Some Carbon-Dioxide Requiring Mutants of *Neurospora crassa*

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SUMMARY

This paper describes some *Neurospora* mutants which grow on minimal medium when the gas-phase contains 30 % CO₂ (v/v); they are referred to as 'CO₂ mutants'. One is an arginine mutant (*arg-2*; 33442) having a mutation at a different locus from the other arginine CO₂ mutant (*arg-3*; 30300); a second (*arg-11*; 30820) requires arginine + a purine + a pyrimidine for growth in the absence of CO₂; a third (44601) requires arginine + a purine + a pyrimidine + a carboxylic acid for growth in the absence of CO₂. The mutations in mutants 30820 and 44601 are allelic and the carboxylic acid requirement of mutant 44601 is attributable to its genetic background. The remaining CO₂ mutants described here were isolated directly from wild-type *Neurospora*, and their nutritional requirements in the absence of CO₂ subsequently determined. Two of these mutants, which are allelic, are adenine-requiring mutants sited at the adenine-3 locus, although the adenine-3A and -3B mutants 38709 and Y 112-M2 are not CO₂ mutants. Another two of these new CO₂ mutants, which may be allelic, require carboxylic acids in the absence of CO₂; these mutants are similar in their nutritional requirements to the succinate mutants described by Lewis (1948) and Strauss (1956).

INTRODUCTION

Charles (1962) showed that two mutants of *Neurospora crassa* grew vigorously on minimal medium when the gas phase was air + 30 % (v/v) CO₂. These mutants were the pyrimidine-requiring mutant 1298, and the arginine-requiring mutant 30300, both isolated by Beadle & Tatum (1945). Auxotrophic *Neurospora* mutants which are able to grow on minimal medium when the gas-phase contains CO₂, but which are unable to grow on minimal medium when the gas-phase does not contain CO₂, will be referred to as 'CO₂ mutants'. After the discovery of the above CO₂ mutants experiments were made to discover other CO₂ mutants of *Neurospora*. As a result, at least twelve different kinds of CO₂ mutants are now known. Some are well-known mutants which were isolated by Beadle & Tatum (1945), others are CO₂ mutants which have been isolated as such from wild-type *Neurospora* and their alternative nutritional requirements in the absence of CO₂ subsequently determined. The present paper describes several CO₂ mutants which may be profitably considered together. In the absence of CO₂, these respond, respectively, to: adenine; an aliphatic carboxylic acid; arginine + adenine + uridine; arginine + adenine + uridine + an aliphatic carboxylic acid; arginine. From the number and type of CO₂ mutants now known it is concluded that CO₂ mutants may occur at many or all points in metabolism where a CO₂-incorporation reaction occurs. Possible explana-

tions of how CO₂ in the gas-phase causes the mutants to grow on minimal medium will be considered in the Discussion. The arginine-requiring CO₂ mutant is the second such CO₂ mutant to be described, and has a mutation at the arginine-2 locus, whereas the arginine-requiring CO₂ mutant described by Charles (1962) has its mutation at the arginine-3 locus. Study of the mutants described here, and of those already described (Charles, 1962) leads us to suggest that CO₂ for the synthesis of arginine, purines, pyrimidines and aliphatic acids may either pass along a common pathway, or be under the control of a single gene. Some of the results described here were briefly reported elsewhere (Charles & Broadbent, 1964).

METHODS

Neurospora crassa wild-type strains Emerson 5297a and Emerson 5256A and mutants 30820, 46005, 37807, 66702, 35402 and 55901 were kindly provided by Professor D. G. Catcheside, F.R.S. All other mutants, except those isolated in this department, were kindly provided by Mr W. Ogata of the Fungal Genetics Stock Center, Hanover, New Hampshire, U.S.A.

The medium used was the minimal medium of Ryan, Beadle & Tatum (1943) containing 0.5% sucrose and 0.5% sorbose, and solidified with 1% Difco Bacto agar. A sorbose solution was autoclaved (120°, 10 min.) separately and added to the molten medium to give the required concentration immediately before the medium was distributed into Petri dishes. Unless otherwise stated, supplements were added to final concentration 30 mg./l., except that acetate was added to final concentration 500 mg./l.

