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NUTRITIONAL REQUIREMENTS OF *CORYNEBACTERIUM RENALE*

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INTRODUCTION

The cultivation of organisms of *Corynebacterium* in chemically defined media has been investigated by MUELLER (1937 a, b), PAPPENHEIMER et al. (1937), EVANS et al. (1939) and DREW & MUELLER (1951) with *C. diphtheriae*. These studies have shown the requirement needed for excellent toxin-formation of this species was satisfied by media containing 8 amino-acids, 4 accessary growth factors, ammonium sulfate, trace metals, inorganic salts and maltose.

More recently, LACHANCE (1960, 1962) reported on the vitamin and amino acid requirements of the causative agent of potato ring rote, *C. sepedonicum*. The results were similar to those obtained with *C. diphtheriae*.

Nutritional requirements of *C. renale*, agent of the bovine pyelonephritis, have not yet been elucidated. The availability of a chemically defined medium for *C. renale* would be of value in many aspects of studies of micro-organisms. This communication deals with the vitamin and amino acid requirements of *C. renale* particularly in relation to the 3 types of *C. renale* reported by YANAGAWA et al. (1967).

MATERIALS AND METHODS

Strains Fifteen strains of *C. renale* were used in this study; 9 strains were provided by the Hokkaido Branch of the National Institute of Animal Health, Sapporo, the remaining 6 strains were from the stock culture of this department. The types of these strains were as follows: Strains Nos. 8, 15, 24, 34 and 65 were type I; strains Nos. 45, 13, 20, 35 and 75 were type II; strains Nos. 42, 43, 44, 48 and 103 were type III. Serological characteristics of these strains were described in the previous report (YANAGAWA et al., 1967). Strains Nos. 8, 45 and 42 were selected randomly and used as the representatives of the respective types.

Of the strains used, Nos. 8, 13, 42, 43, 44, 45, 48 and 103 were the isolates from material of bovine nephritis while the remaining strains were isolates from the urine of apparently healthy cattle.

Preparation of media Two kinds of media were primarily prepared. One was

a semi-synthetic medium, in which 0.5% casamino acids "vitamin-free" (Difco) were nitrogen source. The casamino acids were dissolved in Salt A and added with 6 vitamins and sodium bicarbonate (tab. 1). Stock solutions of vitamins (1 mg/ml) were made separately in Salt A. The pH was adjusted to 7.4 with sodium hydroxide. Twenty-five ml portions were dispensed into 100 ml flasks, fitted with cotton plugs, wrapped with surgical gauze and sterilized by autoclaving. To these flasks, Salt B (1/20 volume), a mixture of calcium chloride (5 mg/dl) and glucose (3 g/dl) (1/10 volume) were added. All were sterilized just before use. The medium was thus prepared and a 10 ml portion was poured into each tube (12×185 mm).

A synthetic medium was prepared similarly, except that the casamino acids were replaced with 19 amino acids (tab. 1). Ammonium sulfate (10 mg/dl) was also added. Stock solutions of concentrated amino acids (usually 20 mg/ml) were made separately in Salt A.

Inoculation The strains used as inocula were cultivated on the nutrient agar media in petri dishes and incubated for 18 to 24 hr at 37°C. The culture grown was removed,

TABLE 1 *Composition of synthetic medium*

COMPONENT	CONCN.	COMPONENT	CONCN.
BASAL	mg/dl	AMINO ACID SUPPLEMENT	mg/dl
Salt A:		Glycine	2.5
NaCl	62	DL-Isoleucine	5
$NaHPO_4$	28	L-Glutamic acid	25–50
KH_2PO_4	6.8	L-Arginine-HCl	5
KCl	4	L-Proline	2
Salt B:		DL-Tryptophane	2.5
$MgSO_4$	0.5	DL-Methionine	3
$MnSO_4$	trace	DL-Phenylalanine	1
$FeSO_4$	trace	L-Histidine-HCl	5
$CuSO_4$	trace	DL-Valine	12
Salt C:		L-Tyrosine	5
$CaCl_2$	0.5	L-Lysine	1.5
$(NH_4)_2SO_4$	10	DL-Alanine	5
Glucose	300	DL-Serine	5
VITAMIN SUPPLEMENT	μg/dl	DL-Threonine	5
Biotin	5	DL-Aspartic acid	5
Thiamine-HCl	100	DL-Leucine	5
Ca-Pantothenic acid	100	DL-Norleucine	5
Nicotinic acid	100	L-Cystine-HCl	5
p-Aminobenzoic acid	100		
Pyridoxine-HCl	100		
$NaHCO_3$	5 mg/dl		

placed in Salt A, to which glucose had been added, washed twice by centrifugation and resuspened in the same solution to obtain an optical density of 0.15 (approximately 1×10^7 cell/ml). One-tenth ml of the inoculum suspension was added to 10 ml of defined medium and incubated at 37°C. The inocula were used as quickly as possible after preparation.

