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Enzymatic Conversion of Norsolorinic Acid to Averufin in Aflatoxin Biosynthesis

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5'-Hydroxyaverantin (HAVN) was isolated from a mold, *Emericella heterothallica* IFO 30842. *Aspergillus parasiticus* NIAH-26, a UV-irradiated mutant of *A. parasiticus* SYS-4, produced neither aflatoxins nor precursors in yeast extract-sucrose (YES) medium. When the postmicrosome (cytosol) fraction of NIAH-26, which had been prepared from the culture in YES medium, was incubated with norsolorinic acid (NA) in the presence of NADH or NADPH, averantin (AVN) was produced. The reverse reaction from AVN to NA was promoted by the addition of NAD or NADP (dehydrogenase reaction). When the microsome fraction of NIAH-26 was incubated with AVN, HAVN was produced in the presence of NADPH (monooxygenase reaction). HAVN was, furthermore, oxidized to averufin (AVR) by the cytosol fraction of NIAH-26 in the presence of NAD or NADP (dehydrogenase reaction). In the feeding experiments with *A. parasiticus* NIAH-26, aflatoxins were produced from AVN, HAVN, NA, and AVR but not from averufanin or averythrin. These results indicate that the reaction sequence NA↔AVN→HAVN→AVR is involved in the biosynthetic pathway of aflatoxins. The enzyme activities described here were dependent on the culture medium, and no enzyme activities were observed in the nonaflatoxigenic strain *A. oryzae* SYS-2 (IFO 4251).

Aflatoxins are toxic secondary metabolites produced by certain strains of the common molds *Aspergillus flavus* and *A. parasiticus* (2). The biosynthetic pathway of aflatoxins has been studied extensively (2, 9), and the general steps are acetate→anthraquinones→xanthones→aflatoxins. The involvement of anthraquinones such as norsolorinic acid (NA) (13, 14), averantin (AVN) (3), and averufin (AVR) (8, 15, 20) in the biosynthesis were indicated by experiments with blocked mutants and isotopically labeled precursors, and the biosynthetic sequence NA→AVN→AVR is generally accepted (2, 9). Recently, an NA dehydrogenase activity converting NA to AVN has been identified and purified to homogeneity (6). However, the relationship between AVN and AVR has not been enzymatically clarified. On the other hand, averufanin (AVF) (1, 12) was recently reported to be a precursor of aflatoxin B_1 (AFB_1) in the pathway between AVN and AVR (17). Also, averythrin (AVT) (18), another anthraquinone, could appear to be involved in aflatoxin biosynthesis, because the structure of this substance has very similar to that of NA and AVN.

In this study, we isolated a new anthraquinone, 5'-hydroxyaverantin (HAVN), from a mold, *Emericella heterothallica* IFO 30842. The biosynthetic relationship among HAVN, NA, AVN, AVR, AVF, AVT, and aflatoxins was investigated by using cell-free systems and feeding experiments. In the cell-free experiments, microsome and cytosol (postmicrosome) fractions of *A. parasiticus* NIAH-26 were prepared and then examined to analyze the characteristics of each enzyme activity in detail. Also, the dependency of these enzyme activities on the culture medium and the kind of mold was investigated.

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MATERIALS AND METHODS

Microorganisms. *A. parasiticus* NIAH-26, a UV-irradiated mutant of *A. parasiticus* SYS-4 (NRRL-2999), was mainly used. NIAH-26 produced neither aflatoxins nor precursors (24). A wild strain, *A. oryzae* SYS-2 (IFO 4251), was also used, as it produces neither aflatoxins nor their precursors. *E. heterothallica* IFO 30842 was used for the preparation of HAVN. Conidia were freshly prepared by inoculating conidia on malt extract agar medium at 28°C for 1 week and then collected by using 30-μm-pore nylon mesh (23). Malt extract was prepared by boiling 50 g of ground malt in 1 liter of distilled water for 30 min and then filtering the extract through Miracloth (Calbiochem-Behring Corp.). To the resultant malt extract were added 30 g of glucose, 3 g of peptone, and 3 g of agar. Conidia were maintained in aqueous solution (10% glycerol and 0.01% Tween 80) at −80°C.

