CARBON DIOXIDE FIXATION INTO AMINO ACIDS OF PENICILLIUM CHRYSOGENUM[1]

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The relation of CO_2 to the metabolism of *Penicillium chrysogenum* has been studied in three ways in this work: (1) attempts were made to replace CO_2; (2) radioactive $C^{14}O_2$ was used to determine the conditions under which CO_2 fixation occurred; and (3) attempts were made to identify some of the products of CO_2 fixation, especially amino acids.

These methods, singly or in combination, have been used extensively to study CO_2 fixation. The manner in which CO_2 is used by bacteria has been studied by the replacement technique (Lyman *et al.*, 1947; Ajl and Werkman, 1948; Wynne and Foster, 1948). Foster *et al.* (1941) used radioactive $C^{11}O_2$ to obtain direct evidence that molds fix CO_2 into organic acids; recently Martin, Wilson, and Burris (1950) have used $C^{14}O_2$ to study citric acid formation by *Aspergillus niger*.

EXPERIMENTAL METHODS

Replacement experiments. Penicillium chrysogenum, strain NRRL1951-B25, from the departmental stock cultures was used in this study. The sporulation medium was prepared by adding 15 ml sterilized vegetable juice ("V-8") to 15 ml sterilized 3 per cent agar in a 6 oz prescription bottle. The slants were sprinkled with soil from the soil stocks, incubated at 25 C until heavily sporulated, and then refrigerated at 6 C until needed. A suspension of spores in sterile water was used to inoculate a 500 ml Erlenmeyer flask containing 100 ml growth medium. A vegetative inoculum was used in many experiments. Forty-eight hour old mycelium was washed and the pellets were blended for 10 seconds in a Waring blendor. The uniform mycelial suspension was used for inoculation into the culture flasks.

Growth media used were: (1) a corn steep medium containing 2 per cent corn steep liquor solids and 2 per cent crude lactose at pH 4.5; and (2) a synthetic medium containing (in g/L) glucose, 10.0; NH_4NO_3, 5.0; KH_2PO_4, 5.0; $MgSO_4 \cdot 7H_2O$, 0.5; $FeSO_4$, 0.1; $ZnSO_4$, 0.01; $MnCl_2$, 0.01; $CaCl_2$, 0.01. The pH was adjusted to pH 6.5 with NaOH.

The substances tested for the ability to replace CO_2 are listed in table 1. Most compounds were dissolved in water, adjusted to pH 6.5 to 7.0, sterilized by filtering through a bacteriological sintered glass filter, and added to the medium to give a final concentration of 0.1 per cent. Compounds known to be heat stable were sterilized by autoclaving at 120 C for 15 minutes.

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The inoculated flasks were incubated at 25 C on a reciprocating shaker having a stroke of 94 four inch cycles per minute. Flasks with spore inocula were incubated for 48 hours and flasks with vegetative inocula were incubated for 24 hours which was sufficient time to obtain good growth in the control flasks. CO_2 was removed by continuous aeration of the medium with CO_2-free air, obtained by bubbling sterile air through 2 one liter bottles each containing 200 ml of 40 per cent KOH and a third bottle containing sterile water for rehydration of the air. The CO_2-free air was distributed to the culture flasks by means of a manifold attached to the side of the shaker. A duplicate manifold and series of flasks containing sterile water in place of KOH served as a control for aerating the cultures with non-CO_2-free air. The culture flasks were 500 ml Erlenmeyer flasks fitted with rubber stoppers with inlet and outlet tubes. The inlet tubes were capillary and extended below the surface of the medium. An aeration rate of 150 ml air per minute was used.

Growth response to the experimental conditions was measured by determining the dry weights of the mycelium which had been dried at 90 C for 12 hours. Oxalacetic acid was determined manometrically by the aniline citrate method of Edson (1935). The Warburg flasks were shaken at 30 C, and the determinations were completed within 15 minutes incubation time. Since no changes in manometric readings occurred after 10 minutes, the only compound reacting with the aniline citrate to produce CO_2 was oxalacetic acid (Greville, 1939).

