JOURNAL OF BACTERIOLOGY, Mar. 1976, p. 1040-1047
Copyright © 1976 American Society for Microbiology
Vol. 125, No. 3
Printed in U.S.A.

Regulation of Thymidine Metabolism in *Neurospora crassa*

WAIT R. GRISWOLD, VISITACION O. MADRID, PATRICIA M. SHAFFER, DANIEL C. TAPPEN, CHARLES S. G. PUGH, AND MITCHEL T. ABBOTT*

Department of Chemistry, San Diego State University, San Diego, California 92182

Received for publication 6 November 1975

The utilization of thymidine by *Neurospora crassa* is initiated by the pyrimidine deoxyribonucleoside 2′-hydroxylase reaction and the consequent formation of thymine and ribose. Thymine must then be oxidatively demethylated by the thymine 7-hydroxylase and uracil-5-carboxylic acid decarboxylase reactions. This article shows that the 2′-hydroxylase reaction can be regulated differently than the oxidative demethylation process and suggests that the 2′-hydroxylase has, in addition to the role of salvaging the pyrimidine ring, the role of providing ribose not only for the utilization of the demethylated pyrimidine but also for other metabolic processes. One way that this difference in regulation was observed was with the *uc-1* mutation developed by Williams and Mitchell. The present communication shows that this mutation increases the activities of the 7-hydroxylase and the decarboxylase but has no comparable effect on the 2′-hydroxylase. Qualitatively similar effects on these enzymes were brought about by growth of wild-type *Neurospora* in media lacking ammonium ion, such as the Westergaard-Mitchell medium. The 2′-hydroxylase and 7-hydroxylase are also differently affected by the carbon dioxide content of the atmosphere above the growing culture and the growth temperature. Studies with inhibitors indicated that the carbon dioxide effect is dependent on protein synthesis.

Recent studies (34) have indicated that in order for *Neurospora crassa* to utilize pyrimidine deoxyribonucleosides they must be initially metabolized as depicted in Fig. 1. This report describes several factors that may be pertinent to the regulation of the pathway. The first indication of one of these factors came from attempts to facilitate purification of thymine 7-hydroxylase by growing liquid cultures under forced aeration. Although the yield of mycelia was increased over that obtained from shallow liquid cultures, the 7-hydroxylase activity was markedly decreased and, furthermore, the incorporation of thymidine into ribonucleic acid was inhibited (22). However, larger nonaerated cultures with considerable 7-hydroxylase activity could be obtained if the *Neurospora* were grown with stirring in a sealed carboy half-filled with medium (22). Since the atmosphere inside of the carboy became anaerobic during growth, it was suspected that an increase in the 7-hydroxylase activity might, somehow, be initiated by this change in the gas composition.

Another factor that may play a regulatory role is the ammonium ion content of the growth medium. The Westergaard-Mitchell medium permits the efficient utilization of intermediates in the pathway shown in Fig. 1 (39). The data in the present article indicate that mycelia grown in this medium contain higher activities of the enzymes involved in the oxidative demethylation of thymine and that the effect seems to be a consequence of the lack of ammonium ion in the medium. It is also shown that enzymatic activities of the pathway can be affected by the temperature at which the cultures are incubated and by the *uc-1* mutation, which was selected from the pyrimidineless strain *pyr-4* by Williams and Mitchell (39). The *pyr-4,uc-1* strain, in contrast to the *pyr-4* strain, can utilize thymidine, thymine, 5-hydroxymethyluracil, or 5-formyluracil as a sole pyrimidine source, and Williams and Mitchell (39) have suggested that the *uc-1* mutation affects one or more enzymes involved in the pathway. The present communication shows that this mutation effects elevated activities of the enzymes that oxidatively demethylate thymine. An abstract of some of these findings has been published (Fed. Proc. 34:568, 1975).

MATERIALS AND METHODS

Strains. *N. crassa* wild-type strain (1A) and the pyrimidineless mutant *pyr-4* (which lacks orotidylate decarboxylase [28]) were obtained from the Fungal Genetics Stock Center, Arcata, Calif. The characterization and source of the triple mutant *pyr-4,uc-1,uc-2* have been described (34, 39). The double mutant *pyr-4,uc-1* was isolated from a cross between strains 1A and *pyr-4,uc-1,uc-2*. This *pyr-4,uc-1* strain was initially identified by its ability to utilize

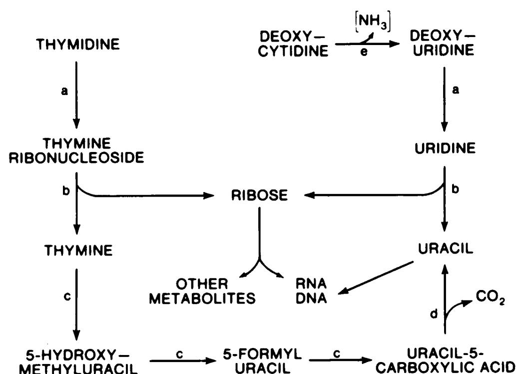


FIG. 1. *Deoxyribonucleoside metabolism in N. crassa: (a) pyrimidine deoxyribonucleoside 2′-hydroxylase, (b) hydrolyase reaction, (c) thymine 7-hydroxylase, (d) uracil-5-carboxylic acid decarboxylase, (e) deaminase reaction. For recent reviews, see references 1, 11.*

thymidine as a sole pyrimidine source, as described by Williams and Mitchell (39), in contrast to the pyrimidineless strains carrying the *uc-2* mutation that cannot effectively metabolize pyrimidine deoxyribonucleosides (34). An additional strain of the double mutant *pyr-4,uc-1* was constructed by crossing strains *uc-1* and *pyr-4*. The *uc-1* strain was also isolated from the cross of strains 1A and *pyr-4,uc-1,uc-2* and was identified by its high 7-hydroxylase activity, wild-type level of 2′-hydroxylase activity, and growth in minimal medium.