Crosses of *Neurospora* were incubated on slopes of the medium of Westergaard & Mitchell (1947). The slopes were inoculated with one mating type and incubated for 10 days at 25°, during which time protoperithecia formed. These were then fertilized with a suspension of conidia of the opposite mating type. Ascospores were germinated by heating them at 60° in sterile distilled water for 45 min. Newmeyer's (1954) rapid plating method for genetical analysis was used to determine the frequency of wild-type recombinants arising from crosses.

Conidia were obtained from slope cultures grown on the medium of Horowitz (1947) for 3 days at 30°. The conidia were quickly rinsed from the surface of the culture with sterile distilled water, and the resulting suspension was passed through a pad of sterile glass wool to remove mycelial fragments and clumps of conidia.

The auxanographic method (Pontecorvo, 1949) was used to determine the nutritional requirements of mutant strains. Usually a suspension of conidia from the mutant to be tested was added to 500 ml. molten agar medium held at 45°. The medium was then immediately distributed in 10 ml. volumes into Petri dishes. The final conidial concentration was usually about 2000/ml. When it was desired to incubate conidia in CO₂-free air they were sometimes spread on the surface of the solidified medium, rather than being dispersed throughout the medium, because other experiments with yeasts indicated that this method allowed more efficient removal of CO₂ by adjacent potassium hydroxide solutions. In preliminary work unsterilized crystals of various compounds to be tested were used in the auxanographic experiments. Important conclusions were confirmed by testing sterile solutions (pH 5.4) of appropriate substances.

The gas-phase surrounding the Petri dish cultures was controlled by incubating the cultures in 5 l. Pyrex vacuum desiccators, as follows. A shelf of $\frac{1}{2}$ in. mesh wire netting was placed over the well of the desiccator. The Petri dishes, with lids removed and with the exposed surface of the medium facing downwards, were placed in layers of three in the desiccator, with the bottom layer of three Petri dishes resting on the wire gauze. Each Petri dish in the succeeding layers was placed to rest equally on two of the Petri dishes in the layer below it, so that diffusion of gases should not be obstructed. When necessary, a gas-phase free from CO₂ was obtained by placing 100 ml. of 25 % (w/v) potassium hydroxide in a beaker in the well of the desiccator, with a wad of glass wool partly immersed in the potassium hydroxide solution to increase the area of the CO₂-absorbing surface. CO₂ pressures greater than that in air were obtained by removing air from the desiccator until an appropriate decrease in pressure was recorded on a mercury manometer, and then allowing CO₂ to enter the desiccator until atmospheric pressure was restored. Restoration of atmospheric pressure was seen by the swelling of an inflatable polythene bag placed in the gas line between the CO₂ generator and the desiccator. CO₂ was generated by the action of dilute hydrochloric acid on marble chips in a Kipp's apparatus, and was passed through a saturated solution of sodium bicarbonate before it was passed into the desiccator. The gas-phase in the desiccators was renewed every 24 hr.

Growth stimulations observed in the auxanographic experiments are described as 'good' or 'weak'. Stimulations which resulted in growth as vigorous as that of wild-type *Neurospora* are described as 'good'. Stimulations which were obvious but which gave growth definitely weaker than that of wild-type *Neurospora* grown under the same conditions are described as 'weak'. All auxanographic experiments were scored by eye; our conclusions were not checked by more objective methods because of the difficulty of controlling the composition of the gas-phase in growth tubes and conical flasks.