Isotopic studies. For experiments with $C^{14}O_2$, the mold was grown from spore inocula in a synthetic medium; the mycelium was harvested, washed with water, and blended for 10 seconds in distilled water. Two types of incubation flasks were used: a large (125 ml) Warburg flask constructed so that $C^{14}O_2$ could be generated within the flask from $BaC^{14}O_3$ (Martin, Wilson, and Burris, 1950); and a 15 ml Warburg flask with double side arms, one side arm for the addition of $NaHC^{14}O_3$ solution and the other side arm for the addition of acid. The flasks were agitated during incubation in a constant temperature water bath at 25 C.

At the conclusion of the incubation period, sufficient H_2SO_4 to reduce the pH of the medium to below pH 1.0 was added to kill the mold and to release residual $C^{14}O_2$ which was removed by aeration. Aliquots of the medium and mycelium were assayed for radioactivity by first converting the carbon to carbonate (Stutz and Burris, 1951) and counting the carbonate by standard methods with a flowing gas counter. In some instances the amounts of activity in the medium and in extracts of the mold were determined by counting aliquots which had been dried in planchets.

The mycelium was extracted for 15 minutes with boiling 80 per cent ethyl alcohol and then for 15 minutes with 5 per cent trichloracetic acid at 90 C. These extractions removed most of the nonprotein material from the mycelium. The residue from the extractions was hydrolyzed with 6 N HCl in a sealed Pyrex test tube for 2 hours at 120 C. The hydrolysate was filtered through filter paper to remove insoluble material, and evaporated to dryness *in vacuo* 3 times to remove HCl. The residue was taken up in M/15 phosphate buffer, pH 6.8, and analyzed for amino acids.

Descending paper chromatography was used for the separation and identification of the radioactive compounds. The methods of Consden, Gordon, and Martin (1944), Dent (1948), and others as adapted by Benson *et al.* (1950) and Housewright and Thorne (1950) were used for the preparation of one- and two-dimensional paper chromatograms. For separation of amino acids the first solvent was phenol (which had been redistilled from 0.1 per cent Al filings and 0.05 per cent $NaHCO_3$) and diluted to contain 18 per cent water (v/v). Butanol-propionic acid-water was the second solvent (Benson *et al.*, 1950). The organic acid fraction was developed with phenol-water (3:1) containing 1 per cent (v/v) of 95 per cent formic acid (Stark, Goodban, and Owens, 1951).

Amino acids were detected by spraying the dried sheets with 0.05 per cent ninhydrin in water saturated butanol and heating the sheets in an electric oven at 90 C for 5 minutes. Organic acids were detected by spraying with a 0.04 per cent solution of brom phenol blue in 95 per cent ethyl alcohol.

Radioactive areas on one-dimensional strips were located by cutting out amino acid and organic acid spots and eluting with boiling water for 15 minutes; the eluates were evaporated to dryness in planchets, and the radioactivity was measured. The location of radioactive areas on two-dimensional chromatograms was determined by preparing a radioautograph of the paper by the method of Benson *et al.* (1950). The paper was then sprayed to detect the compounds, and the radioactive areas of the X-ray film were matched with the spots on the paper.

RESULTS

The various compounds and complex materials which were tested for the ability to replace CO_2 are listed in table 1. Oxalacetic acid and corn steep medium were the only materials which permitted the growth of the mold in the apparent absence of CO_2 from the medium.

The effect of oxalacetic acid was studied further by following its disappearance from the medium during the incubation period. The results in table 2 show that the rate of oxalacetic acid disappearance from the medium was gradual over a 15 hour period and was unaffected by the presence or absence of inoculum. Growth occurred in the inoculated flask that contained oxalacetic acid in the presence and in the absence of atmospheric CO_2.

Complex materials such as casein hydrolysate, yeast extract, beef extract, milk, a water extract prepared from the mold, and the filtrate from 2 day and 5 day cultures of *P. chrysogenum* increased the amount of growth in air two- to fivefold, but there was no response to these materials in the absence of CO_2. Corn steep medium, however, supported growth equally well in CO_2-free air and in ordinary air, and the amount of growth was about double that obtained in synthetic medium.

