that we have found X-prolyl dipeptidyl-aminopeptidase activity in human brain[13]. We have not yet compared molecular properties of the enzymes isolated from cerebrospinal fluid and from serum, but we have preliminary data indicating that the molecular properties of the brain enzyme are different from those of the serum enzyme (unpublished results). We have also found that the homogeneous enzyme from human submaxillary gland hydrolyzes N-terminal dipeptide Arg^1-Pro^2 and subsequent dipeptide Lys^3-Pro^4 from substance P, a putative neurotransmitter[14]. The physiological and pathological significance of this enzyme in cerebrospinal fluid remains for further investigation.

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The use of cell free extracts derived from fungal protoplasts in the study of aflatoxin biosynthesis

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Summary. A supernatant fraction derived from protoplasts of *Aspergillus flavus* was shown to be capable of converting both sterigmatocystin and versiconal hemiacetal acetate to aflatoxin B_1. Versicolorin A was not converted under the same conditions.

The isolation and characterization of the individual enzymes involved in the biosynthesis of aflatoxins is a difficult undertaking as the liberation of these labile enzymes by mechanical means can result in their denaturization[1,2]. In order to overcome this difficulty the technique of digesting the cell wall has been employed, using lytic enzymes derived from *Trichoderma viride*, resulting in the formation of fungal protoplasts[3]. These protoplasts have already been shown to be capable of synthesizing aflatoxins[4], hence the results reported here describe the isolation of a cell-free extract from lysed *Aspergillus flavus* protoplasts capable of converting ^{14}C versiconal hemiacetal acetate to aflatoxin B_1.

Materials and methods. Protoplasts were isolated from 3- and 4-day-old *Aspergillus flavus* mycelium as previously described[4] and collected by centrifugation at 500×g for 10 min. The pellet was shaken with 5 ml 0.1 M phosphate buffer, pH 8.0 and frozen for 30 min at 0 °C. The resulting slurry was then thawed and centrifuged (10,000×g for 30 min) to yield a supernatant fraction which was utilized as the cell-free extract, and a residue fraction. Protein was estimated using the Biuret method (1 ml of extract). The remaining extract (4 ml) was added to a cofactor medium[6] (1 ml) to give a final concentration of FAD (10^{-6} M), EDTA (10^{-3} M), methionine (10^{-3} M), dithiothreitol (10^{-3} M), NADPH (1 µmole), NADH (1 µmole) and the labelled substrate dissolved in NN-dimethylformamide. Labelled substrates were prepared after the method of Yao and Hsieh[7]. The 'cell-free' extract was incubated in a standard Warburg flask at 30 °C and shaken constantly. At

Conversion of added compounds to aflatoxin B_1 by a supernatant fraction isolated from lysed protoplasts of *Aspergillus flavus**

	Substrate added**	Incubation period (h)	Aflatoxin B_1 formed µCi recovered	Specific activity (mCi/mole)	Percent conversion***
A	(G)^{14}C Versiconal hemiacetal acetate	1	0.0000072	0.9	3.6
		18	0.0000192	2.0	9.6
B	(G)^{14}C Versiconal hemiacetal acetate	18	0.0000002	0.02	0.1
C	(G)^{14}C Versiconal hemiacetal acetate	1	0.0000153	1.9	7.7
	(G)^{3}H Sterigmatocystin	1	0.000056	ND	28.0
	(G)^{14}C Versicolorin A	1	zero	–	–
		18	zero	–	–

* All results are an average of essentially reproducible duplicate experiments. ** 0.0002 µCi of sp. act. 3.8 mCi/mole were added in each experiment. *** $\frac{\text{µCi product formed}}{\text{µCi precursor added}} \times 100$. A, Fraction derived from protoplasts of 3-day-old mycelium; B, residue fraction derived from protoplasts of 3-day-old mycelium; C, fraction derived from protoplasts of 4-day-old mycelium. ND, not determined.

various time intervals portions of the incubation mixture were removed and the aflatoxin B_1 was extracted and estimated[8] and counted as described[4]. The added precursor was also isolated and measured in a like manner. Aflatoxin B_1 was isolated from zero time samples in order to ascertain its initial concentration and activity. These results were subtracted from subsequent values in order to allow for physical association of substrate with product and for aflatoxin B_1 not synthesized de novo.

