The Synthesis of Serine by *Leuconostoc mesenteroides* P 60

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SUMMARY

Though CO₂ is essential for the growth of *Leuconostoc mesenteroides* strain P 60 in the absence of serine, ¹⁴CO₂ is not incorporated into serine by the growing organism. The synthesis of serine was further studied by using suspensions of a variant strain of *L. mesenteroides* P 60 which formed serine more actively than the parent strain. Glycine, formate, glucose, *p*-aminobenzoic acid and pyridoxal were required for the synthesis of serine by these suspensions. There was an additional requirement for CO₂ only when the organisms had been incubated previously in phosphate-buffered glucose. Leucovorin satisfied the requirement for both CO₂ and *p*-aminobenzoic acid.

INTRODUCTION

Evidence for the biosynthesis of serine from glycine by *Leuconostoc mesenteroides* strain P 60, and the part played therein by vitamin B₆ and growth factors of the folic acid group, has largely been derived from nutritional experiments (Steele, Sauberlich, Reynolds & Baumann, 1949; Wold & Sirny, 1953; Lascelles, Cross & Woods, 1954; Sauberlich, 1954). In order that this organism shall grow in the absence of serine not only must the concentration of glycine and *p*-aminobenzoic acid (*p*-AB) be increased well above those found to be optimal for growth in the presence of serine, but pyridoxal must also be added to the medium, and the gas phase enriched with CO₂. Lyman *et al.* (1947) reported a similar replacement of amino acids for the growth of *Streptococcus faecalis* strain R, for which CO₂ and vitamin B₆ substituted for aspartic acid, and for the growth of *Lactobacillus arabinosus*, where CO₂ and vitamin B₆ substituted for arginine, phenylalanine and tyrosine. Carbon dioxide also increases the growth of *Streptobacterium plantarum* strain 10S in a medium containing homocysteine in place of methionine, whereas maximum growth is achieved in N₂ in the presence of methionine (Cross, 1954). This replacement of methionine by homocysteine + CO₂ parallels more closely the replacement of serine by CO₂ + glycine, in that, in both cases, CO₂ is apparently substituting for a 'labile methyl group'.

It has already been reported briefly that ¹⁴CO₂ is not incorporated into serine by growing cultures of *Leuconostoc mesenteroides* P 60 (Cross, 1953; Lascelles *et al.* 1954); these experiments will be fully described here. The situation is analogous to that found with *Streptobacterium plantarum* 10S which does not incorporate ¹⁴CO₂ into the methyl group of the synthesized methionine when growing in the absence of the amino acid.

The main object of the present work was the further study of the role of CO₂ in serine synthesis under the more controlled conditions possible with suspensions of

organisms; the initial design of experiments was guided largely by the observations of Lascelles & Woods (1954) with suspensions of *Streptococcus faecalis* R which form serine in a medium containing glycine, formate, glucose, pyridoxal and pteroyl-glutamic acid.

METHODS

The variant strain of Leuconostoc mesenteroides P60. This was derived from *L. mesenteroides* P60 (*Streptococcus equinus* P60) (ATCC 7881), which was maintained as described by Lascelles *et al.* (1954). After 15 subcultures in medium *L* supplemented with 10^{-2} M-glycine, 10^{-6} M-*p*-aminobenzoic acid and 2×10^{-7} M-pyridoxal in an atmosphere of 5% (v/v) CO₂ + 95% (v/v) N₂ at 37° the culture showed an enhanced ability to grow in the absence of added serine.

The variant strain was maintained in liquid medium *L* supplemented as above, subcultured every week and stored at 4°.

Growth experiments with strains of Leuconostoc mesenteroides P60. Inocula for nutritional experiments to compare the two strains were derived from fresh stock cultures of the organisms: in medium *B*₁ (Nimmo-Smith, Lascelles & Woods, 1948) for the parent strain, and in medium *L* supplemented as already described for the variant strain. Both strains were subcultured in supplemented medium *L* and incubated for 24 hr. at 37° in 5% (v/v) CO₂ + 95% (v/v) N₂. The cultures were diluted 1/50 for use as an inoculum. In other respects the basal medium and procedure were as described by Lascelles *et al.* (1954). Each tube of test medium was inoculated with 0.1 ml. of the inoculum. The procedure in experiments involving the parent strain only was exactly as described by Lascelles *et al.* (1954).