Growth measurement Growth was estimated turbidimetrically using Tokyo Koden type 7 A photoelectric colorimeter equipped with a 570 mμ filter.

RESULTS

1 Vitamin requirements

A preliminary test showed that the 3 representative strains of each type, No. 8 (type I), No. 45 (type II) and No. 42 (type III) would grow abundantly in the vitamin-free casamino acid medium supplemented with sodium bicarbonate and 12 growth factors. These growth factors were p-aminobenzoic acid, folic acid, biotin, nicotinic acid, pantothenic acid, riboflavin, thiamine, inositol, erythritol, lipoic acid, pyridoxine and choline.

Individual vitamins were next examined and in this way, it was found that 6 of the 12 vitamins could be eliminated because their absence caused no decrease in growth. Further experiments showed, as indicated in table 2, that strain No. 8 grew profusely when biotin, pantothenic acid and thiamine were present. These 3 vitamins acted as growth stimulants. The other vitamins did not materially affect growth response. In the case of strain No. 45, p-aminobenzoic acid, nicotinic acid and biotin were essential for growth; pantothenic acid and thiamine were required for maximal growth. Strain No. 42 required thiamine, biotin, nicotinic acid, pantothenic acid and pyridoxine essentially, without even one of these vitamins there was no growth.

Conclusively, biotin, thiamine and pantothenic acid were the commonly required vitamins for growth of the 3 representative strains of each type. Strain No. 8 required these

TABLE 2 *Vitamin and $NaHCO_3$ requirements of the 3 types of C. renale*

MEDIUM	GROWTH OF THREE TYPES*		
	No. 8 (type I)	No. 45 (type II)	No. 42 (type III)
Basal	0	0	0
Complete	0.75	0.60	0.65
Omission from complete			
Biotin	0.12	0.09	0.05
Ca-Pantothenic acid	0.32	0.20	0.09
Thiamine-HCl	0.22	0.19	0.02
Nicotinic acid	0.75	0.09	0.06
p-Aminobenzoic acid	0.75	0.06	0.66
Pyridoxine-HCl	0.74	0.60	0.09
$NaHCO_3$	0.26	0.16	0.12

* Results expressed as optical density, measured after 48 hr incubation at 37°C

vitamins only as growth stimulants. Strain No. 45 required more vitamins either essentially or stimulatory. No. 42 was the most exacting in vitamin requirement.

It is also indicated in table 2 that sodium bicarbonate is necessary for maximum growth of each strain.

2 Inoculum size and serial subcultures

Perhaps the most rigorous test of the nutritional adequacy of a medium resides in its ability to support the initiation of growth from a minimum inoculum and to maintain a vigorous cell population upon serial subcultures. The ability of semi-synthetic media to initiate growth from a small inoculum was examined by preparing 10-fold serial dilutions of inoculum with the respective semi-synthetic media for each type. Growth measurements reported in table 3 revealed that the semi-synthetic media were capable of supporting abundant growth from a small inoculum such as 1 : 1,000 dilution of the original bacterial suspension whose optical density was 0.15.

The effect of serial subculture on the cell yield and the antigenicity of each strain was examined by serial inoculation in the semi-synthetic and synthetic media at 5 day intervals. One per cent (by volume) of inoculum was used at each transfer. No pronounced change in the optical density or the antigenic type was noted in 10 serial subcultures.

