Carbohydrate Metabolism by the Acetic Acid Bacteria

Part V. On the Vitamin Requirements for the Growth

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Vitamin requirements for the growth of the acetic acid bacteria were investigated extensively on a taxonomical viewpoint and the following new findings were pointed out. Neither *Acetobacter* nor Intermediate strain required vitamin for the growth.

Gluconobacter required generally pantothenic acid. And some strains belonging to it did moreover somewhat of thiamine, nicotinic acid and *p*-aminobenzoic acid, although there was a difference of requirements between strains even in the same species. Riboflavin, pyridoxine, vitamin B₁₂, folic acid, biotin and inositol were unnecessary for the growth of the acetic acid bacteria. A taxonomical division of the acetic acid bacteria based on the vitamin requirements agreed well with that on basis of the oxidative activities for carbohydrates.

Using many species and strains of the acetic acid bacteria, the carbohydrate metabolisms of them have been investigated by the authors comparative biochemically.^{1,2)} Also in this paper, the vitamin requirements for growth of them were studied on a taxonomical viewpoint.

Until now, the vitamin requirements of the acetic acid bacteria have been reported by the several investigators,^{3~9)} although on only species by species incoherently without regard to other properties.

Besides, one of the authors' purposes in this paper was to make clear the relationship

between the vitamin requirements and the taxonomical findings based on the oxidative activities for various carbohydrates by the acetic acid bacteria. And the other one was to find out the cause of the physiological and biochemical variations¹⁰⁾ of them, for instance on the ketogenic and pigment-producing characteristics, as described in the previous report.²⁾

MATERIALS AND METHODS

Organisms. Most strains used were supplied by Institute for Fermentation, Osaka, Japan (IFO), and a few isolated from the vinegar workshop and other were also examined. The stock cultures were maintained on 5% malt agar slants containing 1.5% of calcium carbonate.

The vitamin-free minimal medium. The composition of the minimal medium used is shown in

1) K. Kondô and M. Ameyama, *Bull. Agr. Chem. Soc. Japan*, **22**, 369 (1958).

2) M. Ameyama, H. Fujisawa and K. Kondô, *J. Agr. Chem. Soc. Japan*, **39**, 427 (1965).

3) L. A. Underkoffler, A. C. Bantz and W. H. Peterson, *J. Bact.*, **45**, 183 (1943).

4) W. Lisky, W. B. Esselen Jr., B. S. Teper and G. Miller, *Food Research*, **18**, 250 (1953).

5) J. O. Foda and R. H. Vaughn, *J. Bact.*, **65**, 79 (1953).

6) M. R. R. Rao and J. L. Stokes, *J. Bact.*, **65**, 405 (1953).

7) A. N. Hall, G. A. Thomas, K. S. Tiwari and T. K. Walker, *Arch. Biochem. Biophys.*, **46**, 485 (1953).

8) C. Rainbow and G. W. Mitson, *J. gen. Microbiol.*, **9**, 371 (1953).

9) G. O. Brown and C. Rainbow, *J. gen. Microbiol.*, **15**, 61 (1956).

10) J. L. Schimwell, *Antonie v. Leeuwenhoek*, **24**, 187 (1958); R. Steel and T. K. Walker, *J. gen. Microbiol.*, **17**, ~~445~~ 445 (1957); *J. Inst. Brew.*, **63**, 510 (1957).
J. Tošić and T. K. Walker, *J. Soc. Chem. Ind.*, **115**, 180 (1946).

