Mutants of *Neurospora crassa*, *Escherichia coli* and *Salmonella typhimurium* Specifically Inhibited by Carbon Dioxide

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SUMMARY

Mutants of *Neurospora crassa*, *Escherichia coli* and *Salmonella typhimurium* are described which are inhibited by CO₂ at concentrations which do not inhibit the parental strains from which the mutants were derived. Sensitivity to inhibition by CO₂ is caused by single gene mutations. The CO₂ inhibitions are reversed by specific substances; for example, the CO₂ inhibition of a methionine-requiring mutant of *N. crassa* is reversed by purines, and the CO₂ inhibition of a prototroph of *E. coli* is reversed by methionine or vitamin B₁₂. The nature of the defects in the CO₂-inhibited mutants is discussed.

INTRODUCTION

Several mutants of *Neurospora crassa* and *Escherichia coli* grow in minimal medium without their specific growth factors when the gas phase is air containing 20 to 30% (v/v) of carbon dioxide (Broadbent & Charles, 1965; Charles & Roberts, 1968). Mutants which are stimulated by CO₂ in this way are called 'CO₂ mutants' (Charles & Broadbent, 1964); they are not usually stimulated by bicarbonate. The mechanism of the stimulation is not known with certainty and may not be the same for all CO₂ mutants. In some CO₂ mutants the mutations affect enzymes which catalyse CO₂-incorporation reactions, and it is probable that high concentrations of CO₂ may compensate for decreased enzymic activity by a mass action effect; this may be the mechanism of the stimulation in some purine-requiring and arginine-requiring mutants which are CO₂ mutants. In other CO₂ mutants, for example certain mutants of *E. coli* which require isoleucine+valine, the mutations apparently affect enzymes which do not catalyse CO₂-incorporation reactions and it is not known how CO₂ stimulates their growth.

CO₂ mutants have also recently been isolated from *Salmonella typhimurium* (H. P. Charles, unpublished).

The present report describes mutants of a new class; these are inhibited rather than stimulated by CO₂. These mutants will be called 'CO₂-inhibited mutants'. They have been found in *Neurospora crassa*, *Escherichia coli* and *Salmonella typhimurium*. They are inhibited by concentrations of CO₂ which stimulate the CO₂-requiring mutants and which do not inhibit the parental strains from which the CO₂-inhibited mutants are derived.

METHODS

Strains. The *Neurospora crassa* wild-type strains used were EMERSON 5256*A* and EMERSON 5297*a*, kindly provided by Professor D. G. Catcheside (Department of Genetics, John Curtin School of Medical Research, Australian National University, Canberra, A.C.T., Australia), and 74-OR 23-1*A* and 74-OR 8-1*a*, kindly provided by Dr F. J. de Serres (Oak Ridge National Laboratory, U.S.A.). The *N. crassa* methionine-requiring strains *met-1* (35599), *met-2* (K44), *met-3* (36104), *met-4* (39816), *met-5* (9666), *met-6* (35809), *met-7* (4894), *met-8* (P53) and *met-9* (C124), the lysine-requiring strains *lys-2* (37101*a*) and *lys-3* (28815) and the para-aminobenzoic acid-requiring strain *pab-1* (1633*A*) were kindly provided by Mr W. Ogata (Fungal Genetics Stock Center, Dartmouth College, Hanover, New Hampshire 03755, U.S.A.). The methionine-requiring strains *mac* (65108) and *me* (S2706) were kindly provided by Dr Noreen E. Murray (M.R.C. Molecular Genetics Unit, Department of Molecular Biology, King's Buildings, West Mains Road, Edinburgh 9).

The strain of *Escherichia coli* from which the CO₂-inhibited mutants were isolated was strain AB 1621 (F⁻; T6-*r*, *thi*, *mtl*, *xyl*, *str-r*, *ara*, *gal*, *lac*, T4-*r*), kindly provided by Professor E. A. Adelberg (Department of Microbiology, Yale University, New Haven, Connecticut 06510, U.S.A.).

The CO₂-inhibited mutant of *Salmonella typhimurium* was isolated from strain LT2, kindly provided by Dr P. E. Hartman (Johns Hopkins University, Baltimore, Maryland, U.S.A.).

Media. The minimal medium used for *Neurospora crassa* was that of Vogel (1956) solidified with agar (Difco Bacto, 1.2 % w/v) when necessary. Sorbose (5 g./l.) was added to the solid minimal medium to restrict the growth of colonies. The complete medium used for *N. crassa* was the conidiation medium of Horowitz (Ryan, 1950).

