Carbon Dioxide as a Growth Factor for Mutants of *Escherichia coli*

By H. P. CHARLES AND GILLIAN A. ROBERTS

Department of Microbiology, the University of Reading

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SUMMARY

This report shows that some auxotrophic mutants of *Escherichia coli* can grow on a minimal medium without growth factors when the gas phase is supplemented with carbon dioxide. Mutants which respond to CO₂ are called CO₂ mutants. The CO₂ mutants of *E. coli* resemble those already known in *Neurospora*. When the gas phase is not supplemented with CO₂ most of the mutants respond to other specific growth factors such as arginine, uracil, adenine, succinate or isoleucine + valine, depending upon the locus of their mutation. One mutant is an obligate CO₂ mutant. The CO₂ effects shown by these mutants are discussed in relation to the general problem of CO₂ effects in micro-organisms.

INTRODUCTION

Carbon dioxide is necessary for the growth or the initiation of growth of bacteria and other micro-organisms (Valley & Rettger, 1927; Rockwell & Highberger, 1927). This is sometimes referred to as 'the CO₂ effect' (e.g. Wilson & Miles, 1964). CO₂ also has other interesting effects. For example, it causes morphogenetic changes in organisms as different as bacteria (Ivanovics, 1937), fungi (Bartnicki-Garcia & Nickerson, 1962) and coelenterates (Loomis, 1961). Recently, a new kind of CO₂ effect was discovered in which CO₂ acts as a growth factor for mutants of the mould *Neurospora* (Charles, 1962; Reissig & Nazario, 1962; Charles & Broadbent, 1964; Broadbent & Charles, 1965). The mutants require up to 30% CO₂ (v/v) in the gas phase for growth to occur, whereas the wild type requires less CO₂ than is present in ordinary air (0.02–0.04%). When these mutants are not supplied with supplementary CO₂ they behave as normal auxotrophic mutants, each requiring a particular growth factor such as arginine, adenine, uridine or succinate, depending upon the site of the mutation. Mutants which are stimulated by CO₂ are called CO₂ mutants (Charles & Broadbent, 1964). The way in which CO₂ stimulates the growth of the mutants has not been definitely established, and the mechanism may be different in different mutants. A problem arising from the research with the *Neurospora* mutants was whether the effects observed are peculiar to *Neurospora*, or whether they are of wider occurrence. This report shows that CO₂ mutants of the bacterium *Escherichia coli* occur. The bacterial mutants are described and compared with those of *Neurospora*. Apart from their intrinsic interest, CO₂ mutants may be useful because analysis of the mechanism of the CO₂ stimulation may contribute to the understanding of CO₂ effects in other organisms.

METHODS

The following strains of *Escherichia coli* K 12 were used:

F- AB 1621 (*thi*⁻, *mtl*⁻, *xyl*⁻, *str*^r, *ara*⁻, *gal*⁻, *lac*⁻)

Hfr AB 2575 (*ilv*⁻, *str*^r) which transfers genes in the order *O-ara, lac, gal, ...*

Hfr P 10 (*thr*⁻, *leu*⁻, *thi*⁻, *T*₈^r, *str*^r) transferring genes in the order *O-thi-met-mtl... .*

Hfr B 7 (*met*⁻, *str*^r, *T*₈^r) transferring genes in the order *O-try-his... .*

Strains AB 1621 and AB 2575 were kindly provided by Professor E. A. Adelberg, and strains P 10 and B 7 by R. Lavallé.

The minimal media used were those described by Vogel & Bonner (1956), and Davis & Mingioli (1950). Unless stated otherwise the minimal media contained glucose (0.5% autoclaved separately) and were supplemented with thiamine (2 mg./l.) because the F⁻ strain requires thiamine. The medium was solidified when necessary with Difco Bacto agar (12 g./l.). The complete medium contained the following (g./l.): tryptone (Oxoid), 10; yeast extract (Difco), 5; KH₂PO₄, 3; glucose, 5; agar, 15 (when required).

Cultures were grown and maintained on slopes of complete agar medium. The temperature of incubation was 37°. Strains to be used in mating experiments were streaked on plates of complete medium to give discrete colonies and stored at 4°. Single colonies were picked for the experiments; their phenotypes were checked before use.

Nutritional requirements of mutants were determined by the auxanographic method (Pontecorvo, 1949); about 3 × 10⁶ bacteria/ml. were dispersed in the minimal agar medium.