Media and chemicals. All stocks were maintained on Fries minimal medium (13), supplemented with uridine (0.2 g/liter) for mutants. Westergaard-Mitchell medium (36) was used for growth tests. Appropriate modifications were made with respect to agar content and pyrimidine and nucleoside supplements. When colonial growth was desired, the normal carbon source of 2% sucrose was replaced by 0.1% sucrose and 1.0% sorbose (7). Crosses of *Neurospora* were carried out on slants of corn meal medium (7). Ascospores were germinated by heating at 60 C in sterile distilled water for 1 h.

The sources of the radioactive compounds were described previously (19, 22, 26). The specific activities (curies per mole) of the compounds used are as follows: 3.0 for [2-^{14}C]thymine, 2.0 for [7-^{14}C]uracil-5-carboxylic acid, and 0.50 for α-[1-^{14}C]ketoglutarate. All other fine chemicals were obtained from commercial sources as previously described (22, 26).

Growth of mycelium for enzymatic assays. Conidia were obtained from cultures that were grown for 10 days (at 28 C in constant light) on 50 ml of Fries minimal medium, solidified with 2% agar, in 125-ml Erlenmeyer flasks. The conidia were suspended in water and adjusted to a concentration of 10^7 conidia per ml. One milliliter of this solution was used for inoculation of each 100 ml of liquid medium.

The nonaerated growth of *Neurospora* in a 10-liter carboy half-filled with medium and closed with a rubber stopper, containing a greased glass sleeve through which a J-shaped glass stirring paddle extended, has been described (22). This apparatus was modified so that several portions of an 8-liter culture could be removed while the gradual change of the atmosphere in the carboy was preserved. The modifications include a glass spigot for withdrawing mycelial samples and tubing for connecting the atmosphere inside of the carboy to the atmosphere above 4 liters of 0.1 N H_2SO_4 in another sealed 10-liter carboy. When a sample of mycelium was removed through the spigot, a volume of 0.1 N H_2SO_4 was simultaneously added to the carboy containing acid so that the ratio of the volume of the growth medium to the volume of the atmosphere in the two carboys remained 1:1. The carboy with media also contained a valve for the withdrawal of gas samples so that the amounts of O_2 and CO_2 could be determined by the Scholander technique (33).

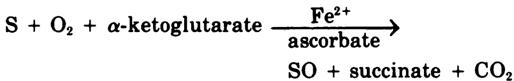
To culture smaller amounts of *Neurospora* under nonaerated conditions, a 125-ml Erlenmeyer flask was used. It was half-filled with medium and sealed with a rubber stopper. The rubber stopper contained a glass tube sealed with a serum cap to permit additions to be made to the culture and analysis of its atmosphere. The flask was shaken during the growth period on an Eberbach reciprocating shaker (model 6000) at 120 excursions per min. For the growth of *Neurospora* in the presence of a CO_2-trap, a polypropylene tube (15 by 50 mm), containing 3 ml of 20% KOH, was suspended above the medium. To grow *Neurospora* under aerated conditions, the flask was capped with a loosely fitted cotton plug instead of with a rubber stopper. In contrast to the incubations in carboys, which were always carried out at 28 C, those in Erlenmeyer flasks were carried out at 34 C unless otherwise indicated.

Pad-sections of mycelia were prepared and assayed as previously described (34).

Preparation of cell-free extracts. The mycelia were harvested by suction filtration and washed twice with 0.05 M tris(hydroxymethyl)amino-methyl)aminomethane-hydrochloride buffer, pH 8.0, which contained 0.1 mM ethylenediaminetetraacetic acid, 1 mM ascorbate, and 1 mM reduced glutathione. The ratio of milliliters of buffer used for each washing to the grams (moist weight) of mycelia was about 40. The moist weight was usually 10 times that of the dry weight, which was determined by drying a portion of the mycelia at 55 C to a constant weight. The washed mycelia were frozen into thin pads between two blocks of dry ice. After breaking the frozen pads into small pieces, 300 mg (moist weight) was combined with 4 ml of the tris(hydroxymethyl)aminomethane buffer and 3 g of acid-washed glass beads (22) in the chamber (5-ml capacity) of the Micro-homogenizer attachment to the Sorvall Omni-Mixer and homogenized for 11 min at 5,000 rpm while the chamber was immersed in an ice-brine mixture at −2 C. The length of the homogenizing period was varied in experiments similar to those previously described (22) to show that no more protein could be extracted by prolonging the grinding and that the enzymatic activity was stable to the procedure. The homogenized material was subjected to centrifugation at 10,000 × *g* for 1 h at 1 C to obtain the crude extract. Protein was determined

with the Folin-Ciocalteau reagent.

