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Stereochemistry during Aflatoxin Biosynthesis: Conversion of Norsolorinic Acid to Averufin

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A reaction sequence, norsolorinic acid (NA) → averantin (AVN) → 5′-hydroxyaverantin (HAVN) → averufin (AVR), is the early part of a biosynthetic pathway for aflatoxins. In this study, we determined the stereochemical relationship among these metabolites by using chiral high-performance liquid chromatography. In cell-free experiments using the cytosol fraction of *Aspergillus parasiticus* NIAH-26, (1′*S*)-AVN was exclusively produced from NA in the presence of NADPH. Also, only (1′*S*)-AVN, and not (1′*R*)-AVN, served as a substrate for the reverse reaction from AVN to NA. When the microsome fraction of NIAH-26 was incubated with (1′*S*)-AVN in the presence of NADPH, two HAVN diastereomers and one AVR enantiomer were formed, whereas these substances were never produced from (1′*R*)-AVN. Moreover, (1′*S*,5′*R*)-AVR was exclusively formed from both HAVN diastereomers by the cytosol fraction in the presence of NAD. The feeding experiments using this mutant showed that aflatoxins were produced from (1′*S*,5′*R*)-AVR but not from (1′*R*,5′*S*)-AVR. These results indicate that the enzymes involved in this pathway show strict stereospecificity to their substrates and that the configuration of (1′*S*,5′*R*)-AVR leading to the formation of aflatoxins is due to the stereospecificity of NA dehydrogenase which catalyzes the reaction between (1′*S*)-AVN and NA.

Aflatoxins constitute a family of toxic secondary metabolites produced by certain strains of the common molds *Aspergillus flavus* and *Aspergillus parasiticus*. Aflatoxins are acutely toxic, teratogenic, and potent carcinogenic and mutagenic agents (11). The contamination of foodstuffs by aflatoxins can have serious effects on the health of animals and humans. In spite of the known danger, however, there are currently no effective control procedures for preventing feed or food contamination by aflatoxins. As part of an effort towards developing some form of control procedure, the biosynthetic pathway of aflatoxins has been extensively studied (4, 6–9, 14, 19, 23, 27–29, 30, 33). We recently reported that averantin (AVN), which has been produced from norsolorinic acid (NA) (4, 14), is successively converted to 5′-hydroxyaverantin (HAVN) and then to averufin (AVR) in the early part of aflatoxin biosynthesis (Fig. 1). Three enzymes, NA dehydrogenase, AVN monooxygenase, and HAVN dehydrogenase, are involved in these reactions (33).

Aflatoxins and some of their precursors are optically active substances, while other precursors are either racemates or have no chiral center on their molecules (13, 14). Since the chirality of aflatoxins may be related to their toxicity, we investigated which step(s) may determine the configuration of aflatoxins or their precursors by using chiral high-performance liquid chromatography (chiral HPLC) analysis in this and other studies (31). The configuration of a small amount of each substance in a mixture composed of heterogeneous substances could be precisely monitored by chiral HPLC methods.

The substances formed during the conversion of NA to AVR have been isolated from various molds (3, 16, 22, 33) as well as *A. flavus* and *A. parasiticus*. Although NA has no chiral carbon atom, AVN isolated from *A. parasiticus* (9) and HAVN isolated from *Emericella heterothallica* (34) have negative signs of specific optical rotation. AVR isolated from *Aspergillus versicolor* had a (+) sign (3, 24), whereas Katsube et al. reported that AVR was a racemate (20). Townsend and Christensen reported that the configuration of (−)-AVN isolated from *A. parasiticus* corresponded to 1′*S* (26) and that the configuration of (+)-AVR corresponded to (1′*S*,5′*R*) (21). In the current study, we examined the configurational changes of the substances during the conversion of NA to AVR in cell-free experiments using cytosol or microsome fractions from a mutant of *A. parasiticus*. We also examined the stereospecific formation of aflatoxins from AVR in feeding experiments using this mutant.

MATERIALS AND METHODS

Microorganism. *A. parasiticus* NIAH-26, a UV-irradiated mutant of *A. parasiticus* SYS-4 (NRRL 2999), was used. This mold induced all enzymes during the conversion of NA to aflatoxins in a YES liquid culture medium (2% yeast extract, 20% sucrose), although it produced neither aflatoxins nor anthraquinone or xanthone precursors (27–30, 32, 33).

