by many bacteria has always seemed unusually high, but if pyridoxal (or pyridoxamine) is taken as the standard, the amount of these compounds needed places them among the very active growth factors. Another oxidation product of pyridoxine, a lactone, has recently been reported by Scott *et al.* (348) to promote the growth of *L. casei*.

p-Aminobenzoic acid (PAB). Since Woods' (452) discovery of the antagonistic action between PAB and sulfanilamide and the consequent identification of PAB as a growth factor, many papers have appeared dealing with various aspects of the relationship between the two compounds. The literature on the subject is beyond the scope of this review and only one or two phases of the problem will be mentioned. While most papers feature PAB as the antagonist of the sulfa drugs, several other compounds have been found to inhibit their action. Methionine, guanine, adenine, hypoxanthine, xanthine, and urethane reverse the action of sulfonamides under some conditions (Bliss and Long, 35; Harris and Kohn, 117; Snell and Mitchell, 377; Johnson, 134a; Kohn and Harris, 161). Combinations of PAB with adenine, hypoxanthine, or xanthine were more effective than would be expected from the amounts of the two compounds used (377). Many sulfur-free compounds (*e.g.*, 4,4′-diaminobenzophenone, phosphanilic

acid, 4,4′-diaminobenzil, 2,2′-dihydroxybenzil) that are structurally related to PAB and antagonize its growth-promoting action have been prepared and tested by Kuhn and associates (175a, 176, 177). Auhagen (11) reported that on a molar basis *p*-aminobenzoyl-*l*-glutamic acid (PABG) was 8 to 10 times as potent as PAB in reversing the action of sulfanilamide on *Streptobacterium plantarum*, but Williams (443) was not able to confirm this result with a similar organism, *Lactobacillus plantarum*. Williams found PAB was 20 times as active as PABG for a closely related organism, *Lactobacillus arabinosus*, and hundreds of times more potent than PABG for *Escherichia coli*, *Clostridium acetobutylicum*, *Streptococcus pyogenes*, *Diplococcus pneumoniae*, and *Acetobacter suboxydans*.

The existence of other antagonists to the sulfa drugs than PAB has been reported in yeast extract (Loomis *et al.*, 205) and in bacterial cultures (Green, 107; Green and Bielschowsky, 108; and Mirick, 244). Sevag and Green (351) concluded that the insensitivity of resistant strains of staphylococci is not associated with the formation of PAB. However, Landy *et al.* (192–194) and Spink *et al.* (391) have shown that strains of *Staphylococcus aureus* which are resistant to sulfonamide drugs produced on the average from 40 to 70 times as much PAB as non-resistant strains of the same organism. The exclusion of PAB on the basis of unlike chemical properties and quantitative data does not appear to rest on too firm a basis because of the uncertainty regarding the properties and quantitative determination of bound PAB.

Rubbo *et al.* (335) reported that *p*-aminophenylacetic acid was ten times as active as PAB for *Clostridium acetobutylicum*, but Wyss *et al.* (470a) and Lampen and Peterson (183) obtained an activity of only about 0.1% of that given by PAB. When tested on *Acetobacter suboxydans*, which requires PAB, *p*-aminophenylacetic acid showed 2 per cent of the activity of PAB. Many other compounds related to PAB have been tested by the above groups of workers but most of these had little activity.

Biotin. Two forms of biotin have been reported to exist in nature and many papers dealing with their isolation, structure and potency have appeared (Kögl and associates, 157–159; du Vigneaud and associates, 419, 421). The first obtained in crystalline form from egg yolk by Kögl and Tönnis (158), is designated α-biotin by Kögl and ten Ham (157) and the second, obtained by du Vigneaud and associates (421) from liver is called β-biotin by the Dutch investigators. In a recent review on biotin, Melville (240a) points out that the structure proposed for α-biotin has not been confirmed by synthesis as has been done with β-biotin and also notes that the conclusion of Kögl and associates regarding the structure of β-biotin is not in agreement with the work of the American investigators. The existence of two forms of biotin therefore appears to be an open question. Aside from differences in structure, a difference in activity is said to distinguish the two compounds. α-Biotin is reported to be only one-half as active for yeast as β-biotin. No reports comparing the activity of the two biotins for bacteria have been noted.

Various compounds related to β-biotin have been tested for their bacterial activity. The methyl ester is as active as the free acid for some bacteria, *e.g.*,

Staphylococcus aureus, *Clostridium butylicum*, but by others, *e.g.*, *Lactobacillus casei*, *Streptobacterium plantarum*, it is used with difficulty (Möller, 255; Stokes and Gunness, 395; and Tomlinson and Peterson, 416). Synthetic biotin had the same potency as natural biotin, *dl*-biotin was only 50% as potent, and *l*-biotin and *dl*-allobiotin had only slight activity (due perhaps to contamination with the active *d*-biotin) for *L. casei* and *L. arabinosus* (Stokes and Gunness, 396a).