TABLE 3 *Effect of inoculum size on the growth of C. renale in semi-synthetic media*[*1]

INOCULUM[*2]		GROWTH AFTER INDICATED INCUBATION HOURS								
		No. 8 (type I)			No. 45 (type II)			No. 42 (type III)		
Dilution	Initital optical density	24	48	72	24	48	72	24	48	72
Original	0.15	0.70[*3]	0.84	0.90	0.33	0.53	0.70	0.08	0.65	0.80
1 : 10	0.05	0.65	0.84	0.90	0.25	0.54	0.75	0.01	0.55	0.80
1 : 100	—	0.45	0.85	0.88	0.15	0.28	0.74	0	0.25	0.80
1 : 1000	—	0.35	0.83	0.90	0.08	0.15	0.74	0	0.05	0.45

[*1] Media used
for type I; Basal+Casamino acid+Vitamins (B+T+Pant)
" II; " (B+T+Pant+N+P)
" III; " (B+T+Pant+N+Py)
B: Biotin T: Thiamine-HCl Pant: Pantothenic acid
N: Nicotinic acid P: p-Aminobenzoic acid Py: Pyridoxine-HCl

[*2] Washed cells from 18~24 hr culture on the nutrient agar were used as inoculum.

[*3] Optical density

3 Amino acid requirements

When a mixture of 19 amino acids was substituted for the vitamin-free casamino acids in the semi-synthetic medium, excellent growth was obtained. Next, individual amino acids were eliminated and in each case the effect on the bacterial growth of the resulting medium was expressed in terms optical density measured after incubation for 48 hr at 37°C (tab. 4).

It was shown that glutamic acid, valine and isoleucine were essential for the growth of the 3 types of *C. renale*. For strain No. 8 (type I) only these 3 amino acids were essential. Phenylalanine accelerated its growth. Strain No. 45 (type II) required the above 3 amino acids essentially. The growth was accelerated with the addition of methionine, phenylalanine and histidine. Growth of strain No. 42 (type III) was most exacting. In addition to the 3

TABLE 4 *Amino acid requirements of the 3 types of C. renale*[*1]

MEDIUM	GROWTH OF 3 TYPES		
	No. 8 (type I)	No. 45 (type II)	No. 42 (type III)
Basal	0	0	0
Complete	0.64[*2]	0.58	0.56
Omission from complete medium			
Glycine	0.60	0.55	0.52
L-Alanine	0.64	0.59	0.50
DL-Threonine	0.35	0.37	0.33
L-Serine	0.61	0.33	0.44
DL-Valine	0.02	0.04	0.05
DL-Leucine	0.66	0.60	0.60
DL-Isoleucine	0.04	0.06	0.05
L-Norleucine	0.62	0.26	0.28
L-Aspartic acid	0.58	0.22	0.38
L-Glutamic acid	0.09	0.10	0.08
L-Arginie-HCl	0.35	0.40	0.18
DL-Lysine	0.40	0.44	0.35
L-Cystine	—[*3]	0.40	0.35
DL-Methionine	0.33	0.14	0.13
DL-Phenylalanine	0.18	0.15	0.14
L-Histidine-HCl	0.25	0.17	0.61
L-Tryptophane	0.60	0.55	0.08
L-Tyrosine	0.62	0.27	0.44
L-Proline	0.66	0.52	0.58
$(NH_4)_2SO_4$	0.20	0.22	0.22

[*1] Media used
for type I; Basal+19 amino acids+Vitamins (B+T+Pant)
" II; " (B+T+Pant+N+P)
" III; " (B+T+Pant+N+Py)
For further explanation see the foot-note of table 3.

[*2] Optical density after 72 hr incubation at 37°C

[*3] Cystine was not added to the medium used for type I.

amino acids commonly required, this strain essentially required tryptophane. Methionine, phenylalanine and arginine were needed for maximal growth. Moreover, strain No. 42 was characteristic in that it did not require histidine.