When it was required to incubate cultures in a gas phase of air supplemented with CO₂ the procedure was as follows. Cultures in Petri dishes (lids removed) were stacked in 5 l. vacuum desiccators in layers of three with the agar facing downwards, each dish resting equally on 2 dishes below it; CO₂ was generated by the action of hydrochloric acid on marble chips in a Kipp apparatus, and allowed to enter each desiccator after the appropriate amount of air had been withdrawn. Removal of the lids of the Petri dishes was later found to be unnecessary. Equally good CO₂ effects were obtained when the bacteria were dispersed throughout the agar as when they were spread on the surface.

Most CO₂ mutants will grow without a supplement of CO₂ when other specific growth factors such as succinate or arginine are added to the minimal medium; they will be referred to as CO₂ mutants or as succinate, arginine, etc., mutants, as convenient.

The loci of the mutations were determined by the interrupted mating technique (Hayes, Jacob & Wollman, 1956) as follows. Overnight broth cultures of the strains to be mated were diluted 1/10 in fresh broth at 37° and 10 ml. volumes were incubated in a shaking water bath for about 1.5 hr; 0.5 ml. of the Hfr culture and 9.5 ml. of the F⁻ culture were then mixed in a 50 ml. Erlenmeyer flask and the mixture gently rocked in the water bath for the duration of the experiment. Samples (0.1 ml.) of the mating mixture were taken immediately after mixing and then at 5 min. intervals, diluted 100 times in minimal medium without glucose and agitated for 60 sec. by using a rotary mixer to stop the mating process. Samples (0.1 ml.) of the suspensions

were then plated in duplicate on selective media to determine the numbers of recombinants. Transduction experiments were done as described by Glansdorff (1965).

Isolation of CO₂ mutants

Experiments 1-4. Mutations were induced in *Escherichia coli* strain AB 1621 with ultraviolet radiation and the mutants enriched by penicillin treatment (Gorini & Kaufman, 1959). The procedure included a 16-18 hr period of intermediate incubation during which the bacteria multiplied, and their mutations became phenotypically expressed, before they were treated with penicillin. The medium used for this intermediate incubation was varied in Exp. 1-4 (see Table 1) to allow for the possibility that some media might select for or against some kinds of CO₂ mutants. For example, in Expt. 3, CO₂ was used to supplement the minimal medium by slowly bubbling a gas mixture composed of 70 % (v/v) air + 30 % (v/v) CO₂ through the incubating suspension. After penicillin treatment CO₂-requiring mutants were detected as follows. Suitable dilutions of the washed bacterial suspensions were spread on complete medium to give about 200 colonies per plate after incubation. After incubation for 24 hr the colonies on each plate were printed on to two plates of minimal medium by the replica plating technique (Lederberg & Lederberg, 1952). One of each pair of plates was incubated in ordinary air and the other in a gas phase of air + 20 % (v/v) CO₂. After incubation for 24-72 hr the pairs of plates were compared and colonies which had grown only in the presence of 20 % CO₂ were purified by streaking and tested further.

Experiment 5. Mutations were induced in *Escherichia coli* AB 1621 with *N*-methyl-*N*-nitroso-*N'*-nitroguanidine (MNNG) by using a modification of the method of Goodgal, Margolin & Sanderson (1964). Overnight growth in broth (5 ml.) was washed and resuspended in the same volume of minimal medium enriched with 2.5 % (v/v) complete medium. A sample, 0.1 or 0.2 ml., of an unsterilized solution of MNNG (4 mg./ml.) was added and the suspension incubated for 30-60 min. Complete medium (4.5 ml.) was added and incubation continued for about 2 hr. Suitable dilutions were then spread on plates of complete medium and incubated for 3-5 days in a gas phase of air + 20 % (v/v) CO₂. The colonies which grew on each plate were printed on to two plates of minimal medium and CO₂ mutants detected as in Expt. 1-4. Enrichment of mutants was not necessary.

RESULTS

Table 1 shows the results of the mutant isolation experiments. As described in Methods, various supplemented media were used for the period of intermediate incubation in the penicillin selection procedure. The results in Table 1 show that the kinds of mutants obtained depended upon the medium used for the period of intermediate incubation. Thus the only medium which gave a good yield of histidine mutants and isoleucine + valine mutants was minimal medium through which a mixture of 70 % (v/v) air + 30 % (v/v) CO₂ was slowly and continuously bubbled. Mutants requiring arginine, a pyrimidine, or arginine + a pyrimidine, were only obtained when the minimal medium was supplemented with arginine + uridine + adenine.

