THE GROWTH OF CLOSTRIDIUM SEPTICUM AND ITS INHIBITION

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Chemical relatives of essential metabolites have been found to be agents for the inhibition of the growth of bacteria, and some of these have been used in the therapy of bacterial infections (Welch, 1945; Roblin, 1946; Woolley, 1946a). In the course of studies on the chemotherapy of gas gangrene we have investigated the nutritional requirements of *Clostridium septicum* with the intention of testing the effect of chemical relatives of growth factors upon its growth. Bernheimer (1944) has shown that a strain of this organism requires, among other things, pantothenic acid for growth. We (Ryan *et al.*, 1945) have shown that this requirement is due to an inability to synthesize the pantoyl moiety of the pantothenate molecule. The other portion of the molecule, β -alanine, is normally synthesized and coupled with the added pantoyl moiety to form pantothenate. The present paper reports studies on the effect of chemical relatives of these precursors and of pantothenate upon the *in vitro* growth of two strains of *C. septicum*, one of which is able to synthesize all parts of the pantothenate molecule.

EXPERIMENTAL

The chemically defined basal medium which was finally selected for the growth of *C. septicum* is a modification of that proposed by Bernheimer, and directions for its composition are as follows:

To 500 ml of distilled water add:

- 10 g glucose
- 20 g casamino acids
- 2.65 g monopotassium phosphate
- 7.14 g disodium phosphate
- 75 mg calcium chloride

Treat the solution for 30 minutes at room temperature with 5 g of norite A and filter through hyflo.

Add:

- 20 mg *l*(-)-tryptophane, dissolved in a small amount of water with heat
- 150 mg *l*(-)-cystine, dissolved in a small amount of water with hydrochloric acid and heat
- 20 mg glutamine
- 800 mg potassium bicarbonate
- 1 μ g biotin, crystalline-free acid
- 1 mg thiamine hydrochloride

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- 1 mg nicotinic acid
- 1 mg pyridoxine (pyridoxal or pyridoxamine can be substituted in an equal quantity without affecting the growth of *C. septicum*)
- 5 ml ferric sulfate solution made by dissolving 357 mg in a liter of distilled water with hydrochloric acid
- 2 ml salt solution, made by dissolving 50 mg $\text{Cu SO}_4 \cdot 5 \text{ H}_2\text{O}$, 50 mg $\text{Zn SO}_4 \cdot 7 \text{ H}_2\text{O}$, 20 mg $\text{Mn Cl}_2 \cdot 4 \text{ H}_2\text{O}$, 22.5 mg $\text{Mg SO}_4 \cdot 7 \text{ H}_2\text{O}$, and 1 ml of concentrated hydrochloric acid in 100 ml of distilled water

Bring to one liter and adjust the pH to between 7.0 and 7.5 with sodium hydroxide.

This medium may be stored under toluene in a refrigerator for at least 5 days. We have routinely prepared fresh medium every 2 or 3 days. Before use the proper amount of neutralized cysteine hydrochloride to make a 0.2 per cent solution was added. The cysteine was neutralized to phenol red in a small amount of water with 1 molar sodium hydroxide. The solution was added immediately to the culture medium, which was then sterilized by autoclaving for 10 minutes at 15 pounds' pressure. Under optimal conditions at 37 C, growth on this basal medium was complete in about 18 hours. In order to obtain the size of the final crop of bacteria, culture tubes were routinely measured after about 40 hours.

The yield of bacteria was determined routinely by the use of a densitometer. When necessary, cultures were diluted with 0.9 per cent sodium chloride to bring the density within the range of the instrument. A micro-Kjeldahl determination of bacterial nitrogen was made on washed cultures of strain 59 Li of *C. septicum* which had grown in complete medium for 20 hours. The densitometer was then calibrated in terms of mg of nitrogen found in such cultures, and readings have been expressed as mg N per 100 ml of culture. The average deviation of this method was about 5 per cent.

The number of viable organisms per ml in 40-hour cultures of strain 59 Li, determined by colony counts on blood agar plates, was 2×10^7 organisms, equivalent to 0.15 mg of bacterial N. Direct hemocytometer counts confirm this equivalence. However, during the early part of the logarithmic phase of growth, direct microscopic observation showed that about one-quarter of the organisms were in the form of the long unsegmented cells frequently found among bacteria whose growth is incompletely inhibited. The presence of these long cells required a very thorough shaking of the first dilution flask in the plating procedure in order that fragmentation would not cause the appearance of greater numbers of colonies as the dilutions progressed.

Consistent colony counts were obtained on both blood agar and complete medium plates when these were inverted over alkaline pyrogallol in Bray dishes. However, in plates made up with our basal medium and washed agar, results under similar conditions were very inconsistent. The same was true when the plates were incubated in desiccators which had been evacuated and refilled with gases such as nitrogen and hydrogen, with or without added carbon dioxide (Rockwell, 1921). Irregular results were also obtained when the plates, after cooling, were filled to the brim with fresh agar medium containing 0.2 per cent

cysteine but no cells. Apparently on the chemically defined medium the gaseous requirements of strain 59 Li are rather precise.