RESULTS

CO₂ mutants of *Neurospora* were sought by two methods, both of which were successful. First, auxotrophs were obtained from other workers and tested to see whether they grew on unsupplemented medium when the gas-phase contained 30 % (v/v) CO₂ and did not grow on unsupplemented medium in CO₂-free air. Secondly, experiments were done to see whether CO₂ mutants could be isolated as such from wild-type *Neurospora* by using a modification of the filtration-enrichment method (Woodward, de Zeeuw & Srb, 1954; Catcheside, 1954). This second method depended on the fact that wild-type conidia grew vigorously in minimal liquid medium swept through with a current of CO₂-free air, whereas conidia from CO₂ mutants did not, even when in mixed suspension with wild-type conidia. Thus the wild-type conidia, once they had produced hyphae, could be removed from the suspension by filtration through a glass-wool pad. This resulted in a considerable increase in the proportion of CO₂ mutants remaining in the suspension. This method was tested and found effective with mixed ultraviolet (u.v.)-irradiated suspensions of conidia from wild-type *Neurospora* and from CO₂ mutant 33442. The most effective procedure was then applied to suspensions of conidia from wild-type *Neurospora*. Conidia were obtained from the Emerson wild-type strains; in some experiments strain 5297 a was used, and in others strain 5256 A. The u.v.-irradiation

was such as to kill about 97 % of the conidia. The method for isolating CO₂ mutants, and the mutants obtained by it, will be described in a later paper.

CO₂ mutants at the arginine-11 locus

Growth requirements of mutant 30820 (arg-11). The first CO₂ mutant discovered in the present series of experiments was mutant 30820. When the gas-phase contained more than 2 % (v/v) CO₂ this mutant grew almost as vigorously on minimal medium as did wild-type *Neurospora*. In the absence of CO₂ no growth was observed even after incubation for 9 days, whereas wild-type *Neurospora* showed vigorous growth within 1 day. Mutant 30820 was obtained by Beadle & Tatum (1945) in their early experiments with *Neurospora*; it was studied in detail by Srb (1950), who reported that it required arginine (or citrulline) + *either* a purine *or* a pyrimidine.

Table 1. *Growth of the arginine-11 mutant 30820 of Neurospora crassa on various supplemented media in different gas-phases with respect to CO₂*

Supplement to minimal medium	Gas-phase			
	CO ₂ -free	Ordinary air	Air containing 1-2 % (v/v) CO ₂	Air containing 30 % (v/v) CO ₂
	Relative amounts of growth			
None	0	±	+ to ++	+++
Adenine* and/or uridine†	0	±	+ to ++	+++
Arginine‡	0	+	+++	+++
Arginine + uridine or arginine + adenine	0	+	+++	+++
Arginine + adenine + uridine	+++	+++	+++	+++

* Or adenosine, adenosine-5'-phosphate; † or cytidine, uracil; ‡ or citrulline.

The only other reference to the nutritional requirements of mutant 30820 in the literature is by Perkins (1959), who reported a personal communication by Newmeyer that 'for optimal growth arginine-11 (30820) requires arginine (or citrulline) plus *both* a purine *and* a pyrimidine'. Since we suspected that even the small amount of CO₂ present in ordinary air (0.02-0.03 %, v/v) affected the nutritional requirements of mutant 30820, experiments were done to see whether this mutant had different nutritional requirements depending on whether the gas-phase was CO₂-free air or air containing CO₂. In these experiments, the conidia of the mutant were spread on the surface of solid minimal medium, rather than being dispersed throughout the medium.

The results of these experiments are given in Table 1. When the CO₂ content of the gas-phase was increased the nutritional requirements of the mutant became simpler. In CO₂-free air the mutant grew only when arginine + a purine + a pyrimidine were present in the medium; growth was then as vigorous as that of wild-type *Neurospora*. In ordinary air (0.02-0.03 %, v/v, CO₂) arginine and citrulline separately were stimulatory, as established by Srb (1950), but the stimulations were very weak and growth only became visible after 9 days. For vigorous growth in ordinary air the mutant still required arginine + a purine + a pyrimidine. In air containing 1-2 % (v/v) CO₂ arginine (or citrulline) gave fair growth and arginine +

either a purine or a pyrimidine gave vigorous growth. When the gas-phase contained more than 2% (v/v) CO₂ the conidia grew vigorously on minimal medium and stimulations were difficult to detect by the auxanographic method. In the absence of arginine, purines and pyrimidines separately or together were not stimulatory under any of the gas-phases. Thus the nutritional requirements of mutant 30820 are not fully expressed when the gas-phase is ordinary air because the small quantity of CO₂ in the air partially satisfies the requirements of the mutant.