Preparation of suspensions of the variant strain of Leuconostoc mesenteroides P60

Medium. Organisms deficient in both *p*-aminobenzoic acid and vitamin B₆ were obtained by growth on medium (C) based on that described by Lascelles & Woods (1954) for the growth of *Leuconostoc citrovorum*. It contained in each litre of final medium: acid-hydrolysed casein (vitamin free, prepared by the method of Snell & Rannefeld, 1945) equivalent to 5 g. original casein; DL-cysteine, 100 mg.; DL-alanine, 870 mg.; DL-tryptophan, 100 mg.; glucose, 10 g.; acetic acid (glacial), 4.0 ml.; (NH₄)₂SO₄, 8 g.; KH₂PO₄, 2 g.; MgSO₄·7H₂O, 40 mg.; MnSO₄·4H₂O, NaCl and FeSO₄·7H₂O, 20 mg. each; adenine, guanine and uracil, 10 mg. each; nicotinic acid, 1 mg.; riboflavin, 500 µg.; thiamin monohydrochloride and calcium pantothenate, each 600 µg.; biotin, 1 µg. *p*-Aminobenzoic acid was added at a final concentration 5×10^{-10} M; the pH value was adjusted to 6.9.

p-Aminobenzoic acid could be omitted from the medium when thymidine was added (Lascelles *et al.* 1954). However, organisms deficient in *p*-aminobenzoic acid were obtained when suboptimal concentrations of this vitamin were present, and this procedure was preferred in order to spare stocks of thymidine.

Growth. Medium C (200 ml. quantities in 300 ml. conical flasks) was autoclaved for 7 min. at 10 lb./sq.in. and warmed at 37° before inoculation with 0.1 ml. (about 2×10^8 organisms) from a 24 hr. culture on the supplemented medium *L* at 37° in 5% (v/v) CO₂ + 95% (v/v) N₂. The flasks were incubated for 10–11 hr. at 37° in air. The culture then contained about 0.05 mg. dry wt. organisms/ml., less than 10% of the maximum density attainable in the medium supplemented with optimal

concentrations of pyridoxal and *p*-aminobenzoic acid. The organisms were centrifuged down, and washed in the culture volume of 2×10^{-2} M-phosphate buffer (pH 6.9).

General procedure for the study of serine synthesis

Reaction mixture. The washed organisms were suspended at a concentration equivalent to 0.2–0.4 mg. dry wt./ml. in 2.5 ml. quantities (in 25 ml. Pyrex conical flasks) of the solution of substrates. The complete solution (*D*) normally contained for synthesis of serine: glycine, 2×10^{-2} M; glucose 10^{-2} M; sodium formate, 10^{-2} M; pyridoxal, 10^{-6} M; *p*-aminobenzoic acid, 10^{-5} M; phosphate buffer, pH 6.9, 10^{-1} M (prepared by mixing 10^{-1} M solutions of Na_2HPO_4 and KH_2PO_4). Modifications in particular experiments are noted in the text. Incubation was at 37° and usually for 24 hr. over which period the dry wt. of organisms decreased about 15% in typical systems. For atmospheres other than air, adapted 'Kilner' fruit-preserving jars (Nimmo-Smith *et al.* 1948) were used. 'Boiled control' flasks contained heated organisms (4 min., 100°) suspended in the complete mixture.

In some experiments the organisms were incubated in 2×10^{-2} M-glucose and 10^{-1} M-phosphate buffer (pH 6.9) before testing their synthetic ability. This was carried out with the same concentration of organisms as above in a final volume of 85 ml. for 3 hr. at 37° in air. The organisms were centrifuged down and re-suspended in solution *D* modified as required.

Microbiological assay of serine. The preparation of material for the assay of serine and the assay itself with *Leuconostoc mesenteroides* P 60 was carried out as described by Lascelles *et al.* (1954) and Lascelles & Woods (1954). The growth response to DL-serine and to typical samples corresponded over a wide range of concentration (Fig. 1). Results are expressed as mm-L-serine (the assay organism responds only to L-serine). There was little variation in the quantity of serine synthesized when the concentration of organisms was increased beyond the range used (equiv. 0.25–0.4 mg. dry wt. organisms/ml.) so that the results are not significantly affected by such changes. The over-all accuracy of the method was about $\pm 10\%$ for concentrations of L-serine in the range 0.05–1.0 mm. The procedure was checked with typical samples by assay in the absence of pyridoxal (Lascelles *et al.* 1954).