Success in making colony counts on the basal medium was attained by the use of the semisolid agar procedure developed by Redowitz (1941) for cultures of *Lactobacillus acidophilus*. This involved the dilution of a culture of *C. septicum* with basal medium containing cysteine. One ml of the appropriate dilution was added to 9 ml of basal medium containing cysteine and 0.15 per cent washed agar. This had been melted and kept at 44 C in a test tube. This mixture was taken up and ejected from a 1-ml blow-out pipette 10 times, and then 1 ml was transferred with the same pipette to the next tube of semisolid agar. From the last tube 1 ml of medium was discarded after mixing, and in this way tenfold dilutions were obtained. The tubes were incubated at 37 C for about 24 hours, when they showed cottony but discrete colonies. About 30 colonies is the maximum number that can be conveniently counted in 9 ml of semisolid medium about 3 inches deep. Determinations by this method of the number of bacteria in nine samples from a single culture of *C. septicum* gave an average value of $3.7 \pm 0.4 (\sigma_M) \times 10^7$ per ml.

Stock cultures of the bacteria were grown on an egg-meat medium at 37 C and then stored (presumably as spores) at room temperature. Inocula were prepared by transferring a loopful of the egg-meat medium to test tubes containing about 15 ml of the complete medium. This medium consisted of 0.25 per cent sodium chloride, 0.1 per cent sodium bicarbonate, 0.34 per cent disodium phosphate, 0.082 per cent monopotassium phosphate, 0.5 per cent glucose, 2 per cent Difco tryptose, 0.3 per cent Difco yeast extract, and 0.1 per cent neutralized cysteine hydrochloride; the pH was 7.4. Growth in this medium was allowed to take place between 10 and 18 hours, at which time the experimental tubes were inoculated with a loopful of bacterial suspension. The data in the tables and figures of this paper are averages of at least duplicate determinations.

Two strains of *C. septicum*, 59 Li and 44, were obtained from Dr. Alan Bernheimer, to whom we are indebted. Early in our studies of strain 59 Li it was found that under standard conditions the length of the lag period varied considerably on our basal medium. Indeed, frequently among a series of mated tubes no growth appeared in some. It was found that this behavior could be completely eliminated by incorporating the pyrimidine, uracil, in the medium. When this was done, growth was regularly complete in 18 hours, and growth failures were not encountered. A culture of 59 Li which had grown up in the absence of uracil was plated out, and strain 59 Li A was isolated. This new strain showed consistent growth that was independent of uracil. The genetics of this situation is the subject of a separate communication (Ryan *et al.*, 1946). Strain 59 Li A was used instead of its parent in most of the following experiments.

RESULTS

Nutritional requirements. We have confirmed the observation of Bernheimer (1944) that the vitamins, pyridoxine, nicotinic acid, thiamine, and biotin, are required for the growth of strain 59 Li of *C. septicum*. In addition, strains 59

Li and 59 Li A, but not strain 44, require *d*-pantothenic acid (3 μ g per ml). The latter requirement can be satisfied by the addition of sodium *dl*-pantoate or pantoyl lactone to the medium (Ryan *et al.*, 1945). Our basal medium, however, differs from that of Bernheimer in several respects. The carbon treatment and filtration remove not only a slight precipitate but also such contaminants of the casamino acids as pantothenate, pantoate, or pantoyl lactone. Strain 59 Li, which requires pantothenic acid or the pantoyl moiety, showed no growth whatsoever on our basal medium until one of these compounds was added. Bernheimer, on the other hand, showed that in the absence of pantothenic acid strain 59 Li grew on his medium to about 35 per cent of its maximum yield. In the following experiments with 59 Li A, which has the same pantothenate requirements as its parent strain, 59 Li, calcium *d*-pantothenate was present in the medium in a concentration of about 3 μ g per ml. Unless otherwise specified strain 44 was grown in medium without added pantothenate.

In our hands cysteine hydrochloride proved a better reducing agent for bacterial growth than thioglycolic acid. A concentration between 0.1 and 0.2 per cent was optimum.

Autoclaving the basal medium was found to be much simpler than making the series of sterile additions proposed by Bernheimer. Such treatment resulted in a growth of strain 59 Li A (14.4 mg N per 100 ml) equal to that obtained on Bernheimer's medium (with 0.2 per cent cysteine, 13.4 mg N per 100 ml). Nevertheless when our basal medium to which cysteine had been added was not autoclaved but was sterilized by filtration through a sintered glass bacterial filter crucible, growth was increased to 19.8 mg N.

This difference is not associated with the toluene used for preservation of the basal medium which is boiled off during autoclaving. The stimulation appeared after sterile filtration of fresh medium, to which toluene had never been added, as well as after filtration of medium which had been preserved under toluene for 2 days. The increased crop of bacteria on the sterile filtered medium was also not due to the removal of some toxic factor during filtration, because we failed to find such stimulation when autoclaving followed or preceded sterile filtration. On the other hand, the time of autoclaving bears a definite relation to the yield of bacteria. In an experiment in which sterile-filtered medium after 48 hours supported growth equivalent to 17.1 mg N per 100 ml, autoclaving for 20, 10, and 5 minutes resulted in final growths of 15.0, 16.2, and 17.7 mg N per 100 ml, respectively. This, and the fact that a slight insoluble precipitate is formed upon autoclaving, suggests that the effect of autoclaving might be due to the loss of some factor in the medium. Glutamine, for example, would be expected to be almost completely destroyed upon autoclaving. However, when glutamine was sterile-filtered separately and added to autoclaved medium, no increase in growth after 48 hours was noted (15.9 and 16.5 mg N per 100 ml). Moreover, the hypothesis of loss by autoclaving does not seem tenable in view of the fact that the final crop of bacteria did not increase in media where the concentration of vitamins, casamino acids, sugar, or salts was doubled or halved. The concentration of these substances in the whole medium was optimum for the growth of strain 59 Li A of *C. septicum*.