Standard samples of metabolites. The structures of the substances used in this study are shown in Fig. 1. HAVN was first isolated as a fungal metabolite in this study. *E. heterothallica* IFO 30842 was cultured (stationary culture) in a malt extract medium supplemented with 3% glucose and 0.3% peptone at 24°C for 14 days. The pigments were extracted from the mycelia with acetone. The acetone extracts were applied to a silica gel column, and the column was washed with a solution of benzene and acetone (9:1, vol/vol). HAVN was eluted from the column with 100% acetone. HAVN was further purified by repeated silica gel column chromatography with benzene-ethyl acetate (2:1, vol/vol) and then benzene-ethyl acetate (1:1, vol/vol). Recrystallization from methanol gave HAVN as an orange powder. AVF was also prepared from *E. heterothallica* IFO 30842. AVN (3) was prepared from the mycelia of the AVN-accumulating mutant *A. parasiticus* NIAH-204 (24). NA (11), AVR (1), and AVT (18) were prepared from the mycelia of *A. versicolor* (Vuillemin) Tiraboschi and purified by column chromatography on silica gel and recrystalliza-

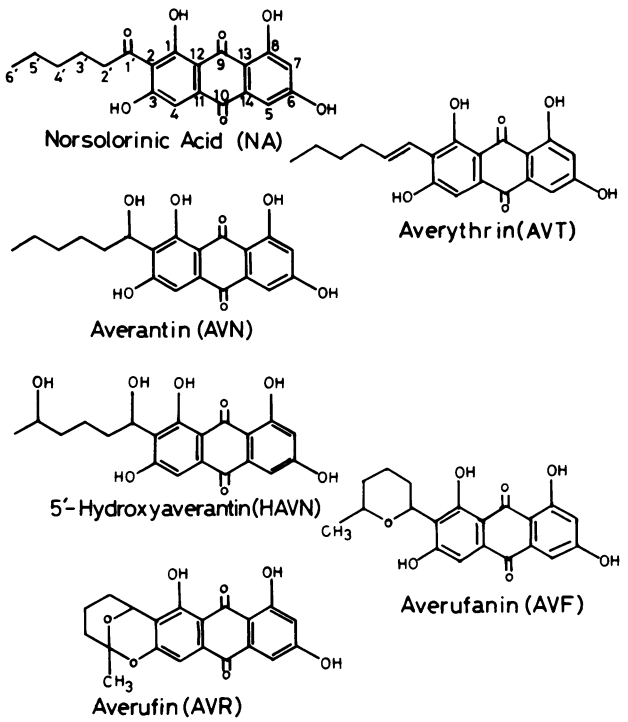

FIG. 1. Structures of the substances used in this study.

tion. A standard sample of nidurufin (2'-hydroxyaverufin [NID]) was also isolated from the mycelia of *A. versicolor* (Vuillemin) Tiraboschi (1). A kit (Makor Chemical Ltd., Jerusalem, Israel) was used as standards for AFB_1, AFB_2, aflatoxin G_1 (AFG_1), and AFG_2. The concentration of the metabolites in methanol was determined from UV absorption spectra by using molar absorption coefficients as follows: NA, 465 nm, 7,760 (7); AVN, 453 nm, 6,658 (3); HAVN, 466 nm, 7,100 (this paper); AVR, 454 nm, 10,500 (7); AVF, 468 nm, 7,600 (17a); AVT, 450 nm, 8,500 (18).

Preparation of microsome and postmicrosome fractions. Cell extract (postmitochondrial fraction) was prepared from the mycelia of NIAH-26 which had been cultured in YES (2% yeast extract, 20% sucrose) as described previously (21). This fraction was centrifuged at 105,000 × *g* for 90 min, and the supernatant (postmicrosome or cytosol fraction) to which glycerol (final concentration, 10%) was added was stored at −80°C until use. The pellet (microsome fraction) was washed once by centrifugation in a solution containing 0.2 M potassium phosphate buffer (pH 7.5) and 1 mM phenylmethylsulfonyl fluoride, and subsequently suspended in a solution containing 0.1 M potassium phosphate buffer (pH 7.5) and 10% glycerol. The resultant microsome fraction was stored at −80°C until use.