A gas phase of 80 % (v/v) air + 20 % (v/v) CO₂ was the most stimulatory for the CO₂ mutants of *Escherichia coli*. Higher proportions of CO₂ were not tested because they inhibited the parental strain. All but one of the mutants responded to other growth factors when a supplement of CO₂ was not provided. The mutants were of at

least 10 kinds, distinguishable by their responses to growth factors other than CO₂. As with *Neurospora* the response of some of the *E. coli* mutants to CO₂ was annulled by certain metabolites. Mutants responding to a particular growth factor showed the same pattern of response to precursors of that growth factor (when available), suggesting that their mutations were at the same locus. One mutant of each kind was chosen for genetical studies. Nutritional and genetical data are shown in Table 2. Because nutritional experiments were done on solid media, quantitative data for the stimulatory effect of CO₂ and other growth factors are not available, but Table 2 shows the time taken by the various mutants when incubated in 20% CO₂ to equal the growth of the parental strain after 18 hr. The different kinds of mutants will now be considered individually.

Table 1. *Escherichia coli*: CO₂ mutants obtained in mutant isolation experiments

Experiment no.	Mutagenic treatment	Supplement added to minimal medium for the period of intermediate cultivation	Kinds of CO ₂ mutants obtained*	No. of mutants of each kind obtained
1	U.v. irradiation followed by penicillin selection procedure	Nutrient broth (5% v/v)	Purine	4
			Succinate	2
			Isoleucine + valine	1
2		Yeast extract + yeast nucleic acid + tryptone (100 mg./l. of each)	Purine	1
			Succinate	1
			Isoleucine + valine	1
3		No supplement, but medium was gassed with a mixture of 30% CO ₂ + 70% air	Histidine	5
			Succinate	1
			Isoleucine + valine	11
4		Arginine + adenine + uridine (10 mg./l. of each)	Purine	7
			Arginine	1
			Pyrimidine	1
			Arginine + pyrimidine	1
5	MNNG† without penicillin selection	Not applicable	Purine	1
			Succinate	2
			Isoleucine + valine	4
			Methionine,	1
			Obligate CO ₂ mutant	1

* The mutants are classified according to their nutritional requirements when incubated in ordinary air.

† MNNG = *N*-methyl-*N*-nitroso-*N'*-nitroguanidine.

Purine mutants

When the gas phase was not supplemented with carbon dioxide the purine mutants were stimulated by adenine, guanine, hypoxanthine or xanthine. This showed that they have a defect in one of the enzymes synthesizing the purine ring. When grown in minimal medium in the presence of 20% CO₂, or in complete medium, for 3 days, the mutants produced a yellowish brown coloration. The crude culture filtrate of a mutant grown for 24 hr in minimal medium supplemented with a growth-limiting concentration of hypoxanthine (5 mg./l.) showed a strong ultraviolet absorption maximum at 300 mμ which was not shown by a similar filtrate of the culture in which the parental strain had grown. This pigment is probably 5-aminoimidazole ribonucleotide (AIR).

or a derivative, which has an absorption maximum at 305 m μ (Love & Gots, 1955). Interrupted mating experiments showed that the mutations mapped between the *lac* and *gal* loci in the region of the *ade f* locus (Stouthamer, de Haan & Nijkamp, 1965). Gots (1965) showed that the enzyme specified by this locus catalyses the conversion of AIR to CAIR (5-aminoimidazole-4-carboxylic acid ribonucleotide). This is the only CO₂-incorporation reaction in purine biosynthesis, and the explanation of the CO₂ effect shown by these mutants may be that the carboxylating enzyme in the mutants requires a higher concentration of CO₂ for activity than does this enzyme in the parental strain. The purine mutants were inhibited by aspartate when growing in CO₂, but they were not inhibited by aspartate when any one of the stimulatory purines was present. Some of the *Neurospora* CO₂ mutants also respond to purines, excrete a similar pigment, and map at a locus which may specify the carboxylating enzyme (Broadbent & Charles, 1965).

