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STUDIES PERTAINING TO THE PURIFICATION AND PROPERTIES OF THYMINE 7-HYDROXYLASE

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SUMMARY

1. It has been shown that the thymine 7-hydroxylase activity of cell-free extracts prepared from cultures of *Neurospora crassa*, grown under forced aeration in liquid media, is usually very low or nondetectable. *In vitro* and *in vivo* studies suggest that this lack of activity is a consequence of the Neurospora cells containing less of the hydroxylase when grown under the aerated conditions. Nonaerated conditions of growth have been developed which permit the preparation of highly active extracts from large batches of Neurospora. Thymine 7-hydroxylase has been purified 50-fold from these extracts with a resultant specific activity of 40 units/mg of protein.

2. The purification procedure used yielded preparations of thymine 7-hydroxylase with markedly reduced amounts of the enzymes which catalyze the conversions of thymidine to thymine ribonucleoside and uracil-5-carboxylic acid to uracil and CO_2.

3. These enzyme preparations have been shown to couple the hydroxylation of thymine to the decarboxylation of α-ketoglutarate so that the products of the reaction, *i.e.* 5-hydroxymethyluracil, succinate and CO_2, are produced in a 1:1:1 molar ratio.

INTRODUCTION

In studies with liver slices the methyl group of thymine appeared to undergo an oxidative attack producing 5-hydroxymethyluracil and uracil-5-carboxylic acid[1,2]. 5-Hydroxymethyluracil has also been implicated as an intermediate in the conversion of thymidine to the uracil and cytosine of RNA by mycelial pads of *Neurospora crassa*[3-5]. The cell-free conversion of thymine to 5-hydroxymethyluracil has been demonstrated using enzyme preparations obtained from Neurospora. The thymine 7-hydroxylase activity of these extracts was stimulated by NADPH and O_2, and thus the enzyme appeared to be a mixed function oxidase[6]. Additional studies showed that the requirement of the hydroxylase for NADPH was eliminated when Fe^{2+}, α-ketoglutarate and ascorbate were included in the enzyme incubation mixture[7]. Prior to

the discovery of the requirements for these cofactors it seemed that the growth of Neurospora under conditions of forced aeration[6], in contrast to its pad-like growth on and near the surface of liquid media which was contained in erlenmeyer flasks[6] or Roux bottles[8], was a satisfactory means of obtaining enough of the mold to permit significant purification of the hydroxylase. However, upon the development of an accurate assay for thymine 7-hydroxylase it was revealed that Neurospora which was grown while air was vigorously bubbled through the media contained only a small percentage of the hydroxylase activity of the nonaerated cultures. Experiments are described in this report which have documented this aeration effect and have provided some insight into its nature. As a result, conditions have been developed for the growth of Neurospora in large batches which are a good source of thymine 7-hydroxylase. A purification procedure is described which has facilitated the obtainment of adequate amounts of enzyme of sufficient purity for studying the role of α-ketoglutarate in the hydroxylase reaction. The purification procedure has also resulted in the obtainment of preparations of thymine 7-hydroxylase which have much reduced amounts of the enzymes responsible for the 2′-hydroxylation of thymidine[9] and the decarboxylation of uracil-5-carboxylic acid[10], *i.e.* the first and last enzymes of the pathway which appears to effect the oxidative demethylation of thymidine.

MATERIALS AND METHODS

Source of materials

[2-^{14}C]Thymine (Schwarz Bioresearch, Inc.) and [6-^{3}H]thymine (Amersham/Searle) were each mixed with amounts of nonradioactive thymine to effect specific activities of 3.0 and 6.2 mC/mmole, respectively. [2-^{14}C]Thymidine (Calbiochem) was combined with non-radioactive thymidine to yield a specific activity of 3.0 mC/mmole. Radioactive α-ketoglutarate was purified by chromatography on columns of silicic acid as described by PRIOR FERRAZ AND RELVÂS[11]. [5-^{14}C]α-Ketoglutarate (Amersham/Searle) and [1-^{14}C]α-ketoglutarate (Calbiochem) were adjusted before use to specific activities of 3.0 and 0.50 mC/mmole, respectively. Aged calcium phosphate gel, thymine, 5-hydroxymethyluracil, thymidine and thymine ribonucleoside were obtained from Calbiochem. Silicic acid, 100 mesh, was obtained from Mallinckrodt. Glass pavement marking beads (3M Company) were acid washed and thoroughly rinsed with water before use. Chymotrypsinogen A, ovalbumin and ribonuclease A were supplied by Worthington Biochemical Corp. The Sigma Chemical Co. provided α-ketoglutarate, GSH, ascorbate, succinate and other organic chemicals. Nuclear Chicago solubilizer reagent, which contains a quaternary ammonium base, was purchased from Amersham/Searle Corp.