In order to study the effect of the carbon source on the enzyme activities, microsome and cytosol fractions were prepared from the mycelia of this mold which had been cultured in YEP medium, containing 20% peptone instead of sucrose. Microsome and cytosol fractions were also prepared from the mycelia of a nonaflatoxigenic strain, *A. oryzae* SYS-2, which had been cultured in YES medium.

The protein concentration was determined by the method of Bradford (5).

Assay of enzyme activities. In order to investigate dehydrogenase activity in enzyme fractions toward NA and AVN, the cytosol fraction (0.7 mg of protein per ml) was incubated at 37°C for 1 h in a reaction mixture containing 90 mM phosphate buffer (pH 7.5), 10% glycerol, and 60 μM NA or AVN. For the monooxygenase activity, catalyzing the reaction from AVN to HAVN, the microsome fraction (2.4 mg of protein per ml) was incubated at 30°C for 1 h in the same reaction mixture as used for the cytosol but which contained 60 μM AVN instead of NA. For the dehydrogenase activity from HAVN to AVR, the cytosol fraction (0.8 mg of protein per ml) was incubated at 37°C for 1 h in the same reaction mixture which contained 60 μM HAVN as the substrate. When specified, 0.2 mM NADPH, NADP, NADH, or NAD was added to the reaction mixture. The total volume was 50 μl in a microtube (1.5 ml). After termination of the reaction by the addition of 3 μl of formic acid and mixing with a Vortex mixer, the mixture was centrifuged at 10,000 × *g* for 2 min. Then, 20 μl of the supernatant was spotted on the origin of a silica gel thin-layer chromatography (TLC) plate (silica gel 60, catalog no. 5721; Merck & Co., Inc., Rahway, N.J.) and dried. The reaction products (NA, AVN, HAVN, and AVR) were examined on TLC against standard compounds with two kinds of developing solutions: chloroform–ethyl acetate–90% formic acid (6:3:1, vol/vol/vol) (solution A) and benzene-ethyl acetate (7:3, vol/vol) (solution B). Anthraquinones isolated by TLC were observed under short-wave (254 nm) UV light, and fluorescence photographs were taken with a FUNA UV light (254 nm, type SL-800F), CONTAX 167 camera, KENKO SO 56.2 YA-3 and SL-39 UV filters, and Tmax 400 film (Eastman Kodak Co., Rochester, N.Y.). When specified, fluorescence photographs were taken under long-wavelength (365 nm) UV light.

The production of AVR from AVN or HAVN and that of NA from AVN were also identified by using high-performance liquid chromatography (HPLC). Water-saturated chloroform (40 μl) was added to the remainder (33 μl) of the reaction mixture for the enzyme assay described above and mixed with a Vortex mixer, and the mixture was centrifuged at 10,000 × *g* for 2 min. The lower chloroform layer was injected into an HPLC apparatus (Shimadzu HPLC LC-6A system) equipped with a combined octyldecyl silane column [Shimpack CLC-ODS(M) and G-ODS(4)]. The column was developed at a flow rate of 1 ml/min with an 80% aqueous methanol solution. The temperature of the column oven was 40°C, and the A_{250} was monitored. The retention times of AVR, AVF, and NA were compared with those of standard samples.

Feeding experiments. The tip culture method (24) was used for the feeding experiments. A spore suspension (5 μl) was inoculated into 250 μl of YES medium containing various substances. After 4 days of incubation, the aflatoxins excreted from the mycelia into the medium were examined by TLC. Water-saturated chloroform (40 μl) was added to the tube containing 35 μl of culture medium and mixed with a Vortex mixer. A 20-μl amount of the lower chloroform layer was spotted on the origin of a silica gel TLC plate and developed in solution A. Fluorescence photographs of aflatoxins were taken with a FUNA UV light (365 nm, type SL-800F), CONTAX 167 camera, SHOTT KV418 and Nikon IF440 filters, and Kodak TMY-400 film.

RESULTS

Characterization of HAVN. The purified pigment from *E. heterothallica* IFO 30842 was identified as HAVN, with a melting point of 247 to 250°C; $[\alpha]_D^{20}$ −156° (*c* = 0.5, dioxane). The physicochemical properties of HAVN were as follows.

