A, the RSA should be 0.9 if labeling is the same as that expected from averufin (Lin et al., 1973). In our experiments the quite low RSA for aflatoxin made from acetate (0.007) compared to the relatively high RSA (0.475) for aflatoxin made from the labeled versicolorin A pigment offers additional strong evidence that this pigment is incorporated essentially intact and is not broken down into acetate units before incorporation into aflatoxin B_1.

Hsieh et al. (1973) report 45–58% conversion of sterigmatocystin to aflatoxin B_1 and conclude that sterigmatocystin or a closely related metabolite is an intermediate in the biosynthesis of aflatoxins. The 46% conversion of versicolorin A to aflatoxin B_1 found in our experiments suggests that this pigment is as efficiently converted to aflatoxin B_1 as is sterigmatocystin and offers experimental proof to the theory hypothesized by Heathcote et al. (1973) in which they propose versicolorin A as a precursor to aflatoxin B_1.

ACKNOWLEDGMENT

A. O. Franz, Jr., is acknowledged for quantitative aflatoxin analyses, and P. Scallet for technical assistance.

LITERATURE CITED

Adye, J., Mateles, R. I., *Biochim. Biophys. Acta* **86**, 418 (1964).
Bassett, C., Buchanan, M., Gallagher, R. T., Hodges, R., *Chem. Ind. (London)*, 1659 (1970).
Bennett, J. W., Goldblatt, L. A., *Sabouraudia* 11, 235 (1973).
Biollaz, M., Buchi, G., Milne, G., *J. Am. Chem. Soc.* **90**, 5017 (1968a).
Biollaz, M., Buchi, G., Milne, G., *J. Am. Chem. Soc.* **90**, 5019 (1968b).
Biollaz, M., Buchi, G., Milne, G., *J. Am. Chem. Soc.* **92**, 1035 (1970).
Burkhardt, H. J., Forgacs, J., *Tetrahedron* **24**, 717 (1968).
Butler, W. H., in "Aflatoxin: Scientific Background, Control and Implications", Goldblatt, L. A., Ed., Academic Press, New York, N.Y., 1969, p 223.
Donkersloot, J. A., Mateles, R. I., Yang, S. S., *Biochem. Biophys. Res. Commun.* **47**, 1051 (1972).
Heathcote, J. G., Dutton, M. F., *Tetrahedron* **25**, 1497 (1969).
Heathcote, J. G., Dutton, M. F., Hibbert, J. R., *Chem. Ind. (London)* **21**, 1027 (1973).
Holker, J. S. E., Underwood, J. G., *Chem. Ind. (London)*, 1865 (1964).
Hsieh, D. P. H., Lin, M. T., Yao, R. C., *Biochem. Biophys. Res. Commun.* **52**, 992 (1973).
Hsieh, D. P. H., Mateles, R. I., *Appl. Microbiol.* **22**, 79 (1971).
Lee, L. S., Bennett, J. W., Cucullu, A. F., Stanley, J. B., *J. Agric. Food Chem.* **23**, 1132 (1975).
Lin, M. T., Hsieh, D. P. H., *J. Am. Chem. Soc.* **95**, 1668 (1973).
Lin, M. T., Hsieh, D. P. H., Yao, R. C., Donkersloot, J. A., *Biochemistry* **12**, 5167 (1973).
Pons, W. A., Jr., Cucullu, A. F., Franz, A. O., Jr., Goldblatt, L. A., *J. Am. Oil Chem. Soc.* **45**, 694 (1968).
Rodricks, J. V., Lustig, E., Campbell, A. D., Stoloff, L., Henry-Logan, K. R., *Tetrahedron Lett.*, 2975 (1968).
Schroeder, H. W., Kelton, W. H., *Appl. Microbiol.* **30**, 589 (1975).
Thomas, R., in "Biogenesis of Antibiotic Substances", Vanek, Z., Hostalex, Z., Ed., Academic Press, New York, N.Y., 1965, p 155.
Turner, W. B., Ed., "Fungal Metabolites", Academic Press, London and New York, N.Y., 1971, p 181.

Received for review February 26, 1976. Accepted June 9, 1976. Presented at the 27th Southeast-31st Southwest Combined Regional Meeting of the American Chemical Society, Memphis, Tenn., Oct 29–31, 1975. Use of a company or product name by the Department does not imply approval or recommendations of the product to the exclusion of others which may also be suitable.

