BIOTIN AND ARGININE REPLACEMENTS IN THE NUTRITION OF CLOSTRIDIUM SPOROGENES[1]

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Recent papers from this laboratory have dealt with the growth requirements of *Clostridium sporogenes*. Shull and Peterson (1948) described a chemically defined medium adequate for light growth. In addition to glucose, amino acids, salts, buffer, and sodium thioglycolate, biotin or oleic acid was essential, and nicotinic acid and *para*-aminobenzoic acid were stimulatory. They reported also that heavy growth resulted after the addition of an unidentified factor present in partial protein hydrolyzates. In a second publication, Shull, Thoma, and Peterson (1949) showed that the unidentified factor could be replaced by suitable concentrations of certain combinations of amino acids such as arginine, tyrosine, and phenylalanine. Oleic, vaccenic, linoleic, and ricinoleic acids could replace biotin if an emulsifying agent such as "tween 40" was included in the basal medium.

The present paper gives a further report about the vitamin and amino acid requirements of *C. sporogenes* and the conditions under which certain substances replace vitamins and amino acids in the nutrition of this organism.

METHODS

American Type Culture no. 10,000 was employed throughout this work. The media designated as P, PA, and IP have been described by Shull and Peterson (1948). P contains glucose, mineral salts, biotin, *para*-aminobenzoic acid, nicotinic acid, sodium thioglycolate, tryptophan, cystine, an acid hydrolyzate of casein, and phosphate buffer. Medium PA contains a mixture of crystalline amino acids in place of the hydrolyzed casein. IP is P to which 1 per cent "casitone," a pancreatic digest of casein, has been added. Two other media, Q and QA, were derived from medium P and medium PA, respectively, by the addition of 0.25 per cent L-arginine hydrochloride and 0.20 per cent L-tyrosine. Medium PO is the same as P except that 4 mμg of biotin has been replaced by 100 μg of oleic acid and 5 mg of "tween 40," a polyoxyethylene derivative of sorbitan monopalmitate. Medium QO bears a similar relation to Q.

The methods of preparing inoculum, conditions of fermentation, and methods of determining relative growth were the same as those described by Shull and Peterson (1948). The ammonia and glucose determinations (table 5) were the same as those used previously (Shull *et al.*, 1949).

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RESULTS

Biotin and structural relatives. In the preceding paper (Shull *et al.*, 1949) it was reported that the biotin requirement of *C. sporogenes* could be met by oxybiotin. A second replacement has been found in desthiobiotin. The effects of biotin, oxybiotin, and desthiobiotin separately or in various combinations on the growth of the organism are given in table 1. These data indicate that oxybiotin, in a biotin-free medium, showed a rather constant activity over a wide range of concentrations. On a molar basis, assuming that only one isomer is active, the potency of *dl*-oxybiotin at levels from 1 to 6 mμg per 10 ml ranged from 90 to 69 per cent of that of biotin. The biotin activity of *dl*-desthiobiotin, however, appeared to change from 44 to 100 per cent as the concentration was raised over a 6-fold range. When oxybiotin or desthiobiotin was added to a medium containing biotin, great variations in combined activity occurred. The biotin analogues were most active when 1 mμg was used with 2 mμg of biotin per 10 ml. Under these conditions oxybiotin and desthiobiotin had biotin equivalences of 187 and 190 on the molar basis. It is possible that the organism can use the *l*-form of oxybiotin or desthiobiotin, but only when it is provided with a suboptimal amount of biotin. A similar variation in the activity of desthiobiotin has been shown for other clostridia by Perlman (1948)

Serial transfer daily for 1 week was successful when desthiobiotin or oxybiotin replaced biotin in medium Q.

Other data not included in table 1 showed that biotin sulfone, which is a biotin antagonist for certain lactic acid organisms (Dittmer and Du Vigneaud, 1944) did not inhibit *C. sporogenes*. In fact, 90 μg of sulfone per 10 ml replaced biotin in a medium free of the latter. Three hundred mμg of sulfone had no effect with or without biotin. The fact that *C. sporogenes* belongs to a group of organisms that are active reducing agents suggests that the sulfone is converted to the natural substance.

