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INHIBITION OF THE MUTAGENICITY OF AMINO ACID PYROLYSIS PRODUCTS BY HEMIN AND OTHER BIOLOGICAL PYRROLE PIGMENTS

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Summary Inhibition of the mutagenic activities of the amino acid pyrolysis products by hemin and other biological pyrrole pigments was investigated using the Ames' Salmonella/microsome system. Hemin, biliverdin and chlorophyllin showed inhibition to all the six mutagens tested, and protoporphyrin to three of them. Hemin was the most effective among these pigments; e.g., the mutagenicity of 1.8 nmole Trp-P-1 (3-amino-1,4-dimethyl-5H-pyrido[4,3-b]indole) was suppressed to 50 % by 1-3 nmole of hemin. Hemin appears to interact with the metabolically activated form of Trp-P-1 and as a result to inhibit the mutagenicity.

INTRODUCTION

Recent demonstration of the existence of strongly mutagenic compounds in the pyrolysates of amino acids and of certain foods (1,2,3) has aroused concern over the possibility that man has been, and will continue to be, exposed to these mutagens.

Modification of mutagenesis has been a subject of current interest (4). Search for natural existence of modifying factors (or compounds) for these pyrolysate-mutagens is obviously important to evaluate the hazard these mutagens can inflict on man. We have recently reported the enhancing effect of cysteine and its derivatives on the mutagenicity of tryptophan pyrolysis products, 3-amino-1,4-dimethyl-5H-pyrido[4,3-b]indole (Trp-P-1) and 3-amino-1-methyl-5H-pyrido[4,3-b]indole (Trp-P-2) (5). We now wish to describe inhibitory effects of hemin and related pigments on several amino acid pyrolysis mutagens.

MATERIALS AND METHODS

Reagents Trp-P-1, Trp-P-2, 2-amino-6-methyl-dipyrido[1,2-a:3',2'-d]imidazole (Glu-P-1), 2-amino-dipyrido[1,2-a:3',2'-d]imidazole (Glu-P-2), 2-amino-9H-pyrido[2,3-b]indole (Amino-α-carboline), and 2-amino-3-methyl-9H-pyrido[2,3-b]-indole (Aminomethyl-α-carboline) were gifts from Dr. M. Nagao. The first two

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compounds are the mutagens found in the pyrolysis products of tryptophan, the next two those of glutamic acid, and the last two those of soy bean. Hemin was from Wako Pure Chemicals, disodium protoporphyrin IX and chlorophyllin (Cu-Na salt) from Nakarai Chemicals, and biliverdin and bilirubin from Sigma.

Inhibition of mutagenesis by pigments The reversion of Salmonella typhimurium TA 98 (His^- to His^+) by treatment with the mutagens was detected by the method of Ames et al.(6) as modified by Yahagi et al.(7). Liver S-9 was prepared from rats induced with polychlorinated biphenyl. Since the pigments used were sensitive to light, the inhibition experiments were carried out in a dimly lighted room and the reaction mixtures were kept from light by covering the test tubes with aluminum foil. The order of mixing the components was the mutagen, the pigment, S-9 mix, and bacteria. The total volume was 0.7 ml. 10 µl of S-9 was used except where indicated otherwise. The mixture was incubated at 37°C for 20 min with gentle shaking, soft agar (2 ml) was added to it, and the whole mixture was poured into an agar plate. The revertant colonies were counted after 2 days of incubation in the dark at 37°C. At least two independent experiments were done for each set of reaction. In these experiments, it was always confirmed by microscopic examination that the mutant colonies were observable on the surface of a lawn of unmutated bacteria; this ensured that the observed effect was not a mere killing.

Preparation of activated Trp-P-1 An aqueous solution (0.10 ml) of 5 nmole Trp-P-1 was mixed with S-9 mix (0.50 ml) containing 10 µl of S-9, and the mixture was incubated at 37°C for 20 min with shaking. Acetone (0.60 ml) was then added, and the mixture was allowed to stand in an ice-bath for 15 min. The mixture was centrifuged at 3000 rpm for 10 min at 0°C. The supernatant was collected and evaporated to dryness. The residue, which contained the activated Trp-P-1, was dissolved in water and was subjected to the mutagenesis assay.