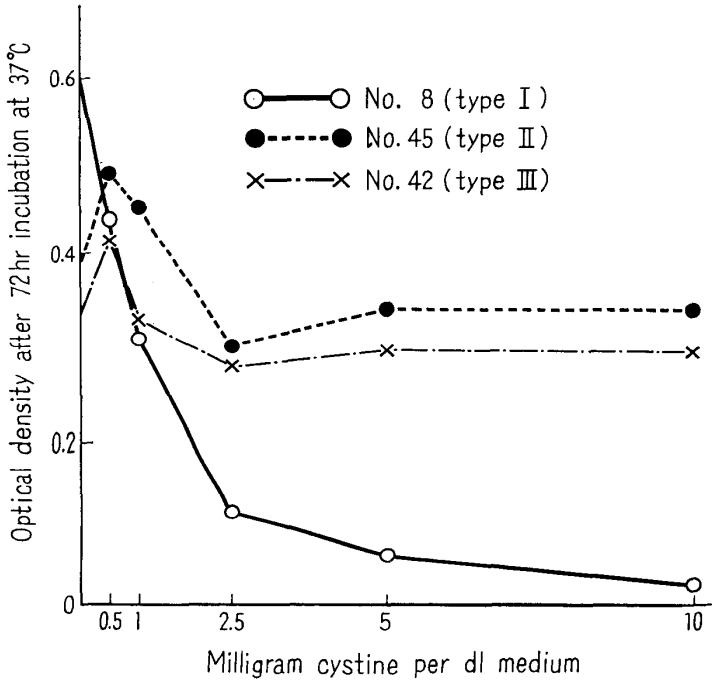
Therefore, it was found that the strains of types III and II required more amino acids than that of type I.

Ammonium sulfate was found necessary for maximum growth of each strain.

4 Quantitative aspects of some amino acids

a) Cystine Attempts to simplify composition of the medium by omission of non-essential amino acids were not successful until it was noted that the growth of strain No. 8 was inhibited by the presence of cystine. Maximum growth of strain No. 8 occurred in the absence of cystine, as shown in table 4. Strains Nos. 45 and 42 were also inhibited by the presence of cystine but not very seriously. The effect of the concentration of cystine present is shown in figure 1. No cystine was best for strain No. 8, only 0.5 mg/dl was sufficient for strains Nos. 45 and 42.

FIGURE 1 *Effect of cystine on the growth of the 3 types of C. renale in chemically defined media**

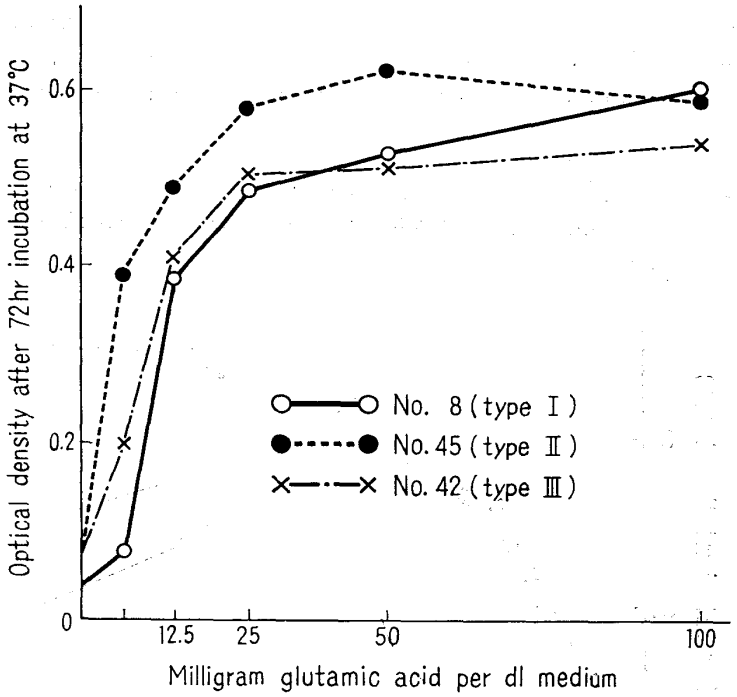


* The media used are indicated in the foot-note of table 4.

b) Glutamic acid The effect of glutamic acid concentration on the growth of the 3 representative strains was tested in synthetic medium. A concentration of glutamic acid more than 25 mg/dl was required for the growth of all the 3 strains (fig. 2).