It appears, rather, that the effect of autoclaving may be due to the production of some toxic substance(s) from material in the medium. It is known that glucose, when autoclaved under alkaline conditions, will form many different compounds (Evans, 1929). Table 1 lists the yields of strain 59 Li A obtained when glucose was sterilized separately and then added to the medium. The medium was made up as previously mentioned except that the glucose and phosphate were omitted at first. In the case of the autoclaved and sterile-filtered controls the glucose and phosphate which were not carbon-treated were added to the medium just before it was sterilized. The experiments consisted of the sterile additions of the specified substances to the remainder of the medium, which was autoclaved with or without phosphate. Autoclaving was at 15 pounds for 10 minutes, and sterile filtration was through a sintered glass bacterial filter. The controls yielded somewhat less growth than when our medium was prepared as usual, but sterile filtration was definitely better than autoclaving. Likewise, whenever glucose was sterile-filtered, growth was increased slightly. However, when the glucose was autoclaved separately and then added to the medium,

TABLE 1

Growth of strain 59 Li A after 48 hours in media to which glucose was added after being separately sterilized

TREATMENT OF GLUCOSE	MG N PER 100 ML
Autoclaved in medium.....	10.8
Sterile-filtered with medium.....	16.1
Autoclaved separately.....	15.3
Sterile-filtered separately.....	11.4
Autoclaved with phosphate.....	15.3
Sterile-filtered with phosphate.....	12.8

there was a considerable increase in growth. Apparently autoclaving glucose separately or with phosphate produced some stimulating factor, which was not pyruvic acid (Smiley, Niven, and Sherman, 1943), but when glucose was autoclaved with the rest of the medium, inhibiting factors were produced as well. Our analysis of this interaction is admittedly incomplete, and certainly more substances than glucose are involved. Nevertheless, we interpret the effect of sterile filtration as due to the absence of formation of inhibitory materials, whereas the limit of growth on autoclaved medium is balanced by both inhibitory and stimulatory factors, which are formed during autoclaving. Strain 44, on the other hand, does not show a difference in the amount of growth in sterile-filtered as compared with autoclaved medium.

During the growth of strain 59 Li A the pH of the basal medium fell from neutrality to 5.1. Cessation of growth was not due to the acidity of the medium alone, for partially grown cultures when washed and transferred to fresh medium of pH 5.1 continued their growth. Moreover, increasing the buffer capacity of the medium by doubling the salt concentration did not increase the yield of bacteria. However, the optimum pH is between 7.0 and 7.5 when growth equiv-

alent to 16.6 and 17.1 mg N per 100 ml was obtained after 48 hours. At pH's of 6.5 and 8.0 somewhat less growth, or 14.4 and 14.1 mg N per 100 ml, resulted.

The factor(s) limiting final growth can be modified by the addition of natural products such as Difco tryptose or Fleischman's yeast extract, as table 2 shows. Yeast extract seems more active for strain 59 Li than tryptose, but strain 44 may be more sensitive to the inhibitors it contains. Although the 2-day yields shown in table 2 were influenced by tryptose, the rate of growth was not (figure 1).

TABLE 2

Yield of bacteria in mg N per 100 ml of medium plus tryptose or yeast extract after 48 hours

STRAIN CONCENTRATION MG PER ML	59 LI A		44	
	Tryptose	Yeast extract	Tryptose	Yeast extract
0	14.8	15.7	8.5	8.5
1	14.7	16.8	9.0	10.1
3	—	19.4	—	—
10	18.2	17.7	16.7	12.0
20	21.9	18.4	19.3	13.8
40	13.9	—	17.2	—
100	15.6	3.0	15.3	5.7

FIG. 1. THE RATE OF GROWTH OF STRAIN 59 LI A IN THE PRESENCE (OPEN CIRCLES) AND ABSENCE (SOLID CIRCLES) OF 20 MG TRYPTOSE PER ML

The time has been designated from an arbitrary zero point since the lag period in tryptose is much shorter than in its absence.

In 14 determinations of the generation time of strain 59 Li A during the logarithmic phase of growth on medium supplemented with 20 mg tryptose per ml, an average of 70 minutes was obtained, whereas 62 minutes was the average generation time of the controls. Tryptose does, however, influence the length of the lag period. The time required to reach a growth equivalent to 2 mg N per 100 ml averaged about 1,100 minutes in three experiments on our basal medium, but the presence of 20 mg of tryptose per ml shortened this time to about 500 minutes.