The data in table 3 show that the presence of the nutrient salts in the synthetic medium was necessary for maximum fixation of $C^{14}O_2$ by *P. chrysogenum*. Table 4 shows that both a carbon source and a nitrogen source were necessary for maximum fixation of $C^{14}O_2$. In the absence of NH_4NO_3 but without glucose the fixa-

TABLE 1

Substances tested for ability to replace CO_2

Organic Acids:

Formate	Oxalacetate	*alpha*-Ketoglutarate
Acetate	Malate	Isocitrate
Lactate	Fumarate	Citrate
Pyruvate	Succinate	Oxalate

Nitrogen Compounds:

Urea	Ureides
Arginine	Methionine
Aspartate	Phenylalanine
Glutamate	

Complex Substances:

Yeast Extract	Culture Filtrate
Casein Hydrolysate	Corn Steep
"V-8" Vegetable Juice	Milk
Orange Juice	Corn Steep Ash
Mold Extract	

Mixtures:

Succinate + Casein Hydrolysate
Succinate + Urea
Succinate + Casein Hydrolysate + Urea
Succinate + Casein Hydrolysate + Urea + Methylene Blue
Succinate + Casein Hydrolysate + Urea + Methylene Blue + Mold Extract
Beef Extract + Yeast Extract
Casein Hydrolysate + Yeast Extract

TABLE 2

Disappearance of oxalacetic acid from inoculated and uninoculated flasks of a synthetic medium (pH 7.0) aerated with CO_2-free air and with ordinary air. Spore inoculum of Penicillium chrysogenum

TIME IN HOURS	μL OXALACETIC ACID IN 100 ML MEDIUM*			
	Inoculated		Uninoculated	
	Air	CO_2-free air	Air	CO_2-free air
0	4,600	4,400	4,400	4,800
3	2,600	2,300	2,500	2,600
6	1,800	1,700	1,700	1,800
9	1,100	1,000	1,200	1,100
12	900	800	1,000	900
15	400	700	800	600

* Oxalacetic acid determined manometrically as CO_2. In the main compartment, 0.3 ml 50 per cent citric acid and 2.5 ml test solution; in the side arm, 0.4 ml aniline citrate.

tion of $C^{14}O_2$ was less than 10 per cent of the fixation obtained in the complete medium, and without glucose but with NH_4NO_3 the amount of fixation was reduced to a negligible amount.

The influence of pCO_2 on fixation was examined, using concentrations of 0.03 per cent and 0.3 per cent. The amount of fixation in the higher concentration of $C^{14}O_2$ was about 5 times the amount at the lower concentration. Since the actual concentration of CO_2 in the culture flask probably is much higher

TABLE 3

Fixation of $C^{14}O_2$ in Penicillium chrysogenum in synthetic medium (pH 6.5) with and without mineral salts. Blended mycelia inoculum incubated for 2 hours at 25 C

SALTS	ACTIVITY			CARBON IN MYCELIUM	FIXATION PER MG CARBON
	Medium	Mycelium	Total		
	counts/min			*mg*	*counts/min*
Present	118,000	332,000	450,000	4	112,500
Absent	134,000	255,000	389,000	5	77,800

TABLE 4

Fixation of $C^{14}O_2$ in Penicillium chrysogenum in synthetic medium with and without carbon and nitrogen sources. Blended mycelial inoculum incubated for 1 hour

MEDIUM		ACTIVITY			CARBON IN MYCELIUM	FIXATION PER MG CARBON	RELATIVE FIXATION VALUE
NH_4NO_3	Glucose	Medium	Mycelium	Total			
		counts/min			*mg*	*counts/min*	
+	+	32,400	13,000	45,000	13.2	3,400	100
−	+	3,000	2,000	5,000	19.0	260	8
+	−	—	—	—	21.2	—	0