Growth of N. crassa

Inoculation cultures of *N. crassa* strain 1A (wild type) were grown for 5 days at 28° in 125-ml erlenmeyer flasks on 50 ml of minimal media[12] which contained 2% agar. A 100-ml suspension of conidia in water was prepared from each flask, and 50 ml of the conidia suspension were added to a 10-l carboy which contained 5 l of the liquid minimal media. The carboy was closed with a rubber stopper. A J-shaped glass stirring paddle extended through a greased glass sleeve which was inserted into the stopper. Neurospora grown under these conditions is referred to, in this report, as a

nonaerated culture. Neurospora designated as an aerated culture was grown in the same manner except that the rubber stopper contained two additional holes which allowed cotton filtered air to enter and leave. The air was introduced, at a rate of 5 l/min, through a glass tube which extended to the bottom of the carboy. To prevent dehydration of the media, sterile water was added during the growth period. Both nonaerated and aerated cultures were stirred at 310 rev./min and grown for 3 days at 28°.

Enzyme purification

At the time of completion of the 3-day growth period, the nonaerated cultures of Neurospora were harvested by filtration through cheesecloth. Unless indicated otherwise, all steps in the collection and rinsing of the Neurospora mycelia and the subsequent enzyme purification procedures were performed at 0–4°. The mycelia obtained from two carboys were suspended in 1 l of buffer (0.05 M Tris–HCl, pH 8.0) which contained 0.1 mM EDTA, 1 mM ascorbate and 1 mM GSH. The mycelia were again collected by filtration through cheesecloth which was tightened around the Neurospora to squeeze out excess buffer. The Neurospora was subjected to this rinsing and filtration process two more times, and a yield of approx. 60 g, moist weight (7 g, dry weight), was obtained. After spreading the Neurospora into a thin pad it was frozen with dry ice. Usually the mycelia from 12 carboys were harvested together, and the frozen pads were either used immediately as a source of the hydroxylase or stored for as long as 11 weeks at −30° before being homogenized.

To obtain the crude extract the frozen Neurospora was broken into small pieces, combined with 120 g of glass beads and 150 ml of the Tris buffer which contained EDTA, ascorbate and GSH. The mixture was placed in the stainless steel chamber (400 ml capacity) of a Servall Omni-Mixer, and homogenized for 2 min at 7000 rev./min with the chamber immersed in ice. At the completion of this 2-min period, the homogenate was cooled by placing the chamber in a mixture of brine and ice and reducing the speed to 1000 rev./min for 10 sec; then two additional 2-min periods of homogenizing at 7000 rev./min were carried out with the chamber immersed in brine and ice. The homogenate was centrifuged at 8500 × *g* for 1 h. The protein concentration of the resulting crude extract was usually found to be in the range of from 7 to 10 mg per ml as determined with the Folin–Ciocalteu reagent. The extract was made 1 mM in EDTA, stirred for 15 min at 0° and then fractionated utilizing calcium phosphate gel[8].

The calcium phosphate gel was prepared by centrifuging a slurry of calcium phosphate at 8500 × *g* for 15 min (in a centrifuge bottle, Servall catalogue No. 259, suitable for use as a chamber for the Servall Omni-Mixer). The crude extract was added to the resulting pellet so that the ratio of dry wt. of gel to protein was generally 2.5:1. The pellet was dispersed in the extract with a stirring rod and the resulting mixture was blended with the Omni-Mixer at 8000 rev./min for 5 sec and then at 800 rev./min for 5 sec and again at 8000 rev./min for 5 sec. The centrifuge bottle was immersed in an ice–brine mixture during the blending period. At the completion of this procedure, the mixture was further equilibrated by slowly stirring it for an additional 10 min at 0°. A calcium phosphate gel supernatant fraction was isolated after centrifuging the mixture at 8500 × *g* for 15 min. The supernatant fraction was adjusted to pH 6.5 with dilute HCl and added to a second calcium phosphate gel pellet,

prepared as above so that a gel to protein ratio of 5:1 was established. This mixture was blended and equilibrated as described for the first pellet. It was necessary to do preliminary "pilot" fractionations to determine optimal gel to protein ratios and pH adjustments since they varied with the batch of gel employed. The calcium phosphate gel and the protein adsorbed to it were separated from the solution by centrifugation at 8500 × *g* for 15 min. The resulting pellet was rinsed with 150 ml of water and then twice more with 55-ml portions of 0.1 M sodium phosphate buffer (pH 8.0). Each of the three rinse solutions was blended with this pellet using the Omni-Mixer as described above. The two eluants obtained with phosphate buffer were combined and are referred to as the calcium phosphate gel eluant. To concentrate the hydroxylase activity a saturated solution of ammonium sulfate was rapidly stirred into this eluant until it was 75% saturated with ammonium sulfate, and the resultant precipitate was immediately removed by centrifugation at 8500 × *g* for 10 min. The precipitate was dissolved in a volume of buffer (0.05 M Tris–HCl, pH 8.0, which contained 1 mM GSH, 1 mM ascorbate, and 10 mM KCl) so that the protein concentration was 10–15 mg/ml. 20 ml of this solution was applied to a Sephadex G-150 column (2.5 cm × 87 cm) and was eluted with 0.05 M Tris–HCl (pH 8.0), which was 1 mM in GSH, 0.1 mM in ascorbate, and 0.5 mM in EDTA, at 19 ml per h. 10-ml fractions were collected and monitored both with respect to ultraviolet absorption (280 nm) and thymine 7-hydroxylase activity. The fractions containing the hydroxylase activity were combined and are referred to as the Sephadex G-150 enzyme preparation. This fraction was concentrated with ammonium sulfate, as described above, so that a protein concentration of 10 mg/ml was established. A 2.0-ml sample of this concentrated fraction was applied to a Sephadex G-100, superfine, column (2.5 cm × 41 cm). This column was eluted at a rate of 8.5 ml per h and monitored in the manner described for the Sephadex G-150 column. In preliminary experiments, Sephadex G-100 columns were calibrated with standard protein solutions of chymotrypsinogen A, ovalbumin and ribonuclease A to determine, for each protein, the ratio of its elution volume to the void volume of the column. Using these ratios and the corresponding molecular weights of the proteins, a graph was constructed so that the apparent molecular weight of the thymine 7-hydroxylase could be obtained by interpolation[13].