Enzyme assays of crude extracts. Thymine 7-hydroxylase (EC 1.14.11.6) and pyrimidine deoxyribonucleoside 2'-hydroxylase (EC 1.14.11.3) are dioxygenases that catalyze the following type of reaction:



$$S + O_2 + \alpha\text{-ketoglutarate} \xrightarrow[\text{ascorbate}]{Fe^{2+}} SO + \text{succinate} + CO_2$$

The assays used for the cell-free studies were based on the rate of formation of radioactive CO_2 from carboxy-labeled α-ketoglutarate. The standard incubation mixture (0.225 ml) was 10 mM in sodium phosphate, pH 7.5, and contained 0.1 ml of the enzyme preparation, 1 mM α-[1-^{14}C]ketoglutarate, 1 mM ferrous sulfate, 1 mM ascorbate, 0.75 mM thymine or deoxyuridine, and 0.4 mg of catalase per ml (19, 22, 26). Since crude extracts could decarboxylate α-ketoglutarate in the absence of exogenous thymine and deoxyuridine, corrections were made in the calculation of enzyme activities with the values obtained from such control incubations. However, the rate of CO_2 formation in these control incubations usually did not exceed 10% of the rate observed when either thymine or deoxyuridine was present, except when the 7-hydroxylase or 2'-hydroxylase activity was very low.

The assay for uracil-5-carboxylic acid decarboxylase has been described (26).

The precision observed when different experiments were compared is indicated by the standard error of the mean, although it was usually calculated from only several values. When no error bar is associated with a symbol of a figure in the text, this means, unless otherwise indicated, that the error bar is too small to be shown clearly.

RESULTS

Effect of atmosphere of the culture on the 7-hydroxylase and 2'-hydroxylase activities. To determine the extent of the inhibitory effect of aeration on the 7-hydroxylase activity and whether the 2'-hydroxylase was also inhibited, cultures were tested early in nonaerated growth. Figure 2 shows that the 7-hydroxylase activity of the *Neurospora* was initially low and that there was no comparable inhibition of the 2'-hydroxylase. To identify the changes in atmosphere on which the apparent induction of the 7-hydroxylase is dependent, cultures were grown for various periods under atmospheres of different CO_2, O_2, and N_2 contents. But none of the conditions tested effected the dramatic increase in 7-hydroxylase activity shown in Fig. 2. It does not seem likely that this increase in activity is dependent on a decrease in the pH of growth medium, brought about by the rise in CO_2, since the media of nonaerated cultures does not become more acidic during growth than that of aerated cultures. However, CO_2 was shown to be required for the increase in

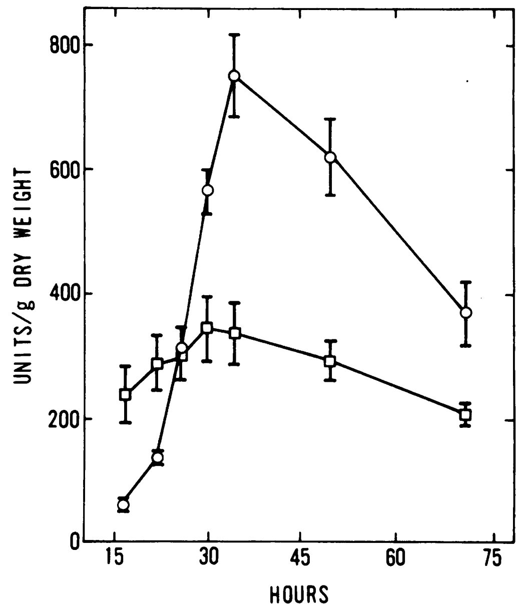


FIG. 2. *Thymine 7-hydroxylase (○) and pyrimidine deoxyribonucleoside 2'-hydroxylase (□) activities during nonaerated growth of N. crassa strain 1A in Fries medium at 34 C in Erlenmeyer flasks. At the indicated times after inoculation, mycelia were harvested for preparation of crude extracts that were assayed as described in Materials and Methods. Each point is the mean of the values obtained from four mycelial samples that were taken from different flasks.*

enzymatic activity by growing cells in the presence of a CO_2-trap (Table 1). In a time study, similar to that represented in Fig. 2, it was shown that the 7-hydroxylase activity did not increase while the *Neurospora* was grown for 60 h in the presence of a CO_2-trap.

The apparent induction of the 7-hydroxylase was prevented by inhibitors of protein and nucleic acid synthesis (Table 2). Since the observed effects of the inhibitors on the 7-hydroxylase activity might be ascribed to the reduction in atmospheric CO_2 or to the apparent induction occurring at a different time during the growth period, a time study was carried out similar to that described in Fig. 2, but some of the flasks were made 0.6 μM with respect to cycloheximide 21 h after inoculation. This concentration did not allow the 7-hydroxylase activity to increase during the subsequent growth period and yet had little, if any, effect on the CO_2 content of the atmosphere at a time when the 7-hydroxylase activity was markedly increasing in the control cultures.