Several papers (Dittmer *et al.*, 74; Dittmer and du Vigneaud, 75; Lilly and Leonian, 203; and Stokes and Gunness, 396a) deal with the activity of compounds structurally related to biotin. None of these compounds have any real growth-promoting effect but several of them have antibiotin activity, and the inhibition can be reversed by the addition of more biotin. Most of the work has been done with desthiobiotin (a sulfur-free compound) and *L. casei*. Biotin sulfone, however, has greater antibiotin activity than desthiobiotin. Some bacteria that require biotin (*L. arabinosus* and *Rhizobium trifolii*) were not inhibited by large additions of desthiobiotin to the medium. In contrast to yeasts, which are able to reform the sulfur ring, bacteria do not seem to have this synthetic ability. Benzimidazole, which is also structurally related to biotin, inhibited the growth of *Streptococcus lactis* R and *Escherichia coli*. Biotin did not counteract the effect of benzimidazole on either organism but guanine and adenine overcame its action on *E. coli*, and uracil removed the inhibition toward *S. lactis* R (Woolley, 456).

By treatment of urine or vitab hydrolysates, Burk and Winzler (47) have obtained biotin-like products that have been designated miotin, tiotin, and rhiotin, depending on their reaction to heat, avidin, yeast, and rhizobium bacteria. As these products have not been isolated and identified, and as many of the properties attributed to them are possessed by known derivatives of biotin, *e.g.* desthiobiotin and diaminocarboxylic acid, little advantage seems to be gained from the introduction of these terms.

Pimelic acid, discovered by Mueller (263, 264) to be a growth factor for the diphtheria bacillus, is apparently a precursor of biotin (du Vigneaud *et al.*, 420). No other biotin-requiring bacterium has been found able to use pimelic acid in place of biotin. Wright (462) and Hutner (129) obtained negative results with *Lactobacillus casei* and *Rhodospirillum rubrum*, respectively.

SYNTHESIS OF GROWTH FACTORS

In table 1, a record indicated by the letter S was made of the synthesis of growth factors which need not be supplied to the bacteria named. Besides these, there is a larger number of bacteria that need no preformed growth factors. Bacteria that can grow on a sugar + salts medium must of course synthesize all the growth factors required in their metabolic processes. A list of all bacteria reported to synthesize one or more growth factors is given in table 3. Probably less than ten species have been tested for their ability to synthesize all of the B vitamins listed in table 1, and less than a dozen have been tested for the presence or absence of as many as four of these compounds. On the other hand, it is generally assumed that all of these compounds are utilized in the growth and

metabolism of all bacteria. The limited information available lends support to this view The most complete data bearing on the subject are those of Thompson (411) He reports data for eight B-vitamins in cells and medium of bacteria from five different genera grown for 24 hours on the same medium. Arranged

TABLE 3

Synthesis of growth factors by bacteria

FACTOR	ORGANISMS, REFERENCES, AND CONCENTRATION RANGE
p-Aminobenzoic acid	*Alcaligenes faecalis* (193); *Aerobacter aerogenes* (193); *Bacillus megatherium* (193); *B. subtilis* (193); *B. vulgatus* (193); *Brucella abortus* (193); *Clostridium botulinum* (193); *C. sporogenes* (193); *C. tetani* (193); **Corynebacterium diphtheriae* (193); *Diplococcus pneumoniae* (193); *Eberthella typhosa* (193); *Escherichia coli* (193); *Klebsiella pneumoniae* (193); *Lactobacillus casei* (193); *L. delbrückii* (193); **Myocbacterium tuberculosis* (193); *M. stercoris* (193); **M. smegma* (193); *Neisseria gonorrhoeae* (191); *Proteus vulgaris* (193); **Pseudomonas aeruginosa* (193); *Salmonella paratyphi* (193); **S. schottmuelleri* (193); *Serratia marcescens* (193); *Shigella dysenteriae* (193); *S. paradysenteriae* (193); **Staphylococcus albus* (193); **S. aureus* (193); staphylococci (resistant strains (391)); *Streptococcus hemolyticus* (193); *S. salivarius* (193); **S. scarlatinae* (193) Range: 0.003γ/ml, cells and culture filtrate, *C. sporogenes*, to 3.3γ/ml, *S. aureus*
Biotin	*Alcaligenes faecalis* (48, 187); *Acetobacter suboxydans* (417); **Aerobacter aerogenes* (187, 411); *Azotobacter vinelandii* (200); *Bacillus anthracis* (187); *B. subtilis* (187); *B. vulgatus* (48); *Bacterium aerogenes* (48); *Clostridium acidi urici* (14); *Eberthella typhi* (187); **Escherichia coli* (48, 101, 187, 241); *Klebsiella pneumoniae* (187); **Mycobacterium tuberculosis* (187); **Phytomonas tumefaciens* (234); *Proteus vulgaris* (48, 411); *Pseudomonas aeruginosa* (187); **P. fluorescens* (411); *Sarcina lutea* (187); *Serratia marcescens* (187, 411); **Staphylococcus aureus* (187); *Thiobacillus thiooxidans* (290) Range: 0.0005γ/ml, cells and culture filtrate, *A. faecalis*, to 0.035 γ/ml, *P. tumefaciens*
Nicotinic acid	*Alcaligenes faecalis* (48); *Aerobacter aerogenes* (411); **Azotobacter vinelandii* (200); *Bacillus mesentericus* (48); *B. vulgatus* (48); *Bacterium aerogenes* (48); **Clostridium butylicum* (411); *Corynebacterium diphtheriae* (424); *Escherichia coli* (48); **Proteus vulgaris* (411); *Pseudomonas fluorescens* (411); *Serratia marcescens* (411); *Shigella paradysenteriae* (169); *Thiobacillus thiooxidans* (290) Range: 0.028 γ/ml, cells and culture filtrate, *E. coli*, to 4.6 γ/ml, *A. vinelandii*