Oleic acid and "tween 40." Shull and Peterson (1948) and Shull, Thoma, and Peterson (1949) grew *C. sporogenes* in a medium devoid of biotin by adding oleic, vaccenic, linoleic, or ricinoleic acid with "tween 40," a polyoxyethylene derivative of sorbitan monopalmitate. Maximal growth was reached consistently in an oleate medium even after 10 successive daily transfers. Moreover, the organism did not lose the ability to use biotin even after repeated subculturing in its absence. No difference in morphology was noted between organisms grown in a medium with biotin (P) or in one containing oleic acid (PO).

When *C. sporogenes* was grown with oleic acid as a substitute for biotin, a small amount of biotin (0.0039 mμg per ml) in excess of that contained in the inoculum was detected by assay with *Torula cremoris* (Williams, 1946). Apparently a little biotin is synthesized by this organism. Broquist (1949) has found that a similar situation exists with *Clostridium butylicum*, *Lactobacillus arabinosus*, and *Lactobacillus fermenti* grown in media containing oleic acid instead of biotin.

Although oleic acid (100 μg per 10 ml) and "tween 40" (5 mg per 10 ml) are more than sufficient to replace biotin, either substance alone is quite toxic. An antagonism between "tween" and biotin was noted by Shull and Peterson

TABLE 1

Activities of desthiobiotin and oxybiotin in combination with biotin

The medium was Q minus biotin. The figures in the last column were calculated as if only one isomer were active.

SUPPLEMENT			GALVANOMETER READING	D-BIOTIN EQUIVALENCE	
d-Biotin	*dl*-Oxybiotin	*dl*-Desthiobiotin		Weight	Moles
mμg/10 ml	*mμg/10 ml*	*mμg/10 ml*		*mμg/10 ml*	*per cent*
0	0	0	98	0	0
1.0	0	0	56	1.0	100
2.0	0	0	33	2.0	100
3.0	0	0	25	3.0	100
0	1.0	0	90	0.5	90
0	2.0	0	70	0.7	69
0	4.0	0	37	1.7	80
0	6.0	0	27	2.6	82
1.0	0.5	0	56	1.0	0
1.0	1.0	0	39	1.6	112
1.0	2.0	0	34	1.9	84
2.0	1.0	0	25	3.0	187
0	0	1.0	96	0.3	44
0	0	2.0	70	0.7	53
0	0	4.0	37	1.7	76
0	0	6.0	23	3.4	100
1.0	0	0.5	56	1.0	0
1.0	0	1.0	42	1.5	88
1.0	0	2.0	30	2.2	106
2.0	0	1.0	24	3.3	190

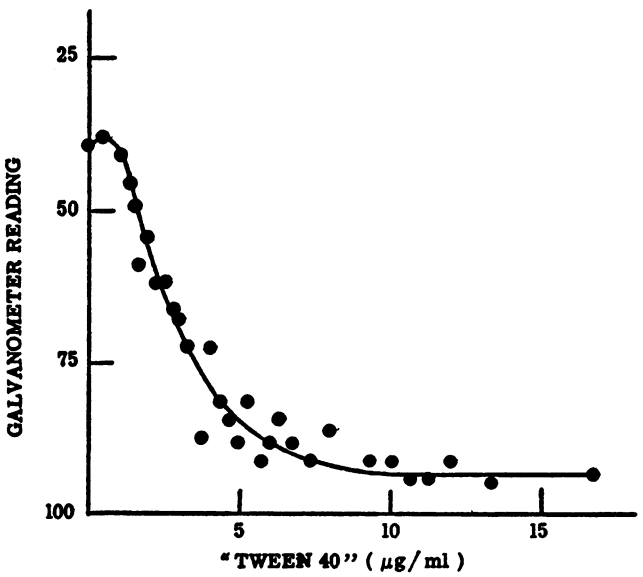

Figure 1. Inhibition of growth by "tween 40," medium Q, 20 hours.