The 7-hydroxylase activity could be reduced without a comparable effect on the 2'-hydroxylase by growth at a lower temperature (compare

TABLE 1. *Effect of carbon dioxide on thymine 7-hydroxylase*[a]

Growth condition[b]	Activity (U/g [dry wt])[c]		Gas analysis (%)		Dry wt (mg)	Protein (mg/g [dry wt])
	7-Hydroxylase	2'-Hydroxylase	CO_2	O_2		
Nonaerated	518 ± 62	210 ± 36	42.2 ± 0.9	0.12 ± 0.07	35 ± 1	204 ± 6
Aerated	37 ± 6	375 ± 40	0.30 ± 0.04	20.1 ± 0.5	156 ± 15	142 ± 8
Nonaerated with CO_2-trap	19 ± 4	166 ± 11	0.21 ± 0.03	0.34 ± 0.09	34 ± 1	148 ± 8

[a] *N. crassa* strain 1A was grown for 40 h in Fries medium at 34 C in Erlenmeyer flasks. Crude extracts were prepared and assayed as described in Materials and Methods.
[b] The procedures for growing the *Neurospora* under nonaerated and aerated conditions and for preventing the atmospheric CO_2 of the growing cultures from increasing are described in Materials and Methods.
[c] The values are the means and standard error of the mean for seven flasks.

TABLE 2. *Effect of inhibitors of protein and nucleic acid synthesis on the apparent induction of thymine 7-hydroxylase activity in N. crassa strain 1A grown under nonaerated conditions*[a]

Additions (μM)	Activity (U/g [dry wt])[b]		Gas analysis (%)[b]		Dry wt (mg)[b]	Protein (mg/g [dry wt])[b]
	7-Hydroxylase	2'-Hydroxylase	CO_2	O_2		
None	554 ± 36	252 ± 29	40.0 ± 0.8	0.17 ± 0.05	37 ± 1	193 ± 15
Cycloheximide (71)	53 ± 5	266 ± 42	30.0 ± 0.6	0.38 ± 0.03	31 ± 2	212 ± 9
Actinomycin D (40)	62 ± 12	306 ± 54	36.2 ± 0.3	0.09 ± 0.03	33 ± 1	209 ± 17
Proflavin (300)	38 ± 18	146 ± 35	28.0 ± 1.4	0.27 ± 0.13	24 ± 2	143 ± 7

[a] *N. crassa* was grown in nonaerated 125-ml Erlenmeyer flasks as described in Table 1. Inhibitors were added to the cultures at 21 h after inoculation. The cultures were harvested at 40 h after inoculation.
[b] Values given are the means and the standard error of the mean for six flasks.

Fig. 3 with Fig. 2), and the CO_2 content of the atmosphere did rise more slowly at the lower temperature. However, the temperature sensitivity of a *Neurospora* mutant that requires CO_2 for growth has been attributed to the decreasing solubility of CO_2 with increasing temperature (6).

Effect of ammonium ion content of growth media on the 7-hydroxylase, 2'-hydroxylase, and decarboxylase activities. The first indication that the ammonium ion content of the medium might have an effect on some of the enzymes in the pathway came from studies, with mycelial pad-sections, of substrate utilization in vivo. They showed that *Neurospora* grown in the Westergaard-Mitchell medium had an increased capacity for the utilization of thymine and uracil-5-carboxylic acid, but not for the utilization of deoxyuridine. That this is a reflection of the activities of the corresponding enzymes of the pathway is shown by Fig. 4. It shows that the 7-hydroxylase and decarboxylase activities were decidedly elevated in *Neurospora* grown in the Westergaard-Mitchell medium in contrast to the 2'-hydroxylase activity. The effect of the Westergaard-Mitchell medium does not appear to be brought about through an increase in CO_2, since the composition of atmospheres during growth on the two media is nearly the same. The comparison of Fig. 4A with Fig. 2 reveals that *Neurospora* cultured in Fries medium had much higher 7-hydroxylase activity when the growth occurred under the nonaerated conditions used with Erlenmeyer flasks rather than with carboys. Since the cultures grown in flasks were maintained at 34 C and those in carboys were at 28 C, the growth

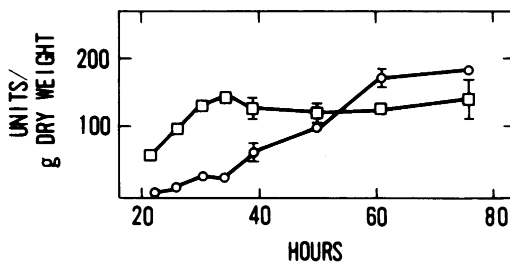


FIG. 3. *Effect of culturing N. crassa strain 1A at a lower temperature on the activities of thymine 7-hydroxylase (○) and pyrimidine deoxyribonucleoside 2'-hydroxylase (□). Cultures were grown as described in Fig. 2, except that the temperature during growth was maintained at 22 C. Points representing mycelial samples harvested after 36 h of growth are the means of the values obtained from six samples that were taken from different flasks. The earlier points are the results of a determination from a single flask.*