Table 2. *Escherichia coli*: some characteristics of CO₂ mutants

Designation of mutants selected for study	Locus of the mutation	Time (hr) required in 20 % (v/v) CO ₂ to equal the growth produced by the parent strain after 18 hr	Nutritional requirement when incubated in ordinary air without a supplement of CO ₂	Growth in CO ₂ inhibited by
A 17	<i>pur-E (ade-f)</i>	18	Purines	—
S 19	<i>suc</i>	18–36*	Succinate, 2-oxoglutarate, lysine + methionine	Aspartate
L 23	Not known	18–48*	Isoleucine + valine, isoleucine + α -keto-isovalerate	Aspartate, purines
H 13	Not known	36 (18 hr in presence of nicotinic acid)	Histidine	Aspartate, purines
M 12	<i>pyr-F</i>	36	Pyrimidines	—
B 22	<i>cap</i>	48	Arginine or citrulline	Pyrimidines
AP 21	<i>cap</i>	18	Arginine or citrulline + a pyrimidine	Pyrimidines
45-I	Not determined	18	Methionine or cysteine	Aspartate
'Unknown'	Not determined	36	Unknown	Purines, histidine, tyrosine

* Different mutants grow at different rates.

Isoleucine + valine mutants

The isoleucine + valine CO₂ mutants are especially interesting because a CO₂-fixation reaction does not occur in the biosyntheses of isoleucine and valine. The only reaction in the biosynthetic sequences in which CO₂ takes part is a decarboxylation. The genetics and biochemistry of isoleucine + valine mutants have been studied in detail, especially by Ramakrishnan & Adelberg (1964, 1965*a, b*). The double amino acid requirement arises because the same four enzymes catalyse the last four reactions in the biosyntheses of both isoleucine and valine. The four genes specifying these enzymes are closely linked and are probably adjacent on the bacterial genome. The CO₂ mutants of *Escherichia coli* map in this region in interrupted mating experiments. It was not possible to determine by nutritional experiments which reaction is defective

in the mutants because most of the intermediates are not available. However, the last enzyme of the sequence must be functional because the mutants grow when supplied with isoleucine + α -keto-isovalerate, the latter substance being the immediate precursor of valine. The growth response to CO_2 was inhibited by purines and aspartate, which did not inhibit the parental strain and did not inhibit the mutant when it was growing in the presence of isoleucine + valine. In *Neurospora*, CO_2 mutants responding to isoleucine + valine were not obtained, despite several large mutant-isolation experiments.

Succinate mutants

Six *Escherichia coli* mutants required succinate when the gas phase was not supplemented with CO_2 . These mutants have not all been studied in detail, and they may differ amongst themselves. The important facts are as follows. In the presence of glucose the mutants responded to CO_2 , succinate or 2-oxoglutarate. When glucose was omitted from the medium the mutants did not use succinate or 2-oxoglutarate as carbon and energy sources, but the parental strain did. The mutants did not grow when other Krebs-cycle intermediates, or acetate, were supplied as carbon sources, even when succinate (50 mg./l.) was present, or when the gas phase contained 20 % (v/v) CO_2 , whereas the parental strain used several of the Krebs-cycle intermediates. These observations show that neither succinate nor CO_2 substituted fully for the product of the defective enzyme(s).

The mutants also grew in minimal medium when supplied with lysine + methionine, instead of succinate or CO_2 , whether or not glucose was present. This suggested that the mutants may be defective in the synthesis of succinyl-CoA, which is an intermediate in the biosynthesis of lysine and methionine (Gilvarg, 1957; Rowbury & Woods, 1964). The fact that succinate was stimulatory only in the presence of glucose may indicate that the mutants required glucose to make acetyl-CoA, which might be used in turn for converting succinate to succinyl-CoA.

Succinate mutants similar to those described here have been isolated by other workers, who did not examine the effect of CO_2 on their mutants. Davis *et al.* (1959) described a mutant of *Escherichia coli* which required succinate or lysine + methionine + threonine for growth. It differed from our mutants in requiring threonine and in failing to respond to 2-oxoglutarate. Because it responded to succinate but not to 2-oxoglutarate they suggested that the mutant might be blocked in the conversion of 2-oxoglutarate to succinate. Davis *et al.* (1959) showed that their mutant grew on minimal medium, and did not require succinate or lysine + methionine + threonine when it was incubated anaerobically in glucose minimal medium. They concluded that in the parental strain succinate could be formed from glucose anaerobically by reduction of 4-carbon acids, but aerobically only by oxidation of 2-oxoglutarate. Stouthamer (1967) showed that succinate mutants of an *Aerobacter* species also grew anaerobically in minimal medium without succinate. The succinate mutants described in the present paper show the same effect. Interpretation of the growth-promoting effect of anaerobic conditions requires caution because some succinate mutants may be CO_2 mutants and growth on minimal medium in anaerobic jars may be a response to CO_2 , either added deliberately as a mixture of 95 % (v/v) H_2 + 5 % (v/v) CO_2 (Stouthamer, 1967) or produced by the bacterial cultures themselves. The fact that the gas phase of the cultures was O_2 -free may be irrelevant. Experiments which we have done to

determine whether CO₂ is responsible for the stimulatory effect of anaerobic conditions have been inconclusive because of technical difficulties.

