The Synthesis of Citrovorum Factor by *Leuconostoc mesenteroides* P 60

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SUMMARY

When incubated with *p*-aminobenzoic acid, formate and glucose, suspensions of a variant strain of *Leuconostoc mesenteroides* strain P 60 formed two factors possessing folic acid activity. One of these factors behaved like rhizopterin in supporting the growth of *Streptococcus faecalis* strain R but not of *Lactobacillus casei*. The other, like leucovorin, supported the growth of *Leuconostoc citrovorum*. The synthesis of 'citrovorum factor' was enhanced 5- to 20-fold by the presence of CO₂ in the gas phase. Sulphathiazole inhibited the synthesis of both factors, the inhibition being overcome competitively by *p*-aminobenzoic acid. Leucovorin was destroyed by suspensions of *L. mesenteroides* P 60 in phosphate-buffered glucose. The diminution of activity for *L. citrovorum* (decrease of citrovorum factor) was decreased when the incubation was carried out in a CO₂-enriched atmosphere. Ascorbic acid, which behaved similarly to CO₂ in partially preventing inactivation of leucovorin by suspensions of *L. mesenteroides* P 60 variant strain, also increased the synthesis of citrovorum factor from formate and *p*-aminobenzoic acid and partially satisfied the requirement of the growing *L. mesenteroides* P 60 for CO₂.

INTRODUCTION

Carbon dioxide will replace serine for the growth of *Leuconostoc mesenteroides* P 60 provided that glycine is present at relatively high concentration; yet, under these conditions, ¹⁴CO₂ is not incorporated into the serine synthesized (Cross, 1960). The growth of *L. mesenteroides* P 60 also occurs in the absence of both serine and CO₂, when the *p*-aminobenzoic acid in the medium is replaced by leucovorin (Lascelles, Cross & Woods, 1954). This suggested that the group of reactions in which the intervention of CO₂ is necessary might be those leading from *p*-aminobenzoic (*p*-AB) acid to the final coenzyme form of folic acid. The present work provides evidence of the dependence of the '*L. citrovorum* factor' (citrovorum factor) concentration in suspensions of a variant strain of *L. mesenteroides* P 60 upon the presence of carbon dioxide in the gas phase.

METHODS

Leuconostoc mesenteroides P 60. The two strains used were those used by Cross (1960). The variant strain was derived from *L. mesenteroides* P 60 (*Streptococcus equinis* P 60; ATCC 7881) by repeated subculture in the absence of added serine. The variant strain synthesizes serine more efficiently in growing cultures and in

suspensions than does the parent strain. The media, conditions of growth and the method of assessment of growth were as described previously.

Suspensions of organisms deficient in *p*-aminobenzoic acid and in vitamin B₆ were also obtained and incubated as previously described. The complete solution for the synthesis of citrovorum factor normally contained: glucose, 10^{-2} M; sodium formate, 10^{-2} M; *p*-aminobenzoic acid, 10^{-5} M; phosphate buffer, pH 6.9, 10^{-1} M. In some experiments, in which the synthesis of serine and citrovorum factor were being observed concurrently, the reaction mixture (solution *D*) also contained: glycine, 2×10^{-2} M and pyridoxal, 10^{-6} M (Cross, 1960). Modifications in particular experiments are noted in the text.

In experiments in which the organisms were incubated in 2×10^{-2} M-glucose and 10^{-1} M-phosphate buffer (pH 6.9) before testing their synthetic ability, the procedure was again exactly as previously described (Cross, 1960).

Microbiological assay of citrovorum factor. The preparation of material for the assay of citrovorum factor and the assay with *Leuconostoc citrovorum* (ATCC 8081) was carried out as described by Lascelles & Woods (1952, 1954). Leucovorin was used as a standard and gave a dose-response curve similar to that of the sample under test (Fig. 1). The effective range of the assay was from 0.2 to 1.5 $\mu\mu$ mole leucovorin with an accuracy of about $\pm 10\%$. Results are expressed in terms of $m\mu$ M-leucovorin.