The chromatographic identification of serine by using Dowex-50 resin. Serine synthesized by suspensions of the variant strain of *Leuconostoc mesenteroides* P 60 was separated chromatographically on Dowex-50 resin (Moore & Stein, 1951). The amino acid fractions were located by estimation of amino-nitrogen with ninhydrin (Moore & Stein, 1948; Lascelles & Woods, 1954).

Growth experiments with isotopically labelled substrates. To investigate the incorporation of ^{14}C -labelled CO_2 , sodium bicarbonate and sodium formate into serine by growing cultures of *Leuconostoc mesenteroides* P 60 the organism was grown in 200 ml. quantities of medium *L* supplemented with 10^{-2} M-glycine, 10^{-6} M-*p*-aminobenzoic acid and 2×10^{-7} M-pyridoxal (added after autoclaving) in 500 ml. flat-bottomed flasks fitted with a side-arm. The medium, with the addition of [^{14}C]-sodium formate (10^{-2} M) when required, was autoclaved 10 min. at 10 lb./sq.in. The inoculum was 0.2 ml. of an 18 hr. culture in medium *B*₂ derived in turn from stock *B*₁. Each flask was filled with N_2 to a partial pressure of about 95% atmospheric by alternate evacuation and washing with that gas. Then $^{14}\text{CO}_2$ was generated from the calculated quantity of [^{14}C]barium carbonate + excess HCl in a Rittenberg

tube attached to the side arm (to give a final concentration of 2–5 % (v/v) CO_2 in the flasks); or, alternatively, a solution of [^{14}C]sodium bicarbonate was introduced through a tap funnel to a final concentration of $5 \times 10^{-3}\text{M}$. Incubation was for 30–46 hr. at 37° . The apparatus was then flushed with N_2 and the organisms centrifuged down and washed with 10^{-2}N-HCl and water. After drying *in vacuo* over P_2O_5 the yield was about 80–110 mg. Control experiments were performed with and without additions to the basal medium of inactive substrates and DL-serine, and uninoculated flasks were incubated for several days. These experiments indicated that the sterile techniques adopted in using this apparatus were satisfactory.

The isolation of glycine and serine as their 2:4-dinitrophenyl derivatives. Organisms grown in the presence of isotopically labelled nutrients were hydrolysed with 6N-HCl at 105° in sealed tubes for 16 hr. and the HCl then removed *in vacuo*. The product was allowed to react with 1-fluoro-2:4-dinitrobenzene and the mixture of dinitrophenyl derivatives fractionated on 'Celite 545' (Johns Manville Co. Ltd., London, S.E. 11). The DNP-serine and DNP-glycine fractions were purified and their content estimated. After dilution with previously prepared DNP-L-serine and DNP-glycine the samples were recrystallized until they showed no further change of radioactivity. The procedure followed throughout was that of Campbell & Work (1952).

Radioactivity measurements. Samples were mounted at 'infinite thickness' on 1 sq.cm. polythene disks (Popják, 1950) and measurements were made with a thin mica end-window Geiger counter. Combined errors amount to about $\pm 5\%$. Results are recorded in counts/min. under these conditions.

Chemicals used. The pteroylglutamic acid used was 'Folvite' (Lederle Laboratories, Pearl River, New York, U.S.A.). [^{14}C]Barium carbonate, sodium bicarbonate (in aqueous solution) and sodium formate, were supplied by the Radiochemical Centre, Amersham, Buckinghamshire, England. The source of all other materials, and the preparation and storage of stock solution, were described by Lascelles *et al.* (1954)

RESULTS

*The incorporation of [^{14}C]formate into serine by growing cultures of *Leuconostoc mesenteroides* P60*

The relationship between the concentrations of glycine and CO_2 required by *Leuconostoc mesenteroides* P60 for growth in the absence of serine (Lascelles *et al.* 1954) led to the suggestion that CO_2 might serve as a source of the one-carbon residue, necessary for the conversion of glycine to serine (Lascelles *et al.* 1951). Though the observations with suspensions of the variant strain of the organism (see below) appear to suggest a different role for CO_2 , the most direct evidence against the possibility that CO_2 is such a one-carbon donor was that obtained by using ^{14}C -labelled CO_2 .