A wide variety of growth factors were tested in an attempt to duplicate the stimulation caused by tryptose. Among these were tested, individually and in combination, 19 amino acids, asparagine, guanine, adenine, xanthine, uracil, yeast nucleic acid, choline, glutathione, pyruvic acid, oleic acid, folic acid concentrate, vitamin B₆ and B₆ conjugate, pimelic acid, *p*-aminobenzoic acid, inositol, riboflavin, pyridoxal, pyridoxamine, welchii factors (Ballentine *et al.*, 1944), and iron.² None of these substances increased the yield of bacteria on our medium.

It is possible that some polypeptide growth factor, like strepogenin (Sprince and Woolley, 1945; Womack and Rose, 1946) is present in tryptose and responsible for its effect on *C. septicum*. The addition of 1 mg of Difco casamino acids per ml did not reproduce the tryptose stimulation. It is also possible that tryptose may bind some trace element present in toxic quantities (Hutner, 1946), although this seems improbable in view of the absence of effect when the salt composition of the medium was doubled or halved. Tryptose contains material(s) active in supporting the growth of uracil-requiring strains of *Neurospora crassa*, *Escherichia coli*, and *Shigella paradysenteriae*. This material cannot, however, be responsible for the effect of tryptose on the uracil-independent strain of *C. septicum*, 59 Li A. The effect of tryptose may be due to its content of several factors influencing the lag period and the final growth independently. The growth of strain 44 on basal medium is irregular in the sense that an appreciable fraction of a series of similar cultures fail to grow. Bernheimer (personal communication) suggests that carbon dioxide or bicarbonate is important for the initiation of growth of strain 44. In our autoclaved medium the bicarbonate would be lost. However, the incorporation of 20 mg of tryptose per ml, but not of purines or pyrimidines, results in the growth of all cultures. It may be that there is some substance in tryptose required for the growth of strain 44, and that growth in its absence is due to mutation to independence of this factor just as in the case of the uracil requirement of strain 59 Li (Ryan *et al.*, 1946). Moreover the absence of glutamine from the basal medium results in a similarly erratic growth of strain 59 Li. Glutamine itself is destroyed under the conditions of pH and temperature used in sterilization, whereas glutamic acid is a component of the basal medium. The effect of glutamine may be due to the existence of organisms independent of some contaminating heat-stable factor (Ballentine *et al.*, 1947). Neither the genetic nor the chemical nature of these effects has been investigated.

One of the vitamin analogues, sodium β -pyridine sulfonate, which was studied as a possible inhibitor of growth (McIlwain, 1940) actually increased the bacterial crop. The average 48-hour yield of five experiments with 59 Li A in which 10 mg of this compound per ml were present was 17.1 mg per 100 ml, compared to the control yield of 12.8 mg N per ml. The presence of 1 mg per ml resulted in a similar increase. Raoul (1945) found that high concentrations of 3-pyridine

² The authors wish to thank the following for supplying them with certain growth factors: Dr. R. J. Williams for the folic acid concentrate, Dr. J. Piffner for the vitamin B₆ conjugate, and Dr. E. E. Snell for the pyridoxal and pyridoxamine.

sulfonic acid inhibited the growth of *E. coli* and *Bacillus proteus*, although lower concentrations favored growth. Since the addition of nicotinic acid in concentrations from 0.25 to 4.0 μg per ml did not result in increased growth, it is improbable that the stimulatory action of β -pyridine sulfonate is due to its substitution for the analogous vitamin, nicotinic acid. The action of sodium β -pyridine sulfonate is probably different from that of tryptose or of sterile filtration, for when combined with these treatments the effects seem to be additive. The same is true of the action of tryptose and sterile filtration (table 3). A combination of the last two yielded the maximum growth of strain 59 Li A of *C. septicum* that we have obtained—24 mg of bacterial nitrogen per 100 ml of culture medium.

The incomplete nature of our basal medium is also apparent in another way. There are a minimum number of cells of *C. septicum* (between 10^2 and 10^3) which when inoculated into 10 ml of liquid basal medium will result in growth. Since

TABLE 3

Growth of strain 59 Li A in mg N per 100 ml after 48 hours in basal medium to which combinations of supplements had been added and which was sterilized by different methods

METHOD OF STERILIZATION	SUPPLEMENT	MG N PER 100 ML
Autoclaved	None	13.7
Autoclaved	20 mg tryptose per ml	18.8
Autoclaved	10 mg sodium- β -pyridine sulfonate per ml	16.8
Autoclaved	Tryptose and sodium- β -pyridine sulfonate	19.6
Filtered	None	17.1
Filtered	20 mg tryptose per ml	24.0
Filtered	10 mg sodium- β -pyridine sulfonate per ml	18.8

the inocula were centrifuged and resuspended in basal medium three times, it is not likely that the effect of inoculum size is due to a carry-over of some required material from the complete medium. In 10 ml of basal medium rigidified with 0.15 per cent semisolid agar, single colonies are frequently found in tubes containing 10^{-7} dilutions. Thus, it is possible that the cells are slowly able to synthesize some required catalytic substance which can be lost in the medium. In very dilute bacterial suspensions this material is lost too rapidly for growth to take place. On the other hand, in semisolid agar medium the material may not be carried away rapidly enough to bring the concentration in the environment of the cell to a subthreshold level. It is also possible that the relationship between growth and inoculum size is not due to a stimulatory substance but rather to an inhibitor, which seems to be the case in the tubercle bacillus (Davis and Dubos, 1946).