Standard sample of metabolites. This study was the first to synthesize racemic AVN from NA as follows. A suspension of NA in acetic acid was added to platinum dioxide (1, 15) that had been activated in a hydrogen gas environment. The reaction was continued at room temperature by mixing with a magnetic stirrer for 4 to 6 days. The progress of the reaction was monitored by silica gel thin-layer chromatography. The reaction products were then separated from the catalyst by filtration. The racemic AVN was purified by silica gel preparative thin-layer chromatography by using a solution of chloroform-ethyl acetate (2:1, vol/vol). (1′*R*)-(+)-AVN$_1$ was prepared from racemic AVN by using a

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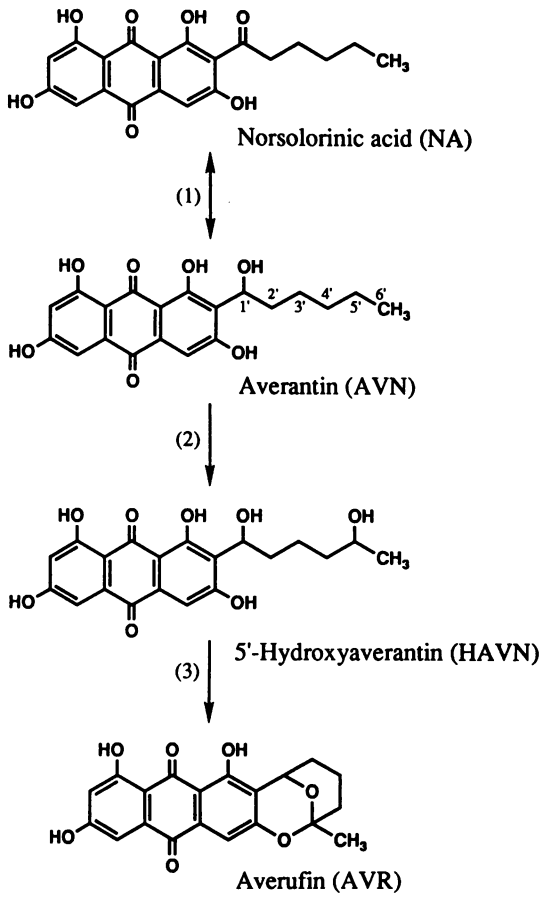

FIG. 1. Partial pathway from NA to AVR in the early stages of aflatoxin biosynthesis and structures of the substances related in this study. Step 1 was catalyzed by NA dehydrogenase (cytosol enzyme), step 2 was catalyzed by AVN monooxygenase (microsome enzyme), and step 3 was catalyzed by HAVN dehydrogenase (cytosol enzyme).

Chiralpak AD HPLC column (0.46 by 25 cm; Daisel Chemical Industry Ltd., Tokyo, Japan). (1'*S*)-(−)-AVN_2 was prepared from an AVN-accumulating mutant, *A. parasiticus* NIAH-204; HAVN was prepared from *E. heterothallica* IFO 30842 (33); and NA (17) and racemic AVR (20) were prepared from *A. versicolor* (Vuillemin) Tiraboschi. Each enantiomer of AVR was prepared from racemic AVR by using a Chiralcel OD column (0.46 by 25 cm; Daisel Chemical Industry). Averufanin (AVF) (3, 13, 18) and averythrin (AVT) (13, 25) were prepared from *E. heterothallica* IFO 30842 and *A. versicolor* (Vuillemin) Tiraboschi, respectively. These substances were used as standards for the artificially dehydrated products from HAVN and AVN, respectively. The concentration of the metabolites in methanol was determined from UV absorption spectra by using molar absorption coefficients (5, 13, 33) as follows (in M^{-1} cm^{-1}): NA, at 465 nm, 7,760; AVN, at 453 nm, 6,658; HAVN, at 466 nm, 7,100; AVR, at 454 nm, 10,500.

The circular dichroism (CD) spectrum of AVR enantiomers or AVN_2 were measured with a J-720 spectropolarimeter (Japan Spectroscopic Co., Ltd.).