Microbiological assay of 'Lactobacillus casei factor'. The preparation of material was carried out as for the citrovorum factor assay. The assay with *Lactobacillus casei* (ATCC 7469) was performed by the method of Nimmo-Smith, Lascelles & Woods (1948), slightly modified in that a final volume of 2.0 ml./tube was found adequate to obtain reproducible results. As in other growth experiments, extent of growth was estimated with an EEL photoelectric colorimeter (Evans Electro-selenium Ltd., Harlow, Essex) using 0.25 in. tubes and a neutral density filter.

Microbiological assay of factors active for Streptococcus faecalis R. The material for assay was prepared as above. The test organism was *S. faecalis* Rogers (ATCC 8043) maintained as described by Lascelles & Woods (1954); the assay procedure was that of Lascelles & Woods (unpublished). Medium *G* of Lascelles & Woods (1954), modified by the exclusion of thymine and pteroylglutamic acid, and the addition of pyridoxine (1 mg./l.) was the basal medium. The latter, together with the test solution or pteroylglutamic acid as standard, was distributed to 1.9 ml. final volume in hard glass tubes (125 $\times$ 16 mm.), and autoclaved for 7 min. at 10 lb./sq.in. The inoculum was derived from a fresh stab culture in medium *B*₁ which was sub-cultured into medium *B*₂ (Nimmo-Smith *et al.* 1948) and incubated 18 hr. at 37°. This culture was diluted fifty-fold, and each tube inoculated with 0.1 ml. (about 10^7 organisms). Incubation was for 20–24 hr. at 37° in air.

Standards containing pteroylglutamic acid (1 to 16×10^{-10} M) were set up in each assay; the content of factors active for *Streptococcus faecalis* R is recorded as $m\mu$ M equivalent pteroylglutamic acid by direct comparison of the response obtained at two concentrations with the dose-response curve given by synthetic pteroylglutamic acid.

The response curve for samples approximated to that for pteroylglutamic acid (PtG) at lower concentration (Fig. 2). The deviation at higher levels will be discussed in the text.

Chemicals. The pteroylglutamic acid was 'Folvite' (Lederle Laboratories, Pearl River, New York, U.S.A.). The source of all other materials, and the preparation and storage of stock solutions, were described by Lascelles *et al.* (1954).

Fig. 1

Fig. 2a

Fig. 2b

Fig. 1. Response of *Leuconostoc citrovorum* to leucovorin (●—●) and to a typical sample (○—○) containing active factor synthesized by suspensions of *L. mesenteroides* P 60 variant strain.

Fig. 2a. Response of *Streptococcus faecalis* R to pteroylglutamic acid (○—○) and to a typical sample (●—●) containing active factor synthesized by suspensions of *Leuconostoc mesenteroides* P 60 variant strain.

Fig. 2b. Response of *Streptococcus faecalis* R to I, N^{10} -formylpterico acid (○—○) and a typical sample (●—●) containing active factor synthesized by suspensions of *Leuconostoc mesenteroides* P 60 variant strain; and II, pterico acid (+—+). N^{10} -formylpterico and pterico acids were added, as $4 \times 10^{-9}M$ and $4 \times 10^{-8}M$ solutions respectively diluted from $10^{-4}M$ solutions in 'boiled control' samples. The sample was added as a 1 in 60 dilution of the incubated system.

RESULTS

The synthesis of citrovorum factor by suspensions of Leuconostoc mesenteroides P 60 variant strain

Lascelles & Woods (1954) showed that during the synthesis of serine from glycine and formate by suspensions of *Streptococcus faecalis* R, there was a concurrent formation of a factor or factors active for the growth of *L. citrovorum*. A similar appearance of this factor attended the production of serine by suspensions of *L. mesenteroides* P 60; glucose, formate and *p*-aminobenzoic acid were essential substrates (Table 1; Figs. 3-5).

The growth of *Leuconostoc mesenteroides* P 60 in the absence of serine and leucovorin is dependent on the enrichment of the atmosphere with CO_2 (Lascelles *et al.* 1954). No effect of such a gas phase on the synthesis of serine by suspensions of *L. mesenteroides* P 60 variant stain were detected, but the increase in citrovorum factor synthesis was considerable, varying from 5- to 20-fold in a number of experiments.