Assay procedures

A 0.1-ml aliquot of the enzyme preparation was pipetted into a 10 mm × 75 mm test tube which contained the substrate and cofactors in 0.125 ml of 0.04 M Tris–HCl (pH 8.0). The resultant standard incubation mixture was 0.22 mM in [2-^{14}C]-thymine, 0.5 mM in α-ketoglutarate, 1.0 mM in ascorbate, 0.5 mM in $FeSO_4$ and 1 mM in GSH. These values listed for the ascorbate and GSH concentrations in the standard incubation mixture do not take into account the amount of these compounds which were added to enzyme preparations during the purification procedure.

After the addition of the enzyme preparation, the uncapped test tube was placed in a Dubnoff incubator and shaken at 30°. The reaction was stopped by heating it to 100° for 3 min. Following removal of the coagulated protein, 16 μg each of non-radioactive thymine and of 5-hydroxymethyluracil were added as chromatographic markers to a 40-μl aliquot of the supernatant fluid which was then chromatographed in two dimensions[2]. The solvent employed for both dimensions was ethyl acetate–

formic acid–water (70:20:10, by vol.)[14]. A thin window Geiger tube was used for measuring the radioactivity of the chromatographically separated thymine and hydroxymethyluracil. Duplicate assays of a given enzyme preparation usually agreed within ± 10% of the mean. The position of radioactive compounds was determined by radioautography[6].

For measuring the specific activity of an enzyme preparation, the protein concentration of the incubation mixture and the time of incubation were varied to determine conditions under which the rate of hydroxylation was linear and proportional to the enzyme concentration. This rate was always determined at a time in the incubation period when the amount of thymine hydroxylated did not exceed 10%. The other incubation conditions were those described above for the standard incubation mixture. A unit of thymine 7-hydroxylase is defined as the amount of enzyme which catalyzes the hydroxylation of 1 nmole of thymine per min.

The standard incubation conditions were also used to assay enzyme preparations for their ability to catalyze the conversion of thymidine to thymine ribonucleoside[9] except that thymidine, at an initial concentration of 0.25 mM, was substituted for the thymine in the reaction mixture. In the manner indicated above the protein concentration and the time of incubation were selected so that the initial rate of thymine ribonucleoside formation was proportional to the amount of enzyme present in the incubation mixture. Similarly, a unit of the enzyme effecting the 2′-hydroxylation of thymidine is defined as the amount of enzyme which catalyzes the hydroxylation of 1 nmole of thymidine per min under the standard assay conditions.

To determine the stoichiometry of succinate formation in the thymine 7-hydroxylase reaction, standard incubation mixtures were used which contained [6-^{3}H]-thymine and [5-^{14}C]α-ketoglutarate. At the completion of the incubation period, protein was removed from the incubation mixture by the addition of 0.9 ml of absolute ethanol and centrifugation. An aliquot of the incubation mixture was subjected to chromatography, as described above, to determine the extent to which thymine was converted to 5-hydroxymethyluracil. α-Ketoglutarate does not separate well in this solvent system and so additional chromatography was required. Two-dimensional chromatography was carried out using the *n*-butanol–glacial acetic acid–water solvent (50:25:25, by vol.)[14] on another aliquot of the reaction mixture to which α-ketoglutarate (R_F 0.44) and succinate (R_F 0.79) had been added as chromatographic markers. This solvent system yielded chromatograms on which the location of 5-hydroxymethyluracil (R_F 0.48) overlapped that of α-ketoglutarate, but the radioactivity measurements distinguished between the ^{3}H and ^{14}C labels in these compounds. Radioautography[6] was used to determine the location of the radioactive α-ketoglutarate and succinate on the developed chromatograms. The portions of the two sets of developed chromatograms, which contained thymine, 5-hydroxymethyluracil, α-ketoglutarate and succinate, were each cut out, shaken with 3 ml water after which 14 ml of a dioxane-scintillation solution[4] were added. The mixture was shaken and subjected to simultaneous measurements of ^{14}C and ^{3}H using a Packard Tri-carb scintillation counter as previously described[9]. Identical chromatograms, which were not used for measurements made with the scintillation counter, were sprayed with "aniline–glucose solution," No. 1 (ref. 15), for the detection of succinate or with the "*p*-dimethylaminobenzaldehyde–acetic anhydride solution", No. 2 (ref. 15), for the detection of α-ketoglutarate. These chromatograms were aligned with

the corresponding radioautograms to determine if the authentic marker compounds coincided with the radioactive compounds.