Results and discussion. The table shows that the supernatant fraction of lysed protoplasts plus added cofactors is capable of converting versiconal hemiacetal acetate and sterigmatocystin to aflatoxin B_1. This result was supported by a commensurate loss of precursor. The much higher conversion of sterigmatocystin clearly reflects its close proximity to aflatoxin B_1 in the metabolic pathway.

As the residue fraction, which contains mitochondria and cell membranes, was not capable of such conversions it would seem that the enzymes involved in this section of the biosynthesis pathway are present in the microsomal fraction isolated from the lysed protoplasts. Hsieh and Matales[5] have shown that aflatoxins are acetate derived and probably synthesized extramitochondrially. Singh and Hsieh[1] demonstrated the conversion of ^{14}C sterigmatocystin to ^{14}C aflatoxin B_1 in the post-mitochondrial fraction of a cell-free extract derived from *A. parasiticus* ATCC 15517, they also suggest the involvement of an oxygenase in the conversion of sterigmatocystin to aflatoxin B_1. In our preparation, removal of the FAD from the incubation mixture results in an essentially zero incorporation of ^{14}C into aflatoxin B_1 indicating the presence of oxygenase(s).

It is possible that low conversion rates observed in our experiments may be due to the absence of an NADPH/NADH regenerating system. 'Cell-free' extracts of protoplasts derived from 4-day-old mycelium appear to be able to convert more substrate to aflatoxin B_1 than the 'cell-free' extracts of 3-day-old mycelium protoplasts. This may indicate a greater abundance of enzymes involved in substrate conversion in the 4-day-old material.

It was not possible to demonstrate the conversion of versicolorin A to aflatoxin B_1 even though the experiment was repeated a number of times. This result was unexpected as versicolorin A is a well-documented precursor of aflatoxin B_1[9] and because intact protoplast are capable of its conversion to aflatoxin B_1[4] it seems likely that some activating factor, present in the residue fraction, is required for its conversion. Work is currently in progress in order to clarify this point.

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Tryptophan in *Kinixys crosa* hemoglobins

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Summary. The hemoglobins, TH_I and TH_{II}, isolated from the red cells of the testudinid *Kinixys crosa* each have 3 tryptophan residues/18,000 daltons. Total number of amino acid residues for the proteins are therefore 156 and 155/18,000 daltons respectively.

The isolation and characterization of the hemoglobins in the hemolysate of the red cells of the testudinid, *Kinixys crosa* has recently been described[1]. The amino acid compositional data, which lacked information about the tryptophan content, showed these hemoglobins to be unusual in at least 2 respects: a) The protomer or repeating unit in the tetramer in each case appears to be a single polypeptide chain. b) This chain is larger than the normal vertebrate α or β chain.

For a complete picture of the amino acid compositional status of these proteins, it is of some importance to determine the tryptophan content in both.

Materials and methods. The hemoglobins TH_I and TH_{II}, as well as the corresponding globins, were prepared from the isolated red cells of *Kinixys crosa* as already described[1].

Table 1. Tryptophan content of TH_I and TH_{II}

Amino acid	TH_I nmoles	Ratio*	Integer	TH_{II} nmoles	Ratio**	Integer
Trp	139	2.50	3	161.8	2.72	3
Gly	438	7.85	8	438	7.36	7
Leu	1095	19.59	20	1138	19.13	19
Lys	668	11.95	12	654	10.99	11

* 55.9 nmoles = 1; ** 59.5 nmoles = 1.

Analysis for tryptophan. The globins (about 1 mg each) were hydrolyzed in 1 ml of 3 N mercatoethanesulfonic acid

Table 2. Amino acid compositions of *Kinixys crosa* hemoglobins

Amino acid		TH_I	TH_{II}
	His	10	11
	Lys	12	11
	Arg	4	4
	Asx	12	13
	Thr	10	7
	Ser	11	9
	Glx	12	16
	Pro	6	5
	Gly	8	7
	Ala	14	16
Half	Cys	2	1
	Val	16	14
	Met	-	1
	Ile	5	5
	Leu	20	19
	Tyr	4	5
	Phe	7	8
	Trp	3	3
Total		156	155
Mol.-wt		17,417	18,008