Biosynthesis of Aflatoxin. Conversion of Norsolorinic Acid and Other Hypothetical Intermediates into Aflatoxin B_1

Dennis P. H. Hsieh,* Ming T. Lin,[1] Raymond C. Yao, and Rajendra Singh

Five compounds were separately incubated with resting mycelium of the aflatoxin (AF) producing strain of *Aspergillus parasiticus*, under conditions found optimal for converting averufin (AR) into AFB_1. They were: four tritiated derivatives of C_{18} anthraquinone and anthrone synthesized to simulate possible intermediates in the pathway of AF biosynthesis, and the ring-labeled [^{14}C]norsolorinic acid (NA) produced from [1-^{14}C]acetate by a mutant culture of *A. parasiticus* deficient in AF biosynthesis. Results indicate that only a trace amount of labels from one of the four tritiated compounds was incorporated into AFB_1, whereas over 2% of the label from NA was accountable in the repeatedly purified AFB_1 which possessed a specific activity of 0.19 relative to that of [^{14}C]NA. The efficiency of conversion of NA into AFB_1 as compared to that of AR, an orange pigment (OP), versicolorin A (VA), and sterigmatocystin (ST), along with the structural relationship of NA and AR and the pattern of accumulation of NA by the mutant of *A. parasiticus*, suggest that NA is an intermediate in the pathway of AF biosynthesis one step (or steps) before AR. Experimental evidence is thus provided for the pathway for AFB_1 biosynthesis as 10 acetate $\rightarrow$ NA $\rightarrow$ AR $\rightarrow$ OP $\rightarrow$ VA $\rightarrow$ ST $\rightarrow$ AFB_1.

The remarkable molecular structure of aflatoxins (AF's) and their significance as foodborne carcinogenic mycotoxins have prompted intensive studies on their occurrence, chemistry, and biological effects. Their biosynthesis by *Aspergillus parasiticus* has also been a subject of continued investigation.

Several experimental approaches have been employed by different investigators in the elucidation of the pathways for AF biosynthesis. Based on the structural characteristics of AF's and possible analogy to the synthesis of other fungal secondary metabolites, several plausible pathways were advanced with no experimental evidence (Moody, 1964; Holker and Underwood, 1964; Heathcote et al., 1965; Thomas, 1965). Particularly notable is the

Department of Environmental Toxicology, University of California, Davis, California 95616.

[1]Present address: Department of Plant Biology, University of Brasilia, 70000 Brasilia, DF, Brazil.

derivation of the substituted coumarin moiety from the polyketide hypothesis, acetate → anthraquinones → xanthones → coumarins, and the involvement of averufin (AR), versicolorin A (VA), and sterigmatocystin (ST) as biosynthetic intermediates (Thomas, 1965; Mateles and Wogan, 1967; Biollaz et al., 1970; Moss, 1972).

Biollaz et al. (1970) determined the label distribution in the molecule of ^{14}C-labeled AFB_1 synthesized from [1-^{14}C]- and [2-^{14}C]acetate (Hsieh and Mateles, 1971) and provided experimental support to the polyketide hypothesis. They suggest the possible involvement of a C_{18} polyhydroxynaphthacene compound and aversin in the biosynthesis of AFB_1. Use of mutants of *A. parasiticus* impaired in AF synthesis has led to the finding that averufin (AR; Donkersloot et al., 1968) and norsolorinic acid (NA; Lee et al., 1971; Detroy et al., 1973) were accumulated in large quantities in place of diminished AF's, indicating the possibility that the two compounds produced by the deficient mutants are precursors of AFB_1. Of the aforementioned possible intermediates, we have shown that AR and ST can be readily converted into AFB_1 by the wild type *A. parasiticus* (Lin et al., 1973; Hsieh et al., 1973). VA was also found to be readily convertible into AF's (Lee et al., 1976).

Biosynthesis of AFB_1 was also found to be inhibited by the organophosphorus insecticide dichlorvos (Rao and Harein, 1972; Hsieh, 1973). The inhibition of the biosynthesis was accompanied by accumulation of an orange pigment (OP) which was readily converted into AFB_1 (Yao and Hsieh, 1974), indicating that this pigment compound could be another possible biosynthetic intermediate. Schroeder et al. (1974) have tentatively identified the OP as versiconal acetate.