Cultures of *Leuconostoc mesenteroides* P60 were grown for 30–46 hr. in medium *L* supplemented with glycine, *p*-aminobenzoic acid and pyridoxal in atmospheres containing 2–5 % (v/v) $^{14}\text{CO}_2 + 95\%$ (v/v) N_2 ; and in N_2 with the addition of $5 \times 10^{-3}\text{M}$ [^{14}C]sodium bicarbonate to the medium. Very little activity was found in the whole dried organisms. The dried organisms were hydrolysed with 6N-HCl and the hydrolysate fractionated by paper chromatography. No activity was detected

in either serine or glycine, though incorporation of CO₂-carbon into serine would have been observed by this technique had the dilution been as great as 1 in 400. In one experiment serine and glycine were isolated as their 2:4-dinitrophenyl derivatives. These showed no activity.

On the other hand, when *Leuconostoc mesenteroides* P 60 was grown for 46 hr. in the presence of unlabelled sodium bicarbonate under the same conditions but with addition of 5×10^{-3} M-[¹⁴C]sodium formate (1.3×10^6 counts/min./sq.cm. at infinite thickness), the DNP-L-serine isolated from the hydrolysate of the organisms was labelled (5.6×10^4 counts/min./sq.cm. at infinite thickness). DNP-glycine isolated from the organisms showed no significant activity. Assuming that all the activity in the serine was located in the β -carbon atom, the dilution of the exogenous formate-carbon entering the position was about 1 in 6. Such dilution was to be expected since exogenous formate does not even stimulate growth of the organism in the absence of serine.

Growth characteristics of the variant strain of Leuconostoc mesenteroides P 60

The requirements of the variant strain for serine, and for glycine in the absence of serine, differ from those of the parent strain (Lascelles *et al.*, 1954) in that (a) some growth occurred in the absence of serine in the presence of only 5×10^{-4} M-glycine, under which conditions there was stimulation by CO₂; (b) the serine requirement was satisfied by 10^{-3} M-glycine in the absence of CO₂ (Table 1). The parent strain does not grow significantly in the absence of serine unless the glycine concentration is increased above the basal concentration of 5×10^{-4} M and the atmosphere enriched with CO₂. Such differences might be expected if the organism had been 'trained' to synthesize serine more efficiently. This would seem to have been the case since suspensions of the variant strain yielded twice the concentration of serine produced by the parent strain under the same conditions.

Table 1. *Response of parent and variant strains of Leuconostoc mesenteroides P 60 to glycine, serine and CO₂*

Basal medium L was supplemented with 10^{-6} M-*p*-AB, 5×10^{-4} M-glycine and 2×10^{-7} M-pyridoxal.

Additions to basal medium	Atmosphere	Growth at 44 hr. (EEL reading)	
		P 60	Variant
None	N ₂	1.5	8.0
10^{-3} M-glycine		4.0	21.0
2×10^{-3} M-DL-serine		26.0	22.5
None	5 % (v/v) CO ₂ + 95 % (v/v) N ₂	1.0	16.0
10^{-3} M-glycine		26.5	22.5
2×10^{-3} M-DL-serine		29.5	28.5

As with the parent strain, formate did not replace CO₂ in stimulating growth of the variant. The growth-promoting effects of *p*-aminobenzoic acid, leucovorin (which replaced both *p*-aminobenzoic acid and CO₂) and pyridoxal in the presence and absence of serine were qualitatively identical with those observed with the parent strain.

The synthesis of serine by suspensions of Leuconostoc mesenteroides P60

Suspensions of the variant strain in solution *D* synthesized about 0.25 μ moles L-serine/ml. medium in 24 hr. when incubated at 37° either in air or in N₂ (Fig. 2). Glycine, glucose, formate, *p*-aminobenzoic acid and pyridoxal were all essential for synthesis (Table 2). Enrichment of the atmosphere with CO₂ had no effect upon serine formation (Tables 2 and 3). The requirement for glycine in concentrations of about 100 times those of the serine formed reflects the replacement of serine for growth of *Leuconostoc mesenteroides* P60.