* Denotes organisms that have been reported to be good producers of the factor; *i.e.*, approximately one-half or more of that reported for the best producer.

TABLE 3—*Continued*

FACTOR	ORGANISMS, REFERENCES, AND CONCENTRATION RANGE
Pantothenic acid	**Aerobacter aerogenes* (411); **Azotobacter vinelandii* (200); **Clostridium butylicum* (411); *Phytomonas tumefaciens* (234); *Proteus vulgaris* (411); *Pseudomonas fluorescens* (411); *Rhizobium meliloti* (217); *Serratia marcescens* (411); *Thiobacillus thiooxidans* (290) Range: 0.0303 γ/ml, cells and culture filtrate, *P. vulgaris*, to 0.99 γ/ml, *A. vinelandii*
Pyridoxine	*Aerobacter aerogenes* (411); **Clostridium butylicum* (411); *Lactobacillus arabinosus* (38); *L. pentosus* (38); *Leuconostoc mesenteroides* (38); *Proteus vulgaris* (411); **Pseudomonas fluorescens* (411); **Serratia marcescens* (411); *Thiobacillus thiooxidans* (290) Range: 0.0049 γ/ml, cells and culture filtrate, *P. vulgaris*, to 0.0242 γ/ml, *P. fluorescens*
Riboflavin	*Alcaligenes bookerii* (415); *A. faecalis* (48); *A. viscosus* (330); **Acetobacter suboxydans* (330, 417); *Achromobacter delicatulium* (415); *A. radiobacter* (330); *Aerobacillus polymyxa* (330); *Aerobacter aerogenes* (330, 411, 415); *A. cloacae* (415); *A. oxytocum* (415); *Azotobacter agile* (393a); *A. chroococcum* (330, 393a); *A. vinelandii* (200, 330, 393a); *Bacillus albolactis* (415); *B. cereus* (415); *B. cohaerens* (415); *B. graveolens* (415); *B. globigii* (330); *B. mesentericus* (48); *B. mycoides* (330, 415); *B. niger* (330, 415); *B. rotans* (415); *B. ruminatus* (415); *B. subtilis* (330, 415); *B. vulgatus* (48, 415); *Bacterium aerogenes* (48); *B. brassicae* (381); *B. herbicola*; (330); **Clostridium acetobutylicum* (330, 471); *C. acidi urici* (14); **C. butylicum* (411); **C. butyricum* (425a); *C. felsineum* (330); *C. pasteurianum* (425a); *C. roseum* (330); *Corynebacterium diphtheriae* (67, 73, 88, 330, 424); *Eberthella typhi* (330); *Escherichia coli* (48, 330, 415, 461); *Flavobacterium sulfuricum* (330); *Klebsiella pneumoniae* (415); *Lactobacillus arabinosus* (330); *L. brassicae* (381); **L. delbrückii* (425a); *L. helveticus* (2); *L. pentosus* (381); *Leuconostoc mesenteroides* (381); *Micrococcus casei* (415); *M. cereus* (415); *M. citreus* (415); *M. freudenreichii* (415); *M. percitreus* (415); *M. perflavus* (415); *M. subflavus* (415); *M. ureae* (415); **Mycobacterium tuberculosis* (40, 333, 401); *M. smegmatis* (330); *Neisseria catarrhalis* (330); **Phytomonas tumefaciens* (234, 330); *Proteus vulgaris* (48, 330, 411, 415); *Pseudomonas aeruginosa* (330); *P. fluorescens* (292, 294, 330, 411); *P. pyocyaneus* (292, 294); *Rhizobium trifolii* (437); *Salmonella schottmuelleri* (330); *Sarcina lutea* (415); *Serratia marcescens* (330, 411, 415); *Shigella dysenteriae* (330); *Shigella paradysenteriae* (80); *Spirillum serpens* (330); *Staphylococcus albus* (330, 415); *S. aureus* (289, 330 415); *S. flavus* (289); *Streptococcus bovis* (415); *S. lactis* (381); *Thiobacillus thiooxidans* (290) Range: 0.02 γ/ml, *Bacterium herbicola*, to 8.5 γ/ml, *Acetobacter suboxydans*