In the present study, we have attempted to incorporate radioactivity into AFB_1 from four tritiated anthraquinone and anthrone derivatives chemically synthesized to simulate the C_{18} compounds suggested by Biollaz et al. (1970) that may be involved in AF biosynthesis and ^{14}C-labeled NA produced from [^{14}C]acetate by a deficient mutant of an AF producer. The conversion data were compared with that for acetate, AR, OP, VA, and ST as reported previously to determine the precursor–product relationship between each of these compounds and AFB_1. The structures of these related compounds are shown in Figure 1.

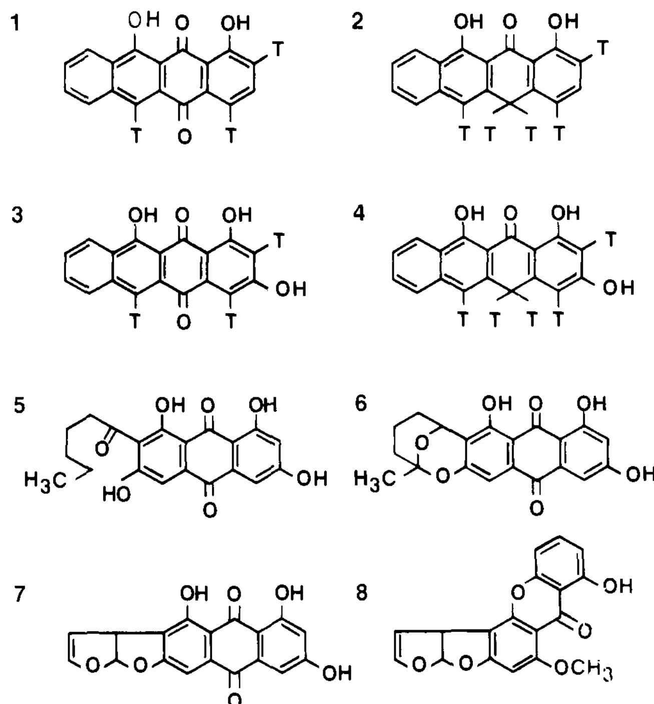

Figure 1. Structures of some possible intermediates in aflatoxin biosynthesis: 1–4, tritiated compounds I, II, III, and IV; 5–8, norsolorinic acid averufin, versicolorin A, and sterigmatocystin.

MATERIALS AND METHODS

Organisms. The fungi used in this study were an aflatoxin-producing strain, *A. parasiticus* ATCC 15517, and a NA producing mutant strain, *A. parasiticus* NRRL A-17,996. The latter was obtained from J. W. Bennett of the Southern Region Research Laboratories, New Orleans, La. Conidia were harvested from well-sporulated cultures on mycological agar (Difco) and were stored in 0.01% aqueous sodium lauryl sulfate solution.

Media and Culture Conditions. In each experiment, two chemically defined liquid media were used to cultivate the organisms in shake flask cultures. The minimum mineral (MM) medium of Adye and Mateles (1964) containing ammonium sulfate and 50 g of glucose per liter was used for the growth of the cells. The nitrogen-free resting cell (RC) medium of Hsieh and Mateles (1971) containing 90 μmol of glucose per 10 ml was used for incorporation studies and accumulation of radiolabeled compounds.

The fungal mycelia were grown in 100 ml of MM medium in 500-ml baffled conical flasks inoculated with 10^6 conidia. Incubation was carried out in a gyrotory waterbath shaker at 30 °C with continuous shaking at 100 rpm for the first day and 200 rpm, thereafter. After incubation for 48 h, the mycelial pellets were collected on cheesecloth and washed with N-free RC medium.

^{14}C-Labeled Precursors. Sodium [1-^{14}C]acetate (AC, 50 Ci/mol) was purchased from New England Nuclear, Cambridge, Mass. The tritiated polyhydroxy anthraquinone and anthrone derivatives (compounds I, II, III, and IV) were synthesized by Milne (1969) and provided to us by Dr. G. Büchi of the Massachusetts Institute of Technology.