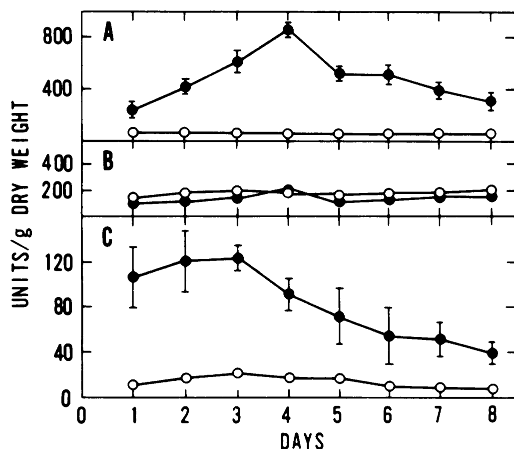


FIG. 4. *(A) Effect of growth media on thymine 7-hydroxylase activity during nonaerated growth of N. crassa strain 1A, at 28 C in carboys. Each point is the mean of the values obtained from six mycelial samples that were taken from different carboys. (B) Effect of growth media on pyrimidine deoxyribonucleoside 2′-hydroxylase activity. The crude extracts used in the experiments depicted in (A) were also assayed for pyrimidine deoxyribonucleoside 2′-hydroxylase activity. (C) Effect of growth media on uracil-5-carboxylic acid decarboxylase activity. Some of the crude extracts used in the experiments depicted in (A) were also assayed for uracil-5-carboxylic acid decarboxylase activity. Each point is the mean of the values obtained from three mycelial samples that were taken from different carboys. The crude extracts were prepared and assayed as described in Materials and Methods. Growth in Fries medium (○), growth in Westergaard-Mitchell medium (●).*

temperature appears to be one of the factors responsible for this difference.

Table 3 indicates that the component of the Fries medium that is responsible for the lower 7-hydroxylase activity is the ammonium ion. The data show that when *Neurospora* was grown in Westergaard-Mitchell medium that had been modified to contain a concentration of ammonium ion equivalent to that in Fries medium, the 7-hydroxylase activity was found to be similar to that of *Neurospora* grown in Fries medium. In other experiments it was shown that when Fries medium was modified so that it contained no ammonium ion, the *Neurospora* from this medium contained just as high 7-hydroxylase activity as did the control cultures grown in Westergaard-Mitchell medium. The Fries medium was made "ammonia-less" by replacing ammonium tartrate and ammonium nitrate with the corresponding potassium salts. Since this medium had the same ionic strength as did Fries medium, it is apparent that the effect is not simply one of ionic strength. In preliminary experiments, Fries medium was modified so that: (i) it contained potassium ion equivalent to the Fries medium that had been altered so that it contained no ammonium ion, (ii) it had the same sucrose concentration as the Westergaard-Mitchell medium, or (iii) it had the same initial pH, i.e., 6.5, as did the Westergaard-Mitchell medium. None of these modifications resulted in *Neurospora* containing 7-hydroxylase activity comparable to that obtained when the growth occurred in Westergaard-Mitchell medium.

Although the ammonium ion content of the medium did not appear to affect the 2′-hydroxylase activity in *Neurospora* grown under nonaerated conditions in carboys (Fig. 4B), a pronounced effect was noted when the cells were cultured under the aerated conditions described in Materials and Methods for Erlenmeyer growth. This was shown with cultures, of strain 1A, which were grown in parallel in the Westergaard-Mitchell and Fries media. The cultures were assayed daily during a 4-day growth period. The 2′-hydroxylase activity of the cultures from Westergaard-Mitchell medium ranged from about 60 to 70 U/g (dry weight); the activity of cultures from Fries medium ranged between 250 and 350 U/g (dry weight).

Effect of uc-1 mutation on the 7-hydroxylase, 2′-hydroxylase, and decarboxylase activities. Figure 5 shows that the *uc-1* mutation brings about an increase in the activities of the 7-hydroxylase and the decarboxylase without

TABLE 3. *Effect of ammonium ion in the growth medium on the activity of thymine 7-hydroxylase*[a]

Addition	Activity, thymine 7-hydroxylase (U/g [dry wt])[b]
Fries medium	
None	22 ± 5
Westergaard-Mitchell medium	
None	680 ± 23
NH_4Cl[c]	50 ± 22
NaCl[d]	860 ± 63

[a] *N. crassa* strain 1A was grown for 3 days in nonaerated carboys that contained the indicated media. Extracts were prepared and assayed as decribed in Materials and Methods.

[b] The values are the means and the standard error of the mean for four carboys.

[c] Sufficient ammonium chloride added to make the concentration of ammonium ion equal to that in Fries medium.

[d] Sufficient sodium chloride added to make the concentration of chloride ion equal to that in the Westergaard-Mitchell medium that had been modified by the addition of ammonium chloride.