The minimal medium used for *Escherichia coli* and *Salmonella typhimurium* was that of Vogel & Bonner (1956). It was supplemented with thiamine (2 mg./l.) for *E. coli*, and was solidified when necessary with Difco Bacto agar (1.2 % w/v). The complete medium used contained the following (g./l.): tryptone (Oxoid), 10; yeast extract (Difco), 5; KH₂PO₄, 3; glucose, 5; agar (when required), 15.

Unless otherwise stated, all cultures of *Neurospora crassa* were incubated at 30° and all cultures of *Escherichia coli* and *Salmonella typhimurium* at 37°.

The effect of CO₂ on growth was tested by incubating cultures in Petri dishes in 5 litre vacuum desiccators containing an appropriate mixture of air and CO₂ as described by Charles (1964).

Isolation of mutants. The CO₂-inhibited mutant of *Neurospora crassa*, F4, was obtained from ultraviolet-irradiated conidia of the wild-type strain 74-OR 23-1*A* by the filtration enrichment procedure (Catcheside, 1954; Woodward, De Zeeuw & Srb, 1954). The filtration procedure was modified in that the irradiated conidia were incubated throughout the filtration period in a gas phase of air supplemented with CO₂ (about 30 %, v/v). This modification was introduced in order to suppress growth of any CO₂-inhibited mutants which might be present in the population and which would otherwise grow and be removed from the suspension by filtration. The duration of the incubation period in the filtration enrichment experiment was 48 h. and the suspension was filtered approximately every 5 h. during this period. Samples of the final filtrate were plated on conidiation medium, and after incubation for 3 days conidia from the

colonies which appeared were tested for ability to grow on minimal medium in air and in air supplemented with 30 % (v/v) CO₂. The growth factor requirements of auxotrophs were determined by the auxanographic method (Pontecorvo, 1949). All auxotrophs obtained were tested to determine whether or not the response to their respective growth factor was inhibited by the presence of 30 % CO₂ (v/v) in the gas phase.

The CO₂-inhibited mutants of *Escherichia coli* and *Salmonella typhimurium* were isolated as follows. Samples of an ultraviolet-irradiated cell suspension were plated on complete medium and incubated in air. Colonies which grew were then printed by the replica plating technique (Lederberg & Lederberg, 1952) on to each of two plates of minimal medium, one of which was incubated in air and the other in air supplemented with 20 % (v/v) CO₂. Plates were observed after incubation for 24 h. and any colonies which failed to appear on the plates incubated in CO₂-supplemented air were picked from the corresponding air-incubated plates and retested to confirm their CO₂-inhibited phenotype.

Crosses. Crosses between strains of *Neurospora crassa* were made on cornmeal agar medium (Smith, 1954) supplemented with the growth factor requirement of the protoperithecial parent. They were incubated at 25° and analysed about 2 weeks after ejection of the ascospores. Germination of the ascospores was induced by placing them at 60° for 45 min. Germinated ascospores were individually transferred to tubes of conidiation medium and the resulting cultures were tested for phenotype after incubation for 3 to 4 days.

RESULTS

One mutant of *Neurospora crassa* which showed inhibition by CO₂ (30 %, v/v, in air) was a newly isolated lysine-requiring mutant, F4, derived from wild-type strain 74-OR 23-1 A by the procedure described in Methods. This mutant shows the characteristics of CO₂-inhibited mutants in their simplest form. The sensitivity of the mutant to inhibition by CO₂ was not due to the mutation which causes the lysine requirement, because a cross of the mutant to wild-type (strain 74-OR 8-1 a) yielded some segregants which were inhibited by CO₂ (30 %, v/v) but which did not require lysine. These CO₂-inhibited segregants were prototrophic in ordinary air. Crosses of CO₂-inhibited prototrophic segregants to wild-type strains gave only wild-type and CO₂-inhibited prototrophic segregants in equal proportions, showing that CO₂-sensitivity was determined by a single gene mutation. This suggested that an enzyme in the mutant did not function effectively in the presence of 30 % (v/v) CO₂. The function of this enzyme might be revealed if the CO₂-inhibition could be reversed by supplying a nutrient in the medium. By auxanographic experiments it was found that cytidine reversed the CO₂-inhibition of mutant F4 and of the prototrophic segregants derived from it and caused them to grow as well as wild-type in the presence of 30 % (v/v) CO₂. Other metabolites, including other pyrimidines and pyrimidine derivatives, did not reverse the CO₂ inhibition. Later experiments showed that only one of several samples of cytidine reversed the CO₂ inhibition, indicating that an impurity in the active sample was responsible for reversal of the inhibition. The sample of cytidine was too small to permit identification of its active component and it was not possible to obtain more cytidine of the same batch. The possibility that the impurity was a vitamin was unlikely since all available vitamins were tested on the mutant and failed to reverse the inhibition.