(1948), who reported that as little as 0.5 mg of "tween 40" per 10 ml prevented growth even with biotin in 5,000 times the optimal requirement. Figure 1 illustrates the effect of "tween 40." With the usual inoculum, which gives an initial cell concentration of 1.5×10^7 per ml, the first significant effect of "tween" was with 1 μg per ml. Complete inhibition was obtained with about 10 μg per ml. Approximately the same "tween" to cell ratios were found to hold with other amounts of inoculum. The addition of live cell suspensions or cell autolyzates before autoclaving did not counteract the effect of "tween." "Tween 40" was no less inhibitory when it was added to the medium after separate sterilization.

The cells routinely used for inoculum were grown in the presence of 4 mμg of biotin per 10 ml of medium. One-tenth ml of a saline resuspension of these cells gave a low blank (G = 95) in biotin-free medium Q. Cells grown in a high-biotin medium (50 μg per 10 ml) stored enough biotin to allow full growth

TABLE 2

Growth of C. sporogenes in long periods

The basal medium was Q minus biotin. Inoculations were made from a culture in medium IP, without centrifuging and resuspending the cells. The usual resuspended inoculum gave an 18-hour reading of 95 to 100 (compare with column 5).

SUPPLEMENT			GALV. READ., RANGE OF 3 RUNS				
Biotin	"Tween 40"	Oleic acid	0 time	18 hours	23 hours	41 hours	72 hours
mμg/10 ml	*mg/10 ml*	*μg/10 ml*					
0	0	0	100	82–91	83–91	87–91	94–99
8	0	0	100	33–35	30–32	38–44	56–59
0	0	100	100	100–104	81–103	93–102	98–104
0	5	0	100	100–102	102–104	102	97–100
8	0	100	100	87–105	51–103	37–46	43–49
8	5	0	100	98–99	101	79–101	93–102
0	5	100	100	47–52	52–55	84–89	90–98

(G = 25) with the same inoculation procedure. The effect of "tween 40" as an inhibitor was no less marked when high-biotin cells were used as inoculum.

In order to discover whether or not "tween 40" has a direct effect on biotin, a solution containing 8 μg of biotin and 500 μg of "tween" per ml was allowed to stand 24 hours at room temperature. The solution was diluted 1,000-fold, and an aliquot was added to medium Q, for comparison with a biotin standard series. No diminution of biotin potency was noted.

Oleic acid in a medium free of biotin was said by Shull and Peterson (1948) to give erratic responses unless an agent such as "tween 40" was used. Table 2 is the record of several experiments in which biotin, "tween 40," and oleic acid were used alone and in combinations. Although the inoculum was heavy, no appreciable growth resulted in 140 hours with no supplement or when "tween" was the only addition. With biotin alone or with the "tween"-oleic-acid combination maximal growth was reached in 18 to 23 hours. Replicates deviated little from a mean. Erratic growth was encountered in tubes with oleic acid alone or

with oleic acid and biotin. In all media that contained biotin, heavy growth was sustained if it occurred at all. In biotin-free oleate media, however, rapid lysis seemed to follow the growth peaks.

It is possible that in any normal population of *C. sporogenes* widely different ranges of biotin-synthesizing ability and oleic acid tolerance obtain. It may also be that mutations to biotin independence or oleic acid resistance occur at low but definite frequencies. To test the permanent or transient nature of the abnormal cultures a series similar to that described in table 2 was grown, and frequent subcultures were made to several media to try to isolate a variant that would show a different relationship to biotin or oleic acid. No transfer from any

TABLE 3

Variations in the activity of ornithine

The medium was QA minus arginine. The weight equivalences in column four were read from a standard L-arginine hydrochloride curve (see figure 2). The molar values were calculated as if there were no optical specificity. For example: (1.6 − 0.5) ÷ 211 = 0.0052 moles of L-arginine·HCl; 5.0 ÷ 169 = 0.030 moles of DL-ornithine·HCl; (0.0052 ÷ 0.030) × 100 = 17 per cent.