A maximum effect of CO_2 was observed at a partial pressure of 20 % atmospheric (Fig. 6). The enhancement of citrovorum factor synthesis cannot be ascribed to a

Table 1. *Synthesis of factors with folic acid activity by suspensions of Leuconostoc mesenteroides P 60 variant strain*

Incubation was in the basal solution *D* (with omissions as below) for 24 hr.

Omissions from basal solution	Gas phase	' <i>S. faecalis</i> R factor' found (m μ M)	'Citrovorum factor' found (m μ M)
None		350	40
None (heated control)		5	1
Glycine	20 % (v/v) CO ₂	300	40
Pyridoxal	+	350	40
Formate	80 % (v/v) N ₂	20	3
<i>p</i> -AB		5	1
Glucose		30	3
None	N ₂	350	4

change in the pH value of the medium (Table 2). Over a range of initial pH values from 7.1 to 6.1 the decrease in pH during incubation in N₂ was about 0.3–0.6 of a unit. This increased to between 0.5 and 0.8 of a pH unit when incubation was in 20 % (v/v) CO₂ + 80 % (v/v) N₂. Though citrovorum factor formation was apparently increased by an acid environment, the synthesis in N₂ at initial pH 6.1 was only about 40 % of that in 20 % (v/v) CO₂ + 80 % (v/v) N₂ at initial pH 7.1. The increased synthesis in more acid media cannot account for the carbon dioxide effect.

Fig. 3

Fig. 4

Fig. 3. Rates of synthesis by suspensions of the variant strain of 'citrovorum factor' (○—○) and 'Streptococcus faecalis R factor' (●—●) in solution *D*. Curves I and III were obtained by incubation in N₂; curve II by incubation in 20 % CO₂ in N₂.

Fig. 4. Effect of formate concentration on the synthesis of 'citrovorum factor' (○—○) in 20 % CO₂ in N₂, and 'Streptococcus faecalis R factor' (●—●) in N₂ by suspensions of the variant strain. Incubation for 24 hr. in solution *D* omitting pyridoxal and glycine.

It seemed possible that CO₂ might be involved in the formation of the glutamic acid moiety of citrovorum factor. Both DL- and L-glutamic acid were inactive however, over the concentration range 10⁻¹ to 10⁻⁴ M.

The glucose concentration is also a critical factor in the production of citrovorum factor; peak synthesis occurred at *c.* 10⁻² M-glucose.

Table 2. Effects of pH value and CO₂ on synthesis of folic acid group factors by suspensions of *Leuconostoc mesenteroides* P 60 variant strain

Suspensions incubated in solution D minus glycine and pyridoxal for 24 hr.

pH value of suspension			'Citrovorum factor' ($\mu\mu$ m) found.		'S. faecalis R factor' ($\mu\mu$ m) found.
After incubation in			After incubation in		
Initial	N ₂	CO ₂	N ₂	CO ₂	
7.1	6.6	6.8	4.5	33.0	380
6.7	6.4	6.2	7.0	33.0	125
6.8	5.9	5.6	10.5	35.5	75
6.1	5.5	5.8	18.5	31.5	55

As for maximum serine synthesis, 5×10^{-3} M-formate was the minimum concentration which gave an optimum yield of citrovorum factor (Fig. 4). However, production of the latter increased with *p*-aminobenzoic acid concentration up to 10^{-6} M, a hundred times that required for most rapid formation of serine (Fig. 5). Pteroylglutamic acid was not converted to citrovorum factor by suspensions.

The ability of the organisms to synthesize citrovorum factor varied with their physiological age. For best activity the organisms for suspension were harvested after 10–11 hr. as previously described (Cross, 1960).

Fig. 5

Fig. 5. Effect of *p*-aminobenzoic acid concentration on the synthesis of 'citrovorum factor' (●—●) (incubation in 20% CO₂ in N₂) and 'Streptococcus faecalis R factor' (○—○) (incubation in N₂) by suspensions of the variant strain incubated in solution D omitting glycine and pyridoxal, for 24 hr.

