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

(obtained from Union Chimique Belge, Brussels) at 110 °C for 72 h following the procedure described recently[2].
Amino acid analyses were carried out at the Central Amino Acid Analysis Laboratory, Institute of Biochemistry, Uppsala, Sweden, using a Durrum 500 amino acid analyzer.
Results and discussion. Amino acid analysis data for tryptophan and representative amino acids for both proteins are given in table 1. It should be observed that the hydrolysis with mercaptoethanesulfonic acid yields results which are in good agreement with those obtained earlier using constant boiling hydrochloric acid as the hydrolyzing medium. Using a recovery rate of 91% for tryptophan following a 72-h hydrolysis of proteins in mercaptoethane sulfonic acid[2], we conclude that TH_I and TH_{II} each contains 3 residues of tryptophan per repeating unit or protomer.
The complete amino acid compositional data for both hemoglobins are summarized in table 2. TH_I and TH_{II} are made up of 156 and 155 amino acid residues respectively. These give a mol.wt of approximately 18,000 daltons for the repeating unit of each protein.

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Phosphoenolpyruvate carboxylase assay on polyacrylamide gels[1]

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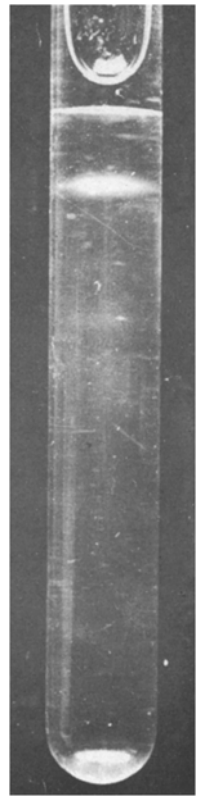
Summary. A simple technique to identify PEP carboxylase on polyacrylamide gels is described. This method involves in the formation of white ring of calcium phosphate precipitate on the reaction site, revealing the presence of enzyme protein.

Any method to detect a specific enzyme protein directly from a mixture of proteins separated on polyacrylamide gels would save considerable time and efforts involved in the routine methods of detecting the protein band with either co-electrophoresis of the purified sample or assaying for their activity after carefully eluting all the protein bands separately. Any such method would also confer a great advantage in analyzing isoenzyme types if present.
There are several methods described to detect specific enzyme proteins, such as amylases[3], acid and alkaline phosphatases[4], adenosine triphosphatase[5], malic dehydrogenase, glutamic dehydrogenase, lactic dehydrogenase[6], ribonuclease[7], malic enzyme[8], etc. on polyacrylamide gels directly. Generally the methods used for direct identification of a particular enzyme is to incubate the unstained gels after electrophoretic run in the appropriate reaction mixture along with a dye or compound that would form a coloured complex with the product(s) of the reaction mediated by the enzyme in the region of its migration on the gel. The coloured complex formed could be immobilized with suitable fixatives at the reaction site.
Phosphoenol pyruvate carboxylase (PEP-carboxylase) has gained more importance recently after the introduction of C_4 dicarboxylic acid pathway by Hatch and Slack[9] in tropical grasses. Several workers have attempted to describe various techniques to identify this protein more easily. Babson et al.[10] have first described a method to detect oxaloacetate on the gels, utilizing the diazonium dye, 6-benzamido-4-methoxy-m-toluidine diazonium chloride. Later, Karn et al.[11] described a complex method of identifying the PEP-carboxylase on the starch gel-electrophoresis. However, the colour complex formed during the incubation, with the substrate spreading quickly beyond the reaction site. Therefore, the 'developed' gels had to be 'fixed' to avoid the spreading immediately. This involved the use of 2 reaction mixtures, one for developing the activity and another for fixing the dye.
A simple technique to identify the PEP-carboxylase on the polyacrylamide gels is described herein. Phosphoenol pyruvate carboxylase catalyzes the following reaction:
Phosphoenol pyruvate + CO_2 → Oxaloacetate + Pi.
The Pi released during the carboxylation of phosphoenol pyruvate can be precipitated as white calcium phosphate in the presence of excess calcium chloride. Using this principle, the following experimental protocol was perfected for detecting the PEP-carboxylase in the polyacrylamide gels.
Protocol. Sorghum leaf mesophyll cells were homogenized in Tris-HCl buffer, pH 7.8, 100 mM, containing $MgCl_2$ and HCO_3, 10 mM each; polyvinyle pyrrolidone, 2%; EDTA, 1 mM and B-mercaptoethanol, 15 mM. The homogenate was clarified at 30,000 × g for 45 min. The clarified supernatant (approximately 100 µg in 0.1 ml) was applied to 7.5% polyacrylamide gels[12] and the electrophoretic run was carried out at 4 °C with a current of 5 mA per gel, until the marker dye bromophenol blue reached about 1 cm from



Polyacrylamide gel-electrophoresis of PEP-carboxylase of *Sorghum vulgare* stained for enzyme activity. The white precipitate reveals the site of the enzyme protein in the gel.