Ring-labeled [^{14}C]NA was prepared by semicontinuous feeding of [1-^{14}C]acetate into the RC cultures of the NA-producing mutant using a technique similar to that for the preparation of [^{14}C]AR (Lin et al., 1973). NA accumulated in the mycelium was extracted with acetone and repeatedly purified by paper chromatography using Chrom AR 500 sheets (Mallinkrodt Chemical Works) developed respectively with the following solvent systems: chloroform–acetone–*n*-hexane (85:15:20, v/v, CAH); benzene–ethyl alcohol–water (46:35:19, v/v, BEW); and benzene–petroleum ether–acetone (90:8:2, v/v, BPA). The purity of [^{14}C]NA was confirmed by thin-layer chromatography (TLC) using Absorbosil-1 plates (Applied Science Lab, State College, Pa.) developed with CAH. The autoradiograph of the TL chromatogram showed a single spot, and the specific activity of [^{14}C]NA during the last two purification steps remained practically constant.

Incorporation of Radioactivity into AFB_1. Acetone and ethanol (95%) were used as vehicles for NA and compounds I to IV, respectively. Compound in 0.2 ml of solvent was placed in a 50-ml baffled conical flask to which was then slowly added 9.8 ml of RC medium containing 90 μmol of glucose. After addition of 1 g of 48-h old mycelial pellets, the flasks were shaken at 30 °C for 24 h. The same amount of solvent was added to the RC cultures containing [^{14}C]AC to check the effect of the solvent on the de novo synthetic activity of the mycelium. Autoclaved mycelium was incubated with each compound in the same

Table I. Incorporation of Radioactivity from Labeled Compounds into Aflatoxin B_1 by *Aspergillus parasiticus* ATCC15517

Labeled compd	dpm added[a]	% taken up[b]	dpm in AFB_1[c]
[^{3}H]I	0.73×10^6	42	*d*
[^{3}H]II	2.99×10^6	46	5 370 (0.18%)[e]
[^{3}H]III	8.46×10^6	77	*d*
[^{3}H]IV	5.21×10^6	79	*d*
[ring-^{14}C]-Norsolorinic acid	63.4×10^3	58	1 390 (2.19%)
[1-^{14}C]Acetate	10.8×10^6	85	16 700 (0.15%)

[a] The specific activities of the labeled compounds were 38, 680, 130, 812, 0.39, and 5.9 dpm/ng in the order listed. [b] The percentage of added radioactivity that disappeared from the medium. [c] After two TLC purifications (CAH and EPW). [d] Negligible activity. [e] The percentage of added radioactivity accountable in pure AFB_1.

manner to serve as a control.

Extraction, Purification, and Analysis of Metabolites. AF's and other metabolites in the resting cells and spent medium in each flask were exhaustively extracted with acetone and chloroform, respectively. The solvents in the pooled extracts were evaporated in vacuo at room temperature and their residues redissolved in 0.2 ml of chloroform. Metabolites in the crude extracts were purified with two-dimensional TLC using Adsorbosil-1 plates developed in CAH followed by ethyl acetate–2-propanol–water (EPW, 10:2:1). The R_f values of AFB_1 in the above two TLC systems were 0.54 and 0.85, respectively. The R_f values for NA were 0.69 and 1.0, respectively, and for compound II, the only one convertible into AFB_1, they were 0.98 and 1.0, respectively. For the determination of specific activity and relative percent incorporation, the AF's were purified by repeated TLC using the Adsorbosil-1 plates developed in CAH, EPW, and chloroform–methanol (CM, 98:2), for each compound. Metabolites on TLC were identified by co-chromatography with standards and ultraviolet absorption. Quantities of AF were measured with a Schoeffel spectrodensitometer Model SD 3000 (Schoeffel Instrument Corp., Westwood, N.J.).

For the determinations of radioactivity, samples were pipetted or scraped from TLC plates into glass vials containing 15 ml of a toluene based scintillation fluid. The radioactivity was counted in a Packard Model 2425 Tri-Carb liquid scintillation spectrometer with a counting efficiency of 84–88%, for all samples measured. To prepare an autoradiograph, a TLC plate was covered with No-Screen Medical x-ray film (Eastman Kodak Co.) and stored in darkness for 2–7 days before developing and fixing.