SUPPLEMENT		GALV. READ.	L-ARGININE HYDROCHLORIDE EQUIVALENCE	
DL-Ornithine·HCl	L-Arginine·HCl		Weight	Moles
mg/10 ml	*mg/10 ml*		*mg/10 ml*	*per cent*
5.0	0.0	99	—	—
5.0	0.5	81	1.6	17
5.0	2.0	70	3.2	19
15.0	0.0	97	—	—
15.0	0.1	85	1.1	5
15.0	0.3	71	3.0	14
15.0	0.5	65	4.2	20
15.0	1.7	69	3.5	10
30.0	0.0	93	0.2	1
30.0	0.5	66	3.9	9
30.0	0.7	54	7.2	18
30.0	2.0	56	6.6	12

actively growing culture acted differently, in any medium, from *C. sporogenes* no. 10,000.

Amino acids. High concentrations of certain combinations of amino acids were found by Shull *et al.* (1949) to replace partial protein digests stimulating the growth of *C. sporogenes.* Arginine or ornithine in combination with tyrosine or phenylalanine produced heavy growth of the organism. Some later experiments with ornithine are summarized in table 3. In a medium high in tyrosine and devoid of arginine, ornithine gave little stimulation, even up to 30 mg. The maximal growth was effected by ornithine and arginine only when the arginine content of the medium was within certain limits. With 5 mg of DL-ornithine hydrochloride the arginine equivalence reached 19 per cent when 2 mg of L-arginine hydrochloride were added. With 15 mg of ornithine 0.5 mg of arginine

permitted the full ornithine effect. Thirty mg of ornithine were most efficient in promoting growth when arginine was present at about 0.7 mg. With this and higher amounts of ornithine erratic results were noted. DL-α,γ-Diaminobutyric acid, a lower homologue of DL-ornithine, was found to have no stimulatory activity in medium PA with several combinations of arginine and tyrosine.

Recently Broquist (1949) reported that the requirement of *Lactobacillus fermenti* for arginine could be satisfied by ornithine if biotin was in the medium, but in an oleate medium ornithine could substitute for arginine only in an atmosphere of CO_2. Similar experiments were run with *C. sporogenes*, but ornithine was unable to replace arginine when the CO_2 pressure was varied from 450 to 1,075 mm of Hg.

TABLE 4

Comparison of the L-*arginine activities of several compounds*

A standard L-arginine·HCl series was included in each experiment (see figure 2). No assumption about optical specificity was made in calculating the figures in column six.

EXPERIMENT NO. AND MEDIUM	SUBSTANCE ADDED		GALV. READ.	L-ARGININE HYDROCHLORIDE EQUIVALENCE	
	Name	Amount		Weight	Moles
		mg/10 ml		*mg/10 ml*	*per cent*
1					
Med. QA minus arg.	DL-Arginine·HCl	3.0	71	3.9	130
		12.0	38	15.1	126
2					
Med. QA minus arg.	D-Arginine·HCl	0.1	95	0.5	500
		1.0	87	1.4	140
		10.0	50	10.7	107
		20.0	37	20.0	100
3					
Med. QA minus arg.	DL-Citrulline	2.0	80	3.3	139
		6.0	54	8.9	123
4					
Med. Q minus arg.	DL-Citrulline	10.5	44	12.0	95
		21.0	39	17.9	71
		42.0	37	19.2	38

Table 4 is a summary of several experiments in which compounds chemically related to L-arginine were tested. Comparisons were made by reference to a standard arginine curve, an example of which is shown in figure 2. DL-Arginine hydrochloride was more active than the L-form in all cases. A fair estimate of its potency is 128 per cent on the molar basis, making no assumption about optical specificity. In minute amounts D-arginine was apparently several times more active than the L-isomer. As the optimal concentration was approached, the D-form became equivalent to the L-form. DL-Citrulline, in media with or

without arginine, ranged from 140 to 38 per cent arginine equivalence within limits of 2 and 42 mg.