Fig. 6

Fig. 6. Effect of partial pressure of CO₂ on the destruction of leucovorin and the synthesis of 'citrovorum factor' by the variant strain. Leucovorin remaining (●—●) from an initial concentration of 1000 m μ M after incubation with organisms suspended in phosphate buffer and 10^{-3} M-glucose; 'citrovorum factor' synthesized (○—○) in solution D omitting glycine and pyridoxal. Incubation was for 24 hr. at 37° in both cases.

The synthesis of factors active for Streptococcus faecalis R by suspensions of Leuconostoc mesenteroides P 60, variant strain

The supernatant fluids from suspensions of the variant strain in solution D incubated in N₂ (which contained serine and a little citrovorum factor) were also active in supporting the growth of *S. faecalis* R, and in replacing pteroylglutamic acid

The concentration of active factor in terms of equivalent pteroylglutamic acid was about one hundred times that of the citrovorum factor present, in terms of equivalent leucovorin (Fig. 3). Since it is likely that the (-)-L-isomer of leucovorin (the active isomer) has activity for *S. faecalis* R equal to that of pteroylglutamic acid (Broquist *et al.* 1952; Cosulich, Smith & Broquist, 1952), and the citrovorum factor synthesized by *L. mesenteroides* P60 would appear to be at least closely related to leucovorin, it seems that this activity for *S. faecalis* R cannot be ascribed to the small quantity of citrovorum factor present. Furthermore, suspensions incubated in 20 % CO₂ yielded the same, or in some experiments a lower, concentration of '*S. faecalis* R factor' than those incubated in N₂, in contrast to the greater activity of the former supernatant fluids for *L. citrovorum* (Table 1).

In other respects the requirements for '*Streptococcus faecalis* R factor' synthesis are identical to those for formation of citrovorum factor (Table 1; Figs. 3-5). Glucose, *p*-aminobenzoic acid and formate were essential components of the system; omission of glycine and pyridoxal from solution *D* had no significant effect. The production of '*S. faecalis* R factor', like that of citrovorum factor, reaches a maximum at 10⁻⁵M-*p*-aminobenzoic acid and 5 × 10⁻³M-formate. The synthesis, however, increases with glucose concentration up to 10⁻¹M; there is no peak activity at a lower concentration as with citrovorum factor.

Neither after incubation in CO₂ nor in N₂ was any activity for *Lactobacillus casei* demonstrable beyond that which could be ascribed to citrovorum factor in the samples. The response of *Streptococcus faecalis* R to the preparations was not due, therefore, to the presence of pteroylglutamic acid, which also supports growth of *L. casei* (Broquist *et al.* 1952).

The response of *Streptococcus faecalis* R to the supernatant fluid from a suspension of *Leuconostoc mesenteroides* P60 variant strain in glucose, phosphate buffer, formate and *p*-aminobenzoic acid incubated in N₂ was compared to the response to pterioic acid and rhizopterin (Fig. 2*b*). The pterioic acid and rhizopterin added as standards were diluted from 10⁻⁶M solutions in supernatant fluids from 'boiled control' suspensions of the variant strain in the above medium. The dose/response curve for the active samples much more nearly approached that for rhizopterin than that for pterioic acid.

*The activity of suspensions of Leuconostoc mesenteroides P60
previously incubated in glucose*

After pre-incubation, *Leuconostoc mesenteroides* P60 variant strain incubated in solution *D* did not yield significant quantities of citrovorum factor whether CO₂ was present or not; however, their ability to synthesize '*Streptococcus faecalis* R factor' was not impaired (Table 3).

*The response of Leuconostoc mesenteroides P60 to factors formed by
suspensions of the variant strain*

It was not possible to draw any conclusions from direct tests of the ability of samples to support the growth of *Leuconostoc mesenteroides* P60 since these samples contained relatively high concentrations of *p*-aminobenzoic acid which is the most potent member of the folic acid group known for this organism. However, the relative efficiencies of samples in antagonizing sulphathiazole-inhibition were as

Table 3. *Synthesis of folic acid group factors by suspensions of Leuconostoc mesenteroides P 60 variant strain*

Organisms incubated in basal solution D for 24 hr. after pre-incubation where indicated.