RESULTS

The results of the incorporation of radioactivity from

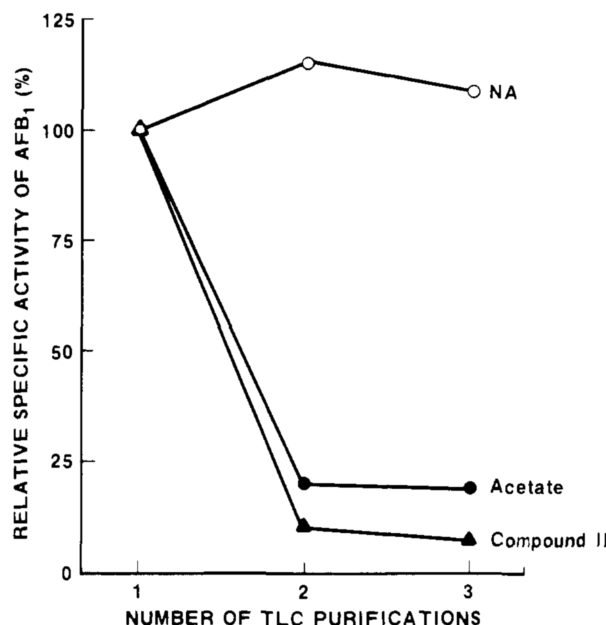

Figure 2. Change in the specific activity of labeled aflatoxin B_1 in the course of thin-layer chromatographic purifications. TLC systems: Adsorbosil-1 plates developed with (1) chloroform–acetone–*n*-hexane (85:15:20, v/v), (2) ethyl acetate-2-propanol-water (10:2:1, v/v), and (3) chloroform-methanol (98:2, v/v).

^{14}C-labeled AC and NA and the four ^{3}H-labeled compounds into AFB_1 by *A. parasiticus* are summarized in Table I. After purification with the two-dimensional TLC (2d-TLC), AFB_1 retained 2.19% and 0.18% of radioactivity from [^{14}C]NA, and compound II, respectively, as compared to 0.15% from [^{14}C]AC, under the experimental conditions. The other three tritiated compounds were not converted into AFB_1. Autoclaved mycelia did not show any incorporation of activity indicating the involvement of enzymes in these reactions.

Special care was taken to separate AFB_1 from other radioactive residues. Changes in the specific activity of AFB_1 derived from various precursors during the repeated TLC purifications are shown in Figure 2. After two TLC purifications using CAH and EPW as respective developing solvents, the specific activity of AFB_1 remained practically constant and there were no significant changes in the specific activity during the third TLC purification using CM as developing solvent. Thus, the 2d-TLC was considered adequate in separating AFB_1 from other radioactive residues.

To determine the precursor–product relationship between AFB_1 and each of the precursors, the conversion efficiency was compared in terms of relative specific activity (RSA) (Hsieh and Mateles, 1970; Lin et al., 1973) of AFB_1 derived from various precursors including AR, OP, VA, and ST from other studies (Yao and Hsieh, 1974; Lee

Table II. Relative Specific Activity (RSA) of Aflatoxin B_1 Derived from Various Labeled Precursors

Labeled precursor	Initial concn, mM	Radioact.[a] incorp., %	RSA of AFB_1 Exptl	RSA of AFB_1 Theor. max.[b]	Reference
Acetate	1.0	0.94	0.19	9	Hsieh and Mateles, 1971
Compound II	0.01	0.18	0.007	1 (?)	
Norsolorinic acid	0.05	2.2	0.19	0.9	
Averufin	0.04	49.4	0.34	0.9	Yao and Hsieh, 1974
Compound OP	0.06	13.7	0.09	0.9	Yao and Hsieh, 1974
Versicolorin A	0.06	41.5	0.475	0.9	Lee et al., 1976
Sterigmatocystin	0.04	65.0	0.42	1.0	Yao and Hsieh, 1974

[a] The percentage of radioactivity of the added precursor recovered in the purified AFB_1. [b] Number of moles incorporated per mole of AFB_1.

et al., 1976). As shown in Table II, while very low conversion efficiency was found with compound II, NA was converted into AFB_1 more efficiently than OP in terms of AFB_1, despite the lower incorporation percentage of labels from NA into AFB_1. Since these polycyclic compounds would be converted into AFB_1 without undergoing any major degradation if they are involved in AFB_1 biosynthesis, the very low conversion efficiency for compound II would indicate that it is not involved in the main pathway whereas NA is possibly an intermediate. The relatively low incorporation percentage of labels from NA and compound II cannot easily be attributed to the possible low solubility and hence poor uptake of the precursor by the mycelium, because in each case a significant portion of the added radioactivity disappeared from the medium. The mycelia of *A. parasiticus* seem to have little difficulty in taking up these polycyclic compounds in view of their high activity in converting AR, OP, VA, and ST into AFB_1 (Lin et al., 1973; Hsieh et al., 1973; Yao and Hsieh, 1974; Lee et al., 1976).