The determination of the stoichiometry of CO_2 production involved the same type of incubations being carried out as did the succinate determinations except [1-^{14}C]α-ketoglutarate was utilized. The 10 mm × 75 mm test tube which contained the standard incubation mixture was sealed in a 12-ml centrifuge tube which contained a 1 cm × 4 cm rectangle of Whatman 3 MM filter paper, saturated with NCS reagent. The details of this procedure for trapping CO_2 and the subsequent determination of its radioactivity with the use of a scintillation counter have been previously described[10].

Control studies included deproteinization at zero time and replacement of the enzyme preparation with the incubator buffer. In other standard incubation mixtures either thymine or α-ketoglutarate was omitted.

The effect of the pH of the incubation mixture on the thymine 7-hydroxylase activity was studied using a calcium phosphate gel eluant preparation which had been prepared and concentrated (5 mg of protein per ml) with ammonium sulfate by older procedures[8]. An aliquot of the enzyme preparation was adjusted to the desired pH with dilute HCl or NaOH, and then a 0.1-ml portion of it was added to the incubation mixture in which was the appropriate buffer containing the cofactors and substrate. Incubations were carried out for 10 min, at pH values ranging from 5.5 to 8.0 using 0.05 M Tris–maleate buffer, from 8.0 to 9.0 using 0.05 M Tris–HCl and from 8.0 to 10.0 using 0.05 M glycine–NaOH buffer.

Silicic acid column chromatography

To further establish the identity of the enzymatic product of [5-^{14}C]α-ketoglutarate a 0.1-ml aliquot of a standard incubation mixture, which had been incubated for 9 min, was chromatographed on a column of silicic acid. The column, 14.5 mm × 130 mm, was prepared from 8 g of silicic acid as described by PRIOR FERRAZ AND RELVÂS[11] and eluted with benzene–*tert.*-butanol (9:1, by vol.) according to LINDSTEDT *et al.*[16]. Before chromatography 5 mg of α-ketoglutarate and 3 mg of succinate were added to the radioactive enzymatic products. A 0.2-ml aliquot of each fraction was dried in a sample vial and subjected to radioactivity measurements in a scintillation counter as described above. The remainder of each fraction was titrated with 0.02 M NaOH.

Comparison of nonaerated and aerated cultures

The procedure described above for the preparation of the crude extract was carried out on simultaneously grown nonaerated and aerated cultures. The homogenizing procedure was modified so that smaller amounts of Neurospora could be subjected to it. The Neurospora was frozen into a thin pad of 15 g moist weight (approx. 1.8 g dry weight) and was combined with 37 ml of buffer, and 30 g of glass beads. The mixture was then placed in a steel chamber of 100 ml capacity. As described above, the Servall Omni-mixer was used to homogenize the cells but the grinding was usually carried out at 5600 rev./min for 12 min (six 2-min grinding periods). Precautions were taken to insure that the procedures utilized for the preparation and assaying of the crude extracts, obtained from the two types of cultures, were identical.

To determine if the aerated conditions of growth affected the conversion of

thymidine to the pyrimidines of RNA, [2-^{14}C]thymidine was incubated with cultures of *N. crassa*, strain 1A, in cotton stoppered 50-ml Erlenmeyer flasks[4] (the nonaerated cultures) and with other cultures under identical conditions except for the passage of fresh, cotton filtered air over the media during the growth period. The Neurospora obtained from these aerated flasks are referred to as aerated cultures. During the growth period the media of both the aerated and nonaerated flasks was stirred at the same rate, usually about 300 rev./min, with a magnetic stirring bar. Each culture contained 1 drop of conidial suspension and 20 ml of minimal media[12], which was 50 μM in [2-^{14}C]thymidine. After 4 days of incubation under the nonaerated or aerated conditions each mycelial pad was extracted with 80% ethanol and with acetone, dried in a vacuum and incubated with 1 ml of 1 M piperidine for each 8 mg dry weight of Neurospora[4]. The nucleotides in the extract were separated by paper chromatography[4] and then the radioactivity was measured with a thin-window Geiger tube.

RESULTS

Purification of thymine 7-hydroxylase

The growth of Neurospora in carboys under nonaerated conditions has much facilitated the obtainment of amounts of mycelia which are suitable as a source of thymine 7-hydroxylase. The Neurospora contained in 12 carboys (60 l of liquid media) was usually harvested together. After 3 days of growth about 0.7 g dry weight of Neurospora per l media was usually obtained, and this mold contained about 200 units of thymine 7-hydroxylase per g dry weight of mycelia. While the 4 days of growth in Roux bottles[8] usually yielded more Neurospora, about 1.7 g dry weight per l of media, this mold contained less of the hydroxylase, about 40 units per g dry weight. Therefore, an unwieldy number of Roux bottles, approx. 800, would be required for the production of the amount of hydroxylase usually yielded from the growth of 12 carboys. The thymine 7-hydroxylase activity of the frozen Neurospora pads, prepared from the harvested Neurospora, was quite stable; no loss of activity was detected upon storage for as long as 11 weeks. The fragility of the frozen, thin pads and their aid in the maintenance of a low temperature during the homogenizing procedure expedited the preparation of the crude extract from relatively large amounts of Neurospora.