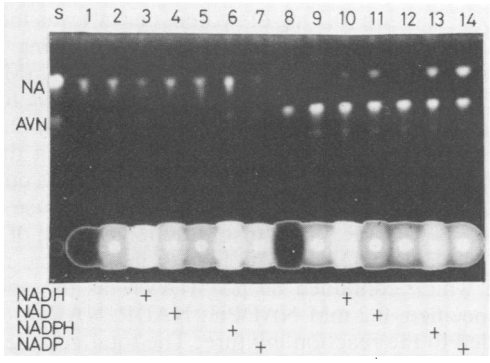

FIG. 2. Reversible reactions between NA and AVN associated with the cytosol fraction. The cytosol fraction was incubated with NA (lanes 1 through 7) or AVN (lanes 8 through 14). Various cofactors were also added where specified. After incubation, the reaction products were analyzed by TLC with developing solution B, and fluorescence photographs were taken under long-wavelength (365 nm) UV light. Lanes 1 and 8 did not contain the cytosol fraction. Lane s contained the standard samples.

λ_{max} (ethanol): 221 nm (ε 26,700); 262 nm (ε 17,400); 294 nm (ε 22,800); 313 nm (ε 21,300); 466 nm (ε 7,100). ν_{max} (KBr): 3,420, 1,665, 1,620, and 1,580 cm^{-1}. δ_H (270 MHz, acetone-d_6): 1.07 (3H, d, J = 5.9 Hz), 1.20 to 1.90 (6H, m), 3.69 (1H, tq, J = 5.9, 5.9 Hz), 5.38 (1H, dd, J = 7.8, 4.9 Hz), 6.59 (1H, d, J = 2.7 Hz), 7.08 (1H, s), 7.18 (1H, d, J = 2.7 Hz), 12.16 (1H, s), 12.71 (1H, s). EIMS (70 eV) lacked the molecular ion peak, but an ion peak which was assigned $M^+ - H_2O$ was detected at m/z 370.

Reaction of HAVN with methyl iodide and K_2CO_3 in acetone resulted in the formation of a tetramethyl derivative which was crystallised as yellow needles from acetone, mp 165 to 167°C. Found: C, 64.84; H, 6.33. Calculated for $C_{24}H_{28}O_8$: C, 64.86%; H, 6.31%. The physicochemical properties were as follows. λ_{max} (ethanol): 220 nm (ε 30,100); 280 nm (ε 33,000); 340 nm (ε 5,100); 405 nm (ε 3,800). ν_{max} (KBr): 3,450, 1,665, 1,605, and 1,580 cm^{-1}. δ_H (270 MHz, $CDCl_3$): 1.17 (3H, d, J = 5.9 Hz), 1.40 to 1.60 (4H, m), 1.72 (1H, m), 1.96 (1H, m), 3.52 (1H, br, d, J = 10.8 Hz), 3.80 (1H, m), 3.96 (3H, s), 3.98 (6H, s), 4.03 (3H, s), 5.14 (1H, m), 6.77 (1H, d, J = 2.7 Hz), 7.32 (1H, d, J = 2.7 Hz), 7.53 (1H, s). EIMS (70eV) (relative intensity): m/z 444 (11%, M^+); 426 (21), 411 (13), 257 (100). These data were consistent with the structure of HAVN.

In addition to HAVN, NA, AVT, AVF, and AVN were also isolated from the same mold and identified from the melting point, infrared spectrum, nuclear magnetic resonance spectrum, and mass spectrum.

Reversible conversion between NA and AVN by cytosol enzyme activity. When the cytosol fraction derived from *A. parasiticus* NIAH-26 was incubated with NA in the presence of NADH or NADPH, an additional orange fluorescent spot appeared, which was identified as AVN by comparison with the standard sample (Fig. 2). On the other hand, when the cytosol fraction was incubated with AVN in the presence of cofactors, NA was formed. The amount of NA produced from AVN was greater in the presence of oxidized forms of cofactors than with the reduced forms, indicating that NA was formed through the dehydrogenation of AVN.

These reversible reactions were not observed when the cytosol fraction of this mold which had been cultured in YEP medium was used or when the cytosol fraction of *A. oryzae* SYS-2 which had been cultured in YES medium was used.