Analysis of crosses between CO₂-inhibited prototrophic segregants derived from mutant F4 and various marker strains indicated that the mutation causing sensitivity to CO₂ was located in the right arm of linkage group V. Crosses with the Group VR markers *lys-2* (37101*a*) and *pab-1* (1633*A*) gave recombination frequencies of 23 and 7 % respectively. Crosses of CO₂-inhibited prototrophs with mutants in other linkage groups gave recombination frequencies ranging from 45 to 70 % recombination.

The first CO₂ inhibition to be discovered was in *Neurospora crassa* mutant 35809*a* which has a mutation at the *me-6* locus (Beadle & Tatum, 1945; Perkins, 1959). Under ordinary cultural conditions, when the gas phase is air, the mutant requires methionine for growth and does not respond to methionine precursors (Murray, 1960). When the gas phase was 70 % (v/v) air + 30 % (v/v) CO₂ the mutant did not grow on methionine-supplemented medium. Auxanographic experiments showed that the mutant grew on methionine-supplemented medium, in the presence of 30 % (v/v) CO₂, when adenine or other purines were supplied. Purines therefore reversed the inhibitory effect of CO₂. The *me-6* mutant 35809 thus seemed to have two defects, one expressed as a requirement for methionine and the other expressed as a requirement for adenine in the presence of 30 % (v/v) CO₂. It is difficult to understand how such defects could be caused by a single gene mutation, and it seemed that the mutant had mutations in two different genes. However, the requirement for methionine and the sensitivity to inhibition by CO₂ did not segregate when the mutant was crossed to wild-type, showing that CO₂ sensitivity was caused either by the same mutation as that which caused the methionine requirement (*me-6*) or by a second mutation closely linked to the *me-6* locus. On the assumption that CO₂ sensitivity was caused by a second mutation closely linked to *me-6*, attempts were made to separate it from *me-6* by crossing the mutant to mutants *lys-3* (28815) and *mac* (65108), which carry mutations close to, and on either side of, the *me-6* locus. Prototrophic segregants arising by recombination in the chromosome interval between *me-6* and *lys-3* or *mac* were selected from the crosses and tested for sensitivity to CO₂ inhibition. None of the prototrophic segregants obtained was CO₂-inhibited. It therefore seems that the CO₂ sensitivity of mutant 35809 was caused by the *me-6* mutation.

Recently, a second methionine-requiring mutant *me* (s2706), kindly given to us by Dr Noreen E. Murray, has been found to resemble mutant *me-6* (35809) in its nutritional characteristics. Mutant *me* (s2706) is leaky but shows a good growth response to methionine; the methionine-dependent growth is inhibited by CO₂, and the CO₂ inhibition is reversed by adenine and other purines. The occurrence of a second mutant resembling mutant *me-6* (35809) provides additional evidence that the methionine requirement and CO₂ sensitivity result from a single gene mutation. Methionine mutants representative of the other known *me* loci, *me-1* (35599), *me-2* (K44), *me-3* (36104), *me-4* (39816), *me-5* (9666), *me-7* (4894), *me-8* (P53) and *me-9* (C124) were not CO₂-inhibited.

When growing in CO₂, mutant *me-6* (35809) partially resembles mutant *mac* (65108), which is reported to require methionine+adenine+cysteine for growth (Dubes, 1953; Perkins, 1959). This similarity in growth requirement together with the apparent close linkage of *mac* to *me-6* (Perkins, 1959) suggested that the mutants might be allelic.

The nutrition of *mac* was investigated. It was found that *mac* grew most quickly with methionine+adenine and less quickly with methionine alone. A requirement for

cysteine was not demonstrable. CO₂ did not inhibit the growth of *mac* under any conditions. Thus *mac* differed from *me-6* (35809) in that it required adenine for growth under all conditions. Experiments by Dr N. E. Murray (personal communication) have shown that *mac* (65108), *me-6* (35809) and *me* (s2706) may be allelic.

Of the CO₂-inhibited mutants isolated from *Escherichia coli* only one has been studied. It grew prototrophically in minimal medium in air but its growth was completely inhibited in a gas phase of air + 20 % (v/v) CO₂. The inhibition by CO₂ was reversed by vitamin B₁₂, L- and D-methionine and was partially reversed by cysteine, cystine, thymidylic acid and DL-serine.

The CO₂-inhibited mutant of *Salmonella typhimurium* similarly grew prototrophically in minimal medium in air and failed to grow in air + 20 % (v/v) CO₂. The CO₂ inhibition of this mutant was specifically reversed by lysine and by the lysine precursor, diaminopimelic acid.

The mutations responsible for the CO₂ sensitivity of the bacterial mutants have not been mapped.