The thymine 7-hydroxylase activity of this extract was typically purified as shown in Table I. It can be seen that about half of the activity was lost in the calcium phosphate gel purification procedure. By adjusting the calcium phosphate gel to protein ratios, yields of from 90 to 100% were obtained, but these increases in yields were achieved at the expense of the specific activity of the resultant preparations. The concentration of enzyme preparations using ammonium sulfate brought about the loss of most of the activity if the procedure were not carried out as rapidly as possible. The use of this procedure to concentrate the activity of the calcium phosphate gel eluant resulted in the recovery of most of the activity in a majority of experiments. However, the concentration of the Sephadex G-150 enzyme preparation using ammonium sulfate was less satisfactory. Usually a 40% loss of activity occurred in this step, although no, or only a small reduction of the specific activity was observed. The concentration of the Sephadex G-150 enzyme preparation by ultra-

TABLE I

PURIFICATION OF THYMINE 7-HYDROXYLASE

Fraction	*Vol. (ml)*	*Protein (mg/ml)*	*Total activity (units)*	*Specific activity (units/mg)*	*Yield (%)*	*Purification (-fold)*
1. Crude extract	300	9.2	2160	0.78	100	
2. $Ca_3(PO_4)_2$ gel supernatant	300	4.6	2370	1.7	110	2.2
3. $Ca_3(PO_4)_2$ gel eluant	210	1.9	1260	3.2	59	4.1
4. $(NH_4)_2SO_4$	22	13.8	810	2.7	38	3.5
5. Sephadex G-150	55	2.2	560	4.6	26	5.9
6. $(NH_4)_2SO_4$	3.4	19.0	286	4.4	13	5.6
7. Sephadex G-100	8.4	0.4	240*	42	11*	54

* Value adjusted to take into account that only 2.0 of the 3.4 ml of Fraction 7 was subjected to Sephadex G-100 chromatography.

filtration (Amicon) did not result in a higher recovery of activity. The Sephadex G-150 purification procedure (Fig. 1) usually yielded enzyme preparations with specific activities of from 4 to 12 units/mg. Enzyme preparations with specific activities in the range of 20–40 units/mg were usually obtained from the Sephadex G-100 procedure.

Thymine 7-hydroxylase and the enzymes catalyzing the conversions of thymidine to thymine ribonucleoside[9], 5-hydroxymethyluracil to 5-formyluracil[8] and the latter compound to uracil-5-carboxylic acid[17] were eluted from the Sephadex G-150 column in the same fractions. However, the thymine 7-hydroxylase activity was markedly freed of the activity responsible for the hydroxylation of thymidine

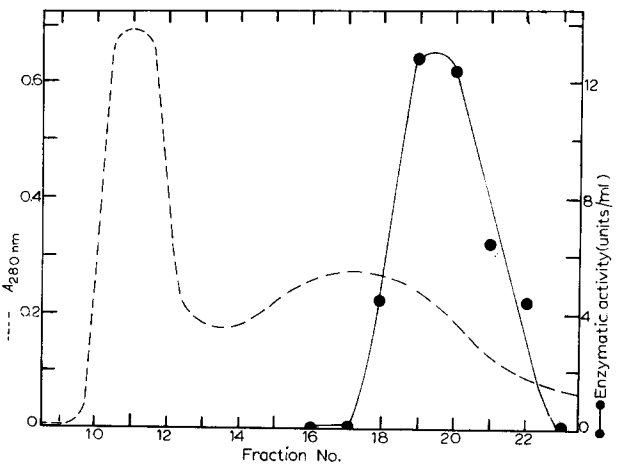


Fig. 1. Elution pattern of thymine 7-hydroxylase activity on a Sephadex G-150 chromatography column (2.5 cm × 87 cm) equilibrated with 0.05 M Tris–HCl (pH 8.0) which was 1 mM with respect to GSH, 0.1 mM with respect to ascorbate, and 0.05 mM with respect to EDTA. The concentrated calcium phosphate gel eluant which was applied to the column had a volume of 20 ml and a protein concentration of 13 mg/ml. The flow rate was 19 ml per h, and 10-ml fractions were collected.

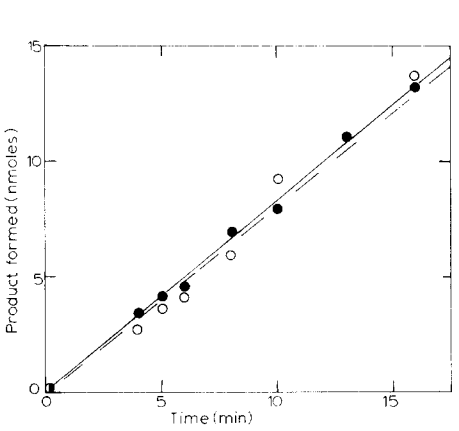
TABLE II

SEPARATION OF THYMINE 7-HYDROXYLASE ACTIVITY FROM THAT CATALYZING THE CONVERSION OF THYMIDINE TO THYMINE RIBONUCLEOSIDE

The Sephadex G-150 enzyme preparation which had been concentrated by precipitation with ammonium sulfate (8.5 mg protein per ml), was assayed for the two enzymatic activities, as described in MATERIALS AND METHODS, before and after being frozen at −79° and then stored at −30° for 8 days.