Table 2. *Effect of various substances on the growth of Neurospora crassa mutant 44601*

Stimulatory		Not stimulatory
Sodium succinate	2-Oxoglutarate	Sodium citrate
Sodium lactate	Tween 80	Glycollic acid
Sodium acetate	Oleic acid	Sodium pyruvate
Sodium hydrogen malate	Caprylic acid	DL-Isocitrate lactone
Sodium fumarate	Glycerol	Glyoxylate
Oxaloacetate	DL-Aspartate	
Stearic acid	Sodium hydrogen glutamate	
Lauric acid	Sodium propionate	
	DL- α -Aminobutyrate	

Growth requirements of mutant 44601. One of the most interesting mutants which we found to be a CO₂ mutant was mutant 44601, from the original experiments of Beadle & Tatum (1945). This mutant has not been described in the literature, except in a recent stock culture list of the Fungal Genetics Stock Center where it was described as requiring arginine + adenine (Barratt & Ogata, 1963). When the gas-phase was CO₂-free air, the nutritional requirements of mutant 44601 were as follows. It did not grow on unsupplemented medium. On medium supplemented with arginine + adenine it grew slowly, growth becoming visible after 3 days. When the medium was supplemented with arginine + a purine + a pyrimidine growth was more vigorous but not nearly as vigorous as that of mutant 30820 or of wild-type strains under these conditions, or as that of mutant 44601 on unsupplemented medium when the gas-phase contained 2% (v/v) CO₂. For most vigorous growth in CO₂-free air mutant 44601 required acetate in addition to arginine + a purine + a pyrimidine. Vigorous growth did not occur when any one of these four substances was omitted from the medium. Acetate could be replaced by many other substances, most of which were carboxylic acids, although glycerol was also stimulatory. A list of active and inactive substances is given in Table 2. It was concluded from the auxanographic experiments that mutant 44601 had the same nutritional requirements in CO₂-free air as mutant 30820, except that it also required a carboxylic acid. Experiments such as the one shown in Fig. 1 confirmed this conclusion.

Evidence for allelism of mutants 30820 and 44601. The next question to be investigated was whether the mutations in mutants 30820 and 44601 were allelic. The mutants were of different mating type, and a cross between them was fertile. In one experiment 102 ascospores were isolated separately into tubes of complete medium and heat-activated; 70 of these ascospores gave rise to cultures, of which 31 had the same nutritional requirements as mutant 30820 and 39 had the same nutritional requirements as mutant 44601. None of the cultures was wild type, and this suggested that the mutations were allelic. In a subsequent experiment 2500 ascospores were heat-activated and plated on unsupplemented medium. More than

2000 of these ascospores germinated, but not one gave a wild-type colony. The two mutations are therefore almost certainly allelic, unless chromosome abnormalities distorted the segregation. It thus became of interest to know whether the additional requirement for a carboxylic acid shown by mutant 44601 was attributable to its having a different allele at the *arg-11* locus or a second mutation not present in

Fig. 1. The effect of sodium acetate on the growth of *Neurospora* mutants 30820 and 44601. Incubation at 25° for 7 days in 20 ml. liquid medium containing arginine + adenine + uridine (all at 30 mg./l.) + various amounts of sodium acetate. Medium held in 150 ml. conical flasks. Incubation without shaking in a gas-phase of ordinary air (0.03% v/v, CO₂).

mutant 30820. A cross between mutant 30820 and the wild-type strain Emerson 5297 a yielded 50 % of auxotrophic segregants, all of which had the same nutritional requirements as mutant 30820. From this cross 97 % of the ascospores germinated. A cross between mutant 44601 a and wild-type strain Emerson 5256 A also yielded about 50 % auxotrophic segregants. However, these segregants differed amongst themselves, some requiring a carboxylic acid, others not, although all required arginine + adenine + uridine.