RESULTS

Hemin strongly inhibited the mutagenesis by Trp-P-1 (Fig. 1, solid line). With only 1 nmole of hemin, the mutagenesis by 1.8 nmole of Trp-P-1 was inhibited to an extent of 50 %. With 100 nmole of hemin, the mutagenesis was completely inhibited. (Explanation for the dashed line in Fig. 1 is given below.)

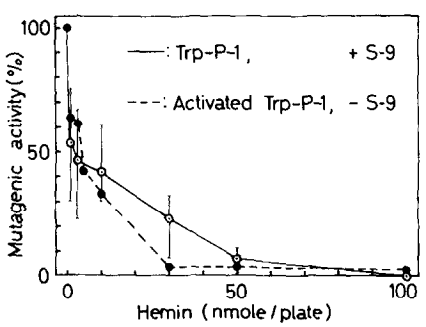

Figure 1. Effect of hemin on the mutagenicities of Trp-P-1 and the activated Trp-P-1. The solid line represents the effect of hemin on the mutagenicity of 1.8 nmole Trp-P-1. The vertical lines indicate the range of observed values in five independent experiments, and the points (⊙) are the averages of these values. The dashed line with points (●) shows the effect of hemin on the mutagenicity of 2 nmole-equivalent activated Trp-P-1.

Table I. Inhibition by pyrrole pigments of the mutagenicity of amino acid pyrolysis products

Mutagen	Amount of mutagen (nmole per plate) [a]	Dose of pigments required for 50 % inhibition (I_{50}) and for > 95 % inhibition (I_{95}), (nmole/plate)							
		Hemin		Chlorophyllin		Biliverdin		Protoporphyrin	
		I_{50}	I_{95}	I_{50}	I_{95}	I_{50}	I_{95}	I_{50}	I_{95}
Trp-P-1	1.8	3	75	100	200	200	750	50	300
Trp-P-2	0.1	20	50	200	300	500	2000	100	300
Glu-P-1	1.7	75	200	100	300	300	1000	(-)	(-)
Glu-P-2	9.1	40	100	150	300	200	750	(-)	(-)
Amino-α-carboline	80	30	100	150	300	500	2000	(-)	(-)
Aminomethyl-α-carboline	250	25	100	200	500	500	2000	50	500

a) Numbers of S. typhimurium TA 98 revertants per plate found in the absence of the pigments were 7800-16900 with Trp-P-1, 975-2170 with Trp-P-2, 18200-33000 with Glu-P-1, 3380-8640 with Glu-P-2, 3400-9750 with Amino-α-carboline, and 1370-6500 with Aminomethyl-α-carboline.

By experiments of this type, inhibitory activities of several biological pyrrole pigments against the amino acid pyrolysis mutagens were found. The amounts of pigments required for 50 % and > 95 % inhibition for each mutagen are listed in Table I. Hemin, chlorophyllin and biliverdin inhibited the activities of all the six mutagens tested. The strength of inhibition was in the order hemin, chlorophyllin and biliverdin for every mutagen. Protoporphyrin inhibited the mutagenicity of Trp-P-1, Trp-P-2 and Aminomethyl-α-carboline. Bilirubin did not inhibit the mutagenicity of any one of these mutagens. Ferrous chloride and ferric chloride were without effect.

It is known that the mutagenicity of Trp-P-1 is highest at a certain concentration of S-9 (8). If the effect of hemin is simply to "consume" the S-9 and as a result to inhibit the activation of Trp-P-1, then an experiment in which hemin is added to Trp-P-1 in the presence of a large, non-optimum amount of S-9 would be expected to result in enhancement, rather than inhibition, of the mutagenicity. This was found not to be the case. As shown in Table II, at three levels of S-9 concentration hemin was inhibitory. Although the efficiency of the inhibition by hemin became lower as the amount of S-9 was increased, no enhancement of the mutagenicity was observed at various doses of hemin.