Inhibitors. Besides studies on the chemical analogues of pantothenic acid and its precursors, a few experiments with derivatives of other vitamins were performed. In concentrations from 1 to 10 mg per ml, γ -(3,4-ureylenecyclohexyl)-

butyric acid³ and desthiobiotin did not appreciably decrease the growth of strain 59 Li A and were not studied further.

Sodium *dl*-pantoyltaurine, the sulfonic acid derivative of pantothenic acid,

TABLE 4

The effect of chemical relatives of pantothenic acid and its precursors on the growth of the pantothenate-independent strain 44 expressed as percentage of the 48-hour bacterial crop produced by controls grown in the absence of these substances

ANALOGUE 10 MG PER ML	NONE	SUPPLEMENTS TO BASAL MEDIUM	
		0.35 μ g Sodium <i>dl</i> -pantoate per ml	0.01 μ g Calcium <i>d</i> -pantothenate per ml
Sodium <i>dl</i> -pantoyltaurine.....	90	102	81
Sodium γ -hydroxy buteryl taurine....	—	83	92
Sodium γ -hydroxy buterate.....	0	0	21
<i>dl</i> -Pantoic amide.....	93	122	113
<i>dl</i> - β -Amino butyric acid.....	—	—	88
Taurine.....	100	107	92

TABLE 5

The effect of chemical relatives of pantothenic acid and its precursors on the 48-hour crop of bacteria produced by the pantothenate-dependent strain 59 Li A

ANALOGUE 10 MG PER ML	PER CENT OF 14.4 MG N PER 100 ML*	SUPPLEMENTS TO BASAL MEDIUM			
		0.35 μ g Sodium <i>dl</i> -pantoate per ml		0.01 μ g Calcium <i>d</i> -pantothenate per ml	
		Per cent of controls	Per cent of 14.4 mg N per 100 ml*	Per cent of controls	Per cent of 14.4 mg N per 100 ml*
Sodium <i>dl</i> -pantoyltaurine.....	73	758	79	619	85
Sodium γ -hydroxy buteryl taurine....	—	116	19	107	67
Sodium γ -hydroxy buterate.....	27	400	35	244	26
<i>dl</i> -Pantoic amide.....	98	1034	88	757	104
<i>dl</i> - β -Aminobutyric acid.....	—	—	—	111	14
Taurine.....	0	34	5	24	2
None—control.....	0	100	11	100	19

* 14.4 mg N per 100 ml was the amount of growth produced on an optimum amount (3 μ g per ml) of calcium *d*-pantothenate in the absence of the pantothenic analogues.

and sodium γ -hydroxy buteryl taurine,⁴ a compound in which both parts of the pantothenate molecule have been modified, were chosen as possible antagonists

³ In low concentrations this compound is known to inhibit the growth of yeast, *Lactobacillus casei* (English *et al.*, 1945) and *Lactobacillus arabinosus* (Axelrod *et al.*, 1946). We wish to thank Dr. J. O. Lampen for supplying us with a sample.

⁴ Sodium γ -hydroxy buteryl taurine, not previously described, was prepared by the fusion for 3 hours at 125 C of γ -hydroxy buteryl lactone and sodium taurine in equimolar quantities. The substance was recrystallized from isopropyl alcohol and ether.

of pantothenic acid. Taurine and *dl*- β -aminobutyric acid were selected as analogues of β -alanine, and sodium γ -hydroxy buterate and *dl*-pantoic amide as relatives of pantoyl lactone. Table 4 shows the effect of these compounds on the growth of strain 44 of *C. septicum*, which is independent of an external supply of pantothenic acid. The only compound with an appreciable effect in the relatively high concentration of 10 mg per ml was sodium γ -hydroxy buterate. The growth of the pantothenate-requiring strain, 59 Li A, on the other hand, is

FIG. 2. THE RELATION OF THE AMOUNT OF GROWTH OF STRAIN 59 LI A AFTER 58 HOURS TO THE CONCENTRATION OF TAURINE IN A MEDIUM TO WHICH 20 MG TRYPTOSE PER ML HAS BEEN ADDED

TABLE 6

Growth of strain 59 Li A in mg N per 100 ml after 58 hours in basal medium plus 20 mg tryptose per ml and in the presence of different concentrations of taurine and pantothenate or its precursors

TAURINE MG PER ML	CALCIUM <i>d</i> -PANTOTHENATE μ G PER ML			SODIUM <i>dl</i> -PANTOATE μ G PER ML		β -ALANINE μ G PER ML
	3	100	1,000	100	1,000	1,000
0	12.6	11.0	9.1	10.6	5.9	7.6
50	3.3	2.8	4.8	3.3	2.6	4.7

inhibited by taurine but not by the other pantothenate analogues (table 5). Indeed, some of the other pantothenate analogues actually supported the growth of 59 Li A in the absence of pantoate and pantothenate, as shown in the first column of table 5. These compounds, sodium γ -hydroxy buterate, *dl*-pantoic amide, and sodium *dl*-pantoyltaurine, also stimulated growth in the presence of suboptimal amounts of pantoate and pantothenate. The percentage to which this stimulation approximated maximum growth is indicated in the third and fifth columns of table 5.