Fig. 1

Fig. 2

Fig. 3

Fig. 1. Response of *Leuconostoc mesenteroides* P60 to DL-serine (●—●) and to a typical acid-treated sample (○—○) containing serine synthesized by suspensions of *L. mesenteroides* P60.

Fig. 2. Rate of synthesis of L-serine by suspensions of the variant strain of *Leuconostoc mesenteroides* P60 in solution *D* incubated in N₂.

Fig. 3. Effect of *p*-aminobenzoic acid (●—●) or leucovorin (○—○) concentration on the synthesis of L-serine by suspensions of the variant strain of *Leuconostoc mesenteroides* P60 in solution *D*. Incubation was for 24 hr. at 37°.

Leucovorin, a mixture of the (+) and (−) L-isomers of calcium *N*⁵-formyltetrahydropteroylglutamate pentahydrate (synthetic '*Leuconostoc citrovorum* factor') has about one half of the activity of *p*-aminobenzoic acid for the growth of *L. mesenteroides* P60 in the absence of serine (Lascelles *et al.* 1954). Leucovorin had the same activity as *p*-aminobenzoic acid for the synthesis of serine by suspensions of

Table 2. *Synthesis of serine by suspensions of the variant strain of Leuconostoc mesenteroides P60*

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

Omissions from basal solution	Atmosphere	L-Serine found (mM)
None	20 % (v/v) CO ₂ + 80 % (v/v) N ₂	0.25
None (heated control)		0.05
Glycine		0.0
Pyridoxal		0.03
Formate		0.03
<i>p</i> -AB		0.08
Glucose		0.0
None	N ₂	0.25

L. mesenteroides P60, variant strain (Fig. 8). Both compounds were optimal at 2×10^{-7} M and half optimal at 10^{-8} M. Though leucovorin contains only 50 % of the natural isomer (Cosulich, Smith & Broquist, 1952) it cannot be concluded that this isomer is twice as active as *p*-aminobenzoic acid for synthesis by the suspensions, as a difference of two in the activities of *p*-aminobenzoic acid and leucovorin would be within the limits of experimental error.

Table 3. *Synthesis of serine by suspensions of the variant strain of Leuconostoc mesenteroides* P60

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

Addition to basal solution	Atmosphere	L-Serine (mm) found when organisms	
		Pre-incubated	Normal
10 ⁻⁶ M- <i>p</i> -AB	N ₂	0.05	0.3
	20 % (v/v) CO ₂ + 80 % (v/v) N ₂	0.2	0.25
10 ⁻⁶ M-Leucovorin	N ₂	0.2	0.25
	20 % (v/v) CO ₂ + 80 % (v/v) N ₂	0.2	0.28

Pteroylglutamic acid, which does not replace *p*-aminobenzoic acid in supporting growth of *Leuconostoc mesenteroides* P60, was also unable to replace it for the synthesis of serine by suspensions of organisms. A concentration of 5×10^{-3} M-formate in the medium was necessary for maximum synthesis of serine though formate had no effect on the growth of the organism either in the presence or absence of serine. These observations parallel those with *Streptococcus faecalis* R (Lascelles & Woods, 1954). The synthetic ability of *L. mesenteroides* P60 variant strain did not vary significantly with physiological age over the range 8–16 hr. growth in medium *C*. There was no destruction of added DL-serine when incubated with suspensions of the variant strain in solution *D*.

The chromatographic identification of serine

To confirm the results of microbiological assay the serine formed by suspensions of the variant strain of *Leuconostoc mesenteroides* P60 was identified chromatographically on columns of Dowex-50 resin by a procedure like that described by Lascelles & Woods (1954) for a similar analysis of samples from *Streptococcus faecalis* R suspensions. Samples from systems incubated without *p*-aminobenzoic acid and without pyridoxal contained less than 15 % of the serine in the complete system, confirming results of microbiological assay.