TABLE 3—*Concluded*

FACTOR	ORGANISMS, REFERENCES, AND CONCENTRATION RANGE
Thiamine	**Alcaligenes faecalis* (48); *Aerobacter aerogenes* (411); *Azotobacter vinelandii* (200); *Bacillus mesentericus* (48); **B. vulgatus* (48, 111); **Bacterium aerogenes* (48); *Clostridium butylicum* (411, 461); *Corynebacterium diphtheriae* (88, 424); *Escherichia coli* (48, 102, 461); hemolytic streptococci (286, 461); *Lactobacillus arabinosus* (461); *L. casei* (461); *Propionibacterium freudenreichii* (408); *P. pentosaceum* (361); **Proteus vulgaris* (48, 411); *Pseudomonas fluorescens* (411); *Rhizobium trifolii* (437); *Serratia marcescens* (411); *Streptococcus durans* (286); *S. faecalis* (286); *S. liquefaciens* (286); *S. zymogenes* (286); *Thiobacillus thiooxidans* (290) Range: 0.0032 γ/ml, cells and culture filtrate, hemolytic streptococci, to 0.150 γ/ml, *B. vulgatus*
Vitamin K	*Bacillus coli* (68, 69, 296); *B. cereus* (4); *B. mycoides* (4); *B. subtilis* (4); *Bacterium aerogenes* (4); *B. bifidum* (296); *B. flexneri* (4); *B. proteus* (4); *B. typhosum* (4); *Erythrobacillus prodigiosus* (4); *Escherichia coli* (4); *Microbacterium lacticum* (296); *Mycobacterium phlei* (460); *M. tuberculosis* (4, 6a); *Sarcina lutea* (4); *Staphylococcus aureus* (4); *Streptococcus, faecium* (69) Range: Expressed as 2 methyl-1,4-naphthoquinone. From 8 γ per gram dry weight of cells, *B. bifidum*, to 152 γ per gram dry weight of cells, *B. subtilis*
Norite eluate factor, folic acid, vitamin B_c	*Aerobacter aerogenes* (465); *Azotobacter vinelandii* (200); *Bacillus lactis acidi, B. brassicae* (124); *Clostridium acidi urici* (14); *C. butylicum* (411); *Escherichia coli* (241); *Lactobacillus arabinosus* (124); *L. gayonii* (124); *L. pentosus* (124); *Leuconostoc mesenteroides* (124); *Proteus vulgaris* (411); *Pseudomonas fluorescens* (411); *Serratia marcescens* (411) Range: Not expressed in terms of a pure compound
Miscellaneous	
Anti-hemorrhagic compounds	*Mycobacterium phlei* (460)
Inositol	*Aerobacter aerogenes* (411); *Clostridium butylicum* (411); *Proteus vulgaris* (411); *Pseudomonas fluorescens* (411); *Serratia marcescens* (411)
Uracil	*Bacterium typhosum* (329); *Staphylococcus aureus* (329)
Vitamins B_{10} and B_{11}	*Mycobacterium tuberculosis* (243)
P factor	*Brucella abortus* (107)

roughly in descending order of growth-factor productivity (cells and medium) the bacteria were as follows: *Pseudomonas fluorescens, Aerobacter aerogenes, Serratia marcescens, Clostridium butylicum, Proteus vulgaris.* In the cells the compound present in smallest amount, biotin, varied from 1.7 (*P. vulgaris*) to 7.1 (*P. fluorescens*) micrograms per gram of dry cells (equivalent to from 2300

to 4000 ml of medium). The compound present in next to largest amount, niotinic acid, ranged from 200 to 300γ. Inositol, which has not been reported to be required by any bacteria, amounted to from 870 to 1700γ.

The distribution of the compounds between cells and cell-free medium was generally in favor of the medium. This was outstandingly so for biotin, where about 90% of the total was found in the medium and only 10% in the cells. Thiamine was distributed about equally between cells and medium. Since the incubation period was only 24 hours, Thompson concluded that the compounds found in the medium represented secretion rather than autolysis of dead cells. Confirming evidence was obtained for biotin in case of *P. vulgaris* by successive analyses during the incubation period. Based on the percentage of the total produced in 48 hours, the biotin in the medium "led" that in the cells.

The effect of adding each growth factor in turn to the medium on the synthesis of the others by *A. aerogenes* was tested with negative results. Likewise the addition of about 1 microgram of riboflavin per ml of medium caused no increase in the riboflavin content of the cells and even slightly more riboflavin was synthesized than when no riboflavin was added. One might say that the cells "preferred" to synthesize their riboflavin rather than to take it from the medium.

Burkholder and McVeigh (48) reported data for the synthesis of four growth factors by six species of intestinal bacteria, grown for 48 hours. Two of the species, *A. aerogenes* and *P. vulgaris*, were the same as those Thompson used. Calculated to a dry matter basis (assumed to be 25%), Burkholder and McVeigh's figures are in general higher than Thompson's; in case of thiamine, 16 to 18 times higher. These differences are not to be stressed, since variation in strain, medium, aeration, incubation period, etc. probably account for them. By selection of strains and modifying the cultural conditions, the productivity can certainly be increased many fold.