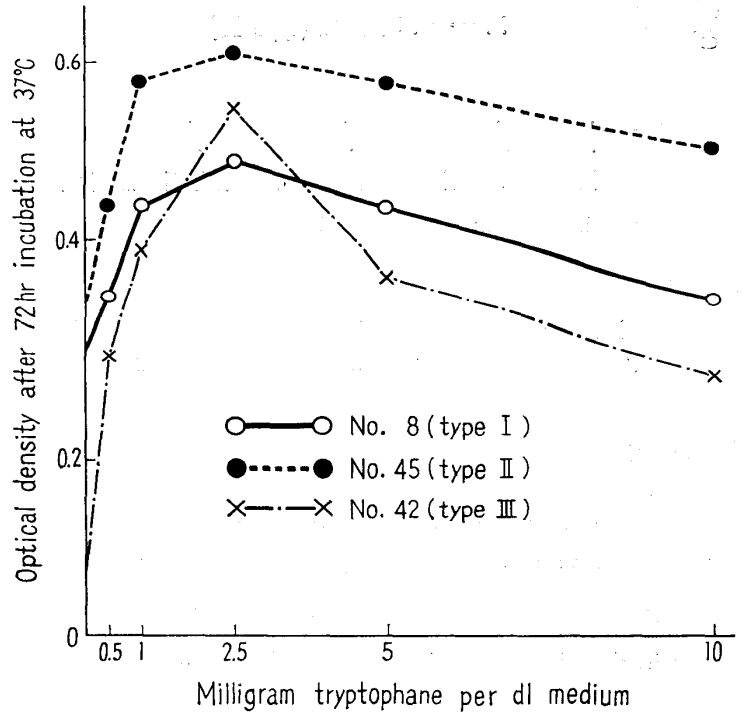
c) Tryptophane The optimum concentration of tryptophane was 2.5 mg/dl for all 3 types. Tryptophane was one of the essential amino acids for strain No. 42 (fig. 3).

FIGURE 2 *Effect of glutamic acid concentration on the growth of the 3 types of C. renale**



* The media used are indicated in the foot-note of table 4.

FIGURE 3 *Effect of tryptophane concentration on the growth of the 3 types of C. renale**

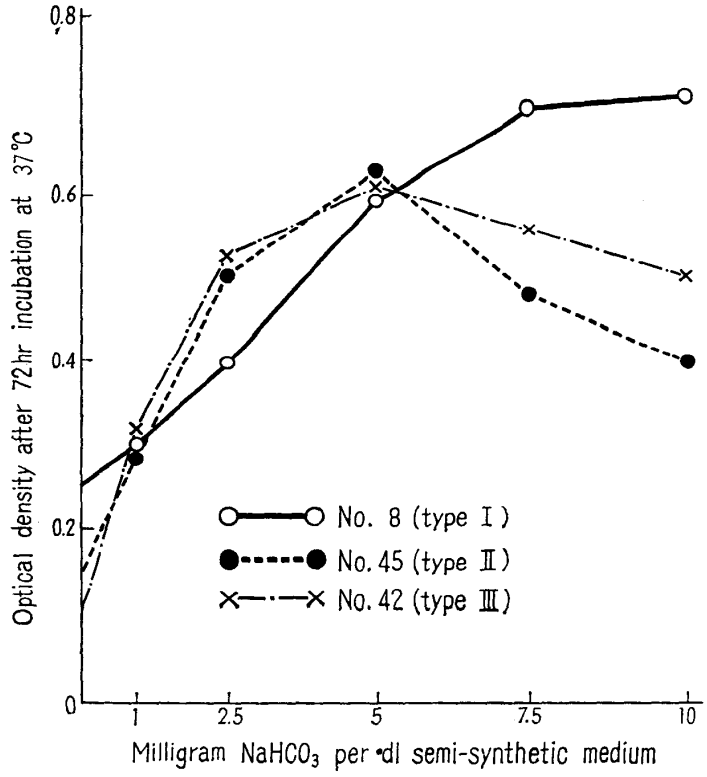


* The media used are indicated in the foot-note of table 4.

5 Sodium bicarbonate

Sodium bicarbonate stimulated growth of the 3 types of *C. renale*. Various levels of this substance were tested to determine the amount needed for optimum growth (fig. 4). Strain No. 8 showed an increased growth rate in a concentration of 10 mg/dl. While strains Nos. 45 and 42 showed optimum growth in the medium with 5 mg sodium bicarbonate per dl added.

FIGURE 4 *Effect of $NaHCO_3$ concentration on the growth of the 3 types of C. renale in a semi-synthetic medium**



* The media used are indicated in the foot-note of table 3.

6 Ammonium sulfate

Omission of ammonium sulfate resulted in a marked decrease in the growth of each strain (tab. 4). The optimum concentration was 10 mg ammonium sulfate per dl for each strain (fig. 5).