Stouthamer (1967) found that the *Aerobacter* mutants were of 2 kinds, one able and the other unable to use succinate as a source of carbon and energy. He concluded that one kind of mutant was blocked in the decarboxylation of 2-oxoglutarate, but that the nature of the lesion in the other kind was unknown. Back & Westaway (1962) also described a mutant of *Escherichia coli* which required lysine+methionine, but they did not state whether it responded to succinate. Various characteristics of the mutants of Stouthamer (1967) and Back & Westaway (1962) are indicative of CO₂ effects. Thus the latter authors say that although their mutant required lysine+methionine for growth, it actually synthesized considerably more lysine and methionine than was available from the medium. Stouthamer (1967) found that growing bacteria oxidized glucose completely, but that washed suspensions only partially oxidized glucose. He also found that the greater the number of bacteria in the inoculum then the shorter the lag period before growth began on medium containing lysine+methionine. All these effects may be due to accumulation of CO₂ in the growth medium by the bacteria with a resulting stimulation of metabolic activity and growth.

The mechanism by which CO₂ stimulates the mutants is not understood. A CO₂-fixation step is involved in several reactions leading to the formation of compounds having 4 carbon atoms, and it is possible that high concentrations of CO₂ stimulate one of these reactions or bring an alternative one into operation. If this be the case, however, malate and oxalacetate should be stimulatory, because they are products of some of the CO₂-fixation reactions.

On the linkage map of *Escherichia coli* (Taylor & Thoman, 1964) the succinate and lysine+methionine loci are shown separately, but close together. The one mutant we have tested also maps in this region, and it seems likely that the succinate and lysine+methionine loci are identical.

Arginine and pyrimidine mutants

The three mutants of *Escherichia coli* to be described in this section were isolated in penicillin selection experiments in which minimal medium supplemented with arginine, uridine and adenine (10 mg./l. each) was used for the period of intermediate incubation (see Methods). Two of the mutants (AP 21, B 22) have defects in the biosynthesis of carbamoyl phosphate (CAP) which is a precursor of arginine and pyrimidines. In *E. coli*, CAP for the biosynthesis of these substances is formed by a CO₂-incorporation reaction catalysed by the enzyme glutamino-carbamoyl phosphate synthetase (GCPS-ase; Piérard, Glansdorff, Mergeay & Wiame, 1965). The synthesis of this enzyme is cumulatively repressed by arginine and pyrimidines and its catalytic activity is subject to feedback inhibition by uridine-5'-monophosphate (UMP). Ornithine counteracts the inhibition by UMP, a mechanism which ensures that CAP is available for arginine biosynthesis when required (Piérard, 1966). The enzyme is specified by the *cap* locus (Piérard *et al.* 1965), which is the same as the *pyr A* locus of Jacob & Wollman (1961) and Beckwith, Pardee, Austrian & Jacob (1962).

The *Escherichia coli* mutant designated AP 21 required arginine+a pyrimidine for growth in the absence of supplementary CO₂. Arginine was stimulatory in the absence of a pyrimidine when incubation was prolonged for 2-3 days, but growth was never as great as with arginine+a pyrimidine. Arginine was replaceable by citrulline. Pyrimi-

dines which stimulated the mutant were uracil, uridine, cytosine and cytidine; thymidine was weakly stimulatory; orotic acid and thymine were inactive. The pyrimidines were not stimulatory unless arginine or citrulline was also present. The growth rate of the mutant in minimal medium when the gas phase was supplemented with 20 % (v/v) CO₂ was as good as that of the parental strain.

Escherichia coli mutant B 22 grew in minimal medium when supplied with arginine or citrulline without a pyrimidine, but growth was better when a pyrimidine was also provided. The mutant responded slowly to CO₂, 2 days of incubation being necessary for a growth response. Mutant B 22 therefore differed from mutant AP 21 in its rapid response to arginine (or citrulline) and its slow response to 20 % (v/v) CO₂.