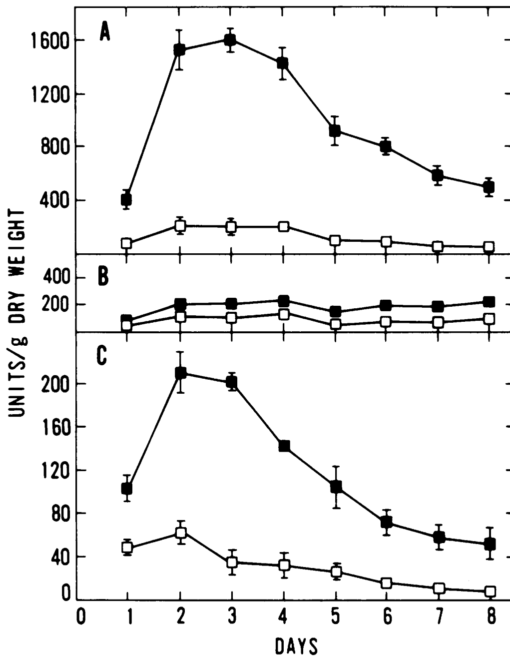


FIG. 5. *(A) Effect of the uc-1 mutation on thymine 7-hydroxylase activity during nonaerated growth at 28 C in carboys. Each point is the mean of the values obtained from five mycelial samples that were taken from different carboys. (B) Effect of the uc-1 mutation on pyrimidine deoxyribonucleoside 2'-hydroxylase activity. The crude extracts used in the experiments depicted in (A) were also assayed for pyrimidine deoxyribonucleoside 2'-hydroxylase activity. (C) Effect of the uc-1 mutation on uracil-5-carboxylic acid decarboxylase activity. Some of the crude extracts used in the experiments depicted in (A) were also assayed for uracil-5-carboxylic acid decarboxylase activity. Each point is the mean of the values obtained from three mycelial samples that were taken from different carboys. Westergaard-Mitchell medium was used to grow strains pyr-4 (□) and pyr-4,uc-1 (■). Crude extracts were prepared and assayed as described in Materials and Methods.*

having an appreciable effect on the 2'-hydroxylase. Data indistinguishable from that shown in Fig. 5 were obtained when the strain of *pyr-4,uc-1* that was used was that isolated from the cross between *uc-1* and *pyr-4* as described in Materials and Methods. The effect of the *uc-1* mutation probably cannot be ascribed to the CO_2 effect since the CO_2 content of the atmosphere above cultures of the *pyr-4,uc-1* strain does not rise more rapidly than that of the *pyr-4* strain.

DISCUSSION

Regulatory roles have been proposed for CO_2 (24) that range from its having effects on the development of several organisms (e.g., 3, 5, 21) to its regulating various areas of metabolism (e.g., 20, 32) and more specifically,to its directly interacting with enzymes and consequently affecting their activities (e.g., 2, 23). The studies with inhibitors of protein and nucleic acid synthesis, the apparent retention of the activity during purification of thymine 7-hydroxylase (18), and the use of an assay for this enzyme (22) that employs a CO_2-trap suggest that CO_2 may in some way effect the induction of the hydroxylase rather than directly interact with the preexisting enzyme. In another study of pyrimidine metabolism of *Neurospora* (25), it was indicated that growth of cultures on solid medium in the presence of CO_2 increased the activities of the pyrimidine-specific carbamyl phosphate synthetase and aspartate transcarbamylase reactions (25, 38). Interestingly, some of the early work (6) implicating the regulatory role of CO_2 on these reactions also showed that the growth of another pyrimidineless mutant of *Neurospora* (*pyr-3*) on thymine was dependent on the presence of an atmosphere that contained 10 to 15% CO_2 and that the presence of exogenous arginine prevented such growth. Arginine (0.57 mM) has also been shown, in preliminary experiments, to prevent thymine 7-hydroxylase activity from increasing during the nonaerated growth of wild-type *Neurospora* in Fries medium in Erlenmeyer flasks.

The effect of CO_2 on the 7-hydroxylase activity does not appear to be confined to wild-type *Neurospora* grown in Fries medium since in preliminary studies with the wild-type strain grown in Westergaard-Mitchell medium and with the *pyr-4,uc-1* strain grown in either Westergaard-Mitchell or Fries medium, much higher 7-hydroxylase activities were found in the mycelial samples from carboys maintained under the nonaerated conditions than in the samples from carboys under the aerated conditions. These studies also showed that the effect of growth medium on the 7-hydroxylase activity is not restricted to the wild-type strain, since the activity of this enzyme in the *pyr-4,uc-1* strain was markedly higher when growth occurred in a nonaerated carboy with Westergaard-Mitchell medium than with Fries medium.

Perhaps Fig. 5 can explain why the *pyr-4* strain is only able to grow on thymidine if growth is initiated (9, 39) with uridine or cytidine. Even when grown under nonaerated conditions, the *pyr-4* strain has low thymine 7-hydroxylase activity, and it may be a limiting factor early in the growth period. The lower 7-hydroxylase activity in the *pyr-4* strain com-

pared to that in the wild-type strain may be a consequence of the growth medium of the *pyr-4* strain being supplemented with uridine. The wild-type strain has been shown, in preliminary experiments, to have lower 7-hydroxylase activity when its growth medium was supplemented with uridine, but not as low as when the supplement was arginine.

Although *Neurospora* can utilize purines and other nitrogen-containing metabolites as a nitrogen source, it apparently cannot utilize pyrimidines for this purpose (4, 29). Thus the effect of "ammonia-less" media in increasing thymine 7-hydroxylase and uracil-5-carboxylic acid decarboxylase activities is probably not a means of providing ammonium ion per se but one of salvaging the heterocyclic pyrimidine ring. Similarly, the apparent induction of the 7-hydroxylase by CO_2 may provide a means of salvaging the ring at a time when, for example, the energy available for de novo pyrimidine synthesis is limited. A different type of regulation might be expected for the pyrimidine deoxyribonucleoside 2'-hydroxylase if it has the additional role of providing ribose for the salvaged pyrimidines, purines, and other intermediary metabolites. Perhaps the demand for ribose is increased when the mycelium has sufficient oxygen and ammonium ion to carry out biosynthetic processes, as it presumably does under the conditions that were shown to increase the 2'-hydroxylase activity.