The data from this second cross were as follows: of 228 ascospores transferred individually to culture tubes and heat-activated, 212 germinated (93%); 183 of the resulting cultures were chosen at random and tested further, of these 94 grew on minimal medium in CO₂-free air and were assumed to be wild type; the remaining 89 were auxotrophs which grew on minimal medium only when the gas-phase contained 30 % (v/v) CO₂. The nutritional requirements of the 89 auxotrophs were determined auxanographically in CO₂-free air: 41 had the same nutritional requirements as mutant 30820, and did not require acetate; 29 had the same nutritional requirements as mutant 44601, requiring acetate + arginine + adenine + uridine; 19 were intermediate types in that they grew to varying extents when supplied with arginine + adenine + uridine and were further stimulated by acetate. This cross did not yield any segregants which required acetate alone.

It is concluded that the mutations in mutants 30820 and 44601 are allelic and that the carboxylic acid requirement of mutant 44601 is caused by its genetic 'background', which apparently causes a requirement for carboxylic acid only in the presence of the 44601 (and presumably the 30820) mutation. It is quite possible that these mutant strains have different genetic backgrounds, because Beadle & Tatum (1945) obtained mutant 30820 from a cross of the wild type strain 1A with X-ray-treated conidia from the wild type strain 19a, and they obtained mutant 44601 from a cross of the wild type strain Abbott 4 with ultraviolet-irradiated conidia from the wild type strain 25a.

Table 3. *Adenine mutants of Neurospora crassa which are not CO₂ mutants*

Locus	Mutant no.
<i>ad-1</i>	3254
<i>ad-3A</i>	38709
<i>ad-3B</i>	Y 112-M2
<i>ad-4</i>	44206
<i>ad-5</i>	71104
<i>ad-6</i>	28610
<i>ad-7</i>	44411
<i>ad-8</i>	Y 193-M22

CO₂ mutants responding to adenine

We suspected that some adenine-requiring mutants might also be CO₂ mutants. Accordingly, mutants representative of seven of the nine known adenine loci were obtained and tested; they are listed in Table 3. None was a CO₂ mutant. However, from our own mutant-isolation experiments we obtained two CO₂ mutants (RE 25, RE 53) which responded to adenine in the absence of CO₂. Both these mutants had the same nutritional requirements and were probably allelic since they did not complement in heterokaryons. Without CO₂ they grew with: adenine, adenosine-5'-phosphate; adenosine; hypoxanthine + histidine; inosine + histidine; they did not grow with hypoxanthine, inosine or histidine separately. A cross between the CO₂ mutant RE 25 and the adenine-3 mutant Y 112-M2 gave no wild-type recombinants from 22,000 ascospores of which more than 90 % germinated. This indicated that the RE 25 mutation was at the adenine-3 locus, and agreed with evidence that the RE 25 mutation was further from the mating type locus than was the arginine-3 locus. However, as shown above, two authentic adenine-3 mutants were not CO₂ mutants. All known adenine-3 mutants produce a purple pigment when the concentration of adenine is insufficient for rapid growth, and high temperatures enhance production of this pigment (Mitchell & Houlahan, 1946; De Serres, 1956, 1958). Conidia of the adenine mutants RE 25 and Y 112-M2 were therefore incubated at 37°, without shaking, in 20 ml. volumes of liquid medium supplemented with a suboptimal concentration of adenine (10 mg./l.). Mutant Y 112-M2 produced large amounts of purple pigment but mutant RE 25 did not produce pigment.

