acid and histidine contents of the haemolymph of diseased full grown larvae were higher than those in the normal haemolymph. Those amino acids which were extremely reduced in amount by the disease were leucine, tyrosine, serine, valine, methionine, phenylalanine, threonine, ornithine and glutamic acid. These amino acids decreased by about 40, 47, 48, 51, 71, 73, 78, 84 and 87% respectively. Alanine and citrulline stayed at relatively constant levels in the haemolymph of healthy and diseased full-grown larvae. The total amount of free amino acids in the haemolymph declined from a high level of 7826 µmoles/100 ml of haemolymph in healthy full grown larvae to a low level of 4788 µmoles/100 ml of haemolymph in diseased larvae.

The results of the present investigation agree with the findings of several investigators that a marked decrease of most amino acids in the haemolymph is observed during nucleopolyhedrosis[5,6] and cytoplasmic polyhedrosis[4]. This decrease of most of the free amino acids in the haemolymph of infected full-grown larvae of *Spodoptera* could indicate that free amino acids are being used to make viral and/or inclusion body protein. These are, of course, only indications; injection of labeled amino acids and determination of the incorporation rate of these amino acids in the virus particles and inclusion bodies could prove this hypothesis.

In the present study the concentrations of lysine and histidine were higher in diseased full grown larvae than in healthy ones. Also, histidine[1,10] and lysine[4,10] accumulated during virosis. The present data differ from those obtained for *Heliothis zea* larvae[6] and for *Peridroma saucia*[5] which showed an increase of glycine during polyhedrosis.


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## Occurrence of unconjugated pterins in a higher plant, *Stizolobium hassjoo*


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*Summary.* 3 unconjugated pterins, D-erythro-neopterin, 6-hydroxymethylpterin and isoxanthopterin, were isolated from the pericarps of a leguminous plant, *Stizolobium hassjoo*.


A pyrazine derivative, stizolamine (1-methyl-3-guanidino-6-hydroxymethylpyrazin-2-one), was isolated from *S. hassjoo* (Leguminosae)[1,2]. The compound is widely distributed in leguminous seeds[3] and is biosynthesized from U-$^{14}$C-GTP[4]. The structural resemblance of stizolamine to pterins, and the commonness of the precursor, led to the inference that stizolamine might originate from a pterin(s) in the plant[4]. As a 1st approach to check the theory we have surveyed unconjugated pterins in the plant and found them in an extract from pericarps of the plant. The present paper describes isolation and characterization of 3 major pterins in the extract.

The pericarps of *S. hassjoo* (8 weeks old) were powdered in liquid nitrogen. The powder (50 g) was put into 10 vol of 5 mM 2-mercaptoethanol at 80 °C and kept at 60 °C for 1 h with occasional stirring. The suspension was then squeezed through a silk cloth and centrifuged at 6000 × g for 10 min. The precipitate was re-extracted as above. The combined supernatant was concentrated to about 50 ml at 40 °C under reduced pressure. After centrifugation, the concentrate was applied to a Dowex 1 X8 column (formate, 3 × 30 cm). On elution with distilled water, the blue fluorescent band was resolved into 3 fluorescent zones. The 1st and 2nd blue fluorescent zones were eluted with distilled water (fractions I and II) and the 3rd purple fluorescent zone with 2 N formic acid (fraction III). The main pterin in each fraction was purified by column chromatography and/or TLC. In all cases, the columns were developed with distilled water. The concentrated fraction I was applied to a QAE-Sephadex A-25 column (formate, 3 × 18 cm). The blue fluorescent eluate was concentrated and subjected to TLC (Avicel SF. ethanol/acetic acid/$H_2O$, 15:1:34, v/v). The band corresponding to D-erythro-neopterin ($R_f$, 0.49) was eluted with 5 mM 2-mercaptoethanol, concentrated and applied to a Sephadex G-25 column (3.5 × 43 cm). On evaporation to dryness, the eluate gave a pale yellow powder (2.7 mg). The material (compound 1) was converted to 6-carboxypterin by alkaline permanganate oxidation. Compound 1 was identified as D-*erythro*-neopterin by TLC (table) and spectral analyses: UV. $\lambda_{max}$, 236, 274, 344 nm ($H_2O$); fluorescence: excited, 284, 362 nm, emission, 440 nm ($H_2O$); GC-MS (TMS): $M^+$, m/e, 613 (pentakis-TMS-neopterin, 8%). The configuration of the side chain was confirmed with CD by comparing with authentic D-erythro-neopterin (0.1 N HCl); $[\theta]_{312}^{max} = +3.00 \times 10^3$, $[\theta]_{270}^{min} = -1.25 \times 10^3$ and $[\theta]_{248}^{max} = +6.25 \times 10^3$. The main pterin in