The growth response of mutants AP 21 and B 22 to 20 % (v/v) CO₂ was inhibited by uracil, uridine, cytosine, cytidine or thymidine. The inhibitions were annulled by arginine or citrulline. The mutants partially resemble the uracil-inhibited mutants U 28 of Gorini & Kalman (1963) and *ura-s* of Piérard *et al.* (1965) which grow in minimal medium and show inhibition by uracil and annulment of this inhibition by arginine.

Transduction experiments showed that the mutations in *Escherichia coli* mutants AP 21 and B 22 were at the *cap* locus which specifies the only CO₂-incorporation enzyme (GCPSase) in arginine and pyrimidine biosynthesis. Enzymological studies showed that GCPSase activity was present in the mutants but at a lower concentration than in the parental strain (G. A. Roberts, unpublished). The fact that both mutants grew without a pyrimidine when supplied with arginine suggests that CAP was available for pyrimidine synthesis but not for arginine synthesis. Perhaps the CAP-synthesizing enzyme in the mutants was altered at a regulatory site, so that ornithine no longer counteracted the feedback inhibition by pyrimidines, and arginine synthesis consequently failed through lack of CAP. An explanation of this kind was proposed by Piérard (1966) to explain the uracil inhibition of *ura-s*. Carbon dioxide may stimulate growth in minimal medium by increasing CAP synthesis, e.g. by a mass action effect or by antagonizing the pyrimidine feedback inhibition, so that sufficient CAP is available for arginine synthesis. A second and quite different possibility is that the increased CO₂ concentration may bring into action another CAP-synthesizing enzyme such as 'carbamyl phosphokinase', which is present in *E. coli* but does not provide CAP for arginine and pyrimidine synthesis under the usual cultural conditions (Thorne & Jones, 1963; Yashphe & Gorini, 1965). This kind of explanation does not account for the pyrimidine inhibitions unless more assumptions are made.

The *Escherichia coli* mutant M 12 was stimulated by the pyrimidines uracil, uridine, cytosine or cytidine. The growth response to CO₂ was slow and was not inhibited by any common metabolite. Arginine and citrulline were slightly stimulatory to growth in 20 % (v/v) CO₂. Interrupted mating experiments showed that the mutation was in the region of the *pyr C*, *D* and *F* loci (Beckwith *et al.* 1962; Taylor *et al.* 1964). *Pyr C* and *D* are close together and some distance from *pyr F*, which is nearer to the *try* locus (Taylor 1965; R. Lavallé, private communication). Preliminary experiments suggested that the M 12 mutation may be at the *pyr F* locus which specifies the enzyme orotidylic acid decarboxylase (Beckwith *et al.* 1962; Taylor & Thoman, 1964). This mutation was not at the *cap* locus, which specifies the only CO₂-incorporation enzyme in pyrimidine (and arginine) biosynthesis.

Histidine mutants

The histidine/CO₂ mutants of *Escherichia coli* showed a better response to histidine than to 20% (v/v) CO₂, suggesting either that adaptation was necessary for utilization of CO₂, or that CO₂ only partially compensated for the defects in the mutants. In the presence of supplementary CO₂, nicotinic acid was stimulatory but it was not stimulatory when the gas phase was ordinary air. This relationship between nicotinic acid and histidine biosynthesis requires further investigation. When growing in CO₂ these mutants were completely inhibited by purines. The mutation has not been mapped, and it is possible that it may map outside the *his* operon. A CO₂-fixation reaction does not occur in the biosynthesis of histidine from its precursor adenosine triphosphate (ATP) so that if CO₂ acts by stimulating a CO₂-incorporation enzyme it must act on the enzyme catalysing the formation of CAIR from AIR, in the biosynthesis of ATP. But the mutation did not map at the locus which specifies this enzyme, and it seems unlikely that a defect in this enzyme would cause a requirement for histidine unless there are unexpected complexities in purine and histidine biosynthesis. Histidine/CO₂ mutants are not known in *Neurospora*.

Methionine mutant

The methionine/CO₂ mutant of *Escherichia coli* responded vigorously to methionine and more weakly to cysteine. It gave a good response to CO₂ within 24 hr. When growing with CO₂ the mutant was partially inhibited by aspartate and completely inhibited by serine; the parental strain was also inhibited by serine, but not as strongly as was the mutant. Methionine/CO₂ mutants are also known in *Neurospora*. The ability of CO₂ to substitute for methionine in these mutants is not understood.