Gas phase	'Citrovorum factor' (m μ M) found when organisms		'S. faecalis R factor' (m μ M) found when organisms	
	Pre-incubated	Normal	Pre-incubated	Normal
N ₂	0.2	0.2	550	550
20 % (v/v) CO ₂ + 80 % (v/v) N ₂	0.2	8.2	550	550

might be expected if a rhizopterin-like compound were formed by incubating suspensions in N₂, together with a leucovorin-like compound when incubation was in an atmosphere enriched with CO₂ (Table 4). Sulphathiazole inhibited growth of the parent strain in the presence of a sample showing activity for *Streptococcus faecalis* R equivalent to 2.2×10^{-8} M-pteroylglutamic acid; but did not inhibit, even at 10^{-3} M-sulphathiazole, a sample (from incubation in 20 % CO₂) which showed also citrovorum factor equivalent to 4.2×10^{-9} M-leucovorin. Rhizopterin is inactive for the growth of *L. mesenteroides* P 60 (Lascelles *et al.* 1954).

Table 4. *Antagonism of sulphathiazole-inhibition of the growth of Leuconostoc mesenteroides P 60 by factors of the folic acid group*

Leuconostoc mesenteroides P 60 was grown in medium L supplemented with 5×10^{-4} M-glycine, 10^{-3} M-DL-serine and 2×10^{-7} M-pyridoxal in air. The added samples were derived from incubated suspensions of the variant strain prepared for assay in the usual way.

Sample A (from incubation in N₂) contains '*Streptococcus faecalis* R factor' equivalent to 2.2×10^{-8} M-PtG and 'citrovorum factor' equivalent to 0.1×10^{-9} M-leucovorin.

Sample B (from incubation in CO₂) contains '*S. faecalis* R factor' equivalent to 1.8×10^{-8} M-PtG and 'citrovorum factor' equivalent to 4.2×10^{-9} M-leucovorin.

Sample added (% (v/v) of final volume)	Sulphathiazole added (M)	Sample added	
		A	B
		Growth (EEL reading at 44 hr.)	
0.5	—	24.0	21.5
0.5	10^{-3}	8.0	18.0
1.0	—	21.5	23.5
1.0	10^{-3}	8.5	24.0
2.0	—	20.5	24.0
2.0	10^{-3}	10.5	30.5

The inhibition of the synthetic ability of suspensions of the variant strain by sulphathiazole

Like the synthesis of serine (Cross, 1960), the synthesis of citrovorum factor and '*Streptococcus faecalis* R factor' by suspensions of *Leuconostoc mesenteroides* P 60 variant strain was inhibited by sulphathiazole; each inhibition was competitively antagonized by *p*-aminobenzoic acid (Table 5). '*S. faecalis* R factor' formed by suspensions of *L. mesenteroides* P 60 variant strain did not annul sulphathiazole-inhibition of the growth of *L. mesenteroides* P 60 in a non-competitive manner. Yet

the Streptococcus factor is the product of a sulphathiazole-inhibited reaction in *L. mesenteroides* P60. The simplest interpretation of these facts is that the Streptococcus factor cannot support the growth of *L. mesenteroides* P60. This is again in accordance with indications that the Streptococcus factor is a rhizopterin-like compound.

Table 5. *Inhibition by sulphathiazole of the synthesis of folic acid group factors by suspensions of Leuconostoc mesenteroides P60 variant strain*

Organisms incubated in solution D minus *p*-AB for 24 hr.