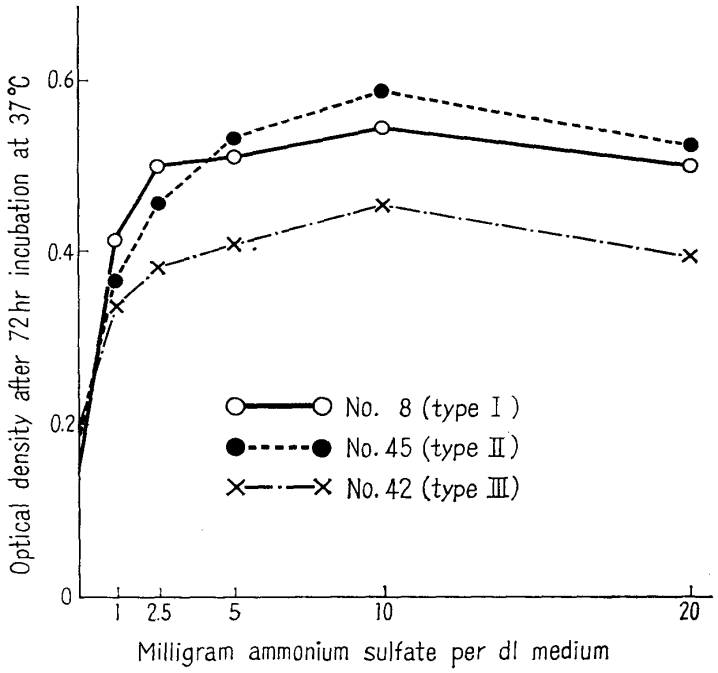
7 Purines and pyrimidines

The effects of purines and pyrimidines on the growth of *C. renale* were investigated with basal medium containing vitamin-free casamino acids. These factors were recognized as neither a stimulant nor inhibitor of the growth of the 3 strains used.

8 Growth of 5 strains of each type in semi-synthetic medium

It was suggested in the above experiments that the growth factors of *C. renale* differed

FIGURE 5 *Effect of ammonium sulfate concentration on the growth of the 3 types of C. renale**



* The media used are indicated in the foot-note of table 4.

among strains of different types. In order to confirm the correlation between the growth factor requirements and the type of *C. renale*, 5 strains were indiscriminately selected from each type, and the growth of these strains was compared in semi-synthetic medium. The semi-synthetic medium used was prepared for each type, according to their own vitamin requirements. The results are shown in table 5, in which the media used are designated A (for type I), B (for type II) and C (for type III). The composition of these media is indicated

TABLE 5 *Growth of 5 strains of each type in semi-synthetic media*[*1]

SEMI-SYNTHETIC MEDIUM	TYPE I					TYPE II					TYPE III				
	8[*2]	15	24	34	65	18	20	35	45	75	42	43	44	47	48
A	###[*3]	###	###	###	###	+	+	+	+	+	−	−	−	−	−
B	###	###	###	###	###	###	###	###	###	###	−	−	−	−	−
C	###	###	###	###	###	##	##	±	+	+	###	##	##	##	##

[*1] Media A, B and C were used for types I, II and III respectively. For the composition refer the foot-note of table 3.

[*2] Strain No.

[*3] Results expressed were from the optical density measured after 48 hr incubation at 37°C.

O.D. more than 0.4 ## O.D. 0.21~0.4 + O.D. 0.11~0.2
± O.D. 0.06~0.1 − O.D. 0~0.05

in the foot-note of table 3.

The strains of type I showed maximum growth in all the media A, B and C.

The strains of type II exhibited their best growth in medium B. Media A and C were inferior to medium B in supporting profuse growth of type II. p-Aminobenzoic acid was found to be the factor most responsible, because the growth was decreased in media A and C lacking p-aminobenzoic acid.

Strains of type III grew only in medium C, without exception. Pyridoxine, which was contained in medium C, was found to be a particularly essential growth factor for strains of type III.

Thus it was again emphasized that each type of *C. renale* had characteristic vitamin requirements.