An obligate CO₂ mutant

One mutant of *Escherichia coli* was an obligate CO₂ mutant in that it did not grow in minimal or complete medium except when the gas phase was supplemented with CO₂. In the presence of 20% (v/v) CO₂ colonies formed on complete medium within 24 hr, but on minimal medium colonies took 48 hr to form. This showed that CO₂ itself did not completely compensate for the defect in the mutant. Growth of the mutant in 20% (v/v) CO₂ was inhibited by purines and tyrosine. In some experiments the inhibitions were complete but in others they were incomplete. The reason for this variation from experiment to experiment is not known.

Obligate CO₂ mutants are also known in *Neurospora* and some are similarly inhibited by purines (Broadbent, 1965; A. Vivian, personal communication.)

DISCUSSION

This investigation shows that CO₂ mutants occur in *Escherichia coli* as well as in *Neurospora*. The phenomena which they reveal are therefore likely to be of general occurrence. The parental strains of *Neurospora* and *Escherichia coli* from which the mutants have been isolated differ in their CO₂ requirements. The *Neurospora* strains grow when incubated in a desiccator containing potassium hydroxide (100 ml., 25%, w/v) but the *Escherichia* strains do not, showing that *Neurospora* requires a much lower concentration of CO₂ than *Escherichia*. A second difference is that growth

of *Neurospora* is not inhibited when the gas phase contains 30 % (v/v) CO₂, whereas *Escherichia* begins to show inhibition when the gas phase contains more than 20 % (v/v) CO₂. The differences between the parental strains are reflected in the CO₂ mutants. *Neurospora* CO₂ mutants do not grow when incubated in minimal medium in the presence of potassium hydroxide, but they grow when the gas phase contains 30 % (v/v) CO₂; when incubated in ordinary air some of the CO₂ mutants grow fairly quickly, probably because of the CO₂ in the environment and because of accumulation of CO₂ by the conidia. In *Escherichia*, CO₂ mutants do not grow when the gas phase is ordinary air and they grow when the gas phase contains 20 % (v/v) CO₂. The fact that some mutants were obtained only once shows that the samples of mutants isolated were small, and it is likely that more kinds of CO₂ mutants await discovery in both *Escherichia* and *Neurospora*. This may explain why the patterns of mutants from the two organisms do not match completely, although there is no reason to expect that they should.

Arginine, purine, pyrimidine and succinate mutants occur in both organisms. The mechanism of the CO₂ stimulation may be fairly easily understood in some of these CO₂ mutants because the relevant biosynthetic pathways all have CO₂-incorporation reactions and some of the mutations which have been mapped fall at the loci which specify the appropriate CO₂-incorporation enzymes. An unexplained fact is that CO₂ mutants occur at three arginine loci in *Neurospora*. Whether CO₂ mutants occur at several arginine loci in *Escherichia* remains to be investigated. Mutation at the *arg-11* locus in *Neurospora* causes simultaneous requirement for arginine, a pyrimidine, a purine and possibly succinate (Newmeyer, 1964; Broadbent & Charles, 1965). The function of this gene is unknown. The facts that the two known *arg-11* mutants are both CO₂ mutants and that CO₂ completely satisfies or annuls all the requirements suggest that the gene may be concerned with a process providing CO₂ for arginine, purine, pyrimidine and succinate biosyntheses. Mutants similar to *arg-11* mutants have not been obtained in *Escherichia*, but even in *Neurospora* mutants similar to the *arg-11* mutants were not obtained in CO₂-mutant isolation experiments.

With *Escherichia* we have the best example of a mutant which is completely dependent on a supplement of CO₂ and unable to use any other growth factor within a reasonable incubation period. The mutant does not grow on complete medium except when extra CO₂ is added to the gas phase. Its requirements may be complex because it responds better to CO₂ on complete medium than on minimal medium. The mutant may have a defect in fatty-acid metabolism, because a small proportion of the bacteria respond to oleic acid and Tween 80 after prolonged incubation.

Isoleucine+valine mutants were obtained with *Escherichia* but not *Neurospora*. They are of special interest because there is no CO₂-incorporation reaction in the biosyntheses of isoleucine and valine; this excludes certain proposed explanations of how CO₂ may stimulate mutants.

Tryptophan and acetate mutants are so far known only from *Neurospora*; the nature of their enzymic deficiencies is not known.