In contrast with ornithine, citrulline does seem to be able to function as an essential amino acid. Perhaps the classical urea cycle of Krebs (1935) is involved, but it may be that the enzymes concerned with the conversion of ornithine to citrulline are not formed readily. The high activities of DL-arginine and DL-citrulline can be accounted for in part if it is assumed that the fermentation of pairs of amino acids by this organism is not restricted by the optical activity of the arginine or its analogue. This is in agreement with the discovery of Woods (1936), who, using the Thunberg technique to study the coupled reactions of

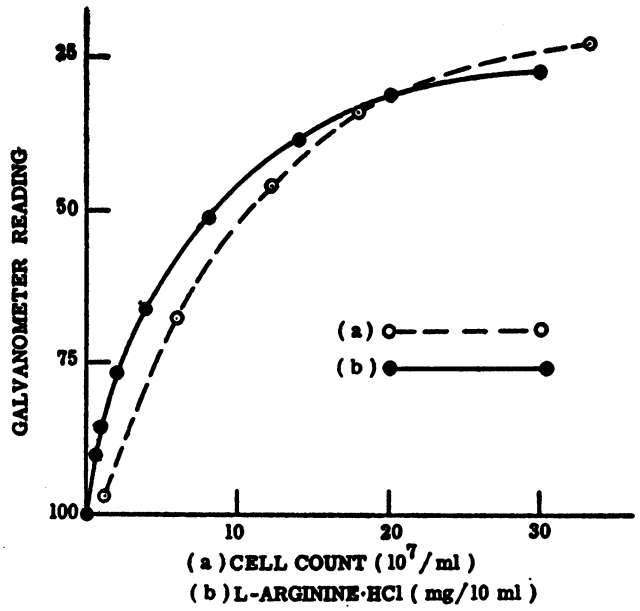

Figure 2. Standard curves. (a) Turbidity as a function of cell numbers in medium Q, 20 hours. Turbidity was read in an 18-mm tube in the Evelyn colorimeter with a 660-mμ filter. Cell counts were made with the Petroff-Hausser counting chamber. (b) Growth response to L-arginine hydrochloride, medium QA minus arginine, 18 hours.

amino acids first described by Stickland (1934, 1935*a*,*b*), reported that racemic ornithine was fully equivalent to the L-isomer as a hydrogen acceptor. The hydrogen donor of his systems, however, had to be the L-form.

Unlike racemic arginine, DL-tyrosine seemed to have only one active isomer. Four and 6 mg, assayed against an L-tyrosine standard in a high arginine medium, had activities of 50 and 41 per cent, respectively (data not shown).

Shull *et al.* (1949) presented evidence that arginine and tyrosine were broken down by this organism even in the presence of glucose. Table 5 shows later work of this sort. It is interesting that little ammonia was produced by growth in a "casitone" medium. The best utilization of glucose per unit of growth occurred with moderate levels (70 mg) of "casitone." The amount of glucose used was

increased, and the amount of ammonia formed was decreased, by the addition of tyrosine to any medium containing a considerable amount of either glucose or arginine or both. The addition of glucose to any of the media greatly reduced the amount of ammonia formed. If arginine was added to, or the amount of arginine was increased, in any of the media, the amount of ammonia formed was usually increased. This effect was least marked when both glucose and tyrosine were present in high concentrations and most pronounced when both glucose and

TABLE 5

The utilization of glucose and the formation of ammonia

The basal medium was P. Incubation was for 18 hours. The cell numbers were obtained from the standard curve (figure 2).