There is evidence that CO₂ does not inhibit growth of the *Neurospora crassa* and bacterial mutants by changing the pH of the medium. Under conditions similar to those of the present experiments, CO₂ caused only trivial changes in the pH of the medium (Broadbent, 1965). Furthermore, the CO₂-inhibited mutants are not more sensitive than the parental strains to changes in this pH. It is therefore unlikely that CO₂ inhibits growth of the mutants by changing the pH of the medium. It is possible that un-ionized CO₂ may penetrate the cells and change the intracellular pH after ionization, but this should cause non-specific inhibition and should not be reversed by single metabolites.

DISCUSSION

Sensitivity to inhibition by CO₂ is caused by single gene mutations at least in *Neurospora crassa*. The CO₂ inhibitions are reversed by single metabolites, and the inhibition of each mutant is reversed by a different metabolite or group of related metabolites. These facts show that the CO₂ inhibitions are specific and affect particular metabolic processes.

Identification of an impurity in one sample of cytidine which reversed the CO₂ inhibition of one of the *Neurospora crassa* mutants (F4) was unfortunately not possible. It may have indicated the nature of the defect in the mutant and have revealed a new growth factor. This is the second unidentified growth factor encountered in the study of mutants showing CO₂ effects; a CO₂-requiring mutant of *Escherichia coli* responds to an unidentified factor in yeast extract (Charles & Roberts, 1968).

The fact that inosine, adenine and other purines reverse the CO₂ inhibition of the methionine-requiring mutant of *Neurospora crassa* (*me-6*, 35809) suggests that CO₂ specifically inhibits an enzyme required for purine biosynthesis. However, the mutation which causes the CO₂ sensitivity does not map at a *purine* locus; both the methionine requirement and the CO₂ sensitivity are caused by mutation at the *me-6* locus. This suggests that the *me-6* locus is concerned with the biosynthesis of a substance required for both methionine and purine biosynthesis. Derivatives of folic acid are required for methionine and purine biosynthesis and it is possible that a reaction in their biosynthesis may be controlled by the *me-6* locus. Mutant *me-6* (35809) may have an incomplete block at this locus, resulting in a requirement for

methionine only, whereas mutant *mac* may have a complete block causing a requirement for both methionine and a purine. CO₂ may cause the purine requirement of *me-6* (35809) by converting the incomplete block into a complete block. Another possible function for the *me-6* locus is control of the biosynthesis of S-adenosyl methionine from its precursors, methionine and adenosine triphosphate. Neither of these suggestions explains why mutation at the *me-6* locus should cause sensitivity to inhibition by CO₂.

The CO₂-inhibited mutant of *Escherichia coli* resembles certain mutants of *Salmonella typhimurium* which also require methionine or vitamin B₁₂ for growth. It differs from them in that its requirement is expressed only in the presence of a high CO₂ concentration. The *Salmonella* mutants have defects in the enzyme *N*⁵-methyltetrahydropteroyl-triglutamate-homocysteine transmethylase (Smith & Childs, 1966) which catalyses the direct methylation of homocysteine in the biosynthesis of methionine (Guest, Friedman & Foster, 1962). Vitamin B₁₂ supports the growth of the *Salmonella* mutants by stimulating the synthesis of methionine from homocysteine by an indirect mechanism (Guest *et al.* 1962; Cauthen & Foster, 1966). It may be that the *E. coli* mutant is unable to effect the direct methylation of homocysteine when the gas phase is air supplemented with CO₂, and thereby becomes dependent upon methionine or vitamin B₁₂.

The CO₂-inhibited mutant of *Escherichia coli* also shows similarities with spontaneously occurring variants of *Mycobacterium tuberculosis* described by Schaefer (1957). Schaefer's variants were inhibited by CO₂ when grown on an oleic acid + albumin medium containing glutamic acid as sole organic nitrogen source. This CO₂ inhibition was reversed by vitamin B₁₂, L- and D-methionine, DL-homoserine, D-serine and D-alanine and 'less regularly' by various other amino acids.

We can propose no explanation of the apparent inhibition of lysine biosynthesis by CO₂ in the CO₂-inhibited mutant of *Salmonella typhimurium*.

The general significance of inhibition by CO₂ is not obvious. Species of micro-organisms differ widely in their relative sensitivity to CO₂. It may be that CO₂ inhibits some micro-organisms by causing non-specific effects such as pH shifts, but it is more difficult to explain large differences in CO₂ sensitivity in the same way. The occurrence of CO₂-sensitive mutants raises the possibility that inhibition of wild-type strains by CO₂ may often be due to effects on single enzymes.

The CO₂ effects shown by the CO₂-inhibited and CO₂-stimulated mutants of *Neurospora crassa*, *Escherichia coli* and *Salmonella typhimurium* suggest that the role of CO₂ in cell physiology is more complex than might be expected from our present knowledge of CO₂ metabolism.

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