Preparation of cytosol and microsome fractions. The cytosol fraction was prepared from mycelia of *A. parasiticus* NIAH-26, which had been cultured in YES medium, by centrifuging the postmitochondrial fraction at 105,000 × *g* for 90 min (33). To remove contaminating pigments, the cytosol protein fraction was further purified through a Sephadex G-25 M column (column PD-10; Pharmacia LKB Biotechnology, Uppsala, Sweden), which was equilibrated and then eluted with a solution containing 20 mM Tris-HCl (pH 7.5), 0.1 M KCl, 5 mM $MgCl_2$, 0.4 mM EDTA, 1 mM mercaptoethanol, and 10% (vol/vol) glycerol. The pellet after centrifugation at 105,000 × *g* was prepared as a microsome fraction (33). The purified cytosol and microsome fractions were stored at −80°C until they were used. The protein concentration was determined by the method of Bradford (10).

Enzyme assay and chiral HPLC analyses. The cytosol fraction (0.2 mg of protein per ml) or the microsome fraction (1.7 mg of protein per ml) was incubated in a reaction mixture consisting of solution A (90 mM potassium phosphate [pH 7.5], 10% [vol/vol] glycerol) supplemented with 55 μM each substrate and 2 mM NADPH, NADP, or NAD. The total volume was 50 μl in a microtube (1.5 ml). After incubation at 37°C (cytosol) or 30°C (microsome), the reaction was terminated by adding 100 μl of water-saturated ethyl acetate and mixing in a Vortex mixer. After centrifugation at 10,000 × *g* for 1 min, the aliquot was transferred to a new microtube and dried by keeping the tube open at room temperature under darkness. At this step, rapid drying in an N_2 gas environment caused dehydration of AVN to AVT and of HAVN to AVF. The residue was then solubilized with methanol and injected into the HPLC apparatus. HPLC was performed at room temperature at a flow rate of 1 ml/min on a Shimadzu HPLC apparatus (LC-6A or LC-9A) equipped with a Chiralpak AD or Chiralcel OD column (0.46 by 25 cm, Daisel Chemical Industry), and the A_{290} was monitored. The solvent system consisted of *n*-hexane–ethanol–trifluoroacetic acid (*n*-hexane–ethanol–TFA; AD column, 95:5:0.2, vol/vol/vol; OD column, 94:6:0.2, vol/vol/vol) unless otherwise stated.

To investigate the NA dehydrogenase activity catalyzing the conversion of NA to AVN, the cytosol fraction was incubated with NA and NADPH. For the reverse reaction from AVN to NA, either AVN_1 or AVN_2 was incubated with the cytosol fraction in the presence of NADP instead of NADPH. The reaction products were analyzed by using a Chiralpak AD column. For the AVN monooxygenase reaction from AVN to HAVN, the microsome fraction was incubated with either AVN_1 or AVN_2 and NADPH. For the HAVN dehydrogenase reaction from HAVN to AVR, the cytosol fraction was incubated with HAVN and NAD. The products of these reactions were analyzed by using a Chiralcel OD column. Since the peaks of AVR were close to those of AVF on a Chiralcel OD chromatogram, the reaction products from AVN or HAVN were further examined by using a Chiralpak AD column with the same elution solution as that described above to verify that AVF was not included in the pathway from NA to AVR. The retention times of the reaction products were compared with those of the standard samples.

Determination of diastereomers of HAVN. Standard samples of HAVN (33) as well as HAVN enzymatically produced from AVN in this study were examined by using a silica gel HPLC column (Shim-pack CLC-SIL, Shimadzu Co. Ltd.) with an *n*-hexane–ethanol–TFA (97:3:0.2, vol/vol/vol) solution. An octadecyl silane (ODS) column (Shim-pack CLC-ODS, Shimadzu Co. Ltd.) with a solution of 50% (vol/vol) aqueous methanol supplemented with 0.2% (vol/vol) TFA was also used. The fragment signals of stan-