Complete inhibition of the growth of strain 59 Li A is not achieved with concentrations of taurine even approximating 1 molar in strength (figure 2). Table

FIG. 3. THE INHIBITION OF THE GROWTH OF STRAIN 44 BY DIFFERENT CONCENTRATIONS OF SODIUM γ -HYDROXY BUTERATE AFTER 58 HOURS IN A MEDIUM CONTAINING 20 MG TRYPTOSE PER ML AND EITHER 1 MG CALCIUM *d*-PANTOTHENATE PER ML (SOLID CIRCLES), 1 MG SODIUM *dl*-PANTOATE PER ML (OPEN SQUARES), 1 MG β -ALANINE PER ML (SOLID SQUARES), OR NO ADDED SUPPLEMENT (OPEN CIRCLES)

FIG. 4. THE INHIBITION OF THE GROWTH OF STRAIN 59 Li A BY DIFFERENT CONCENTRATIONS OF SODIUM SALICYLATE AFTER 48 HOURS IN A MEDIUM DEVOID OF TRYPTOSE BUT CONTAINING EITHER 3.2 μ g CALCIUM *d*-PANTOTHENATE PER ML (OPEN CIRCLES), 100 μ g CALCIUM *d*-PANTOTHENATE PER ML (CLOSED CIRCLES), OR 100 μ g SODIUM *dl*-PANTOATE PER ML (OPEN SQUARES)

6 shows that there may be a small reversal of taurine inhibition by increasing the pantothenate concentration or by the addition of β -alanine. If real, this

effect is slight and is not brought about by the addition of pantoate. The fact that strain 59 Li A grows at all in the absence of added pantoate or pantothenate but in the presence of β -alanine is due to the activity of tryptose in the medium, which in independent assays seemed to contain the equivalent of about 0.28 μ g of pantothenate per mg.

The growth of strain 44 can be completely inhibited by a concentration of approximately 1 molar sodium γ -hydroxy buterate (figure 3). Moreover, the inhibition can be at least partially reversed by the presence of high concentrations of pantothenate. Sodium *dl*-pantoate and β -alanine, in the concentrations tried, had no effect in reversing the inhibition. Apparently an antagonist of sodium γ -hydroxy buterate inhibition is to be found in tryptose, as a comparison of the

TABLE 7

Growth of strains 59 Li A and 44 in mg N per 100 ml after 58 hours in the basal medium plus 20 mg tryptose per ml and in the presence of different concentrations of salicylate and pantothenate or its precursors

SODIUM SALICYLATE MG PER ML	CALCIUM <i>d</i> -PANTOTHENATE μ G PER ML			SODIUM <i>dl</i> -PANTOATE μ G PER ML		β -ALANINE μ G PER ML
	3	100	1,000	100	1,000	1,000
Strain 59 Li A						
0	15.2	14.5	12.4	13.7	11.1	11.7
0.5	4.9	5.2	4.4	5.0	3.2	3.3
1.0	1.5	0.7	1.4	0.5	0.7	0.7
2.0	0.1	0	0	0	0	0
Strain 44						
0	9.3	9.7	8.9	9.0	7.5	—
0.5	2.3	2.5	3.4	2.7	0.6	—
1.0	0.1	2.4	1.8	2.0	1.4	—
2.0	0.2	0.2	1.2	0.2	0.3	—

degree of inhibition by 10 mg of inhibitor per ml in a medium with (figure 3) and without tryptose (table 4) will show. The same seems to be true of taurine inhibition of strain 59 Li A (figure 2 and table 5).

Neither taurine nor sodium γ -hydroxy buterate appeared to be inhibitors worth further study because of the large size of the effective concentrations, and because the relatively poor reversibility by pantothenate indicated considerable nonspecificity. Ivanovics (1942*a* and 1942*b*) has shown, for bacteria able to make their own pantothenic acid, that salicylate acts as a specific inhibitor and interferes with the synthesis of the pantoyl moiety of the molecule. Our data on *C. septicum* suggest that a similar relationship holds.

Strain 59 Li A, which is unable to make its own pantothenate, is inhibited by sodium salicylate but in a way that is not influenced by the pantothenate, pantoate, or β -alanine concentration of the medium (figure 4 and table 7). Investiga-

tion of the length of the lag period and the logarithmic rate of growth indicated that they did not behave differently in this respect than the final amount of growth. Preliminary studies of the inhibition by salicylate of the growth of strain 44 in a medium devoid of tryptose suggested that the inhibition could be reversed by pantoate or pantothenate. These studies were repeated in a medium containing 20 mg tryptose per ml and hence always a small amount (0.28 μ g per ml) of pantothenate. The results are shown in table 7. There appears to be a slight decrease in the salicylate inhibition in the presence of high concentrations of pantoate or pantothenate, although the reversal is not very great. About 1,000 μ g of calcium pantothenate per ml is the maximum concentration that can be used. After such a concentrated solution in our medium is autoclaved, a slight precipitate is formed whose turbidity is subtracted from that measured after the growth of the bacteria.