J. M. Wiame, R. Dothey and R. Harpigny, *International Vinegar Congress, The Hauge* (1957).

J. L. Schimwell, *Antonie v. Leeuwenhoek*, **25**, 49 (1959).

M. M. R. Rao, *Annual Review of Microbiol.*, **11**, 322 (1957).

T. Asai, *Recent Progress in Biochem.*, **6**, 1 (1960).

TABLE I. COMPOSITION OF THE MINIMAL MEDIUM AND THE COMPLETE MEDIUM

Minimal medium	
Glucose	0.5%
Amino acids	total 400 mg% (total N: 56.5 mg%)
L-form	20 mg% each
glycine, valine, leucine, isoleucine, serine, threonine, cystine, cysteine, aspartic acid, glutamic acid, arginine, lysine, histidine, phenylalanine, tryptophan, proline	
DL-form	40mg% each
alanine, methionine	
Bases	1 mg% each
adenine, guanine, uracil, xanthine	
Minerals	
KH ₂ PO ₄	50 mg%
K ₂ HPO ₄	50
MgSO ₄ ·7H ₂ O	20
NaCl	1
FeSO ₄ ·7H ₂ O	1
MnSO ₄ ·4H ₂ O	1
pH	6.0
Complete medium	
Glucose	0.5%
Yeast extract (Oriental Co.)	0.5% (total N: 59.1 mg%)

Table I, conforming to the report by Underkofler et al.³⁾

Vitamins. Ten vitamins examined and their concentrations are shown in Table II. Folic acid, biotin and inositol were used always as a set.

The growth tests were carried out by the omission singly of vitamins from the synthetic medium containing ten vitamins and by the addition singly of them to the minimal medium.

The complete medium composed of 0.5% glucose and 0.5% dried yeast extract (Oriental Co.) was used for comparison with the synthetic medium in all experiments.

Inocula were taken from cultures grown stationarily for 48 hrs in the media consisting of 1% glycerol and 0.5% dried yeast extract, which were dispensed in 2.5 ml quantities into 8 ml centrifugal tubes and autoclaved. Then the inocula were washed twice centrifugally with sterilized water and suspended in the same one. One drop of the suspension served as inoculum for a test tube of the culturing medium.

The culturing media adjusted to pH 6.0 were dispensed in 6 ml quantities into the test tubes (ϕ 18 mm, to use for colorimetry) with metal cup and

TABLE II. CONCENTRATION OF VITAMINS IN THE SYNTHETIC MEDIUM

Abbreviation	Vitamins	Concentrations
B ₁	Thiamine-HCl	40 γ%
B ₂	Riboflavin	40
NA	Nicotinic acid	40
PA	Ca-Pantothenate	40
B ₆	Pyridoxine-HCl	80
PABA	<i>p</i> -Aminobenzoic acid	10
B ₁₂	Vitamin B ₁₂	0.1
FBI	Folic acid	0.03
	Biotin	0.04
	Inositol	0.5

autoclaved (1 kg/cm², 10 min.). The cultures were incubated shaking reciprocally at 30°C for 3 days.

Growth was measured as optical density in the Hitachi EBO-B type photometer with a 660 mμ filter.

RESULTS

Reexamination of the minimal medium was carried out using several acetic acid bacteria.

0.5% glucose was used as a source of carbon and energy, which was reported to influence on the specific nitrogen requirement.⁵⁾ This concentration of glucose was convenient to measure the turbidity and to maintain suitable pH for growth during incubation without any neutralizer.

As a nitrogen source the mixture of amino acids was used. By the way, use of the vitamin-free casamino acid (Nissan Co. and Difco Co.) gave the same results with amino acids mixture. Although tyrosine and hydroxyproline were omitted from the mixture, these were not essential for the growth under the condition mentioned above.

The concentration of the amino acids mixture was adequate between 50 and 150 mg% as nitrogen, as shown in Fig. 1. By the way, Na-glutamate used as a sole nitrogen source for the growth of *A. suboxydans* var. *α* was also adequate in the same concentration (see Fig. 1). The concentration of yeast extract in the complete medium was then also adjusted to the same mg% of nitrogen.

FIG. 1. Relation between the Growth and the Nitrogen Concentration of the Medium.

The medium contained ten vitamins and the substances shown in Table I other than nitrogen source.

FIG. 2. Effect of the Bases.