Ammonium ion has been implicated in the regulation of several enzymes (e.g., 16, 27, 31, 35, 37). One of these is tyrosinase, which may be a determining factor in the formation of protoperithecia by *Neurospora*. Both tyrosinase and sexual differentiation are derepressed in media lacking ammonia (12). Recent studies indicate that both cyclic adenosine 3',5'-monophosphate (cAMP) and inhibitors of cAMP phosphodiesterase can induce tyrosine (8). In preliminary experiments modeled after those carried out with tyrosinase (8) and in other ones in which media and atmosphere were varied, cAMP, dibutyryl cAMP, and caffeine had no stimulating effects on the activities of the 7-hydroxylase and the 2'-hydroxylase. The low concentrations of cycloheximide that induce tyrosine (15) did not have this effect on the 7-hydroxylase. Preliminary attempts have also been made to induce the 7-hydroxylase in Fries medium supplemented with thymine or ascorbate and to replace CO_2 with α-ketoglutarate, but in these cases, too, no stimulating effects were detected. Ascorbate, α-ketoglutarate, and Fe^{2+} have been shown to be involved in the activation of prolyl hydroxylase, which is another α-ketoglutarate dioxygenase (17). Because of the qualitative similarity of the effects of the *uc-1* mutation and of media lacking ammonium ion on the 7-hydroxylase activity, it is interesting that the *ty-1* mutation, which causes female sterility in *Neurospora* and an abnormal regulation of tyrosinase (14), appears to be involved in the regulation of nitrogen metabolism (10). No studies have yet been made of the regulation of the enzymes that deaminate deoxycytidine, hydrolyze uridine and thymine ribonucleoside, and that are involved in the utilization of uracil and ribose.

ACKNOWLEDGMENTS

We wish to thank William M. Thwaites for his generous help in genetical aspects of these studies. The authors are also indebted to A. Steven Dahms and Adela S. Baer for helpful comments.

This work received financial support from Public Health Service grant AM 09314 from the National Institute of Arthritic and Metabolic Diseases and title 4, National Defense Education Act Funds.

LITERATURE CITED

1. **Abbott, M. T., and S. Udenfriend.** 1974. α-Ketoglutarate-coupled dioxygenases, p. 167-214. *In* O. Hayaishi (ed.), Molecular mechanisms of oxygen activation. Academic Press Inc., New York.
2. **Anderson, W. B., and R. C. Nordlie.** 1968. Glucose dehydrogenase activity of yeast glucose 6-phosphate dehydrogenase. I. Selective stimulation of bicarbonate, phosphate, and sulfate. Biochemistry 7:1479-1485.
3. **Barnett, H. L., and V. G. Lilly.** 1955. The effects of humidity, temperature and carbon dioxide on the sporulation of *Choanephora cucurbitarum*. Mycologia 47:26-29.
4. **Bollard, E. G.** 1966. A comparative study of the ability of organic nitrogenous compounds to serve as sole sources of nitrogen for the growth of plants. Plant Soil 25:153-166.
5. **Braverman, M. H.** 1962. Studies in hydroid differentiation. I. Podocoryne carnea, culture methods and carbon dioxide induced sexuality. Exp. Cell Res. 27:301-306.
6. **Charles, H. P.** 1962. Responses of *Neurospora* mutants of carbon dioxide. Nature (London) 195:359-360.
7. **Davis, R. H., and F. J. de Serres.** 1970. Genetic and microbiological research techniques for *Neurospora crassa*, p. 79-143. *In* H. Tabor and C. W. Tabor (ed.), Methods in enzymology, 17A. Academic Press Inc., New York.
8. **Feldman, J. F., and J. P. Thayer.** 1974. Cyclic AMP-induced tyrosinase synthesis in *Neurospora crassa*. Biochem. Biophys. Res. Commun. 61:977-987.
9. **Fink, R. M., and K. Fink.** 1961. Biosynthesis of radioactive RNA and DNA pyrimidines from thymidine 2-^{14}C. Biochem. Biophys. Res. Commun. 6:7-9.
10. **Hanson, B. A., and S. Brody.** 1975. The utilization of purines and other nitrogen compounds *nit* and *ty-1* mutants. Neurospora Newslett. 22:8-9.
11. **Hayaishi, O., M. Nozaki, and M. T. Abbott.** 1975. Oxygenases: dioxygenases, p. 119-189. *In* P. D. Boyer (ed.), The enzymes, vol. 12. Academic Press Inc., New York.
12. **Hirsch, H. M.** 1954. Environmental factors influencing the differentiation of protoperithecia and their relationship to tyrosinase and melanin formation in *Neu-*