	Hydroxylase substrate	*Total activity (units)*	*Specific activity (units/mg)*
Before freezing	Thymine	115	1.8
	Thymidine	190	3.0
After freezing	Thymine	110	1.7
	Thymidine	15	0.2

by storage, in the frozen state, of either the Sephadex G-150 enzyme preparation or this preparation after it had been concentrated with ammonium sulfate (Table II). Both of these enzymatic activities in the calcium phosphate gel eluant can be frozen and stored for months with no detectable loss. It has previously been shown[10] that the calcium phosphate gel fractionation procedure yields thymine 7-hydroxylase preparations which contain no uracil-5-carboxylic acid decarboxylase activity. Preliminary results indicate that the Sephadex G-100 chromatography procedure does not further separate thymine 7-hydroxylase from the other enzymes mentioned



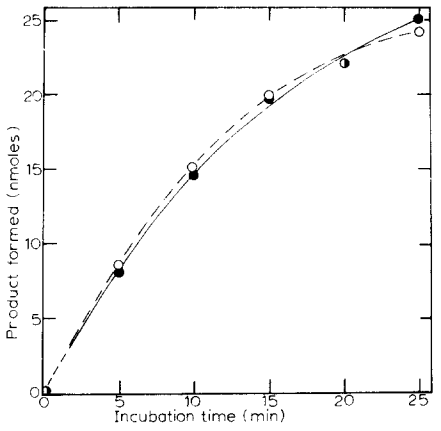


Fig. 2. The relationship of the conversion of α-ketoglutarate to succinate to that of thymine to 5-hydroxymethyluracil. The standard incubation mixture contained [6-^{3}H]thymine, [5-^{14}C]α-ketoglutarate and a Sephadex G-150 enzyme preparation which had been concentrated with ammonium sulfate (2.7 mg of protein per ml of incubation mixture). The amounts of 5-hydroxymethyluracil (●—●) and succinate (○— —○) formed were determined with the use of a scintillation counter as described in MATERIALS AND METHODS.

Fig. 3. The relationship of the conversion of α-ketoglutarate to CO_2 to that of thymine to 5-hydroxymethyluracil. The conditions of incubation are the same as described in Fig. 2, except that [1-^{14}C]α-ketoglutarate was used and the protein concentration of the incubation mixture was 1.6 mg/ml. ●—●, 5-hydroxymethyluracil; ○— —○, CO_2.

above. In other preliminary work involving the Sephadex G-100 chromatography procedure, the apparent molecular weight of thymine 7-hydroxylase was estimated to be 30 000.

The conversion of α-ketoglutarate to succinate and CO_2

The Sephadex G-150 enzyme preparation proved to be suitable for the study of the role of α-ketoglutarate in the thymine 7-hydroxylase reaction. In contrast to the calcium phosphate gel eluant, the Sephadex G-150 enzyme preparations catalyzed little or no degradation of α-ketoglutarate in the absence of exogenous thymine, *e.g.* under the conditions given in Fig. 2, after 30 min of incubation 1% of the α-ketoglutarate was decarboxylated in incubation mixtures to which no thymine had been added. The enzyme described in Fig. 2 did convert the 5-hydroxymethyluracil formed to 5-formyluracil, but after 30 min of incubation this conversion represented 3% of the initial thymine concentration. No uracil-5-carboxylic acid was detected after this incubation period. As previously mentioned[17] a stoichiometric relationship exists between the decarboxylation of α-ketoglutarate and the hydroxylation of thymine. Figs. 2 and 3 show that 5-hydroxymethyluracil, succinic acid and CO_2 are produced in a 1:1:1 molar ratio when thymine and α-ketoglutaric acid are incubated under the standard incubation conditions. Succinic acid was originally identified as the enzymatic product of [5-^{14}C]α-ketoglutaric acid with two-dimensional chromatography using the *n*-butanol–glacial acetic acid–water solvent system. Radioautography revealed that the location of the radioactive, enzymatic product exactly coincided with that of the authentic succinate used as a chromatographic marker. Fig. 4 shows that the enzymatic product and authentic succinic acid were eluted simultaneously from a silicic acid chromatography column.