- A: The basal medium contained ten vitamins and the substances shown in Table I. "- Adenine", "- guanine", "- uracil" and "- xanthine" show the growth in the medium without adenine, guanine, uracil and xanthine, respectively.
- B: The basal medium consisted of ten vitamins and the substances shown in Table I, without four bases. "+ Adenine", "+ guanine", "+ uracil" and "+ xanthine" show the growth in the medium added adenine, guanine, uracil and xanthine, respectively.

Organic bases used were unnecessary for the growth of all strains (see Fig. 2), although Rao and Stokes⁶⁾ and Brown et al.⁹⁾ reported that uracil was the essential growth factor of *A. rancens*, *A. melanogenum*, *A. gluconicum* and *A. suboxydans*.

Minerals used were conformed to the report of Underkoffler et al. And yet a simple mixture of 0.02% KH_2PO_4 , 0.01% $\text{MgSO}_4 \cdot 7\text{H}_2\text{O}$ and 2% KCl was also sufficient for growth of all strains.

Vitamin requirements. Some instances of vitamin requirements are shown in Fig. 3 and 4. The upper parts in both A and B of the figures indicate the influence of omission singly of each vitamin from the synthetic medium containing ten vitamins. And in the lower parts the effect of addition singly of each vitamin to the minimal medium is shown. As mentioned above, folic acid, biotin and inositol were always treated together in both cases.

As shown in these figures, it was understood that vitamins had not only the inhibitive effect, but also co-multiplicable effect for the growth. This fact, as seen in Fig. 5, was recognized, too. And in this figure, it was indicated that each effective vitamin operated only individually, and that the growth was enough supported by only necessary vitamins, since pantothenic acid plus *p*-aminobenzoic acid supported coefficiently growth of *A. suboxydans* var. α but, even in excess amount, only one of the two did not support the growth sufficiently.

Therefore, in Table III, the results of the omission tests are only expressed.

In order to simplify the presentation of the data and to indicate directly the necessary vitamins, in Table IV, growth is recorded in terms of -, 0 and +. For these purposes, on the basis of the growth in the synthetic medium containing ten vitamins, the following arbitrary scale was established and used: - for values less than a half, namely required the vitamin; 0 for equal values; and + for

FIG. 3. The Vitamin Requirements of the Acetic Acid Bacteria.

A: *A. acetosum* var. EB 1000B: *A. aurantium* IFO 3246

In the synthetic medium, ten vitamins were added to the minimal medium.

FIG. 4. The Vitamin Requirements of the Acetic Acid Bacteria.

A: *A. melanogenum* IFO 3292B: *A. industrium* IFO 3260

In the synthetic medium, ten vitamins were added to the minimal medium.

values greater than twice growth.

Oxidizabilities for four typical carbohydrates were quoted in Table IV from the previous paper²⁾ studied about many substrates, for reference. These were manometrically meas-

ured in a conventional Warburg respirometer at 30°C using the resting cells harvested from the malt agar slant cultures containing calcium carbonate. Each vessel contained 1.0 ml of the cell suspension and 1.5 ml of M/15 phos-

FIG. 5. Vitamin Requirements of *A. suboxydans* var. α IFO 3254.TABLE III. VITAMIN REQUIREMENTS OF THE ACETIC ACID BACTERIA
(Optical density $\times 10^3$ at 660 m μ)