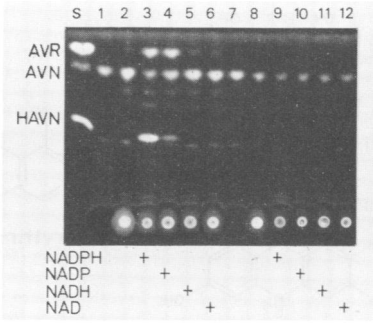

FIG. 3. Production of HAVN and AVR from AVN by microsome enzyme activity. The microsome fraction of *A. parasiticus* NIAH-26 which had been cultured in YES medium (lanes 2 through 6) or YEP medium (lanes 8 through 12) was incubated with AVN. Cofactors were also added where specified. Lanes 1 and 7 did not contain the microsome fraction. After incubation, the reaction products were analyzed by TLC with developing solution A, and fluorescence photographs were taken under short-wavelength (254 nm) UV light. Lane s contained the standard samples.

Formation of HAVN from AVN by microsome enzyme activity. The microsome fraction was prepared from mycelia of *A. parasiticus* NIAH-26 and then incubated with AVN in the presence of NADPH. Three new fluorescent spots were formed (Fig. 3). The lowest spot was identified as HAVN by comparison with the standard sample. The top spot, with an R_f which corresponded to that of AVR, had the same color as that of AVR and was further identified as AVR by HPLC. Though the AVR and HAVN spots were also observed when NADP was added instead of NADPH, the amount of HAVN was much lower than in the presence of NADPH.

In order to analyze the biosynthetic relationship among AVN, HAVN, and AVR, the reaction products were examined after various incubation intervals (Fig. 4). A significant amount of HAVN was formed within 5 min and reached a maximum value after 10 min, decreasing markedly after 90 min. On the other hand, the amount of AVR continued to increase constantly during the reaction time.

In this experiment, a small amount of the pigment corresponding to NID was formed transitorily.

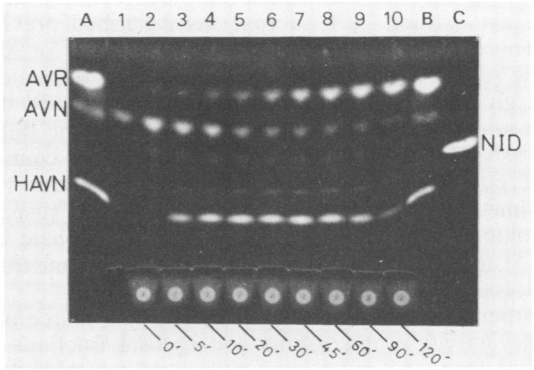

FIG. 4. Time course of the production of HAVN and AVR from AVN. The microsome fraction of *A. parasiticus* NIAH-26 which had been cultured in YES medium was incubated with AVN for various periods of time (minutes) as specified. After incubation, the reaction products were analyzed by TLC with developing solution A, and fluorescence photographs were taken under 254-nm UV light. Lane 1 did not contain the microsome fraction. Lanes A, B, and C contained the following standard samples: A, AVR, AVN, and HAVN; B, AVF, AVN, and HAVN; C, NID.

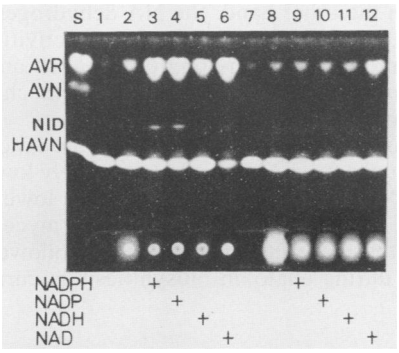

FIG. 5. Production of AVR from HAVN by cytosol enzyme activity. The cytosol fraction of *A. parasiticus* NIAH-26 which had been cultured in YES medium (lanes 2 through 6) or YEP medium (lanes 8 through 12) was incubated with HAVN. Cofactors were also added where specified. After incubation, the reaction products were analyzed by TLC with developing solution A, and fluorescence photographs were taken under 254-nm UV light. Lanes 1 and 7 did not contain the cytosol fraction. Lane s contained the standard samples for AVR, AVN, and HAVN. The R_f values of the spots between AVR and HAVN in lanes 3 and 4 corresponded to authentic NID.