CO₂ mutants which require succinate, or lysine+methionine, when not provided with supplementary CO₂ have been discussed in the Results section. An interesting question arising from the discovery that succinate mutants are CO₂ mutants is whether the reported ability of succinate mutants to grow anaerobically in minimal medium without succinate or lysine+methionine (Davis *et al.* 1959; Stouthamer,

1967), may be due to stimulatory concentrations of CO₂ in the anaerobic jars, rather than to absence of O₂.

The interest of CO₂ mutants lies chiefly in their relevance to problems of nutrition and metabolism. It has long been known that some micro-organisms, and probably all, require CO₂ for initiation of growth (Valley & Rettger, 1927; Rockwell & Highberger, 1927; Rahn, 1941). This phenomenon is often referred to as 'the CO₂ effect'. CO₂ mutants are of special interest in relation to the problems posed by this and other effects of CO₂. When it was realized that generation of a 4-carbon compound from a 3-carbon compound by a CO₂-fixation reaction was necessary for continuous operation of the Krebs cycle, it seemed that the explanation of 'the CO₂ effect' was at hand (Krebs, 1941). However, experiments on nutrition in which attempts were made to replace CO₂ with glutamate, aspartate or Krebs cycle intermediates showed that these substances only partly substituted for CO₂, if at all; and the more stringent the removal of CO₂, or the smaller the inoculum, then the more nutritionally demanding the bacteria became or the longer the lag period before they grew (Lwoff & Monod, 1946, 1947; Wong & Ajl, 1953). With the discovery of CO₂-fixation reactions in other essential processes such as the biosyntheses of arginine, pyrimidines and purines one might have expected the CO₂ effect to be fully explained, but experiments show that in some bacteria it is not possible to replace CO₂ with other growth factors, and in other bacteria growth factors which replace CO₂ do not have a CO₂-fixation reaction in their path of biosynthesis. Other points of interest are: (1) CO₂ requirements are not always satisfied by bicarbonates and carbonates; (2) newly isolated pathogenic bacteria may require relatively high concentrations of CO₂ for growth, which may lessen after serial transfer; (3) effects of CO₂ on morphogenesis are known, for example, high CO₂ concentrations cause some filamentous fungi to adopt a yeast-like mode of growth (Bartnicki-Garcia & Nickerson, 1962), and bring about the development of sex organs in the coelenterate *Hydra* (Loomis, 1961).

It is sometimes implied that CO₂ is required for the initiation rather than the continuation of bacterial growth. It seems more likely that CO₂ is required both for initiation and growth, and that when growth has started the bacteria may generate far more CO₂ than they use so that it is impossible to demonstrate a net requirement for CO₂. Whether CO₂ must be absorbed from outside the cell during growth or whether intracellular CO₂ may satisfy all the requirements is unknown. It may be that intracellular and extracellular CO₂ are freely exchangeable, in which case the distinction between intracellular and extracellular CO₂ would be false. This possibility is worth investigation because it would help to explain some aspects of CO₂ effects.

All the problems encountered in the study of 'the CO₂ effect' are encountered in the study of CO₂ mutants; thus bicarbonates and carbonates do not replace CO₂. It is uncertain whether CO₂ is required for initiation and/or continuation of growth. Inoculum size and lag effects are common and some mutants grow without a supplement of CO₂ or growth factors when the inoculum is concentrated or incubation is prolonged. Some mutants do not respond to growth factors other than CO₂. Most interesting of all, some CO₂ mutants respond in the absence of CO₂ from the gas phase to growth factors which do not have CO₂-incorporation reactions in their biosyntheses. It seems likely, for all these reasons, that CO₂ mutants mimic some of the CO₂ effects shown by different bacterial species. CO₂ mutants may be the most satisfactory experimental systems for analysing CO₂ effects because they show well-

defined CO₂ effects expressed against a common genetic background, and because the biochemical problem is considerably simplified, since each CO₂ effect is probably the result of a change in a single enzyme as compared with the parental strain which serves as a control or reference. By contrast, the CO₂ effects shown by wild-type organisms may result from peculiarities in several enzymes and there will be no reference strain available to show which enzymic characteristics are responsible for the CO₂ effect. That mutants may make other contributions to the understanding of CO₂ effects is shown by a recent observation that some mutant strains of *Escherichia* and *Neurospora* are inhibited rather than stimulated by CO₂; this raises the possibility that some CO₂ effects may result from inhibition rather than stimulation of enzymic reactions by CO₂ (Roberts & Charles, unpublished).

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