CO₂ mutants responding to succinic acid

The carboxylic acid requirement of mutant 44601 was especially interesting because two CO₂ mutants (RE 50, RE 56) from our mutant-isolation experiments were 'succinate mutants' and required any one of the carboxylic acids and similar

substances which stimulated mutant 44601 (see Table 2) except aspartate, glutamate, 2-aminobutyrate and glycerol. These 'succinate' mutants did not require arginine or a purine or a pyrimidine, nor were they inhibited by these substances. In their nutritional requirements they resembled the 'succinate' mutants studied by Lewis (1948) and Strauss (1956, 1957). They responded to several long-chain fatty acids; there is no record in the literature of the effect of these fatty acids on other succinate mutants. Only one succinate locus is recorded on the linkage maps of Barratt, Newmeyer, Perkins & Garnjobst (1954), although Lewis (1948) reported that one succinate mutation (39311) was on the other side of the centromere from the locus of three other succinate mutants, so there may be more than one succinate locus in *Neurospora*. So far we have not obtained a culture of mutant 39311, but we have obtained cultures of the allelic succinate mutants 35402, 37807, 46005, 55901 and 66702. When tested in one experiment these mutants did not respond to 30 % (v/v) CO₂. Mutant 66702 was crossed with one of our succinate CO₂ mutants (RE 50) to see whether the mutations were allelic. Sixty-six ascospores were isolated at random; all of the resulting 66 cultures were succinate mutants and, surprisingly, all were CO₂ mutants, although only about half should have responded to CO₂. This problem was resolved when further experiments showed that the succinate mutant 66702 was a CO₂ mutant, as were the other succinate mutants 35402, 55901, 37807, and 46005. We conclude that our succinate CO₂ mutant RE 50 is allelic with, and probably identical with, the succinate mutants 35402, 46005, 37807, 55901 and 66702. The failure of these five mutants to respond to CO₂ in the first experiment is unexplained. In several subsequent experiments these five mutants have all grown vigorously on minimal medium when the gas-phase contained 30 % (v/v) CO₂. On minimal medium in CO₂-free air mutants 35402, 55901 and 46005 showed no growth, and mutants RE 50, RE 56, 37807 and 66702 showed slow ('leaky') growth.

CO₂ mutants responding to arginine

It has been shown that another arginine mutant (33442) is a CO₂ mutant (Reissig & Nazario, 1962; Charles & Broadbent, 1964). This mutant is not allelic with the first arginine CO₂ mutant (30300), being sited at the arginine-2 locus on chromosome IV (Barratt *et al.* 1954). The characters of mutant 30300 have already been considered in detail (Charles, 1964); mutant 33442 is phenotypically indistinguishable from mutant 30300. Both mutants grew vigorously when the gas-phase was air + 30 % (v/v) CO₂, or when the medium contained arginine or citrulline. Pyrimidines inhibited the response to CO₂ but were not inhibitory when the mutants were growing on arginine or citrulline; that is, arginine and citrulline non-competitively overcame the inhibitory effect of pyrimidines. Our mutant-isolation experiments have yielded eleven arginine-requiring CO₂ mutants. Nine of these have been tested in complementation and crossing experiments. Three of them have mutations at the arginine-2 locus, and 6 have mutations at the arginine-3 locus. This suggests that CO₂ mutants may not occur at other arginine loci, except of course at arginine-11 (30820), which is not strictly an arginine locus. Our isolation experiments have not yielded any arginine-11 mutants.

DISCUSSION

The first question to be considered is whether the new CO_2 mutants reveal anything about the mechanism whereby 30 % (v/v) CO_2 in the gas-phase causes the mutants to grow. The only biosynthetic processes known to require carbamoyl phosphate are the syntheses of arginine and of pyrimidines. When only arginine-requiring and pyrimidine-requiring mutants were known to respond to CO_2 it seemed probable that CO_2 mutants might only be associated with biosynthetic pathways which incorporated CO_2 into carbamoyl phosphate. The discovery of many different CO_2 mutants (Charles & Broadbent, 1964), including the succinate-requiring mutants and the adenine-requiring mutants described here, shows that CO_2 mutants are not restricted to pathways which incorporate carbamoyl phosphate, unless carbamoyl phosphate takes part in more reactions than is at present realized.