Strains	vitamins omitted from the synthetic medium										complete medium
	none	B ₁	B ₂	NA	PA	B ₆	PABA	B ₁₂	FBI	all	
<i>Acetobacter</i>											
<i>A. ascendens</i>											
IFO 3299	028	069	045	037	032	044	048	040	053	048	250
<i>A. aceti</i>											
IFO 3281	030	045	048	060	090	042	060	060	048	021	547
<i>A. acetosum</i> var.											
EB 1000	274	325	294	339	333	342	310	275	263	323	229
<i>A. xylinum</i> var.											
EB 1002	313	268	297	295	274	251	283	170	312	264	242
<i>A. kützingianum</i>											
IFO 3222	031	055	028	029	049	078	044	035	056	027	160
<i>A. rancens</i>											
IFO 3297	024	020	034	024	016	044	022	019	019	016	262
<i>A. acetigenum</i>											
IFO 3277	071	094	103	077	110	103	095	067	088	098	225
<i>A. albuminosum</i>											
IFO 3283	091	071	090	058	073	088	079	095	085	055	264
IFO 3284	050	051	050	038	058	052	054	046	057	029	360
<i>A. turbidans</i>											
IFO 3225	240	240	225	237	242	222	223	219	221	209	109

TABLE III. (Continued)

Strains	vitamins omitted from the synthetic medium										complete medium
	none	B ₁	B ₂	NA	PA	B ₆	PABA	B ₁₂	FBI	all	
Intermediate type											
<i>A. aurantium</i>											
IFO 3245	232	204	211	192	217	183	189	206	215	300	548
IFO 3246	283	219	229	185	210	231	236	240	213	292	598
IFO 3247	255	211	231	206	255	220	250	194	238	315	570
<i>G. liquifaciens</i>											
25	210	199	223	210	229	211	220	224	203	206	410
<i>Gluconobacter</i>											
<i>A. melanogenum</i>											
IFO 3292	204	064	218	033	000	207	189	219	216	000	375
IFO 3293	280	303	293	008	006	275	136	273	282	011	555
<i>A. rubiginosus</i>											
IFO 3244	245	055	150	070	015	160	170	245	185	060	660
<i>A. dioxyceticus</i>											
IFO 3272	301	252	279	284	025	305	003	280	340	000	365
IFO 3273	131	143	148	010	000	134	141	114	130	000	155
IFO 3274	200	170	215	208	008	188	170	195	157	000	201
<i>A. orleanens</i>											
IFO 3259	120	098	100	050	003	105	000	109	150	000	395
<i>A. cerinus</i>											
IFO 3262	356	299	360	249	017	285	008	335	370	011	230
IFO 3263	110	149	175	107	012	169	108	142	183	000	280
IFO 3264	360	343	332	368	000	313	255	370	359	000	406
<i>A. cerinus</i> var. <i>ammoniacum</i>											
IFO 3267	333	070	321	098	010	290	128	330	280	000	450
IFO 3268	185	210	203	225	015	198	198	205	210	015	290
IFO 3269	127	134	128	063	020	126	121	101	127	023	450
<i>A. nonoxygluconicum</i>											
IFO 3276	202	245	295	228	012	250	024	280	263	000	415
<i>A. gluconicum</i>											
IFO 3286	136	145	141	168	011	144	077	147	132	000	218
<i>A. indurium</i>											
IFO 3260	339	340	335	340	033	344	065	340	350	000	255
<i>A. oxydans</i>											
IFO 3287	144	167	158	051	009	135	143	151	155	000	318
<i>A. capsulatum</i>											
IFO 3462	263	179	280	010	016	282	233	274	272	000	350
<i>A. albidus</i>											
IFO 3251	240	250	270	262	020	245	089	295	218	000	369
IFO 3252	068	038	045	025	008	081	090	057	016	000	039
<i>A. suboxydans</i>											
IFO 3289	419	325	330	390	012	309	018	346	390	000	478
IFO 3291	141	128	153	126	001	121	005	126	145	000	265
<i>A. suboxydans</i> var. α											
IFO 3254	360	335	340	363	012	360	053	368	338	014	413
IFO 3255	200	209	200	213	009	196	038	204	216	007	106