$R_f$-values of isolated pterins on TLC

| Compound | Solvent system I | II | III | IV |
|---|---|---|---|---|
| Compound 1 | 0.61 | 0.25 | 0.37 | 0.41 |
| Compound 2 | 0.48 | 0.33 | 0.46 | 0.46 |
| Compound 3 | 0.33 | 0.26 | 0.34 | 0.34 |
| $KMnO_4$ oxidation product of 1 | 0.60 | 0.28 | 0.24 | 0.24 |
| $KMnO_4$ oxidation product of 2 | 0.60 | 0.28 | 0.24 | 0.24 |
| D and L-erythro-neopterin | 0.61 | 0.25 | 0.37 | 0.41 |
| D and L-threo-neopterin | 0.61 | 0.23 | 0.19 | 0.28 |
| 6-Hydroxymethylpterin | 0.48 | 0.33 | 0.46 | 0.46 |
| Isoxanthopterin | 0.33 | 0.26 | 0.34 | 0.34 |
| 6-Carboxypterin | 0.60 | 0.28 | 0.24 | 0.24 |
| 7-Carboxypterin | 0.60 | 0.28 | 0.32 | 0.28 |

Solvent systems: I; 0.1 M Na-phosphate buffer (pH 6.0); II, *n*-propanol/ethyl acetate/$H_2O$ (7:1:2); III, ethanol/5% ammonium borate/3% $NH_4Cl$ (2:1:1); IV, *n*-propanol/1% $NH_4OH$ (2:1). Avicel SF plates (Asahi Chemical Industries).

fraction II (compound 2) was purified by column chromatography in the following order: QAE-Sephadex A-25 (formate, 2×21 cm), phosphocellulose ($H^+$, 1.7×21 cm) and Sephadex G-25 (3.5×43 cm). Yield: 2.1 mg. The compound was also oxidized to 6-carboxypterin by alkaline permanganate and identified as 6-hydroxymethylpterin: TLC (table); UV: $\lambda_{max}$, 273, 345 nm ($H_2O$); fluorescence: excited, 283, 368 nm, emission, 444 nm ($H_2O$); MS: $M^+$, m/e, 193 (2%). GC-MS (TMS): $M^+$, m/e, 409 (tris-TMS-6-hydroxymethylpterin, 100%). The pterin in the fraction III (compound 3) was purified by the following column systems and TLC: QAE-Sephadex A-25 (formate, 2.4×22 cm); CM-cellulose ($H^+$, 2.1×24 cm); TLC (Avicel SF, 0.1 M Na-phosphate buffer, pH 6.0) and Sephadex G-25 (3.5×43 cm). Yield: 1.1 mg. The compound was identified as isoxanthopterin: TLC (table); UV: $\lambda_{max}$, 285, 339 nm ($H_2O$); fluorescence: excited, 293, 350 nm, emission, 407 nm ($H_2O$); MS: $M^+$, m/e, 179 (100%); GC-MS (TMS): $M^+$, m/e, 395 (tris-TMS-isoxanthopterin, 71%).