One possible explanation of CO_2 mutants is that they may have modifications in enzymes which catalyse CO_2 -incorporation reactions. Another possible explanation is that they may have enzymes which are altered in such a way that their activity is restored by CO_2 through some physicochemical effect. For example, CO_2 might alter the configuration of the protein produced by the mutant gene, either by combining with it or by altering the intracellular pH value. According to this kind of explanation CO_2 mutants may occur in any metabolic pathway, regardless of whether the pathway has a CO_2 -incorporation step. Since several of our CO_2 mutants, especially the arginine mutants at two loci, have nutritional requirements which agree with their having blocks in a CO_2 -incorporation process, it is unnecessary to invoke such explanations at present. The adenine-requiring CO_2 mutants are particularly instructive since they are at the adenine-8 locus, which is concerned with two consecutive reactions of purine biosynthesis, the first of these being the reaction of CO_2 or bicarbonate with 5-aminoimidazole ribotide to form 5-amino-4-carboxy-imidazole ribotide (Buchanan, 1960).

Assuming that CO_2 mutants are restricted to CO_2 incorporation reactions, the next question to be considered is how it is that high pressures of CO_2 cause the mutants to grow. One possibility is that the enzymes retain some activity which is expressed in the presence of high concentrations of their substrate, in this case CO_2 . Another possibility is that high pressures of CO_2 permit alternative pathways to function. The large number of CO_2 mutants that we have found raises doubts about the general validity of hypotheses which invoke alternative pathways, since it is necessary to invoke so many alternative pathways, unless one pathway may serve as an alternative pathway for several different CO_2 mutants. Since three of the new kinds of mutants described here, and several described elsewhere (Charles & Broadbent, 1964), do not show specific inhibitions by any well-known metabolites, it is in any case unnecessary to invoke alternative pathways to explain these properties. At present, it is not possible to come to a definite conclusion about the nature of CO_2 mutants. We adopt the working hypothesis that CO_2 mutants have damaged mechanisms for incorporating CO_2 and that high CO_2 pressures force CO_2 down alternative pathways which overflow and provide CO_2 or a derivative of CO_2 to the damaged pathway. If the alternative pathway supplies unchanged CO_2 , it may either be a pathway having a CO_2 -evolving reaction, or be some kind of CO_2 -transporting system.

Whatever hypotheses are adopted to explain CO₂ mutants, it is difficult to understand how CO₂ mutants may occur at all if CO₂, which is presumably a product of metabolic processes, has free access to all parts of the protoplasm, because this waste CO₂ should have the same effect as exogenous CO₂ supplied in the gas-phase. One possibility is that CO₂ formed as a product of metabolism may be eliminated from the cell without having direct access to CO₂-fixing reactions, and the CO₂ required for the CO₂-fixation reactions may be brought into the cell by one or more special transport systems. Alternatively, particular CO₂-incorporating reactions may have access to the CO₂ from particular CO₂-evolving reactions only.

Considering other problems raised by the CO₂ mutants, the first question of interest is why CO₂ mutants occur at two different arginine loci. Both mutants have the same nutritional requirements, in that they respond to arginine or citrulline but not to ornithine, which suggests that both are unable to form carbamoyl phosphate from CO₂. The occurrence of two genes which control the synthesis of carbamoyl phosphate for arginine synthesis may be explained in two ways. First, there may be two steps in the proposed arginine-specific pathway for synthesis of carbamoyl phosphate. Secondly, both genes may affect the same enzyme, in which case one gene might determine the structure of the enzyme, and the other gene might be a 'regulator' gene which controls the 'structural' gene.