DISCUSSION

It is significant that of the possible pantothenic acid antagonists which we have used, three, sodium γ -hydroxy buterate, *dl*-pantoic amide, and sodium *dl*-pantoyltaurine, were able to substitute for pantothenic acid and supported the growth of pantothenate-requiring *C. septicum*. One of these compounds, sodium *dl*-pantoyltaurine, has been used as a chemotherapeutic agent (McIlwain and Hawking, 1943). It is very possible that our pantoyltaurine and pantoic amide were contaminated with some of the pantoyl lactone used in their preparation. As little as between 0.5 and 1.0 μ g pantoyl lactone per mg of analogue is all that would be needed for the effects we observed. It would be necessary, however, to assume an unreasonable degree of contamination with pantoyl lactone to explain the results of Stansly and Alverson (1946). These authors report an antagonism of the salicylate inhibition of the growth of *Escherichia coli* by pantoyltaurine. It is more probable that some hydrolysis of pantoyltaurine occurs yielding *dl*-pantoate and taurine (cf. Sarett and Cheldelin, 1945a). Such a hydrolysis of about 2 per cent of the pantoyltaurine would not release sufficient taurine to inhibit significantly the growth of strain 59 Li and would be consistent with the data we have obtained.

On the other hand, the pantothenate activity of sodium γ -hydroxy buterate cannot be similarly explained, for this compound was not made from pantoyl lactone and cannot be directly transformed into it. Apparently γ -hydroxy buterate has about one hundred-thousandth of the catalytic activity of pantothenate.

There have been many attempts to inhibit specifically the utilization of pantothenic acid in bacteria by the use of chemical analogues of that growth factor (Nielson, Hartelius, and Johansen, 1944; Nielson and Roholt, 1945; Drell and Dunn, 1946; Mead *et al.*, 1946; cf. Roblin, 1946, for earlier references). The specific activities of these compounds not only depend upon their chemical constitution but are correlated with the pantothenic acid requirement of the organism being inhibited. With some exceptions pantothenate-independent

organisms are resistant to inhibition by pantothenate analogues. A possible explanation of this relationship is that suggested by McIlwain (1942), which assumes that pantothenate produced within the cell is more effective in reversing inhibition than pantothenate supplied from the outside. He has been able to show (1945) that pantoyltaurine prevents the binding of pantothenate into functional form. On the other hand, Stansly and Alverson (1946) suggest that the pantoate formed from such compounds as pantoyltaurine (or the pantoyl lactone they contain) may be sufficient to enable an organism which can synthesize pantothenate from the pantoyl moiety to produce sufficient pantothenate to prevent inhibition. However, McIlwain's explanation is in better accord with the finding of Snell (1941*a* and 1941*b*) that pantoyltaurine inhibited the growth of a yeast stimulated with pantothenic acid, but it was ineffective when the yeast grew on β -alanine. Yet Sarett and Cheldelin (1945*b*) did not observe this relationship in a strain of yeast which was inhibited by pantoyltaurine when grown either on β -alanine or pantothenic acid. At the present time, in the absence of much fundamental information such as the permeability of vitamins and analogues, it is difficult to apply a general interpretation to the distribution of pantoyltaurine resistance among bacteria. For example, Woolley and Collyer (1945) have shown that phenyl pantothenone inhibited all of the organisms tested, although the inhibition was reversed by pantothenic acid only in those organisms which required pantothenic acid as a growth factor. Again, both pantothenate-independent and pantothenate-dependent strains of *C. septicum* are resistant to pantoyltaurine. Since interference with pantothenate metabolism is antagonized by amino acids (Ivanovics, 1942; Woolley, 1946*b*), it is possible that the resistance of the two strains of *C. septicum* was due to the high concentration of amino acids in our culture medium.

Investigations of the inhibition of growth of microorganisms by the use of chemical analogues of the pantothenate precursor, β -alanine, have also been numerous. Snell (1941*a* and 1941*b*), for example, failed to secure the inhibition by taurine of the growth of *Saccharomyces cerevisiae* stimulated with β -alanine, and Nielson (1943) and Nielson *et al.* (1944) confirmed this observation, although Sarett and Cheldelin (1945*b*) did find 1 of 17 strains of yeast requiring pantothenic acid or β -alanine for growth which was susceptible to taurine inhibition when grown on β -alanine. In the presence of β -alanine, but not of pantothenic acid, it was found that asparagine (cf. Weinstock *et al.*, 1939, and Atkin *et al.*, 1944) and many *l*-amino acids and natural products would inhibit yeast growth. These inhibitions were reversed by high concentrations of β -alanine. The same relations were found by Nielson and Johansen (1943) and Nielson *et al.* (1944) in the inhibition of yeast growth by many α -amino acids, isoserine, β -phenyl- β -alanine, and β -aminobutyric acid, and by Hartelius (1943) in the inhibition of yeast respiration by β -aminobutyric acid. In *Acetobacter suboxydans*, which requires pantothenic acid or the pantoyl moiety for growth, Sarett and Cheldelin (1945*a*) found that *dl*-alanine inhibited growth in the presence of pantothenic acid. Taurine inhibited the growth of this form more in the presence of the

pantoyl moiety than in the presence of intact pantothenate, and the inhibition was completely overcome by excess β -alanine. These results suggest that the analogues inhibit by competing with β -alanine and hence interfering with the synthesis of pantothenic acid.