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

The absence of any stimulation by CO₂ of serine production by suspensions, in contrast to its ability to replace serine for growth of *Leuconostoc mesenteroides* P60, suggested that the harvested organisms might contain sufficient of some cofactor normally synthesized from CO₂. In case such a cofactor might be degraded by the organism and thus removed, organisms, grown as already described, were incubated

in glucose and phosphate buffer (pH 6.9) before incubation in solution *D*. Suspensions of organisms treated in this way formed less serine, but the activity was largely restored by enriching the atmosphere with CO₂ or by replacing *p*-aminobenzoic acid by leucovorin (Table 3). Stimulation by CO₂ was most marked after 3 hr. pre-incubation.

The inhibition of serine synthesis by sulphathiazole

The synthesis of serine by suspensions of *Leuconostoc mesenteroides* P 60 variant strain was competitively inhibited by sulphathiazole (Table 4). When *p*-aminobenzoic acid was replaced by leucovorin in solution *D* serine synthesis was no longer so inhibited by sulphathiazole at the concentrations tested (Table 5). Leucovorin non-competitively antagonized the inhibition by sulphathiazole of the growth of *Leuconostoc mesenteroides* P 60 (Lascelles *et al.* 1954). These results suggest that a leucovorin-like compound is a product of the utilization of *p*-aminobenzoic acid by this organism.

Table 4. *Inhibition by sulphathiazole of the synthesis of serine by suspensions of the variant strain of Leuconostoc mesenteroides P 60*

Organisms incubated in a basal solution *D* (minus *p*-AB) for 24 hr.

<i>p</i> -AB added (M)	Sulphathiazole/ <i>p</i> -AB ratio found for 50 % inhibition of synthesis
4×10^{-7}	1.8
10^{-6}	1.3
4×10^{-6}	1.6
4×10^{-5}	1.4

Table 5. *Reversal by leucovorin of sulphathiazole inhibition of serine synthesis by suspensions of the variant strain of Leuconostoc mesenteroides P 60*

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

Sulphathiazole added (M)	Leucovorin required for 50 % of optimum synthesis (M)
—	1.0×10^{-8}
10^{-6}	2.5×10^{-8}
10^{-5}	1.5×10^{-8}
10^{-4}	8.0×10^{-8}

DISCUSSION

Though CO₂ and a relatively high concentration of glycine are essential to the growth of *Leuconostoc mesenteroides* P 60 in the absence of serine, ¹⁴CO₂ is not incorporated into the serine of the organism. The simplest explanation, particularly in the light of evidence to be discussed in the following paper (Cross, 1960), is that CO₂ does not act as a donor of the β-carbon of serine. It is possible that exogenous CO₂ acts as a one-carbon donor in the initial stages of growth only until sufficient of some endogenous source of the β-carbon of serine is available. This seems unlikely, since formate does not satisfy the requirement of the organism for CO₂, even though

it is a substrate for the conversion of glycine to serine by suspensions of *Leuconostoc mesenteroides* P 60 and is incorporated into serine by the growing organism. Further, optimum synthesis of serine from glycine by specially treated suspensions of the variant strain is only achieved in the presence of both CO₂ and formate.

Serine synthesis was studied in suspensions of the variant strain since it proceeded more actively with this variant than with the parent strain of *Leuconostoc mesenteroides* P 60. Serine is formed from glycine and formate provided that pyridoxal and either *p*-aminobenzoic acid or leucovorin are supplied. The system is analogous to that described by Lascelles & Woods (1954) with suspensions of *Streptococcus faecalis* R, the requirement for a folic acid group factor in their system being met by pteroylglutamic acid or leucovorin, either of which will also support growth of the organism. Maximum synthesis of serine by suspensions of *Leuconostoc mesenteroides* P 60 variant strain, which had been previously incubated in phosphate-buffered glucose solution, required CO₂ in addition. Presumably some factor essential for serine synthesis, be it substrate or enzyme, had been destroyed during this preliminary incubation. Leucovorin, for example, is destroyed under these conditions (Cross, 1960). The ability to form serine was restored when CO₂ was available. Perhaps conditions in these suspensions of pre-incubated organisms more closely paralleled those in cultures of the growing organism. The role of CO₂ in promoting serine synthesis in suspensions of the organism is examined further in the following paper (Cross, 1960).

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