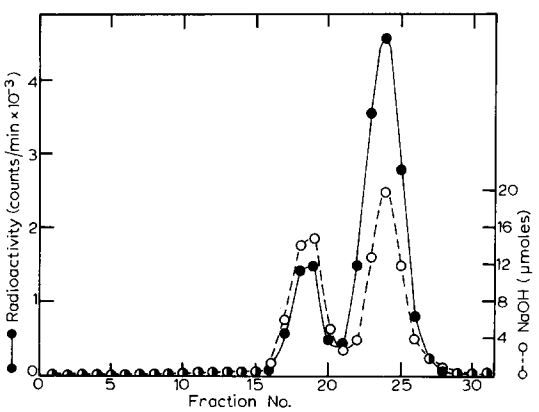


Fig. 4. Identification of enzymic product of α-ketoglutarate using silicic acid column chromatography. The conditions of incubation were the same as those described in Fig. 2, except that the thymine used as substrate was not radioactive and the protein concentration of the incubation mixture was 1.6 mg/ml. The 14.5 mm × 130 mm chromatography column of silicic acid was eluted with benzene–*tert.*-butanol (9:1, by vol.) in 2-ml fractions. The radioactivity of 0.2-ml aliquots of each fraction was measured with a scintillation counter, and the remainder of each fraction was titrated with 0.02 M NaOH. See MATERIALS AND METHODS for further details. The first peak eluted from the column contained the succinic acid.

TABLE III

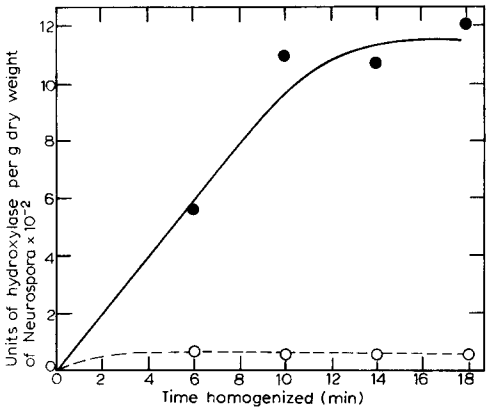
COMPARISON OF THE THYMINE 7-HYDROXYLASE ACTIVITY OF CRUDE EXTRACTS PREPARED FROM NONAERATED AND AERATED CULTURES OF NEUROSPORA

Crude extracts were prepared, as described in MATERIALS AND METHODS, from nonaerated and simultaneously grown, aerated cultures of Neurospora. In the preparation of the extracts, each Neurospora pad was homogenized for a 12-min period.

Culture conditions	*Vol. of extract (ml)*	*Protein concn. of extract (mg/ml)*	*Specific activity (units/mg)*	*Neurospora extracted (dry wt.) (g)*
Nonaerated	35.0	10.6	0.92	1.6
Aerated	35.0	7.1	0	1.3

The effect of aeration on intact mycelial cells in regard to thymine 7-hydroxylase activity

The unsuccessful attempts to use aerobically grown Neurospora (usually about 4 g dry weight of Neurospora were obtained per l of media) as a source of thymine 7-hydroxylase led to the hypothesis that some modification of the atmosphere above the growing culture might produce conditions of growth resulting in higher yields of the hydroxylase. Thus, cultures of Neurospora were grown, with stirring, in carboys which were stoppered to prevent the exchange of the atmosphere above the media with the air outside of the carboys. Table III compares the activity of a typical extract, which was obtained from one of these nonaerated cultures, with that of an extract from an aerated culture which was grown at the same time. Approx. 10% of the aerated cultures have yielded extracts in which considerable activity was observed, but in these cases the corresponding (control) nonaerated cultures contained dramatically more enzyme (Fig. 5). It seemed possible that the much lower hydroxylase activity observed in extracts obtained from aerated cultures might result from the enzyme being more difficult to solubilize or less stable to the grinding procedure. Fig. 5 shows that continuing the time of grinding did not reduce the difference in the activity of the extracts obtained from the two types of cultures and that the activity of the extract from the aerated culture was as stable to the homogenizing procedure as was that from the nonaerated cultures. Using the SCHOLANDER[18] technique measurements were made of the O_2 and CO_2 content of the atmosphere above the growing cultures. The composition of the air above the aerated cultures did not measurably change, because of the rapid rate of air flow. In contrast the atmosphere above the nonaerated cultures, after 72 h of growth, usually contained about 40% CO_2 and 5% O_2. This higher CO_2 content of the atmosphere of nonaerated cultures did not result in their yielding extracts with a lower pH. However, the pH of extracts obtained from both aerated and nonaerated cultures did range from 7.0 to 8.0. This variation in pH was regarded as having little effect on the enzymatic assay on the basis of studies which had been carried out using the concentrated calcium phosphate gel eluant (prepared by the older procedures[8]) to determine the effect of the pH of the incubation mixture on thymine 7-hydroxylase activity (Fig. 6). To investigate the possibility that the cultures grown under aerated conditions contained an inhibitor of the hydroxylase, the crude extract from these cultures was subjected to Sephadex G-25 chromatography as previously described[7]. No stimulation of enzymatic activity was observed as a result of this procedure. The extracts obtained from aerated and



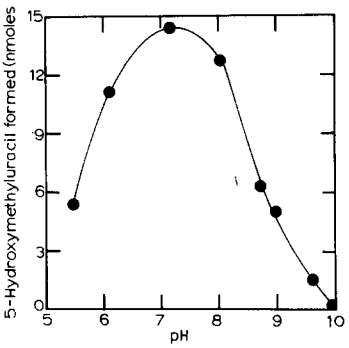


Fig. 5. Effect of the length of the homogenizing period on the thymine 7-hydroxylase activity of extracts obtained from nonaerated cultures. Neurospora from nonaerated (●—●) and simultaneously grown, aerated (○— —○) cultures was homogenized with a Serval Omni-mixer as described in MATERIALS AND METHODS. During the grinding period, aliquots of the homogenates were removed, centrifuged and assayed in the standard incubation mixture. After 18 min of grinding the specific activities of the extracts prepared from the nonaerated and aerated cultures were 6.7 and 0.4.