DISCUSSION

To determine whether a compound is involved in the natural biosynthetic pathway for a fungal secondary metabolite such as AFB_1, using the in vivo conversion experiments such as those described in this study, the results need to be interpreted with careful consideration of at least the following four factors: (1) uptake of the compound by the cells, (2) percentage of labels in the compound incorporated into the product, (3) relative specific activity of the product, and (4) possible involvement of the compound in the metabolic grid (Bu'-Lock, 1965) rather than the true natural biosynthetic pathway. In the present study, data (Table I) show that the uptake of compounds by cells is not a problem to obscure the conversion efficiency of each compound into AFB_1. Therefore, one can be reasonably certain that compound II is not involved in the main pathway leading to the synthesis of AFB_1 because of its low incorporation percentage into AFB_1 and the very low RSA of AFB_1 derived from it as compared to those from AR, OP, VA, or ST. Previously, Hsieh (1969) and Donkersloot (1970) both found none of the four tritiated compounds was convertible into AFB_1 by *A. parasiticus*. The present low level conversion of compound II into AFB_1 is probably attributable to the improvement in culture techniques (Lin et al., 1973). The high conversion eficiency of AR, OP, VA, and ST provides a range of values to be expected from the compounds truly involved in the main pathway of AFB_1 biosynthesis under the experimental conditions. Based upon the values, NA has to be considered as a possible intermediate in the biosynthetic pathway, but further evidence is needed to rule out the possibility of its involvement only in the metabolic grid connected to the formation of AFB_1.

NA has been found to accumulate in relatively large quantities in the mycelium of a mutant of *A. parasiticus*, induced by ultraviolet irradiation, which has lost 80% of its AFB_1 synthetic activity (Lee et al., 1971). The production of NA by the mutant culture followed a pattern very similar to that of AFB_1 (Bennett et al., 1971; Detroy et al., 1973). The molecular structure of NA ($C_{20}H_{18}O_7$) is also closely related to that of AR ($C_{20}H_{16}O_7$), an established precursor of AFB_1. Since we have found that the skeletal carbons of AR are entirely derived from acetate (Fitzell et al., 1975), we have good reason to believe that NA is also uniformly labeled by [1-^{14}C]acetate. One explanation for the significantly lower incorporation percentage of labels from NA into AFB_1 than labels from AR is that NA needs to be converted into AR before its conversion into AFB_1. Since NA is accumulated by the mutant of *A. parasiticus* deficient in AF biosynthesis, it follows that NA is involved in the biosynthetic pathway one step (or steps) before AR. The involvement of NA in AF biosynthesis was further supported by the restoration of AF production by the NA accumulating mutant when AR, OP, VA, or ST was provided to the mutant culture (our unpublished data). Recently we have found that the bisfuran ring structure in AFB_1 is derived from the nonanthraquinone portion of AR (Hsieh et al., 1976). The precursor–product relationship between AR and AFB_1 implicates that AFB_1 biosynthesis involves ten acetate units or a C_{20} polyketomethylene intermediate, a mechanism different from the involvement of only C_{18} compounds as proposed by Biollaz et al. (1970) or the involvement of C_4 and C_{14} compounds as proposed by Heathcote et al. (1973). Since the four tritiated compounds were synthesized to simulate the C_{18} compounds proposed in Biollaz's scheme of AF biosynthesis, it was expected that none of the four would be readily convertible into AFB_1. Thus, the experimental evidence indicates that the biosynthesis of AFB_1 involves ten acetate units and norsolorinic acid, averufin, an orange pigment, versicolorin A, and sterigmatocystin, but none of the derivatives of the C_{18} anthraquinone and anthrone, as intermediates.