Sevag and Green (350) have shown that tryptophane is required for the synthesis of arylamines (PAB ?) by *Staphylococcus aureus*. Lysine, threonine, and alanine appear to be required for the synthesis of pyridoxine by *Lactobacillus arabinosus* (Stokes and Gunness, 396).

Besides the papers dealing with the synthesis of vitamins by pure cultures of bacteria, there is a considerable literature that is concerned with the synthesis of B vitamins by bacteria in the intestinal tract of animals and man. The synthesis of B vitamins in the rumen of cattle is sufficient to meet all the needs of the animal for these vitamins. In the rat, a biotin deficiency cannot be obtained without the use of egg white to bind the biotin that is produced by bacteria in the cecum and lower intestine. The synthesis of the B vitamins in the intestinal tract of animals thus becomes a matter of considerable nutritional importance. Likewise this synthesis is a complicating factor in the interpretation of the data obtained in feeding experiments. A number of papers dealing with the effect of diet, species, and other factors on the intestinal synthesis of growth factors have appeared for the following compounds: biotin (222, 246, 278, 427); nicotinic acid (246, 427); pantothenic acid (221, 246, 427); pyridoxine (221, 246, 262, 427); riboflavin (221, 236a, 246, 262, 427); thiamine (1, 221, 246, 427); inositol (246, 455a); vitamin K (221); and folic acid (246).

BACTERIOLOGICAL ASSAYS FOR GROWTH FACTORS

One of the striking developments in the field of growth factors in recent years is the use of microörganisms for the quantitative determination of these compounds in foods, normal and abnormal tissues, blood, feces, urine, bacteriological media, vitamin products, and many other biological materials. Probably more analyses are made by microbiological than by chemical methods. Many of the B vitamins cannot yet be determined by chemical methods and the advent of microbiological procedures has been of enormous advantage to the industries that manufacture products featuring vitamin content. Of the ten vitamins in the B-group, eight can be readily determined by means of bacteria, four can be assayed by means of yeasts, and four lend themselves well to chemical determination. Inositol can be determined by means of yeast but not with bacteria, because as yet no bacterium has been shown to require this compound in the culture medium.

While the use of bacteria for assay purposes had been suggested previously, the first widely used method was that proposed by Snell and Strong in 1939 (382) for riboflavin by means of *L. casei* (A.T.C.C. 7469). In the next five years nearly a score of papers dealing with this one method were published. During the same period this bacterium has been used successfully for the determination of pantothenic acid, biotin, and *L. casei* factor (vitamin B_c, folic acid) and less satisfactorily for nicotinic acid and pyridoxine. Its growth is only slightly stimulated by thiamine and *p*-aminobenzoic acid, hence it is unsuitable for the determination of these compounds. Nearly forty papers have been published dealing with the use of this one microörganism. This is approximately one-half of all papers published on bacteriological assay methods in the past five years.

Many of the methods that have been published have been found to have serious faults which came to light as the methods were applied to a wide variety of materials. Sensitivity to compounds (*e.g.*, fatty acids) never suspected of playing a part in the nutrition of bacteria, free and bound forms of vitamins occurring in nature, effect of structure on activity (leading to a rational explanation of the action of certain drugs) are some of the products of research on quantitative methods.

Theoretically an assay method should be possible for any compound that is required by a microörganism, but in practice some microörganisms are more satisfactory than others. Some of the necessary conditions that should be fulfilled are as follows:

1. The medium should contain all constituents that are necessary for optimum development and activity of the microörganism other than the factor to be determined. Such a medium is indicated by the production of a low turbidity or acidity in the absence of the factor and optimum growth and formation of products in its presence. With an excess of the factor the development of the microörganism should be equal to that in a natural medium of comparable sugar and nitrogen content.

2. The response of the microörganism (as measured by cell growth, products,

or other index) to increasing quantities of the factor should be regular and preferably proportional. Equal response to all forms of the compound on a molar basis is desirable. A striking example of non-equivalence is illustrated by the difference in response of *L. casei* to pyridoxine, pyridoxal, and pyridoxamine. No satisfactory bacterial assay for all three of these compounds appears to be available at the present time.

Measurement of combined forms of growth factors is particularly difficult. While some of these, *e.g.*, coenzyme I and riboflavin nucleotides, appear to be equivalent on a molar basis to the free compound, in other cases (*e.g.* biotin, pantothenic acid), the bound form is unavailable to the assay organism. The growth factor must then be set free by means of chemical reagents or enzymes. Some growth factors are labile to acid and alkali; and enzymes do not always release the compound from its bound form. The ideal microörganism might be one that has strong amylolytic and proteolytic powers so as to enable it to use all forms of the compound and thus approximate the action of digestive enzymes in animal nutrition. On the other hand, the microörganism should not respond to degradation products of the compound. Up to the present no bacterium possessing all these desirable features has been found.