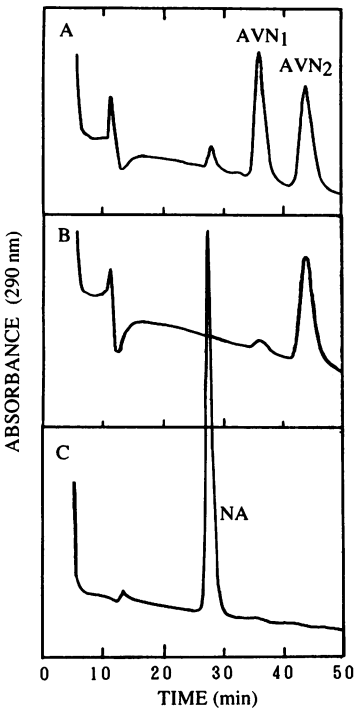

FIG. 2. Chiral HPLC separations of standard samples of AVN and NA by using a Chiralpak AD column and elution with *n*-hexane–ethanol–TFA (95:5:0.2, vol/vol/vol) at a flow rate of 1.0 ml/min. (A) Racemic AVN chemically synthesized from NA (AVN_1 and AVN_2 were assumed to be (1′*R*)-(+) and (1′*S*)-(−) enantiomers, respectively [see Results]); (B) AVN prepared from *A. parasiticus* NIAH-204; (C) NA prepared from *A. versicolor*.

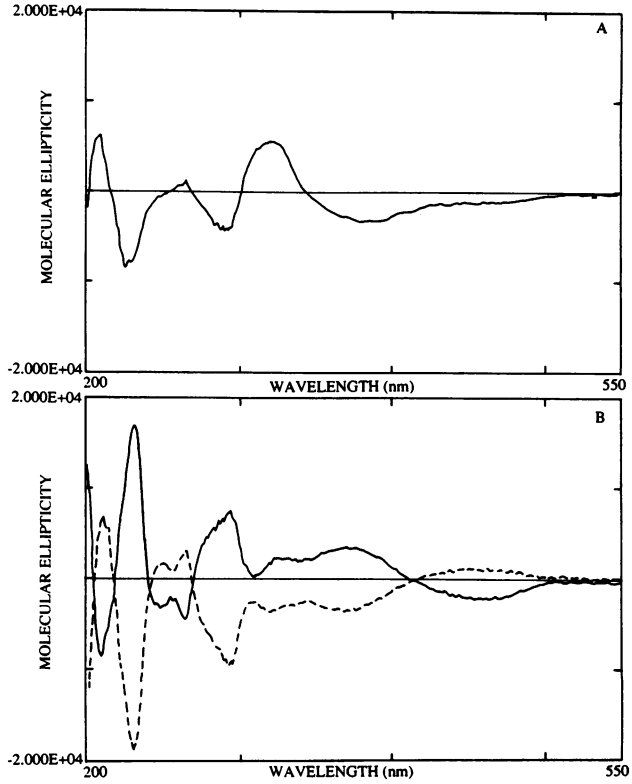

FIG. 3. CD spectra of AVN_2 (A) and AVR (B) enantiomers showing molecular ellipticity at various wavelengths. (1′S)-(−)-AVN_2 (in ethanol, 5-mm path, 0.188 mM). (B) —, (1′*R*,5′*S*)-AVR_1 (in ethanol, 5-mm path, 0.119 mM); ------, (1′*S*,5′*R*)-AVR_2 (in ethanol, 10-mm path, 0.0569 mM).

dard samples of HAVN or AVR were analyzed by using thermospray HPLC-mass spectrometry (Shimadzu LCMS-QP1000EX, Shimadzu LC-9A, STR ODS-2 column) with a 0.05 M aqueous solution of ammonium acetate-methanol-TFA (0.05 M CH_3COONH_4:CH_3OH:TFA; HAVN, 50:50:0.2, vol/vol/vol; AVR, 25:75:0.2, vol/vol/vol) at a flow rate of 1.0 ml/min.

Feeding experiments. The tip culture method (27, 32) was used for feeding experiments. A spore suspension (5 μl) was inoculated into 250 μl of a YES medium containing 30 μM various substances. After 4 days of incubation at 28°C, the aflatoxins excreted from the mycelia into the medium were examined by thin-layer chromatography. Water-saturated chloroform (35 μl) was added to a tube containing 35 μl of a culture medium and mixed with a Vortex mixer. A 25-μl lower chloroform layer was spotted on a silica gel thin-layer chromatography plate and developed in a solution containing chloroform–ethyl acetate–90% formic acid (6:3:1, vol/vol/vol). Photographs for fluorescence of aflatoxins were taken with a FUNA UV light (365 nm, type SL-800F), a CONTAX 167 camera, SHOTT KV418 and Nikon IF440 filters, and Kodak TMY-400 film.