ACKNOWLEDGMENT

The authors are indebted to G. Büchi of Massachusetts Institute of Technology for the tritiated derivatives of anthraquinone and anthrone and to J. Bennett of Tulane University for the norsolorinic acid producing mutant strain *A. parasiticus* NRRL-A-17996.

LITERATURE CITED

Adye, J. C., Mateles, R. I., *Biochim. Biophys. Acta* **86**, 418 (1964).
Bennett, J. W., Lee, L. S., Vinnett, C., *J. Am. Oil Chem. Soc.* **48**, 368 (1971).
Biollaz, M., Büchi, G., Milne, G., *J. Am. Chem. Soc.* **92**, 1035 (1970).
Bu'Lock, J. D., "The Biosynthesis of Natural Products and Introduction to the Secondary Metabolism", McGraw-Hill, New York, N.Y., 1965.
Detroy, R. W., Frier, S., Ciegler, A., *Can. J. Microbiol.* **19**, 1373 (1973).
Donkersloot, J. A., Ph.D. Thesis, Massachusetts Institute of Technology, Cambridge, Mass., 1970.
Donkersloot, J. A., Hsieh, D. P. H., Mateles, R. I., *J. Am. Chem. Soc.* **90**, 5020 (1968).
Fitzell, D. L., Hsieh, D. P. H., Yao, R. C., LaMar, G. B., *J. Agric. Food Chem.* **23**, 442 (1975).
Heathcote, J. G., Child, J. J., Dutton, M. F., *Biochem. J.* **95**, 23 (1965).
Heathcote, J. G., Dutton, M. F., Hibbert, J. R., *Chem. Ind. (London)* **21**, 1027 (1973).
Holker, J. S. E., Underwood, J. G., *Chem. Ind. (London)* **45**, 1865 (1964).
Hsieh, D. P. H., Sc.D. Thesis, Massachusetts Institute of Technology, Cambridge, Mass., 1969.
Hsieh, D. P. H., *J. Agric. Food Chem.* **21**, 468 (1973).
Hsieh, D. P. H., Lin, M. T., Yao, R. C., *Biochem. Biophys. Res. Commun.* **52**, 992 (1973).
Hsieh, D. P. H., Mateles, R. I., *Biochim. Biophys. Acta* **208**, 482 (1970).
Hsieh, D. P. H., Mateles, R. I., *Appl. Microbiol.* **22**, 79 (1971).
Hsieh, D. P. H., Yao, R. C., Fitzell, D. L., Reece, C. A., *J. Am. Chem. Soc.* **98**, 1020 (1976).
Lee, L. S., Bennett, J. W., Cucullu, A. F., Ory, R. L., *J. Agric. Food Chem.*, preceding paper in this issue (1976).
Lee, L. S., Bennett, J. W., Goldblatt, L. A., Lundin, R. E., *J. Am. Oil Chem. Soc.* **48**, 93 (1971).
Lin, M. T., Hsieh, D. P. H., Yao, R. C., Donkersloot, J. A., *Biochemistry* **12**, 5167 (1973).

Mateles, R. I., Wogan, G. N., *Adv. Microb. Physiol.* 1, 25 (1967).
Milne, G., Ph.D. Thesis, Massachusetts Institute of Technology, Cambridge, Mass., 1969.
Moody, D. P., *Nature (London)* **202**, 188 (1964).
Moss, M.O., in "Phytochemical Ecology", Halborne, J. B., Ed., Academic Press, London, 1972, pp 125–144.
Rao, H. R. G., Harein, P. K., *J. Econ. Entomol.* **65**, 988 (1972).
Schroeder, H. W., Cole, R. J., Grigsby, R. D., Hein, H., Jr., *Appl. Microbiol.* **27**, 394 (1974).
Thomas, R., in "Biogenesis of Antibiotic Substances", Venek, Z., and Hostalek, Z., Ed., Academic Press, New York, N.Y., 1965, pp 155–167.
Yao, R. C., Hsieh, D. P. H., *Appl. Microbiol.* **28**, 52 (1974).

Received for review March 11, 1976. Accepted July 28, 1976. The investigation was supported in part by Public Health Service Grant No. ES00612 and Training Grant No. ES00125 from the Division of Environmental Health Services and Western Regional Research Project W-122.