TABLE IV. RELATION BETWEEN THE VITAMIN REQUIREMENTS AND CARBOHYDRATE OXIDIZABILITIES BY THE ACETIC ACID BACTERIA

Strains	Growth in*										Oxidative activity for†			
	vitamins omitted from the synthetic medium									complete medium				
	B ₁	B ₂	NA	PA	B ₆	PABA	B ₁₂	FBI	all		arabinose	succinate	AcOH	EtOH
<i>Acetobacter</i>														
<i>A. ascendens</i>														
IFO 3299	0	0	0	0	0	0	0	0	0	+	2	21	2	113
<i>A. aceti</i>														
IFO 3281	0	0	0	0	0	0	0	0	0	+	0	32	11	49
<i>A. acetosum</i> var.														
EB 1000	0	0	0	0	0	0	0	0	0	0		11	12	33
<i>A. xylinum</i> var.														
EB 1002	0	0	0	0	0	0	0	0	0	0		32	4	43
<i>A. kützingianum</i>														
IFO 3222	0	0	0	0	0	0	0	0	0	+	1	14	1	11
<i>A. rancens</i>														
IFO 3297	0	0	0	0	0	0	0	0	0	+	1	27	1	34
<i>A. acetigenum</i>														
IFO 3277	0	0	0	0	0	0	0	0	0	+	1	23	1	42
<i>A. albuminosum</i>														
IFO 3283	0	0	0	0	0	0	0	0	0	+	1	33	1	49
IFO 3284	0	0	0	0	0	0	0	0	0	+	1	37	1	56
<i>A. turbidans</i>														
IFO 3225	0	0	0	0	0	0	0	0	0	0	1	4	2	13
Intermediate type														
<i>A. aurantium</i>														
IFO 3245	0	0	0	0	0	0	0	0	0	+	10	11	3	11
IFO 3246	0	0	0	0	0	0	0	0	0	+	9	8	2	9
IFO 3247	0	0	0	0	0	0	0	0	0	+	9	5	2	4
<i>G. liquifaciens</i>														
25	0	0	0	0	0	0	0	0	0	+	3	10	9	13
<i>Gluconobacter</i>														
<i>A. melanogenum</i>														
IFO 3292	—	0	—	—	0	0	0	0	—	0	1	0	0	9
IFO 3293	0	0	—	—	0	0	0	0	—	+	1	0	0	12
<i>A. rubiginosus</i>														
IFO 3244	—	0	—	—	0	0	0	0	—	+	1	0	0	16
<i>A. dioxycetonicus</i>														
IFO 3272	0	0	0	—	0	—	0	0	—	0	1	0	0	13
IFO 3273	0	0	—	—	0	0	0	0	—	0	1	0	0	12
IFO 3274	0	0	0	—	0	0	0	0	—	0	1	0	0	14
<i>A. orleanens</i>														
IFO 3259	0	0	—	—	0	—	0	0	—	+	1	0	0	16
<i>A. cerinus</i>														
IFO 3262	0	0	0	—	0	—	0	0	—	0	0	0	0	13
IFO 3263	0	0	0	—	0	0	0	0	—	+				
IFO 3264	0	0	0	—	0	0	0	0	—	0	1	0	0	14

TABLE IV. (Continued)

Strains	Growth in*										Oxidative activity for†				
	vitamins omitted from the synthetic medium										complete medium				
	B ₁	B ₂	NA	PA	B ₆	PABA	B ₁₂	FBI	all	arabinose		succinate	AcOH	EtOH	
<i>A. cerinus</i> var. <i>ammoniacum</i>															
IFO 3267	—	0	—	—	0	—	0	0	—	0	1	0	0	12	
IFO 3268	0	0	0	—	0	0	0	0	—	0	1	0	0	10	
IFO 3269	0	0	0	—	0	0	0	0	—	+	1	0	0	12	
<i>A. nonoxygluconicum</i>															
IFO 3276	0	0	0	—	0	—	0	0	—	+	1	0	0	14	
<i>A. gluconicum</i>															
IFO 3286	0	0	0	—	0	0	0	0	—	0	1	0	0	8	
<i>A. indurium</i>															
IFO 3260	0	0	0	—	0	—	0	0	—	0	2	0	0	10	
<i>A. oxydans</i>															
IFO 3287	0	0	—	—	0	0	0	0	—	+	0	0	0	19	
<i>A. capsulatum</i>															
IFO 3462	0	0	—	—	0	0	0	0	—	0	1	0	0	14	
<i>A. albidus</i>															
IFO 3251	0	0	0	—	0	—	0	0	—	0	1	0	0	13	
IFO 3252	0	0	—	—	0	0	0	0	—	0	0	0	0	9	
<i>A. suboxydans</i>															
IFO 3289	0	0	0	—	0	—	0	0	—	0	1	0	0	13	
IFO 3291	0	0	0	—	0	—	0	0	—	0	1	0	0	13	
<i>A. suboxydans</i> var. α															
IFO 3254	0	0	0	—	0	—	0	0	—	0	2	0	0	7	
IFO 3255	0	0	0	—	0	—	0	0	—	0	1	1	0	2	