9 Growth of strain No. 42 in synthetic, semi-synthetic and non-synthetic media

Because type III required a greater quantity of vitamins and amino acids than the other types, the growth of strain No. 42 of type III was compared using the synthetic, semi- and non-synthetic media. The synthetic medium used contained 19 amino acids and 5 vitamins as already mentioned and shown in the foot-note of table 4. Two kinds of semi-synthetic media whose main nitrogen source was the vitamin-free casamino acids, were used, to one 5 vitamins were added and to the other no vitamins were added. The non-synthetic media used were 0.5% peptone in the basal medium (tab. 1) and nutrient broth. The results are shown in table 6.

The growth in the synthetic medium and the semi-synthetic medium supplemented with the 5 vitamins was not at all inferior to that in the non-synthetic medium.

TABLE 6 *Growth of strain No. 42 (type III) in synthetic, semi-synthetic, and non-synthetic media*

	MEDIA	DAYS AFTER INOCULATION			
		1	2	3	5
Synthetic	Basal+19 amino acids*1+5 vitamins*2	0.10*3	0.26	0.48	0.62
Semi-synthetic	Basal+Casamino acid+5 vitamins	0.16	0.38	0.65	0.60
	Basal+Casamino acid	0.01	0.01	0.02	0.02
Non-synthetic	Basal+Peptone	0.12	0.12	0.14	0.22
	Nutrient broth	0.18	0.22	0.30	0.48

*1 Amino acids were indicated in table 1.
*2 Vitamins used for type III were indicated in the foot-note of table 4.
*3 Optical density

DISCUSSION

The data presented indicate that it is possible to obtain luxuriant growth of the 3 types of *C. renale* in a chemically defined medium and that the nutritional requirement differs among the 3 types of *C. renale* classified by YANAGAWA et al.

(1967). Type I required biotin, thiamine and pantothenic acid for maximum growth. These vitamins acted as growth stimulatory factors. Type II required biotin, nicotinic acid and p-aminobenzoic acid as essential growth factors, and pantothenic acid and thiamine as stimulants. Type III required biotin, nicotinic acid, pantothenic acid, thiamine and pyridoxine, all as essential growth factors. Pyridoxine was required by type III but not by type II while p-aminobenzoic acid was essential for type II but not for type III. Thus, from the viewpoint of complexity of growth factor requirements, the types are classified in the following order: types III, II and I.

Studies on the nutritional requirement of *Corynebacterium* have been done with *C. diphtheriae*, and *C. sepedonicum*, a causative agent of potato ring rote. MUELLER (1937 a, b) reported that pimeric acid, nicotinic acid and β-alanine stimulated the growth of *C. diphtheriae* gravis. The need of pantothenic acid for growth of *C. diphtheriae* gravis and mitis was established by EVANS et al. (1939). DREW & MUELLER (1951) described that thiamine was defined as an additional growth stimulatory factor for the Toronto strain of *C. diphtheriae* because of its accelerating effect upon the rate of growth and toxin-formation. However, the relationships of the growth factor requirements between *C. diphtheriae* gravis, mitis and intermedium were not understood at all. Generally, *C. diphtheriae* intermedium strain is said to require the most growth factors.

As for the growth factors of *C. sepedonicum*, the need for biotin, nicotinic acid and thiamine was reported by STARR (1949) and MACLACHLAN & THATCHER (1951). They described pantothenic acid as being required essentially, and thiamine, inositol and p-aminobenzoic acid stimulated the growth of this species. Recently, LACHANE (1960) noted that nicotinic acid, thiamine and biotin were necessary as essential growth factors.

In comparing the growth factor requirements of *C. diphtheriae*, *C. sepedonicum* with those of *C. renale* as presented by the authors, all 3 species of *Corynebacterium* require biotin, thiamine, pantothenic acid and nicotinic acid. The growth factor requirements of type I of *C. renale* is rather similar to *C. diphtheriae* and *C. sepedonicum* as described above. Types II and III strains of *C. renale* differed in the need of p-aminobenzoic acid, pyridoxine and sodium bicarbonate. However, it is striking that the growth factor requirements of these species is quite similar in general, despite the difference of their natural hosts, man, plants and cattle.

Another interesting point was that sodium bicarbonate stimulated the growth of type III of *C. renale* in particular. MICKELSON (1964) reported that biotin was stimulatory to growth of *Streptococcus pyogenes* and that biotin could be partially replaced with sodium bicarbonate. It was also noted sodium bicarbonate was required under anaerobic conditions by *Staphylococcus aureus* (FILDES et al., 1936).