3. In the assay of natural materials, the responses at different levels should give the same value when this is calculated per gram or other unit of the material. Irregular values are indicative of another factor or factors in the material which may be stimulatory or inhibitory at different levels.

4. Repeated assays of the same material should check, *e.g.*, within 5%. A suitable sample repeatedly analyzed serves as a reference material and should be included in every large series of analyses as a useful check on the assay as a whole.

5. The sample should contain no inhibitory or toxic substances. If such substances are present, the results will probably be variable, as mentioned under 3. Such a substance may be present in the original material or be produced by the treatment of the material, *e.g.*, decomposition products or excess salts.

6. A standard inoculum should be used. To start with a sturdy and stable microörganism is essential. The stock culture should be carried on a medium that maintains the organism in a stable condition, and the inoculum should be developed in the same way each time. Obviously such points as age and size of inoculum are of great importance.

7. The microörganism should preferably be non-pathogenic. If the method is to be widely used and consequently by technicians, a pathogen is dangerous, and necessary precautions reduce the number of assays that can be performed in a given time.

8. The method should be rapid. In research work, the time required for an assay should be 1 to 3 days, and for control work in industry 5 to 20 hours.

Even if these requirements are met to a reasonable degree, there still is no substitute for skill and experience on the part of the analyst. Perfection in the method should not be expected. Even after more than half a century of use and scores of modifications the Kjeldahl method for total nitrogen is still not perfect.

TABLE 4

Bacteriological assay methods

GROWTH FACTOR	TEST ORGANISM	PRINCIPLE OF MEASUREMENT	TIME OF TEST	RANGE OF STANDARD PER ml	REFERENCES
			hours		
p-Amino-benzoic acid	*Acetobacter suboxydans*	Turbidity	48	0–10 mγ	189, 195, 247
	Clostridium acetobutylicum	Turbidity	20–24	0–0·15 mγ	184
	Lactobacillus arabinosus	Acidity	72	0–0.05 mγ	201
Biotin	*Clostridium butylicum*	Turbidity	72	0–0.1 mγ	181
	Lactobacillus arabinosus	Acidity	72	0–0.25 mγ	466
	Lactobacillus casei	Acidity	72	0–1 mγ	74, 188, 353, 354, 395, 416, 445
	Leuconostoc mesenteroides	Turbidity or acidity	72	0–1 mγ	100
	Rhizobium trifolii	Turbidity	72	Yeast extract	440
Choline	*Pneumococcus* Type III	Turbidity	12–24	0–6 γ	12
Coenzymes I and II	*Hemophilus influenzae*	Nitrite production	48	0–0.037 γ	121
	H. parainfluenzae	Turbidity	24–30	Yeast extract = 0–0.18 mg of fresh yeast	160a
	H. parainfluenzae	Turbidity	40–42	2.5–20 mγ	310
	H. parainfluenzae	Turbidity	24–29	0–0.06 γ	261
Nicotinamide	*Shigella dysenteriae*	Turbidity	16–22	0–0.025 γ	132
Nicotinic acid	*Acetobacter suboxydans*	Turbidity	48	0.25–3.0 γ	138
	Bacillus proteus	Turbidity	30	0–0.1 γ	214
	Lactobacillus arabinosus	Acidity	72	0–0.1 γ	8, 60, 109, 131, 173, 239, 387 388
	L. arabinosus	CO_2 liberation by acid formed	3	0–13.3 mγ	10
	L. casei	Acidity	72	0–0.1 γ	188
	Leuconostoc mesenteroides	Turbidity or acidity	72	0–0.1 γ	100

TABLE 4—*Continued*

GROWTH FACTOR	TEST ORGANISM	PRINCIPLE OF MEASUREMENT	TIME OF TEST	RANGE OF STANDARD PER ml	REFERENCES
			hours		
Nicotinic acid (*Cont'd*)	*Dysentery bacillus (Shigella?)*	Acidity	4 days	.001–.010 γ	79
Pantothenic acid	*Lactobacillus arabinosus*	Acidity	72	0–0.02 γ	362
	L. casei	Acidity	72	0–0.20 γ (Ca salt)	20, 50, 188, 202, 275, 276, 307 308, 355, 393, 398, 403, 425, 442, 463
	Leuconostoc mesenteroides	Turbidity or acidity	72	0–10 mγ	100
	Proteus morganii	Turbidity, pH or bacterial nitrogen	24	0–1 mγ	303, 304
(β-Alanine)	*Corynebacterium diphtheriae*	Bacterial nitrogen	70	0–1.5 γ	342
Pyridoxine* (pyridoxal, etc.)	*Lactobacillus casei*	Acidity	72	0–0.1 γ	51a, 188, 369
	Leuconostoc mesenteroides	Turbidity or acidity	72	0–0.25 γ	100
"Pseudopyridoxine"	*Streptococcus lactis* R	Turbidity	16	0–0.3 γ	134, 368, 375
Riboflavin	*Lactobacillus casei*	Acidity	72	0–0.05 γ	7, 16, 17, 20, 56, 87, 133, 140, 188, 318, 319, 347, 382, 402, 404, 406, 428, 429
(small amounts)	*L. casei*	Acidity	72	0–20 mγ	206
	L. jugurt	Acidity	72	0–0.1 γ	51
Thiamine	*Lactobacillus fermentum*	Turbidity	16–18	0–5 mγ	337
	Leuconostoc mesenteroides	Turbidity or acidity	72	0–3 mγ	100
	Propionibacterium pentosaceum	CO_2 evolution	4	0–0.25 γ	356, 361
	Staphylococcus aureus	Turbidity	36	0–0.5 mγ	436
	Streptococcus salivarius	Turbidity	24	0–0.2 mγ	288
Norite eluate factor	*Lactobacillus casei*	Acidity	48	0–300 γ solubilized liver fraction	379