AMOUNTS BEFORE FERMENTATION				AMOUNTS AFTER FERMENTATION				
Glucose	L-Arg.·HCl	L-Tyr.	"Casitone"	Galv. read.	Glucose used		Ammonia formed	
mg/10 ml	*mg/10 ml*	*mg/10 ml*	*mg/10 ml*		*mg/10 ml*	*μμg/cell*	*mg/10 ml*	*μμg/cell*
100	0	0	0	76	8	170	0.5	10
100	0	0	30	61	22	260	0.6	7
100	0	0	70	48	34	270	0.7	5
100	0	0	150	37	42	250	1.5	9
100	25	0	0	60	14	160	4.5	51
100	25	5	0	45	29	220	4.3	32
100	25	10	0	39	37	230	4.2	27
100	25	20	0	31	43	220	4.4	22
0	0	0	0	90	—	—	1.1	61
100	0	0	0	73	9	170	0.3	6
0	0	20	0	76	—	—	2.2	47
100	0	20	0	68	14	209	0.3	4
0	10	0	0	74	—	—	3.2	62
0	20	0	0	73	—	—	4.5	84
0	50	0	0	71	—	—	6.4	111
100	10	0	0	65	8	107	1.9	25
100	20	0	0	60	—	—	3.1	34
100	50	0	0	61	15	174	4.9	57
0	10	20	0	59	—	—	4.2	46
0	20	20	0	64	—	—	5.1	65
0	50	20	0	58	—	—	7.5	80
100	10	20	0	30	35	170	1.5	7
100	20	20	0	24	37	123	2.7	9
100	50	20	0	23	41	124	6.9	21

tyrosine were low in concentration. Moreover, the additon of arginine to any medium seemed to decrease the amount of glucose used. In parallel experiments amino acid assays were performed on fermented and sterile media. Eighty-eight per cent of the arginine was destroyed when it was the only supplement; 97 per cent was destroyed when tyrosine, glucose, or both were present. Seventy-nine per cent of the tyrosine was used when it alone was present, but the addition of glucose reduced the utilization to 47 per cent. With arginine, or arginine and glucose, 93 to 99 per cent of the tyrosine was rendered inactive for the lactic acid assay organism.

Recently Raynaud and Macheboeuf (1946), using the technique of Woods (1936), showed that the presence of glucose did not retard the rate at which a heavy cell suspension of *C. sporogenes* dehydrogenated alanine, but glucose did impede the reduction of hydroxyproline. In our fermentation system arginine seems to be the counterpart of hydroxyproline.

SUMMARY

dl-Oxybiotin or *dl*-desthiobiotin can replace biotin in the nutrition of *Clostridium sporogenes*. Certain combinations of *d*-biotin with *dl*-oxybiotin or *dl*-desthiobiotin provide a stimulus that seems to indicate that both isomers of the biotin analogues are utilized.

When a fatty acid such as oleic is used in lieu of biotin, a substance like "tween 40" must also be added to the medium for normal growth in 16 to 20 hours. Either substance alone or with biotin is toxic in the normal period of incubation. The effect of "tween" in a biotin medium is dependent on the number of cells in the inoculum but not the amount of biotin present. The influence of oleic acid in the biotin medium is bacteriostatic and is overcome in long periods of incubation. Erratic growth occurs in the oleate medium without either "tween" or biotin.

In an arginine-free medium, DL-ornithine is without any appreciable effect, but with a small amount of arginine and a large amount of tyrosine it promotes growth. D-Arginine, DL-arginine, and DL-citrulline replace L-arginine in a medium free of the latter, and behave as if the D-form is fully utilized if a large amount of tyrosine is present. DL-Tyrosine is only half as active as L-tyrosine in a high-arginine medium.

If the amount of glucose used and the amount of ammonia formed are related to the amount of growth produced, it is seen that arginine and glucose have a mutual sparing action as fermentation substrates. In most cases tyrosine increases the utilization of glucose and reduces the amount of ammonia formed.

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