TABLE 5

Fixation of $C^{14}O_2$ by Penicillium chrysogenum into amino acids of the protein fraction. Blended mycelium incubated in synthetic medium (pH 6.5) for 15 minutes, 30 minutes, and 120 minutes

INCUBATION TIME	ACTIVITY IN AMINO ACIDS*		
	Aspartic	Glutamic	Arginine
minutes	*counts/min*	*counts/min*	*counts/min*
15	9,400	2,400	1,600
30	10,700	6,100	5,400
120	49,500	28,800	27,000

* Reported as total activity of the amino acids in the protein hydrolysate and determined by counting the activity in the aliquot which was used for chromatographic analysis.

than that normally found in air, the use of the higher concentration was valid and permitted easier identification of the compounds labeled during short exposures of the mold to $C^{14}O_2$.

Mycelia which had been incubated for 15 minutes and for 30 minutes with $C^{14}O_2$ were examined to determine the amount of activity appearing in the amino

acids. The main activity on the radioautographs of the protein hydrolysate was found to be in three amino acids which were identified by R_f values as aspartic acid, glutamic acid, and arginine. Other amino acids which were present had insignificant or no activity. Alanine was devoid of activity though present in abundance as judged by the appearance of a spot produced by ninhydrin on the chromatogram. The data in table 5 indicate that aspartic acid contained the largest amount of the fixed $C^{14}O_2$, followed by glutamic acid and arginine in that order. Included also in table 5 are the counts in these amino acids after 120 minutes incubation. The total activity in aspartic acid remained the highest, and the ratios of the activity among the three amino acids remained about the same as after 30 minutes' incubation. These observations were confirmed by the analysis of the hydrolysate on a dowex 50 column chromatogram.

DISCUSSION

Of the single compounds tested for the ability to replace CO_2, only oxalacetic acid fulfilled this function. The rate of breakdown of oxalacetic acid in the medium indicated that it probably functioned as a source of CO_2 and not as a true replacement. Because its rate of disappearance was unaffected by the presence of the mold in the medium, it is unlikely that oxalacetic acid was utilized by *P. chrysogenum*. However, this does not prove that oxalacetic acid is not involved in CO_2 assimilation inside the cell since cell permeability must be considered.

Members of the tricarboxylic acid cycle permit growth of *Escherichia coli* at low aeration rates with CO_2-free air (Ajl and Werkman, 1948). These compounds might decrease the total requirement for CO_2 by the amount which is involved in their synthesis and metabolism, and may also provide, through decarboxylation, a source of CO_2 to participate in other reactions. However, with an increase in the rate of aeration with CO_2-free air, *E. coli* does not grow in the presence of these compounds, although the addition of complex materials such as casein hydrolysate, yeast extract, and peptone supports growth (Lwoff and Monod, 1947). *P. chrysogenum* which respires at a lower rate than *E. coli* (on a dry weight basis) grew only in corn steep medium under our conditions of CO_2 removal. The effect of corn steep medium might be an actual replacement of CO_2 or it might be the result of nutrients which stimulated CO_2 production during early growth or before growth began. The replacement of CO_2 by the addition of single compounds or complex substances as occasionally observed might reflect no more than the difficulty in removing completely the CO_2 produced by an organism which has been invigorated by the improved medium. The rate of formation of CO_2 as opposed to the rate at which CO_2 can be removed will determine whether the organism will grow. Furthermore, the nutrients in the medium will determine to some extent the amount of CO_2 required for synthetic purposes.

The evaluation of replacement experiments requires consideration of the following factors: 1. A substance may replace CO_2 because the substance is, or provides, a metabolite which is the product of a fixation reaction. 2. The apparent

replacement may result from a change in the medium which permits the organism to use alternate pathways not requiring one or more of the products of CO_2 fixation. 3. The apparent replacement may be the result of the addition to the medium of substances which permit the organism to produce more CO_2, either through enzymatic decarboxylations of the substances added or through an increased metabolic rate which increases the output of CO_2. 4. Failure of some substances to by-pass CO_2 fixation may be a result of cell impermeability and is not evidence that these materials are not products of CO_2 assimilation.