The significance of CO₂ mutants in the adenine-3 region has already been mentioned. De Serres (1956, 1964) gave evidence that there are two separate loci (*ad-3A*, *ad-3B*) in the adenine-3 region, separated by an unknown region of the chromosome. It is not known which adenine-3 locus is concerned with the CO₂-incorporation reaction. Whichever locus the CO₂ mutations are at, it is clear that that locus must also give mutants which are not CO₂ mutants, because neither of the *ad-3A* and *ad-3B* mutants in Table 3 were CO₂ mutants. This recalls the situation at the *pyr-3* locus, where some mutants are CO₂ mutants and others not (unpublished experiments with Professor V. W. Woodward). De Serres (1963) showed that at the *ad-3B* locus there are two complementation groups, one of which is composed of leaky mutants. Since our mutants are leaky in air (but not in CO₂-free air) it is possible that the CO₂ mutants occur in the 'leaky' *ad-3B* complementation group.

The mutants responding to carboxylic acids pose an interesting problem when one considers the possible nature of the lesions in them, because of the numerous reactions in which carboxylic acids are involved. Since the mutants grow vigorously with sucrose as carbon source when the gas-phase contains 30% (v/v) CO₂ we assume that the tricarboxylic acid cycle still operates in them. It is known that the operation of the tricarboxylic acid cycle does not generate a supply of 4-carbon substances for syntheses and that for this the cell is dependent upon CO₂-fixation reactions (Wood & Stjernholm, 1962). Perhaps one of these CO₂-fixation reactions is damaged in the 'carboxylic acid' mutants. High CO₂ pressures may therefore bring about effective synthesis of a 4-carbon substance by the damaged pathway, or by an alternative CO₂-incorporation pathway, several of which are known. The response to acetate is not difficult to understand because in many organisms acetate is known to be metabolized via the glyoxylate cycle, the net effect of which is to generate succinate from acetate. These 'carboxylic acid' mutants may be identical with the succinate mutants studied by Lewis (1948) and Strauss (1956,

1957). Strauss (1957) presented evidence that the succinate mutants were deficient in a mechanism for fixation of CO₂ into oxaloacetic acid but he did not investigate the effect of increased CO₂ pressures.

Perhaps the most interesting mutants described here are mutants 30820 and 44601. A possible explanation of why mutant 30820 has three requirements is that its mutation is a deletion covering separate arginine, purine and pyrimidine loci; but this seems unlikely because neither purine or pyrimidine loci are known from the 30820 region. An arginine locus (*arg-10*) is very close to the 30820 locus, but mutation at this locus leads to a requirement for arginine, not citrulline (Newmeyer, 1957). We conclude that a single gene at the 30820 locus is concerned with the biosynthesis of arginine, purines and pyrimidines. An obvious suggestion is that the gene controls the supply of CO₂ to all three pathways; the mutant may require a high CO₂ pressure either because it has an enzyme or permease with a decreased affinity for CO₂, or because the high CO₂ pressure brings an alternative pathway into operation. Although the gene at the 30820 locus may be concerned with three biosynthetic pathways, this does not necessarily mean that the three pathways share a common mechanism for providing CO₂. The gene may be a regulator gene which controls the action of separate systems supplying CO₂ to each pathway. If so, the mutations in the separate arginine, pyrimidine and purine CO₂ mutants might be mutations in 'structural' genes concerned with the individual systems. If an explanation of this type is rejected it becomes difficult to explain how distinct arginine, pyrimidine and purine mutants, as well as mutants which require all three substances, are all able to respond to CO₂.

Our experiments show that the 44601 mutation is allelic with the 30820 mutation and that the carboxylic acid requirement of mutant 44601 is attributable to the genetic background. Barratt & Ogata (1964) also stated that the 44601 and 30820 mutations are allelic, but they gave no further details. The significant facts about the carboxylic acid requirement are as follows: it is only expressed when the 44601 and presumably the 30820 mutation is present; it is satisfied by CO₂; in the absence of CO₂ almost the same array of substances satisfy this requirement as satisfy the requirements of the carboxylic acid mutants described above. It therefore appears that in certain genetic backgrounds not only the supply of CO₂ to the arginine, pyrimidine and purine pathways, but also the supply of CO₂ for one mechanism of carboxylic acid synthesis is under the control of the 30820/44601 locus.

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