<i>p</i> -AB added (M)	Sulphathiazole/ <i>p</i> -AB ratio required for 50 % inhibition of synthesis of	
	'Citrovorum factor'	' <i>S. faecalis</i> R factor'
4×10^{-7}	2.0	2.0
10^{-6}	1.6	1.5
4×10^{-6}	1.6	1.3
4×10^{-5}	1.0	1.3

The inactivation of leucovorin by suspensions of Leuconostoc mesenteroides P60 variant strain

The decrease of activity of leucovorin (citrovorum factor) added to suspensions of *Leuconostoc mesenteroides* P60 variant strain was rapid. Carbon dioxide partially prevented the inactivation of leucovorin during incubation, maximum inhibition occurring in atmospheres containing 20–25 % CO₂. This is a partial pressure which approximates to that required for maximum *de novo* formation of citrovorum factor (Fig. 6). Of the leucovorin originally present only 7 % remained after incubation with *L. mesenteroides* P60 variant strain in N₂ whilst 16 % remained in 25 % (v/v) CO₂ + 75 % (v/v) N₂. Leucovorin activity was not lost by incubation at 10⁻⁶M with suspensions inactivated by heating at the outset of the experiment, nor in the course of preparation of samples for assay.

The inactivation of leucovorin was considerable even in the absence of glucose, but was greatly accelerated in its presence. A critical increase in this enhancement of the degradation occurred at about 10⁻²M-glucose; this might explain the diminution in apparent citrovorum factor synthesis with increasing glucose concentration which occurred at this concentration of glucose, resulting in the peak already described.

The replacement of CO₂ by ascorbic acid

Carbon dioxide has the dual capacity of stimulating serine synthesis (Lascelles *et al.* 1954; Cross, 1960) and of preventing leucovorin inactivation by *Leuconostoc mesenteroides* P60. Any hypothesis about a relationship between these two activities would be supported if another compound which produced one of the phenomena also produced the other. With such a possibility in view tests were carried out with L-ascorbic acid which has been reported to increase citrovorum factor production by suspensions and extracts of *Streptococcus faecalis* R (Broquist *et al.* 1953; Nichol, 1954). When examined L-ascorbic acid (10⁻²M) was found to stimulate synthesis of

citrovorum factor from formate and *p*-aminobenzoic acid and partially prevented inactivation of 10^{-6} M-leucovorin by suspensions of *L. mesenteroides* P60 variant strain, though the effect was not so marked as that produced by CO_2 (Table 6). It was also found that (a) recovery of 5×10^{-8} M-leucovorin from 'boiled controls' was almost complete in the presence or absence of ascorbic acid, and that (b) L-ascorbic acid had little effect when it was present only during the preparation of the samples for assay after incubation.

Table 6. Effect of ascorbic acid and CO_2 on the synthesis and degradation of 'citrovorum factor' by suspensions of *Leuconostoc mesenteroides* P60 variant strain

Incubation in 10^{-3} M-glucose and 10^{-3} M-phosphate buffer for 24 hr. In the flasks in which 'citrovorum factor' synthesis was measured 10^{-6} M-*p*-AB and 10^{-3} M-formate were also present; in the flasks in which leucovorin degradation was measured, 10^{-6} M-leucovorin was added before incubation.

L-Ascorbic acid (M) added	Gas phase	'Citrovorum factor' (m μ M) found	
		Synthesized from formate and <i>p</i> -AB	Remaining from 10^{-6} M initial leucovorin
—	N_2	1.4	9.5
10^{-2}	N_2	9.8	45.2
—	20% CO_2 in N_2	40.5	80.0
10^{-2}	20% CO_2 in N_2	68.0	86.0

Ascorbic acid partially replaced CO_2 for the growth of *Leuconostoc mesenteroides* P60 in the absence of serine, but growth only reached one-third of that attained in 5% (v/v) CO_2 + 95% (v/v) N_2 . The activity of L-ascorbic acid was confirmed by its stimulatory action when the organism was grown in the absence of serine with suboptimal concentrations of leucovorin or CO_2 (Table 7). When medium *L* was supplemented with 10^{-3} M-DL-serine no such stimulation occurred.

Table 7. Effect of ascorbic acid on the growth of *Leuconostoc mesenteroides* P60

Growth in medium *L* supplemented with 5×10^{-4} M-glycine, 2×10^{-7} M-pyridoxal and 10^{-6} M-*p*-AB; and with and without 5×10^{-3} M-L-ascorbic acid.