TABLE 4—*Concluded*

GROWTH FACTOR	TEST ORGANISM	PRINCIPLE OF MEASUREMENT	TIME OF TEST	RANGE OF STANDARD PER ml	REFERENCES
			hours		
Folic acid	*Streptococcus lactis* R	Turbidity	24	0–200 γ liver extract B	207, 248
	Lactobacillus casei	Acidity	72	0–0.003 γ folic acid concentrate	188
Vitamin B_6	*L. casei*	Acidity	30–72	0–1 mγ	174, 410
	S. lactis R	Acidity	30–72	0–8 mγ	410
	S. lactis R	Turbidity	16	0–5 mγ	174

* Pyridoxine is inactive for *L. casei* and other lactic acid bacteria, whereas pyridoxal and pyridoxamine are very active. All these terms are retained in this review since no single compound has as yet been agreed on by investigators to express the vitamin B_6 activity of natural materials.

If used with skill and judgment, microbiological assays can be valuable and useful tools.

In table 4 are listed the principal bacteriological methods that have been proposed for the quantitative determination of various growth factors. In compiling this table only those methods are included that have been definitely proposed for the purpose and their application to natural materials worked out to some degree.

Next to *L. casei*, the organism most used for assays is *L. arabinosus*. It is employed almost exclusively for estimating nicotinic acid, and occasionally for biotin and *p*-aminobenzoic acid. Since it also requires pantothenic acid (table 1), presumably it could be used for the assay of this compound. *Leuconostoc mesenteroides* is a bacterium that requires practically the same growth factors as *L. casei*, and recently Gaines and Stahly (99, 100) have suggested its use for five different assays; but the methods have not yet been applied to biological materials. It is only in practice that the reliability and limitations of a method can be ascertained. *L. mesenteroides* has one advantage over *L. casei* in that it can be used for thiamine assay; but it also has a disadvantage in not being suitable for riboflavin because it synthesizes this compound. Its response to the new *L. casei* and *S. lactis* factors and its sensitivity to higher fatty acids have not been determined.

A single organism and a standard medium suitable for as many assays as possible would be highly desirable in control work, and Landy and Dicken (188) proposed such a medium adaptable for the determination of six growth factors by means of *L. casei*. This medium, however, did not contain all of the factors required by *L. casei* in optimal amounts. It can also be much improved by the inclusion of the pure form of newly isolated growth factors, and the addition of stimulatory substances (Sprince and Woolley, 392; Teply and Elvehjem, 410). The most serious known disadvantage to the use of *L. casei* is its sensitivity to

higher fatty acids. To date no completely satisfactory procedure has been developed for the removal of these interfering substances. Unfortunately most lactic acid bacteria seem to be sensitive to small amounts of higher fatty acids but a systematic study of the lactic acid bacteria might reveal a more suitable organism than *L. casei*.

A glance at table 4 shows that turbidity and acidity are the criteria most widely used for measuring the response of the organism. Nitrite production and synthesis of cellular nitrogen have been used in a few cases.

One of the features most strongly favoring bacteriological methods is the small quantity of the compound required for a determination. This ranges from 10^{-7} to 10^{-3} microgram of biotin to 0.05 to 0.5 microgram of nicotinic acid—quantities that are far beyond the reach of most chemical methods.

DESTRUCTION OF GROWTH FACTORS

For some time bacteria have been known to destroy ascorbic acid in the medium and in the intestinal tract of cattle. Foster (95) describes a new species of bacteria, *Pseudomonas riboflavinus*, that oxidizes riboflavin to lumichrome stoichiometrically according to the equation:

$$\underset{\text{riboflavin}}{C_{17}H_{20}O_6N_4} + 5\tfrac{1}{2}O_2 \longrightarrow \underset{\text{lumichrome}}{C_{12}H_{10}O_2N_4} + 5CO_2 + 5H_2O$$

Mirick (244) isolated a soil organism which, on the basis of oxygen uptake, apparently oxidized *p*-aminobenzoic acid to carbon dioxide, water, and ammonia. By culturing the organism in the presence of anthranilic (*o*-aminobenzoic) acid, the cells became adapted to the destruction of this compound. In a medium containing nicotinic acid as the sole source of carbon *Pseudomonas fluorescens* and *Serratia marcescens* destroyed the compound. In a medium devoid of nicotinic acid both organisms synthesized it (Koser and Baird, 161a).