More direct evidence of the role of CO_2 in *P. chrysogenum* has been gained by the use of $C^{14}O_2$. Although the analysis of the mycelium and of the medium has been incomplete, data have been obtained on the relation of CO_2 fixation to growth, and some of the compounds into which CO_2 is incorporated have been identified.

The complete synthetic medium was found necessary for maximum fixation of $C^{14}O_2$ in *P. chrysogenum*. The influence of carbon and nitrogen on the fixation of CO_2 in *P. chrysogenum* is in agreement with studies of $C^{14}O_2$ fixation by animal tissue (Schubert and Armstrong, 1949; Skipper, White, and Bryan, 1949), yeast (Liener and Buchanan, 1951), and *Serratia marcescens* (McLean *et al.*, 1951). That *P. chrysogenum* needs NH_4NO_3 for maximum fixation is presumptive evidence that CO_2 is used in the synthesis of protein and may be one of the reasons why CO_2 is essential for the growth of the mold.

The participation of CO_2 in protein synthesis was shown by finding C^{14} in the protein fraction of the mold after only 15 minutes exposure to $C^{14}O_2$. In the amino acids of *P. chrysogenum* the fixed CO_2 appeared first in aspartic acid, glutamic acid, and arginine. In the rat these amino acids are also the first to become labeled (Delluva and Wilson, 1946). Of interest is the fact that in *P. chrysogenum* alanine had no radioactivity though much alanine was synthesized. Apparently under these conditions, alanine was not closely linked with CO_2 assimilation.

The manner in which $C^{14}O_2$ becomes incorporated into the amino acids is being studied. A possible mechanism is the formation of oxalacetic acid by the Wood-Werkman reaction followed by reductive amination or transamination to form aspartate. Similarly, glutamate might be formed by reductive amination or transamination of *alpha*-ketoglutarate. In a survey for decarboxylases, only an L-glutamic acid decarboxylase was found in *P. chrysogenum*. This enzyme has been found in bacteria, animal tissue, and plant tissue (Gunsalus, 1950), but has not been reported in molds. Tests for decarboxylase activity on arginine and lysine were negative.

The present knowledge of the role of organic acids in the metabolism of *P. chrysogenum* is so slight that only inferences can be drawn regarding the formation of aspartate and glutamate through the mediation of the dicarboxylic acids. However, the organic acid fraction of the mycelium and the medium after 1 hour of incubation in the presence of $NaHC^{14}O_3$ was highly radioactive. These fractions are being studied.

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SUMMARY

Replacement experiments failed to show clearly how CO_2 is involved in the metabolism of *Penicillium chrysogenum*, strain NRRL1951-B25, although they did show that none of the suspected intermediates of CO_2 fixation would substitute for CO_2. Oxalacetic acid which apparently substituted for CO_2 was shown to disappear from the medium at a gradual rate, suggesting that CO_2 released by the decomposition of the acid enabled the organism to grow. Of the many complex materials added, only corn steep liquor supported growth under the conditions of CO_2 removal. Other complex materials stimulated growth in ordinary air but did not support maximum growth in the absence of CO_2. The difference in response of the mold to the complex materials and other compounds added to the medium was discussed and related to the results of other workers. Reasons for the unsuitability of replacement experiments in tracing metabolic pathways were outlined.

The amount of $C^{14}O_2$ fixed by the mold in a synthetic medium varied with the composition of the medium. In the absence of divalent ions (Mg^{++}, Fe^{++}, Zn^{++}, Mn^{++}, and Ca^{++}) the amount of $C^{14}O_2$ fixed in 2 hours was about two-thirds the amount fixed in the presence of these ions. In the absence of the nitrogen source, NH_4NO_3, the amount of $C^{14}O_2$ fixed in 1 hour was less than 10 per cent of the amount fixed in the complete medium, and in the absence of the carbon source, glucose, the amount of $C^{14}O_2$ fixed was reduced to a negligible amount.

The main activity in the protein fraction was present in three amino acids, aspartic acid, glutamic acid, and arginine. The formation of these amino acids was discussed.

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