In *C. septicum* β -aminobutyric acid did not inhibit the growth of either the pantothenate-dependent or pantothenate-independent strains even when it was present in high concentrations. On the other hand, taurine did inhibit the growth of the pantothenate-dependent strain but only when present in very high concentrations. On this account and because pantothenate and β -alanine were so inefficient in their reversal of the inhibition, it is likely that most of the activity of taurine was nonspecific. Nevertheless, the failure of pantoate to antagonize taurine inhibition and the very slight antagonism shown by pantothenate and β -alanine suggest that part of the activity of taurine may be due to its interference in pantothenate synthesis through its competition with β -alanine. In the case of the inhibition of the growth of the pantothenate-independent strain of *C. septicum* by γ -hydroxy buterate, high concentrations were also necessary. Nevertheless, pantothenic acid was able to reverse this inhibition partially. If γ -hydroxy buterate interfered with the synthesis of pantothenate by competing with the pantoyl moiety, we would expect pantothenate to reverse the inhibition and β -alanine to be relatively ineffective. This was realized. We would also expect pantoate to reverse the inhibition. In the concentrations used such a reversal did not occur. Probably only the *d*-pantoate could be suspected of activity. Moreover, on a molar basis calcium *d*-pantothenate is about 20 times as active as sodium *d*-pantoate in supporting the growth of *C. septicum*, and even more so when activity is calculated in terms of undissociated molecules (Ryan *et al.*, 1945). A further increase in the pantoate concentration may have resulted in a reversal. However, once again, inhibition occurred only in the presence of high concentrations of γ -hydroxy buterate and was not completely reversed by very large concentrations of pantothenate. Probably a large part of the inhibition is nonspecific in nature, though some of the inhibition is due to the specific interference with pantothenic synthesis.

Roblin (1946) has suggested that when an inhibitor affects the synthesis of a metabolite, the "minimum effective concentration of the metabolite which prevents the action of the antagonist is effective against any concentration of the latter." In the case of the inhibition of *C. septicum* by γ -hydroxy buterate the continued effect of increasing concentrations of this compound in the presence of high concentrations of pantothenate is probably due to the fact that its action is primarily nonspecific. If, on the other hand, as Nielson *et al.* (1944) propose, the action of β -alanine analogues is due to the fact that they become coupled with the pantoyl moiety to form inactive derivatives of pantothenic acid, then the suggestion of Roblin would not apply.

Our results on the inhibition of the growth of *C. septicum* by salicylate are in general agreement with the conclusions reached by Ivanovics (1942*a* and 1942*b*) and Stansly and Schlosser (1945). The pantothenate-independent strain was inhibited by salicylate, and this inhibition was partially prevented by panto-

ate and pantothenate but not by β -alanine. Such a relationship would be expected if salicylate were interfering with pantothenate synthesis by competition with the pantoyl moiety. The pantothenate-dependent strain should then be inhibited by salicylate only in high concentrations and then in a way that is not reversed by either pantothenate or its split products. The salicylate inhibition of the pantothenate-dependent strain of *C. septicum* was not reversed by these substances. However, the concentration of salicylate required for the complete inhibition of the pantothenate-independent strain is only about one-half of that required for the complete inhibition of the pantothenate-dependent strain (about 0.01 M). Ivanovics was able to show that amino acids could eliminate the specific antivitamin action of salicylate. Perhaps in the case of the pantothenate-independent strain of *C. septicum* the presence of casamino acids (and of tryptose) in the medium antagonized much of the specific inhibitory action of salicylate. The inhibition observed may be largely nonspecific and hence resemble the salicylate inhibition of the pantothenate-dependent strain. These data then do not disagree with the hypothesis that salicylate can interfere with pantothenate synthesis by competing with the pantoyl moiety.

None of the inhibitors described in this paper were successful as chemotherapeutic agents against experimental gas gangrene in mice (Ryan *et al.*, 1946).

SUMMARY

Two strains of *Clostridium septicum* were used in this study. One is unable to synthesize the pantoyl moiety of the pantothenate molecule and hence, for growth, requires either of these substances in the medium. The other strain can make, and is independent of an external supply of, these substances.

An improvement of Bernheimer's chemically defined medium is described. An examination is made of the role of several factors, such as sterile filtration and natural products like tryptose, in increasing growth.

Several chemical analogues of β -alanine, of the pantoyl moiety, and of pantothenate were chosen as possible antivitamins. Of these only two were active, sodium γ -hydroxy buterate inhibiting the pantothenate-independent strain and taurine inhibiting the pantothenate-dependent strain. In addition, sodium salicylate inhibits growth in a way that suggests that it interferes with the synthesis of the pantoyl moiety of the pantothenate molecule.

Three of the analogues, sodium *dl*-pantoyltaurine, sodium γ -hydroxy pantoate, and pantoic amide, have pantothenate activity for the pantothenate-dependent strain.

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