Antioxidative and Antihemolytic Activities of Soybean Isoflavones

Michael Naim, Benjamin Gestetner,[1] Aron Bondi, and Yehudith Birk*

Two properties of soybean isoflavones were examined in in vitro systems: (1) their antioxidative potency by measuring the extent of inhibition of lipoxygenase action and (2) their antihemolytic activity by measuring their ability to prevent peroxidative hemolysis of erythrocytes. The extent of the antioxidative capacity of isoflavones is positively correlated to the number of hydroxyl groups in the isoflavone nucleus. Glycosidation of isoflavones depressed their antioxidative activity considerably. A different susceptibility of erythrocytes of sheep, rats, and rabbits to the antihemolytic activity of isoflavones was found. Very high antihemolytic activity of some isoflavones such as daidzein was exerted toward sheep erythrocytes, very little and only by genistein was exerted toward rat erythrocytes, and no antihemolytic effect was noted at all on rabbit erythrocytes. A hemolysis enhancing activity of small amounts of isoflavones on rabbit erythrocytes, but not on those of sheep and rats, has been observed. The antioxidative and antihemolytic activities of isoflavones toward sheep erythrocytes were differently affected by structural differences of isoflavones.

In a previous paper (Naim et al., 1974) procedures for isolation and separation of isoflavones from soybeans and for their quantitative determination have been described, and studies on their fungistatic action reported. The wide use of soybeans in foods and feeds calls for the study of some of the biological properties of soybean isoflavones in order to find out whether detrimental and/or beneficial effects on humans and animals are exerted by the presence of isoflavones in soybean foods and feeds. Observations on antioxidative and antihemolytic activities exerted by 6,7,4′-trihydroxyisoflavone, a compound isolated from fermented soybeans, were reported by György et al. (1964) and Ikehata et al. (1968). According to some data reported in the literature, isoflavones are absorbed from the digestive tract of animals and can be partly recovered in the plasma of sheep (Lindner, 1967) and guinea pigs (Shutt and Braden, 1968), after ingestion of isoflavone-containing leguminous forages. Therefore, a systematic study of the antioxidative and antihemolytic properties of soybean isoflavones is desired and the present work deals with these activities of free isoflavones and isoflavone glycosides. Occurrence or nonoccurrence of a relationship between the extent of these activities, when examined, may permit us to draw some conclusions with regard to the mode of the antihemolytic activity of isoflavones. In the present work hemolytic assays were performed with red blood cells of rams, rats, and rabbits in order to study a possible influence of species differences in the structure of components of the erythrocyte membrane which are expressed in the effects of isoflavones.

Department of Agricultural Biochemistry, Faculty of Agriculture, Hebrew University of Jerusalem, Rehovot, Israel.

[1]Deceased June 27, 1975.

EXPERIMENTAL SECTION

Materials. Individual isoflavones and isoflavone glycosides were prepared from soybeans as described by Naim et al. (1973). Quercetin and lipoxygenase type 1 were purchased from Sigma Chemical Co. Dimethoxydaidzein was prepared by treatment of daidzein with CH_2N_2. The potent antioxidant quercetin (Crawford et al., 1961) was used as a reference compound for comparing antioxidative and antihemolytic activities of isoflavones.

Inhibition of Lipoxygenase by Isoflavones. The following two procedures were used for determining the extent of inhibition of lipoxygenase activity by isoflavones. (a) The inhibitory effect of isoflavones on the coupled oxidation of β-carotene and polyunsaturated fatty acids by soybean lipoxygenase was measured by spectrophotometric determination of the disappearance of carotene according to Ben Aziz et al. (1971). Tween 80 was replaced by Tween 20 for solubilizing carotene and linoleic acid in the suitable buffers. (b) Uptake of oxygen by linoleate in the presence of lipoxygenase was measured according to the procedure developed by Grossman et al. (1969). Procedure b permits the performance of the lipoxygenase test in the presence of higher concentrations of isoflavones since the measurement of oxygen absorption, unlike the spectrophotometric determination of carotene bleaching, is not interfered by turbidity resulting from the insufficient solubility of isoflavones, even in the presence of Tween.

Determination of Antihemolytic Activity of Isoflavones. The preventive action of isoflavones and of quercetin against the hemolytic effect of H_2O_2 solutions on washed red blood cells of sheep, rats, and rabbits was examined by the in vitro test of Segal et al. (1966) as adopted for our purposes, using citrated blood. The blood