* 0=50~200% growth compared to that in the synthetic medium containing ten vitamins.

—=<50%.

+ =200%<.

† It was represented as the ratio against a value on glucose which was calculated to 10.²⁾

phate buffer (pH 6.0) in the main compartment, 0.3 ml of substrate (30 μ moles) in the side arm and 0.2 ml of 20% KOH in the central well. In order to simplify, the activity for the substrates was expressed in the ratio to that for glucose which was calculated to 10.

DISCUSSION

The current nomenclatures of "*Acetobacter*", "Intermediate strains" and "*Gluconobacter*" have been advanced by Asai et al.¹¹⁾ on basis of the difference on the morphological and

physiological properties, and independently also by the authors on basis of the difference on the oxidative activities for various carbohydrates.²⁾ Then, in this paper these nomenclatures were used.

As seen in Table IV, it was found that the vitamin requirements of the acetic acid bacteria were divided into two main parts.

No vitamins used were required for growth of *Acetobacter*, although it was reported by Foda and Vaughn,⁵⁾ Hall et al.⁷⁾ and so on that *A. rancens*, *A. ascendens*, *A. pasteurianum* etc. required several vitamins.

Intermediate strains did not require any vitamins, too.

11) T. Asai, H. Iizuka and K. Komagata, *J. Gen. Appl. Microbiol.*, **10**, 95 (1964).

Oppositely, *Gluconobacter* required pantothenic acid in general and moreover some strains did also somewhat of thiamine, nicotinic acid and *p*-aminobenzoic acid.

No strain required riboflavin, pyridoxine, vitamin B₁₂, folic acid, biotin and inositol.

While, as shown in Table IV, it has been recognized that *Acetobacter* oxidized succinate strongly, Intermediate strains, which had distinctively the oxidizability for arabinose, did also it considerably, and, on the contrary, *Gluconobacter* did not oxidize it entirely.

Thus, it was concluded with great interest that a taxonomical division of the acetic acid bacteria on basis of the vitamin requirements corresponded clearly to that on basis of the oxidative activities for carbohydrates, for example, succinate and acetate.

Besides, in the requirements of thiamine, nicotinic acid and *p*-aminobenzoic acid, the difference between strains belonging to the

same species was seen here and there. Such fact had been also observed by Rao and Stokes.⁶⁾ Authors considered it was a variation as same as mentioned in the previous report.

In many strains the complete medium stimulated growth more than the synthetic medium containing ten vitamins.

Either by reason of existence of unknown effective substances in yeast extract or of inappropriate balance of vitamins or nutrients in the synthetic medium was not concluded hastily. Because, uracil reported by Rao and Stokes and Brown et al. was not recognized in this paper as mentioned above, and acidic effective substance in yeast autolysate reported by Goldman et al.¹²⁾ was not also confirmed yet in yeast extract used by the authors.

There were not also biotin-requiring strains reported by Hall et al.⁷⁾

12) C. L. Goldmann, W. Libsky, M. Mandel and H. N. Little, *Can. J. Microbiol.*, **4**, 463 (1958).