RESULTS

Stereospecific conversion between NA and AVN by cytosol enzyme activity. Chiralpak AD HPLC was found to effect the separation of racemic AVN. A ternary system consisting of *n*-hexane–ethanol–TFA yielded good separation of NA and each enantiomer of racemic AVN (Fig. 2). We tentatively called the substances corresponding to the early and later peaks AVN_1 and AVN_2, respectively. AVN prepared from *A. parasiticus* was almost completely composed of AVN_2. Its CD spectrum was examined and is shown in Fig. 3A. Since the natural product has a (1′*S*)-(−) configuration (26), AVN_1 and AVN_2 were assumed to be (1′*R*)-(+) and (1′*S*)-(−) enantiomers, respectively.

When the cytosol fraction was incubated with NA in the presence of NADPH, only (1′*S*)-AVN was formed, whereas (1′*R*)-AVN was not detected even after 90 min (Fig. 4A). As for the reverse reaction, AVN to NA, NA was produced from (1′*S*)-AVN, whereas the production of NA from the (1′*R*) antipode was never detected (Fig. 4B).

Conversion from (1′S)-AVN to HAVN diastereomers and (1′S,5′R)-AVR_2. A Chiralcel OD column with *n*-hexane–ethanol–TFA yielded good separation of anthraquinone metabolites. Standard samples of AVR from *A. versicolor* showed two peaks of equal height with retention times of 10.3 and 11.5 min. Both AVR enantiomers (tentatively called AVR_1 and AVR_2) gave similar fragment ion peaks, including a peak at *m/z* 369 which correspond to the $[MH]^+$ ion. Their CD spectra also showed that these substances were enantiomers of each other (Fig. 3B). On the other hand, the enantiomers of AVN were not resolved when a Chiralcel OD column was used, and the retention time of AVN was 19.2 min. Furthermore, a standard sample of HAVN gave peaks at retention times of 48.5 and 52.8 min at a ratio of 2:1, and these substances could be resolved by using either silica gel

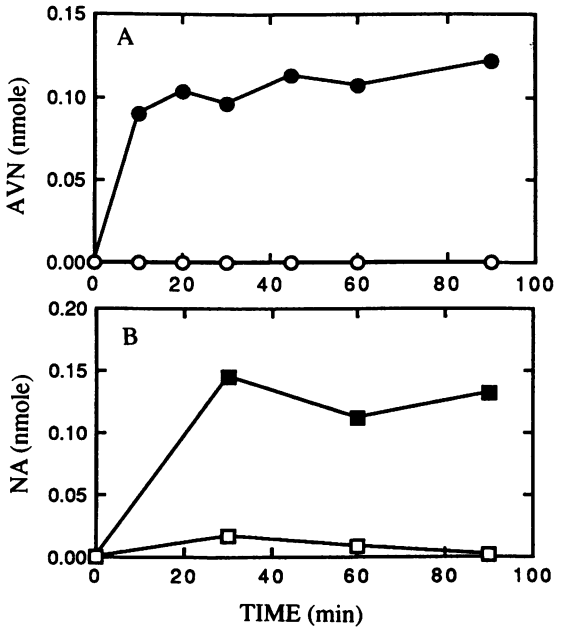

FIG. 4. Stereospecific conversion between NA and AVN, where AVN_1 is (1'*R*)-(+) and AVN_2 is (1'*S*)-(−). (A) Cytosol fraction incubated with NA in the presence of NADPH, with the resultant AVN_1 (○) and AVN_2 (●) measured by Chiralpak AD HPLC analysis as described in the legend to Fig. 2; (B) cytosol fraction incubated with either AVN_1 (□) or AVN_2 (■) in the presence of NADP, with the production of NA measured by Chiralpak AD HPLC analysis.