Known reactions of biotin enzymes are all concerned with carbon dioxide fixation and fatty acid biosynthesis in reference to energy-rich bonds (OCHOA & KAZIRO, 1961). It is said that the reacting ion of carbon dioxide in carboxylation reactions catalysed by biothin enzymes is probably the bicarbonate ion. The need of the bicarbonate ion by *C. renale* will be an interesting problem in future research.

As for amino acids, DREW & MUELLER (1951) reported the rates of growth and toxin formation in a chemically defined medium of *C. diphtheriae*. Cystine and glutamic acid were essential for growth, valine, methionine, proline, tryptophane and leucine and probably glycine were required for maximum growth. On the other hand, the need of aspartic acid, leucine, alanine, proline, methionine, histidine and arginine for maximum growth of *C. sepedonicum* was established by LACHANCE (1962), but this organism did not show an absolute requirement of amino acids.

The essential amino acids common to the 3 types of *C. renale* included glutamic acid, valine and isoleucine. Strain No. 8 (type I) required only these 3 amino acids. Phenylalanine accelerated its growth. Strain No. 45 (type II) required the 3 amino acids essentially; the growth was accelerated with the addition of methionine, phenylalanine and histidine. Growth of strain No. 42 (type III) was most exacting. In addition to the common 3 amino acids, it essentially required tryptophane, also methionine, phenylalanine and arginine were needed for maximum growth. Strain No. 42 was characteristic in that it did not require histidine. Complexity in amino acid requirements are listed in the following order, types III, II and I.

The pattern of amino acid requirements differs among the 3 species of *Corynebacterium*. *C. diphtheriae* and *C. renale* both required glutamic acid and valine. Isoleucine and phenylalanine were required by *C. renale* but not by *C. diphtheriae*. Proline, leucine and glycine were required by *C. diphtheriae* but proved unnecessary for *C. renale*. The amino acid requirement of *C. sepedonicum* is much different from that of *C. renale*. Glutamic acid, valine and isoleucine, all essential for *C. renale*, were not required by *C. sepedonicum*. The observation of the growth inhibition by cystine in type I of *C. renale* was consistent with *C. sepedonicum* studies (LACHANCE, 1962). However, the cystine inhibition was not remarkable in types II and III of *C. renale*.

Omission of ammonium sulfate resulted in a marked decrease in the growth of the 3 types of *C. renale*. The effect of ammonium sulfate is caused by the ammonium ion and/or ammonia nitrogen.

The authors clarified the growth factor and amino acid requirement for luxuriant growth of *C. renale*. Establishment of a minimum medium, which is in progress, will be of value in studying genetical analysis, and biosynthesis

in *C. renale*.

SUMMARY

The growth factor and amino acid requirements of *Corynebacterium renale* were investigated with special reference to the 3 types of this species reported by YANAGAWA et al. (1967). The results are summarized as follows.

1) Strains of type I required thiamine, biotin and pantothenic acid for maximum growth. These vitamins acted as growth stimulatory factors. Strains of type II required biotin, nicotinic acid and p-aminobenzoic acid as essential growth factors. Thiamine and pantothenic acid acted as growth stimulants. Strains of type III which was most exacting, required thiamine, biotin, nicotinic acid, pantothenic acid and pyridoxine as essential growth factors. Without even one of these vitamins type III strains did not grow. Pyridoxine was required by type III but not by type II while p-aminobenzoic acid was essential for type II but not for type III.

2) Sodium bicarbonate stimulated the growth of all 3 types of *C. renale*.

3) Ammonium sulfate was necessary for maximum growth of each type.

4) The essential amino acid common to the 3 types of *C. renale* included glutamic acid, valine and isoleucine. Type I required only these 3 amino acids. Type II required the 3 amino acids essentially; the growth was accelerated with the addition of methionine, phenylalanine and histidine. Growth of type III was most exacting. In addition to the common 3 amino acids, it required tryptophane essentially. Methionine, phenylalanine and arginine were also needed for maximum growth.

Thus, in complexity, the requirements of both growth factors and amino acids was as follows: types III, II and I.

5) Growth inhibition by cystine was particularly noticeable in type I.

6) There was discussion on the comparison of the nutritional requirements of *C. diphtheriae*, *C. sepedonicum* and *C. renale*.

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