Formation of AVR from HAVN by cytosol enzyme activity. The cytosol fraction of *A. parasiticus* NIAH-26 was incubated with HAVN (Fig. 5). When NADP or NAD was also added to the reaction mixture, AVR was formed. Though AVR was also formed in the presence of the reduced forms of the cofactors, the amount of AVR was significantly lower in the presence of NADH than in the presence of NAD. These results indicate that AVR was formed by the dehydrogenation of HAVN. A small amount of NID was also produced from HAVN in the presence of NADPH or NADP.

A limited conversion of HAVN to AVR was observed in the presence of NAD when the cytosol fraction of this mold which had been cultured in YEP medium was used. However, no activity was found when the cytosol fraction of *A. oryzae* SYS-2 was used (data not shown).

No new products were detected when either the cytosol or microsome fraction of *A. parasiticus* NIAH-26 was incubated with AVR or AVF. Also, incubation of the cytosol or postmitochondrial fraction with AVT failed to produce any new substances.

Production of aflatoxins in feeding experiments. When HAVN or AVN was added to NIAH-26 cultures, aflatoxins (mainly AFB_1 and AFG_1) were produced (Fig. 6), and a lower amount of aflatoxins was also produced when AVR or NA was added to NIAH-26 cultures; in contrast, no aflatoxins were detected when AVF or AVT was added.

DISCUSSION

Although *E. heterothallica* is not known to be related to other fungi producing aflatoxins, the precursors of aflatoxins, such as AVN, AVR, and AVF, were isolated from it, together with HAVN. The precursors of aflatoxins such as AVN and AVR were also isolated from other mold genera (1, 7, 10, 16). These facts suggest that at least part of the pathway of aflatoxin biosynthesis is common to several genera of molds irrespective of aflatoxin biosynthesis. However, the function of this pathway in these molds remains unclear. In this study, HAVN was found to be a precursor of aflatoxins. The metabolic scheme proposed in this study,

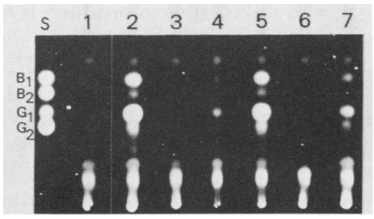

FIG. 6. Production of aflatoxins from various substances in feeding experiments. *A. parasiticus* NIAH-26 was cultured in YES medium (lane 1) or YES containing 20 μM HAVN (lane 2), AVF (lane 3), AVR (lane 4), AVN (lane 5), AVT (lane 6), or NA (lane 7). After 4 days of culture, aflatoxins excreted into the medium were analyzed by TLC. Fluorescence photographs were taken under 365-nm UV light. Lane S contained standard samples of AFB_1, AFB_2, AFG_1, and AFG_2. No significant difference in mycelial weight was found among the experiments.

which is shown in Fig. 7, indicated that two kinds of dehydrogenase and a monooxygenase were involved.

Recently, the enzyme activity for conversion of NA to AVN was observed and the purification to homogeneity of this enzyme was reported (6). In this study, we observed that this reaction was reversible, from AVN to NA. Since the equilibrium between NA and AVN shifted to NA (Fig. 2), it is suggested that the 1′-ketone group of NA could interact with two neighboring hydroxy groups, 1-OH and 3-OH, and may be able to form a stable resonance structure on its molecule. Nicotinamide nucleotides acted as cofactors for these dehydrogenase reactions, though NADP(H) was preferred over NAD(H).

AVN was hydroxylated to HAVN by the microsome enzyme activity. The presence of this enzyme in the microsome fraction was confirmed by the lack of conversion of AVN to HAVN by the cytosol fraction (Fig. 2). The time course experiments with the microsome fraction indicated

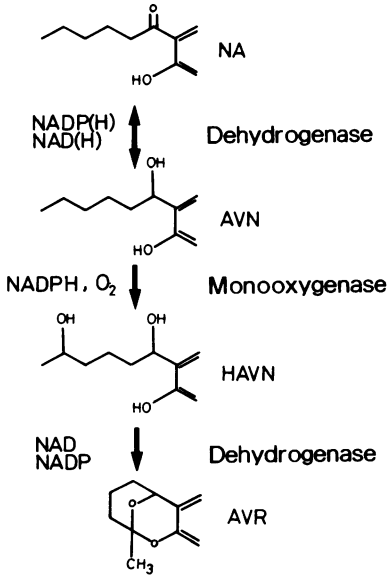

FIG. 7. Partial metabolic scheme for aflatoxin biosynthesis indicated in this study. NA dehydrogenase and HAVN dehydrogenase are cytosol enzymes, whereas monooxygenase is a microsome enzyme.