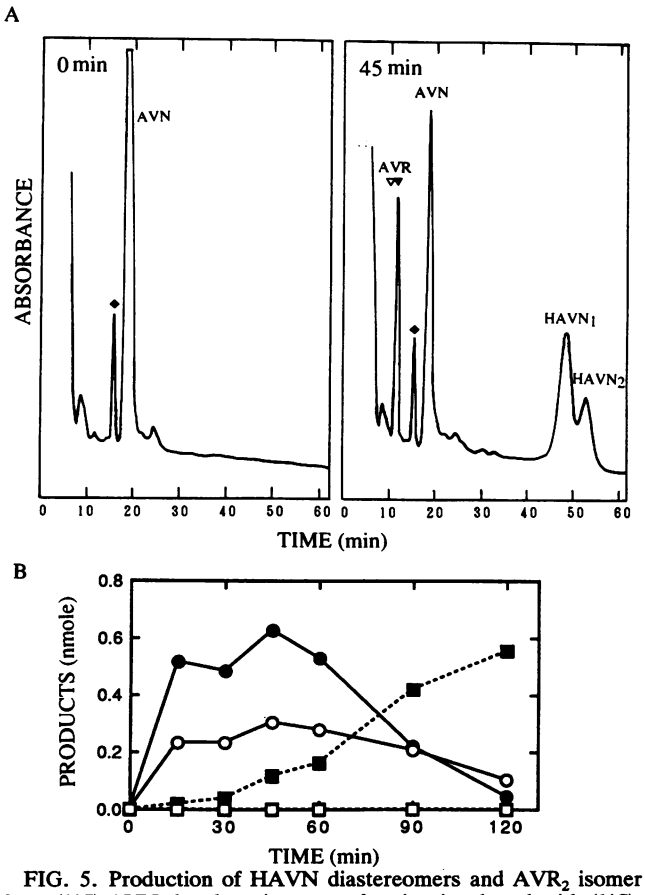

FIG. 5. Production of HAVN diastereomers and AVR_2 isomer from (1'*S*)-AVN_2 by the microsome fraction incubated with (1'*S*)-AVN_2, and products analyzed by using a Chiralcel OD column and elution of *n*-hexane–ethanol–TFA (94:6:0.2, vol/vol/vol) at a flow rate of 1.0 ml/min. (A) HPLC chromatograms of the metabolites at 0 or 45 min showing the retention times of AVN, $HAVN_1$, $HAVN_2$, AVR_1 (▽), AVR_2 (▼), and AVT (◆); (B) reaction product amounts at various reaction times (symbols: ●, $HAVN_1$; ○, $HAVN_2$; □, AVR_1; ■, AVR_2).

or octadecyl silane columns (data not shown). On thermospray HPLC-mass spectrometry, they gave similar fragment ion peaks including the peak at *m/z* 371 which corresponds to the $[MH\text{-}H_2O]^+$ ion. These results indicate that these HAVN molecules were diastereomers and not enantiomers. We designated these two diastereomers $HAVN_1$ and $HAVN_2$, respectively.

When (1'*S*)-AVN_2 was incubated with the microsome fraction of *A. parasiticus* in the presence of NADPH for 45 min, three peaks were formed (Fig. 5A). The retention time of the first peak corresponded to that of AVR_2, and the second and third peaks corresponded to those of HAVN standard samples. The resultant $HAVN_1$ and $HAVN_2$ were also resolved by using either silica gel or octadecyl silane HPLC columns. Although the peak corresponding to AVT was also detected (Fig. 5), AVT seemed to be a dehydrated artifact of AVN (see Materials and Methods). On the other hand, when (1'*R*)-AVN_1 was incubated with the microsome, neither HAVN nor AVR was formed (data not shown).

Figure 5B shows the time course of the production of the two diastereomers of HAVN and of the two possible enantiomers of AVR from (1'*S*)-AVN. The amount of both diastereomers of HAVN increased with time and reached a maximum at 45 min. The ratio of the amount of $HAVN_1$ to $HAVN_2$ was about 2:1, which was similar to the ratio of the standard sample of HAVN. The reaction was followed by a decrease in the amount of HAVN. By contrast, the amount of AVR_2 began to increase after a short time interval and continued to increase during the reaction time, although the formation of AVR_1 was still never detected.

Formation of AVR_2 from HAVN diastereomers. When the standard sample of HAVN was incubated with the cytosol fraction in the presence of NAD, the two diastereomers of HAVN were absolutely converted to AVR_2, not to AVR_1 (Fig. 6), although $HAVN_1$ rather than $HAVN_2$ preferentially converted to AVR_2. The conversion of AVR_1 to AVR_2 and vice versa was never detected in the cytosol fraction (data not shown).

Aflatoxin production from AVR_2 in the feeding experiments. When AVR_2 was added to the culture medium of *A. parasiticus* NIAH-26, aflatoxins (mainly AFB_1 and AFG_1) were produced (Fig. 7). In contrast, scarcely any aflatoxins were produced from the AVR_1 enantiomer. Although aflatoxins were also produced from racemic AVR, (1'*S*)-AVN, and HAVN, the amount of aflatoxins produced from racemic AVR was much smaller than that from AVR_2.