It is well known that folate derivatives are widely distributed in higher plants and phosphate esters of 7,8-dihydro-D-erythro-neopterin and 6-hydroxymethylpterin are intermediates in the biosynthesis of dihydrofolate[5,6]. In spite of these facts, there have been few reports of the presence of unconjugated pterins in higher plants, though 6-hydroxymethylpterin has recently been isolated from the chloroplasts of spinach leaves[7]. By contrast, unconjugated pterins are widely found in both animals and microorganisms[8]. In the present work, in addition to 6-hydroxymethylpterin we have isolated D-erythro-neopterin and isoxanthopterin from the pericarps of *S. hassjoo*. This seems to be the first report demonstrating the presence of the latter 2 pterins in plant materials. *S. hassjoo* seems to be peculiar with respect to accumulation of nitrogenous compounds such as L-DOPA in very large quantities[9], stizolamine[1] and the unconjugated pterins described here. However, the biosynthesis and mechanism of accumulation of these compounds in the plant still remain to be studied.

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## Non-linear kinetics of microsomal styrene monooxygenase after phenobarbital pre-treatment[1]

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*Summary.* Pretreatment of rats with phenobarbital, but not with 3-methylcholanthrene, induces liver microsomal styrene monooxygenase. Under these conditions the kinetic profile is not linear and can be divided into 2 distinct curves. Evidence is presented indicating that the combined treatment with phenobarbital and $CoCl_2$ destroy the high affinity enzyme, suggesting that the native cytochrome is less sensitive to the action of $CoCl_2$.

The first step in the metabolism of styrene is P-450 dependent epoxidation of the olefinic double bond by microsomal monooxygenases[3,4]. The resulting metabolite, styrene 7-8 oxide, is rapidly hydrated by microsomal epoxide hydrase to phenylethyleneglycol and then to mandelic acid, phenylglyoxylic acid, benzoic acid and hippuric acid. Since styrene 7,8 oxide is thought to be a toxic intermediate in styrene metabolism, the epoxide-forming enzyme system assumes an important role as the toxifying component. Several reports have been published by our group on the relationship existing in different animal species and in different organs between toxifying and detoxifying enzymes[5], describing kinetic parameters and the sensitivity of both enzymes to a variety of inducers and inhibitors[6]. In the course of our experiments we noticed that in rats, after in vivo pretreatment with phenobarbital, but not with 3-methylcholanthrene, the epoxide forming enzyme system was significantly induced and it showed non-linear kinetics.

This paper reports a detailed study of this phenomenon, resolving the monooxygenase activity into 2 components, 1 with high affinity for the substance and the other of lower apparent affinity. Evidence is also provided that the de novo synthesized cytochrome is much more sensitive to the action of $CoCl_2$ than the native cytochrome.

*Materials and methods.* Male CDF-COBS rats with a b. wt of 175±20 g were obtained from Charles River Italy (Calco, Como). The rats were given a commercial laboratory chow and water ad libitum and kept in air conditioned quarters (60% relative humidity, 22 °C) with a 12-h light-dark cycle. Animals were pretreated with phenobarbital i.p. at a dose of 80 mg/kg daily for 3 days; $CoCl_2$ was administered as a single dose of 40 mg/kg. In the combined treatments $CoCl_2$ was given 1 day after the last phenobarbital injection. After the last treatment rats were fasted for 16 h before sacrifice. Microsomes were isolated according to Kato and Takayanaghi[7] and their P-450 or P-448 content was measured according to Omma and Sato[8]. Styrene monooxygenase and epoxide hydrase activities were measured using styrene and styrene oxide, respectively, as substrates, as previously described[9]. Proteins were measured according to Lowry et al.[10]. Apparent Km and Vmax values were calculated by Woolf plots, the curves being resolved into 2 components as described by Greenlee and Poland[11].

*Results and discussion.* The Figure shows the Michaelis-Menten curves for styrene monooxygenase from control (curve No. 1) and phenobarbital pretreated (curve No. 2) rats. The kinetic profile in the phenobarbital pretreated animals is not linear and can be divided into 2 distinct