Fig. 6. Effect of the pH of the incubation mixture on the hydroxylation of thymine. The enzyme preparation (4.6 mg of protein per ml) was adjusted to a given pH and was incubated with an appropriate buffer in the otherwise standard incubation mixture. See MATERIALS AND METHODS for details.

nonaerated cultures were also combined in various ratios and assayed in the standard incubation mixture. Even when the standard incubation mixture contained 8 times, by vol., as much of the extract obtained from the aerated culture as it did of that obtained from the nonaerated culture, no inhibitory effect was observed. Since the incorporation of thymidine into the pyrimidines of RNA would appear to be dependent on the presence of thymine 7-hydroxylase, experiments were run to determine if the aerated conditions of growth interferred with this conversion. In all the comparisons made, thymidine was incorporated into the RNA of nonaerated cultures from 1.2 to 4.7 times as much as it was into the RNA of aerated cultures. The data in Table IV are typical of the results obtained.

TABLE IV

THE EFFECT OF AERATION ON THE INCORPORATION OF [2-^{14}C]THYMIDINE INTO THE PYRIMIDINES OF RNA OF *N. crassa*, STRAIN 1A

Nonaerated and aerated cultures of Neurospora were grown in the presence of 50 μM [2-^{14}C]thymidine in 50-ml erlenmeyer flasks at 28°. After 4 days of incubation the nucleotides of RNA were extracted with 1 M piperidine, chromatographed and their radioactivity was measured as described in MATERIALS AND METHODS.

RNA nucleotides	*[2-^{14}C]Thymidine incorporation (μmoles/mg dry wt. of Neurospora $\times 10^{-4}$)*		
	(A) Nonaerated	*(B) Aerated*	*A/B*
UMP	16.4	7.8	2.1
CMP	17.7	8.6	2.1

DISCUSSION

The low levels of the thymine 7-hydroxylase activity detected in aerated cultures of Neurospora as compared to that of nonaerated cultures is probably not attributable to such factors as the presence of an inhibitor which is peculiar to aerated cultures or to the hydroxylase in these cultures being more labile to the procedure used for homogenizing the cells. This effect of aeration appeared to be reflected in the results of studies measuring the conversion of thymidine to the pyrimidines of RNA. However, this *in vivo* effect might involve phenomena such as active transport[19] rather than the level of thymine 7-hydroxylase activity. It should be noted that the effect of aeration on the other enzymes thought to be involved in the demethylation of thymidine has not been studied.

It appeared to be a possibility that more than one of the reactions involved in the demethylation of thymidine and which require α-ketoglutarate might be catalyzed by the same enzyme. The obtainment of enzyme preparations which have lost most of their capacity to hydroxylate thymidine but not thymine argues for two distinct enzymes catalyzing these reactions. These partially purified enzyme preparations have also lost their uracil-5-carboxylic acid decarboxylase activity[10]. Moreover, it appears that the enzymes catalyzing the conversions of 5-hydroxymethyluracil to 5-formyluracil[8] and the latter to uracil-5-carboxylic acid[17] are distinct on the basis that the *UC*-3 mutant of Neurospora, developed by WILLIAMS AND MITCHELL[5], is able to utilize 5-formyluracil but not 5-hydroxymethyluracil as a pyrimidine source.

The demonstration of the stoichiometric conversion of α-ketoglutarate to succinate and CO_2 is consistent with a mechanism[20] which appears to be applicable to a group of hydroxylases which require α-ketoglutarate and O_2 (refs. 7, 9, 21–24) and, possibly, to the enzymatic conversions of 5-hydroxymethyluracil to 5-formyluracil and the latter to uracil-5-carboxylic acid which are also reactions requiring α-ketoglutarate and O_2 (refs. 8, 17). The mechanism involves a peroxide anion of the substrate making a nucleophilic attack on the α carbon of α-ketoglutarate. This hypothesis was based on the findings that a stoichiometric relationship exists between the decarboxylation of α-ketoglutarate and the hydroxylation of the substrates of collagen proline hydroxylase[25] and γ-butyrobetaine hydroxylase[16], that succinic semialdehyde is not an intermediate in the γ-butyrobetaine hydroxylase reaction[26] and that in this reaction molecular oxygen is incorporated into succinate[20]. While this manuscript was in preparation, HOLME *et al.*[27] reported that, in the thymine 7-hydroxylase reaction, succinate had been identified as a product and that there is a stoichiometric relationship between the formation of 5-hydroxymethyluracil and CO_2.

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