DISCUSSION

The chirality of the substances involved in aflatoxin biosynthesis has generally been examined by measuring CD or optical rotatory dispersion spectra. In this and other (31) studies, we were the first to succeed in chromatographically separating each individual enantiomer of the metabolites during aflatoxin biosynthesis. We established the separation

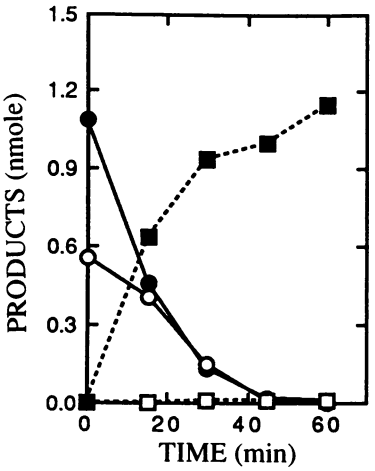

FIG. 6. Conversion of HAVN diastereomers to AVR_1, with the cytosol fraction incubated with an HAVN standard sample at various times (the amount of metabolites was measured by using a Chiralcel OD column). Symbols: ●, $HAVN_1$; ○, $HAVN_2$; □, AVR_1; ■, AVR_2.

methods for racemic AVN, racemic AVR, diastereomers of HAVN, and NA, AVT, and AVF with Chiralcel OD or Chiralpak AD HPLC columns. By using these techniques, we demonstrated that the stereochemistry of the metabolites during the conversion of NA to AVR is due to the stereospecificity of the enzymes involved in this conversion. The configuration of the metabolites during the conversion proposed in this study is shown in Fig. 8.

We demonstrated that the NA dehydrogenase that catalyzes the reversible conversion between NA and AVN showed strict stereospecificity to the position of the 1′ carbon atom of the substrate. Since natural AVN isolated from *A. parasiticus* molds has been reported to have an *S* configuration (26), the resultant AVN enantiomer in these experiments is assumed to be (1′*S*)-AVN_2, and (1′*R*)-AVN_1 is assumed to have not participated in the reaction between NA and AVN (Fig. 3). Since it has been reported that the pure NA dehydrogenase enzyme catalyzes the reactions between NA and AVN in both directions (12), the stereospecific conversion is due to the stereospecificity of the single enzyme for the substrate NA.

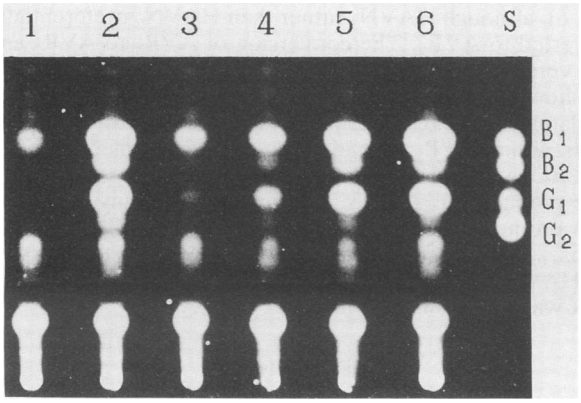

FIG. 7. Fluorescence photographs showing the production of aflatoxins from various substances added to a YES medium in feeding experiments using *A. parasiticus* NIAH-26. Lanes: 1, no supplement; 2, (1′*S*, 5′*R*)-AVR_2; 3, (1′*R*, 5′*S*)-AVR_1; 4, racemic AVR; 5, (1′*S*)-(−)-AVN_2; 6, HAVN standard sample; S, authentic standards of AFB_1, AFB_2, AFG_1, and AFG_2.