Additions to basal medium	Gas phase	Growth (EEL reading in 44 hr.)	
		Without ascorbate	With ascorbate
—	N_2	1.0	7.5
—	0.5% CO_2 in N_2	6.5	18.5
—	5.0% CO_2 in N_2	20.0	22.5
10^{-6} M-leucovorin	N_2	14.5	22.5
10^{-3} M-DL-serine	N_2	22.5	24.0

DISCUSSION

When incubated with *p*-aminobenzoic acid and formate suspensions of a variant strain of *Leuconostoc mesenteroides* P60 form two factors having folic acid activity. *p*-Aminobenzoic acid is also required for the synthesis of serine (Cross, 1960), and these factors are formed concurrently with serine when glycine and pyridoxal are also provided. Though some form of folic acid is clearly required for serine synthesis

by this organism, the present experiments provide no evidence of the identity of the final coenzyme form (CoF).

The two factors formed in the suspensions have been tentatively identified as rhizopterin and leucovorin on the basis of their activities for various micro-organisms. It is unlikely that either of these factors is even an intermediate in the synthesis of CoF from *p*-aminobenzoic acid. Rhizopterin is transformed to citrovorum factor by suspensions of *Streptococcus faecalis* R (Hendlin, Koditschek & Soars, 1953), but it does not support the growth of *Leuconostoc mesenteroides* P 60. It is improbable that this inactivity is due to the impermeability of the cells to the factor, since leucovorin and tetrahydropteroyl-glutamic acid, both containing a pteridine residue, albeit reduced, support growth of the organism (Lascelles *et al.* 1954; Cross, 1956). Leucovorin, likewise, is inactive for a number of micro-organisms, and probably does not mediate in the main pathway to CoF (Woods, 1954). Nevertheless, it may be a sufficiently close by-product of CoF metabolism for its formation to give a measure of the synthesis of the coenzyme.

An inverse quantitative relationship exists between the requirements for leucovorin and glycine for the growth of *Leuconostoc mesenteroides* P 60 in the absence of serine, just as it does between CO₂ and glycine (Lascelles *et al.* 1954). Leucovorin will also satisfy the requirement for CO₂ of suspensions of the pre-incubated cells synthesizing serine (Cross, 1960). One interpretation of these observations is that CO₂ maintains a concentration of CoF sufficient for the synthesis of serine. In support of this view it is now found that the formation of citrovorum factor in suspensions of the variant strain of *L. mesenteroides* P 60 is enhanced by CO₂. If CO₂ is essential for the formation of CoF, then CO₂ should be necessary for the synthesis of serine. But optimum concentrations of serine are formed by suspensions of the freshly harvested variant strain in an atmosphere of N₂ (Cross, 1960). Again, unless *p*-aminobenzoic acid has some function in bacterial metabolism apart from the synthesis of CoF, *L. mesenteroides* P 60 should fail to grow in atmospheres devoid of CO₂ unless the requirement for *p*-aminobenzoic acid were also eliminated. However, growth of the organism in the presence of serine occurs only when *p*-aminobenzoic acid is supplied, and yet it proceeds in N₂ (Lascelles *et al.* 1954).

Carbon dioxide partially inhibits the inactivation of leucovorin by suspensions of the variant strain of *Leuconostoc mesenteroides* P 60. If this inactivation reflects a loss of CoF, then CO₂ may exert its influence on CoF concentration, and therefore on serine synthesis, by inhibiting degradation of the coenzyme. It is assumed that the CoF concentration is sufficiently high, even in the absence of CO₂, for the synthesis of serine by freshly harvested suspensions, and for the requirements of the organism growing in the presence of serine. On the other hand, it is insufficient, in the absence of CO₂, for the synthesis of serine either by growing organisms or by suspensions of organisms previously incubated in phosphate-buffered glucose. In fact no citrovorum factor formation has been detected in suspensions of the pre-incubated organisms, although serine is synthesized under these conditions. It may be that the assay is not sensitive enough to detect small quantities of citrovorum factor consonant with serine synthesis. The hypothesis that the parts played by CO₂ in serine synthesis, in citrovorum factor synthesis and in leucovorin degradation are closely related in *L. mesenteroides* P 60, receives some support from the fact that ascorbic acid can partially replace CO₂ in each of these roles.

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