Trp-P-1 was incubated with S-9 mix and the metabolically transformed compound was separated from the enzymes by acetone precipitation. This compound

Table II. Inhibition at various S-9 doses [a)]

	His^+ revertants (TA 98)/plate		
	Dose of S-9:		
	10 µl	25 µl	50 µl
Trp-P-1 (1.5 nmole) only	7850	1210	682
Trp-P-1 (1.5 nmole) + hemin (3 nmole)	1820	838	517
Trp-P-1 (1.5 nmole) + hemin (100 nmole)	24	392	450

a) The inhibitions at three levels of S-9 were studied at ten doses (1-200 nmole) of hemin, and only the results for the two hemin doses are presented in this table. At all the other doses of hemin, no enhancement of the mutagenicity was observed.

Table III. Mutagenicity of activated Trp-P-1

	His^+ revertants (TA 98)/plate	
	- S-9	+ S-9
Activated Trp-P-1 a), Experiment 1	14700	-
Experiment 2	12700	-
Trp-P-1 (5 nmole)	43	21500

a) Activated Trp-P-1 was prepared from 5 nmole Trp-P-1 as described in Materials and Methods.

showed the mutagenic activity in the absence of S-9 mix (Table III). The activity recovered in the acetone soluble fraction was 60-70 % of the mutagenicity of Trp-P-1 subjected to the incubation. A control experiment was run in which the S-9 mix, without added Trp-P-1, was similarly incubated and subjected to the acetone precipitation, and the enzymatic activity of the acetone soluble fraction was checked. As expected, this fraction did not activated Trp-P-1 in the mutation assay. Since Trp-P-1 itself did not show mutagenicity in the absence of S-9 (Table III), the mutagenic activity found for the acetone soluble fraction (Table III) must be that of the Trp-P-1 derivative(s) formed by the enzymatic transformation of Trp-P-1.

Hemin inhibited the mutagenicity of this activated Trp-P-1 (Fig. 1, dashed line) to similar extents as it did for Trp-P-1 itself.

DISCUSSION

These results have shown that the inhibition by hemin of the mutagenicity of Trp-P-1 is probably due to its chemical interaction with the activated form of Trp-P-1. We have found that either hemin or protoporphyrin can form a complex with Trp-P-1 (to be published elsewhere). The protoporphyrin-Trp-P-1 complex was mutagenically active in the presence of S-9 mix. It is conceivable that a complex formation of this kind can take place between the activated Trp-P-1 and the hemin, and that the resulting complex is inactive.

We consider it unlikely that hemin can interact with the membrane of S. typhimurium and as a consequence the mutagens can no longer enter into the bacteria, because the concentration of hemin required for the inhibition varies greatly from one mutagen to another (Table I).

The method to isolate the activated Trp-P-1 was also applicable to Glu-P-1. In these cases the active compounds appear to be considerably stable. Where such proximate (or ultimate) mutagens are unstable, e.g., in the case of dimethylnitrosamine, this procedure was not applicable.

A result similar to the one found for the activated Trp-P-1 was obtained for the activated Glu-P-1: hemin inhibited the mutagenicity of activated Glu-P-1 (data not shown). This has suggested that the mechanism of the inhibition is the same in Glu-P-1 and Trp-P-1. The mechanisms of inhibition by hemin for the other mutagens may or may not be the same. It is possible that in some of these cases hemin can modify the microsomal enzymic reactions, since it is known that hemin can inhibit the hepatic microsomal benzo[a]pyrene hydroxylation (9) and that hematin can stimulate monooxygenase activities in extra-hepatic tissues (10). The mechanisms operating with the other pigments, chlorophyllin etc, are also yet to be elucidated.

Heme is the prosthetic group of many proteins including hemoglobin and microsomal enzymes. It is derived from protoporphyrin and is metabolized by microsomal heme oxygenase into biliverdin. Hemin has been of biological interest because it plays a regulatory role in protein synthesis in erythrocytes (11) and because it stimulates neurite growth in cultured neuroblastoma cells (12).

The fact that a variety of biological pigments can inhibit the mutagenicity of amino acid pyrolysis products indicates the possibility that humans are equipped with intrinsic mechanisms to protect themselves from these mutagens. Furthermore, the ability of chlorophyllin to inhibit the mutagenesis suggests the presence of protective agents in foods. Relevant to this is the recent report on the inactivation of tryptophan pyrolysate mutagens by vegetable juice (13)

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