that AVN was first hydoxylated to produce HAVN and then HAVN was oxidized to produce AVR. Since the enzyme activity for the production of AVR from HAVN was also associated with the cytosol fraction (Fig. 5), the cytosol enzyme remaining in the microsome fraction was considered to catalyze the reaction from HAVN to AVR. The fact that the production of HAVN from AVN was dependent on NADPH but not on NADH and that the catalytic activity was found in the microsome fraction indicated the involvement of monooxygenase at this step.

HAVN was oxidized to AVR in the presence of NAD or NADP. Though AVR was also formed in the presence of NADH or NADPH, the oxidized forms of cofactors which were assumed to be produced by the putative NADH or NADPH dehydrogenase in the cell-free system may be involved in these reactions. NAD was found to be a more appropriate cofactor than NADP in this reaction.

Dutton (9) proposed the existence of a tentative substance, HVAN, in the reaction between AVN and AVR based on the structures of the precursors. This assumption was validated in the current study. However, the HAVN dehydrogenase was apparently different from NA dehydrogenase in its characteristics. NAD is more suitable as a cofactor than NADP for the HAVN dehydrogenase reaction, whereas NADP(H) is preferred to NAD(H) in the NA dehydrogenase reaction. The HAVN dehydrogenase reaction was slightly promoted in the presence of NAD with the cytosol fraction of *A. parasiticus* NIAH-26 which had been cultured in YEP medium, whereas the NA dehydrogenase reaction did not occur under the same conditions.

As for the reaction mechanism from HAVN to AVR, the 5′ carbon atom of HAVN may be oxidized to a ketone by HAVN dehydrogenase with NAD or NADP, and then internal ketalization among the resultant free carbonyl and two hydroxyls may generate AVR. Recently, McCormick et al. isolated AVF from an aflatoxigenic strain, *A. parasiticus* SU-1 (NRRL 5862), and reported that AVF is a precursor of AFB_1 in the pathway between AVN and AVR (17). Although AVF is probably derived from HAVN by dehydration, it is possible that AVF may operate as a side shunt at the HAVN level during aflatoxin biosynthesis (9). In this study, we were unable to confirm the involvement of AVF in the conversion from NA to AVR in the cell-free study. Also, the production of aflatoxins from AVF was not detected in feeding experiments (Fig. 6). The details of the steps from HAVN to AVR remain to be determined.

Interestingly, in this study, a small amount of NID was transitorily produced from AVN (Fig. 4). NID was also produced from HAVN (Fig. 5) but not from AVR, even when both the cytosol and microsome fractions were used. These results suggest that HAVN may be a direct precursor of NID and the hydroxylation of the 2′-C of HAVN may be catalyzed by a cytosol enzyme depending on NADPH. Though NID has been reported not to be directly involved in aflatoxin biosynthesis (19), the role of NID in the mold remains unclear.

The activities of the NA dehydrogenase and monooxygenase enzymes were not detected when a non-aflatoxin-inducing YEP medium was used. Also, only slight HAVN dehydrogenase activity was detected in the presence of NAD under these conditions. Our previous articles indicated that the activities of two distinct *O*-methyltransferase and oxidoreductase enzymes could not be detected in YEP medium (21, 22). It is possible that the expression of these enzymes is regulated commonly at the transcriptional level by an unidentified factor(s) depending on the kind of carbon sources. On the other hand, the NA dehydrogenase, monooxygenase, and HAVN dehydrogenase activities were not observed in the aflatoxin-nonproducing strain *A. oryzae* SYS-2. This strain seems to have different characteristics than aflatoxigenic strains.

Finally, in the feeding experiments, the amount of aflatoxins produced from AVR was considerably lower than that from HAVN (Fig. 6). Factors such as the lower incorporation rate of AVR than of HAVN into the mycelia might be related to these results. The pathway followed by these substances during aflatoxin biosynthesis is currently being examined.

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