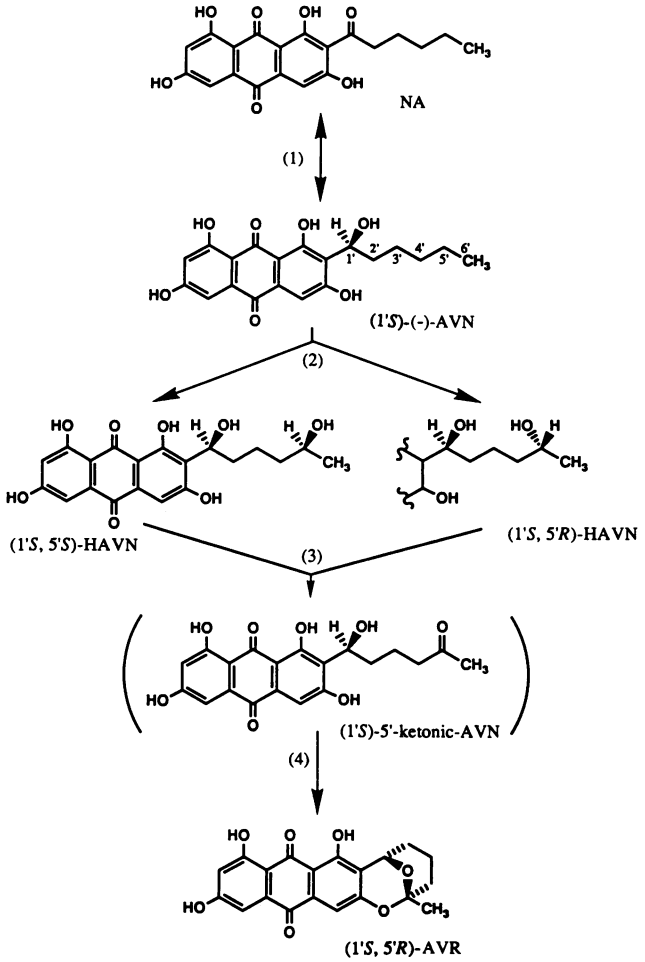

FIG. 8. Stereochemical metabolic scheme of NA to (1′*S*,5′*R*)-AVR_2 in aflatoxin biosynthesis proposing involvement of the keto form of HAVN.

HAVN was formed from (1′*S*)-AVN_2 but not from (1′*R*)-AVN_1, indicating that the AVN monooxygenase catalyzing this reaction also had strict stereospecificity for the 1′ carbon atom of the substrate. Since the configuration of the 1′ carbon is likely conserved, the resultant HAVN diastereomers were assumed to be diastereomers having (1′*S*,5′*S*) and (1′*S*,5′*R*) configurations. We could not, however, identify which peak on the chiral HPLC chromatogram corresponded to which diastereomer. The preferential production and consumption of $HAVN_1$ in the reactions from AVN to HAVN and HAVN to AVR, respectively, indicate that HAVN monooxygenase may have partial stereospecificity on the oxidation at the 5′ carbon of (1′*S*)-AVN_2 (Fig. 5 and 6).

AVR_2 was exclusively formed from both HAVN diastereomers (Fig. 5 and 6). Since both HAVN diastereomers seemed to have the 1′*S* configuration described above, AVR_2 was speculated to have a (1′*S*,5′*R*) configuration. Also, the preparation of (1′*S*,5′*R*)-AVR from *A. parasiticus* has been previously reported (21). However, since the direct

conversion from (1'*S*,5'*S*)-HAVN to (1'*S*,5'*R*)-AVR is stereochemically impossible and since the reaction activity from AVR_1 to AVR_2 was never detected in this study. We expect that the 5' carbon atom of both diastereomers of HAVN may be oxidized to a ketone by HAVN dehydrogenase with NAD, resulting in the loss of chirality on the 5' carbon atom. Intramolecular ketalization may then occur among the resultant ketone and two hydroxyls, to generate (1'*S*,5'*R*)-AVR_2.

On the basis of the feeding experiments, AVR_2, but not AVR_1, served as a precursor of aflatoxins (Fig. 7). This result suggests that other enzymes catalyzing steps following the formation of AVR_2 may also have strict stereospecificity for their substrate(s). In fact, we also determined that the latter parts of the pathway for aflatoxin biosynthesis were strictly regulated by the stereospecific enzymes as reported previously (31). Extremely small amounts of aflatoxins, compared with that from AVR_2, were produced from racemic AVR, indicating that AVR_1 may act as a competitive inhibitor for any enzyme working next to the HAVN dehydrogenase.

The present study showed that (1'*S*,5'*R*)-AVR_2 was exclusively formed in cell-free experiments using the cytosol from *A. parasiticus*. This result is consistent with previous observations that AVR from *A. parasiticus* has optical activity (3, 21, 24). However, AVR prepared from *A. versicolor* was a racemate (20). This observation was confirmed in this study. The difference in the results might depend on the species or strains of molds used in the experiments. Repetitive crystallization of the substance during the isolation procedures of the metabolite might selectively isolate the racemate rather than the isomer. The difference in the stereospecificity of the metabolites and the enzymes involved in this pathway among different species of molds is currently being examined.

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