rospora crassa. Physiol. Plant. 7:72-97.
13. Horowitz, N. H., and G. W. Beadle. 1943. A microbiological method for the determination of choline by use of a mutant of *Neurospora*. J. Biol. Chem. 150:325-333.
14. Horowitz, N. H., M. Flign, H. L. Macleod, and N. Sueoka. 1960. Genetic determination and enzymatic induction of tyrosinase in *Neurospora*. J. Mol. Biol. 2:96-104.
15. Horowitz, N. H., H. M. Feldman, and M. L. Pall. 1970. Derepression of tyrosinase synthesis in *Neurospora* by cycloheximide, actinomycin D, and puromycin. J. Biol. Chem. 245:2784-2788.
16. Horowitz, N. H., M. Fling, H. Macleod, and N. Sueolsa. 1961. Structural and regulative genes controlling tyrosinase synthesis in *Neurospora*. Cold Spring Harbor Symp. Quant. Biol. 26:233-238.
17. Kuttan, R., G. J. Cardinale, and S. Udenfriend. 1975. An activatable form of prolyl hydroxylase in fibroblast extracts. Biochem. Biophys. Res. Commun. 64:947-954.
18. Liu, C. K., C. A. Hsu, and M. T. Abbott. 1973. Catalysis of three sequential dioxygenase reactions by thymine 7-hydroxylase. Arch. Biochem. Biophys. 159:180-187.
19. Liu, C. K., P. M. Shaffer, R. S. Slaughter, R. P. McCroskey, and M. T. Abbott. 1972. Stoichiometry of the pyrimidine deoxyribonucleoside 2'-hydroxylase reaction and of the conversions of 5-hydroxymethyluracil to 5-formyluracil and of the latter to uracil-5-carboxylic acid. Biochemistry 11:2172-2176.
20. Longmore, W. J., C. M. Niethe, and M. L. McDaniel. 1965. Effect of CO_2 concentration on intracellular pH and on glycogen synthesis from glyceral and glucose in isolated perfused rat liver. J. Biol. Chem. 244:6451-6457.
21. Loomis, W. F. 1957. Sexual differentiation in hydra: control by carbon dioxide tension. Science 126:735-739.
22. McCroskey, R. P., W. R. Griswold, R. L. Sokoloff, E. D. Sevier, S. Lin, C. K. Liu, P. M. Shaffer, R. D. Palmatier, T. S. Parker, and M. T. Abbott. 1971. Studies pertaining to the purification and properties of thymine 7-hydroxylase. Biochim. Biophys. Acta 227:264-277.
23. McDaniel, M. L., and W. J. Longmore. 1971. Activation and inhibition of the activities of bovine liver aconitase. J. Biol. Chem. 246:4818-4824.
24. Nahas, G., and K. E. Schaefer (ed.). 1974. Carbon dioxide and metabolic regulations. Springer-Verlag New York Inc., New York.
25. Nazario, M., and J. L. Reissig. 1964. Induction of aspartic transcarbamylase by carbon dioxide. Biochem. Biophys. Res. Commun. 16:42-46.
26. Palmatier, R. D., R. P. McCroskey, and M. T. Abbott. 1970. The enzymatic conversion of uracil-5-carboxylic acid to uracil and carbon dioxide. J. Biol. Chem. 245:6706-6710.
27. Prival, M. J., and B. Magasanik. 1971. Resistance to catabolic repression of histidase and proline oxidase during nitrogen-limited growth of *Klebsiella aerogenes*. J. Biol. Chem. 246:6288-6296.
28. Pynadath, T. I., and R. M. Fink. 1967. Studies of orotidine 5'-phosphate decarboxylase in *Neurospora crassa*. Arch. Biochem. Biophys. 118:185-189.
29. Reinert, W. R., and G. A. Marzluf. 1975. Regulation of the purine catabolic enzymes in *Neurospora crassa*. Arch. Biochem. Biophys. 166:565-574.
30. Ryan, F. J., G. W. Beadle, and E. L. Tatum. 1943. The tube method of measuring the growth rate of *Neurospora*. Am. J. Bot. 30:784-798.
31. Sanwal, B. P., and M. Lata. 1962. Concurrent regulation of glutamic acid dehydrogenases of *Neurospora*. Arch. Biochem. Biophys. 97:582-588.
32. Scheffler, I. E. 1974. Conditional lethal mutants of Chinese hamster cells: mutants requiring exogenous carbon dioxide on growth. J. Cell. Physiol. 83:219-230.
33. Scholander, P. F. 1947. Analyser for accurate estimation of respiratory gases in one-half cubic centimeter samples. J. Biol. Chem. 167:235-250.
34. Shaffer, P. M., C. A. Hsu, and M. T. Abbott. 1975. Metabolism of pyrimidine deoxyribonucleosides in *Neurospora crassa*. J. Bacteriol. 121:648-655.
35. Subramanian, K. N., and G. J. Sorger. 1972. Regulation of nitrate reductase in *Neurospora crassa*: regulation of transcription and translation. J. Bacteriol. 110:547-533.
36. Westergaard, M., and H. K. Mitchell. 1947. *Neurospora*. V. A synthetic medium favoring sexual reproduction. Am. J. Bot. 34:573-577.
37. Whitney, P. A., and B. Magasanik. 1973. The induction of arginase in *Saccharomyces cerevisiae*. J. Biol. Chem. 248:6197-6202.
38. Williams, L. G., S. Bernhardt, and R. H. Davis. 1970. Copurification of pyrimidine-specific carbamyl phosphate synthetase and aspartate transcarbamylase of *Neurospora crassa*. Biochemistry 9:4329-4325.
39. Williams, L. G., and H. K. Mitchell. 1969. Mutants affecting thymidine metabolism in *Neurospora crassa*. J. Bacteriol. 100:383-389.