FUNCTION OF GROWTH FACTORS

The role of certain growth factors, *e.g.*, riboflavin and thiamine, as constituent parts of coenzymes has been mentioned so many times that it need not be reviewed here. By analogy it is generally assumed that the other growth factors serve in a similar capacity. However, most of the recent data in support of this view are general rather than specific in character. Kligler *et al.* (148, 149) reported that nicotinic acid and thiamine are required if glucose is present in the medium, but not if it is absent. Many papers have been published showing that thiamine (119, 321–323, 358, 360, 365); riboflavin (2, 143), pantothenic acid (26, 78, 120), and nicotinic acid (or coenzyme I) (82, 211) increase respiration (oxygen uptake, carbon dioxide production or methylene blue reduction) by cells (*e.g.*, *Staphylococcus aureus*, *Propionibacterium pentosaceum*, *Proteus morganii*, *Lactobacillus mannitopoeus*, dysentery bacilli, *Hemophilus parainfluenzae*) acting on various substrates (*e.g.*, glucose, lactate, pyruvate).

A more specific type of function for a growth factor in catabolic processes has been uncovered by Gunsalus and coworkers (23, 24, 112–115) in relation to the

decarboxylation of tyrosine by strains of *Streptococcus faecalis*. In a series of papers, these workers have shown first, a marked apparent requirement for pyridoxine in the decarboxylation process; second, that pseudopyridoxine is more active than pyridoxine; third, that pyridoxal possesses this increased activity; and fourth, that adenosine triphosphate (ATP) functions with pyridoxal in the decarboxylation process. A synthetic compound, presumably phosphorylated pyridoxal, was prepared and found to function with an enzyme preparation from *S. faecalis*. The exact structure of the compound is still to be determined.

The above results do not correlate entirely with the work of two English investigators (Gale and Epps, 97a, 98) on the coenzyme involved in the decarboxylation of tyrosine and lysine by *E. coli* and *S. faecalis*. These authors report that pyridoxine had no coenzyme activity. In the second paper, they report purification of the coenzyme about 15,000 times and state that their preparation contained no phosphorus. It did contain C, H, and N but the percentage content was not that of pyridoxine. However, they did not test pyridoxal for coenzyme activity nor did they determine the pyridoxine (or pyridoxal) content of their coenzyme preparation. The discrepancies between the results obtained by the two groups of workers will probably be cleared up shortly. Another function for pyridoxal is reported by Schlenk and Snell (344) and by Snell (373) who showed that it is involved in transamination reactions.

In synthetic processes, Sevag and Green (349) showed that pantothenic acid was required for the building of tryptophane by certain strains of *Staphylococcus aureus*; and Stokes and Gunness (396) found that pyridoxamine (or pyridoxal) was needed in the synthesis of lysine, threonine, and alanine by *Lactobacillus arabinosus*, *Lactobacillus casei*, and *Lactobacillus delbrückii*. Stokes (394) explains the interchangeability of thymine and folic acid in the nutrition of various lactic acid bacteria as evidence for the view that the role of folic acid is to function in the synthesis of thymine. This in turn is needed for the building of nucleic acids. *p*-Aminobenzoic acid is not required for the growth of the tubercle bacillus but in high concentrations promotes the formation of an unidentified yellow pigment (Mayer, 238).

Meyer (240) reported that biotin increases enormously the activity of the mucolytic enzyme, lysozyme. The increased lysis, of both living and acetone-dried cells of *Micrococcus lysodeikticus* when 10γ of biotin was added to the substrate, ranged from 8 to 250 times. A note by Laurence (199) reported that lysozyme binds biotin and that the avidin-biotin complex has lysozyme activity.

However, such a relationship of lysozyme to biotin and avidin does not seem to hold as judged by other work. Alderton *et al.* (3) reported that crystalline lysozyme contained little or no biotin or avidin Additions of biotin to pure or impure lysozyme preparations did not enhance the lytic activity of the enzyme. Conversely, avidin preparations were essentially free of lysozyme. Previously Woolley and Longsworth (459a) had found that highly purified avidin was devoid of lysozyme activity.

Two papers (Miller *et al.*, 242; Williams *et al.*, 444) attempting to link growth factors with a number of highly purified enzyme proteins have given negative

results. About 15 of the common enzyme proteins, many of which had been prepared in a crystalline state, were analyzed for seven of the well-known growth factors, *e.g.* biotin, etc. Although small quantities of these compounds were found in the enzyme proteins, the amounts were too small to be considered constituent parts of the molecule except perhaps for inositol in amylase and thiamine in carboxylase. Assuming only a single mole of the factor in one molecule of protein, a molecular weight far greater than that ascribed to these proteins would be required.

While there are more data associating growth factors with breakdown than with synthetic processes, considerable information is being accumulated linking growth factors with the building of the cell as well as with its maintenance. The term growth factor will appear more appropriate as more information is obtained linking it with constructive processes.

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