Preparation of optically pure juvenile hormone I labelled in the ester methyl group with tritium at very high specific activity

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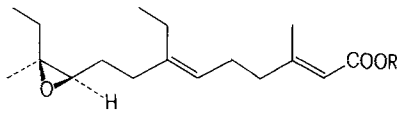
Summary. Radiolabelled juvenile hormone I (**3**) was prepared in an optically pure form by an enzymatic method using the capacity of accessory sex gland homogenates from male *Hyalophora cecropia* to transfer the methyl group of S-adenosyl-L-methionine to the carboxylic acid function of the (10R,11S)-enantiomer of racemic precursor **2**; the specific activity of **3** is higher than 53 Ci/mmole (1.96 TBq/mmole).

There is considerable interest in the availability of insect juvenile hormones (JH) radiolabelled with very high specific activity as a tool for the study of hormone action and metabolism. One of the major problems arising with the synthesis of e.g. JH-I (**1**) by means of classically chemical procedures[2,3], results from the necessity of reacting at least µmole quantities of precursors with corresponding amounts of highly radioactive components. In addition, the purification of the desired product from the reaction mixture may present another difficulty.



1, R = CH_3
2, R = H
3, R + CT_2H

In the silk moth *Hyalophora cecropia* (L.), JH-I (**1**) is accumulated exclusively in the accessory sex glands of the males[4]. As was shown by injection of JH-I into intact males, the process of accumulation is connected with an exchange of the methyl ester function[5], indicating the participation of a methyl transferase enzyme[6,7]. The substrates of this enzyme are the acid **2** (JH-I-acid) and S-adenosyl-L-methionine[8,9].

We now report the preparation of methyl(E,E)-(10R,11S)-10,11-epoxy-7-ethyl-3,11-dimethyltridecadienoate (JH-I, **1**) labelled with tritium in the methyl ester group (formula **3**) at a specific radioactivity higher than 50 Ci/mmole (1.85 TBq/mmole) by using the capacity of a cell-free system from accessory sex glands of newly eclosed male *Hyalophora cecropia* to transfer methyl groups from S-adenosyl-L-methionine to the (10R,11S)-enantiomer of racemic acid **2**.

Accessory glands were removed from 7 male moths within 12 h after eclosion, homogenized in an all glass homogenizer with 2 ml 0.05 M tris-HCl buffer pH 7.2 at 0 °C and centrifuged for 10 min at 12,000 × g and 1 °C. In order to remove possible endogenous JH-I[6] and S-adenosyl-L-methionine, the supernatant was chromatographed at 4 °C on a 1.3 × 38 cm Sephadex G-25 (fine grade, Pharmacia) column with 0.05 M tris-HCl buffer pH 7.2. The effluent was monitored continuously at 280 nm and collected in 1.5-ml fractions at a flow rate of 11 ml/h. The efficiency of separation was checked by previous addition of 2.5 ng (0.1 µCi; 3.7 kBq) [7-ethyl-^{3}H]-JH-I (NEN-Chemicals) to the supernatant: 13% of the radioactivity cochromatographed with the high molecular weight material eluting in the void volume between 19 and 25 ml. The corresponding fractions No. 13–16 were combined. Examination of the ethyl acetate extract from an aliquot by gas chromatography on a 0.32 mm × 50 m glass capillary column coated with FFAP showed that the amount of JH-I, if present at all, was below the detection limit of 50 ng per gland pair equivalent[10]. The remaining protein solution was divided into 5 batches which were quickly frozen in liquid nitrogen and stored at −80 °C; they exhibited full enzymatic activity for at least 5 months.

Preparation of radiolabelled JH-I (**3**) was performed by mixing 200 µl of the crude enzyme solution containing 0.2 gland pair equivalents with 7 µg racemic JH-I-acid (**2**)[11] and 10 µCi (370 kBq) [methyl-^{3}H]-S-adenosyl-L-methionine (58.8 Ci/mmole; 2.18 TBq/mmole) (NEN-Chemicals) in 20 µl ethanol/0.001 N sulphuric acid = 9:1. The vials containing the slightly turbid reaction mixture were shaken gently during 2–3 h at room temperature. The combined ethyl acetate extracts from 6 simultaneous incubations were subjected to TLC on silica gel PF-60 (Merck) with benzene/5% ethyl acetate, followed by high performance liquid chromatography on a 0.4 × 31 cm µ-porasil column (Waters GmbH) with n-hexane/1.25% ethyl acetate/0.15% 2-propanol at a flow rate of 1.5 ml/min. Monitoring of the effluent at 230 nm (limit of detection for JH-I: 50 ng) indicated a total amount of less than 50 ng JH-I containing a radioactivity of 9.04 µCi (334 kBq). Thus the sepcific activity is calculated to be at least 53.2 Ci/mmole (1.97 TBq/mmole).

Optical purity of the radiolabelled JH-I (**3**) was determined after dilution with racemic unlabelled JH-I (ECO Chemical Intermediates), derivatization to methyl d,l-threo-(E,E)-10,11-dihydroxy-7-ethyl-3,11-dimethyltridecadienoate (JH-I-diol)[12] followed by reaction with (+)-α-methoxy-α-trifluoromethylphenylacetic acid chloride to the corresponding diastereomeric esters[13,14]. Upon resolution on a 0.4 × 31 cm µ-porasil column (Waters GmbH) with n-hexane/8% ethyl acetate/0.03% 2-propanol at a flow rate of 1.5 ml/min, 96% of the totally recovered radioactivity eluted with the faster moving diastereomer derived from (10R,11S)-JH-I (**1**). Considering the fact that the epoxide ring opening to JH-I-diol is 97% stereospecific[15], we conclude that the radiolabelled material prepared is optically pure JH-I (**3**).

We do not yet have any information on the stability of JH-I (**3**) labelled with tritium at such a high specific radioactivity during prolonged storage. The relatively simple procedure of the enzymatic preparation, however, should make it possible to prepare new batches whenever required. The main advantage of this methodology is considered to reside in the need of only small quantities of radioactivity in order to obtain substantial amounts of highly specifically radioactive and optically pure JH-I for further studies.

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Immunochemical identity of dipeptidyl aminopeptidase IV from pig serum, liver, submaxillary gland and kidney

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Summary. The enzymes which were extracted by autodigestion from the microsomal fractions of the pig kidney, liver and submaxillary gland and from the serum showed an immunochemical identity by a double immunodiffusion test. But the kidney enzyme had a different pI-value from the pI-values of the enzymes of other organs.

Dipeptidyl aminopeptidase IV, discovered and designated as glycyl-proline-2-naphtylamidase by Hopsu-Havu and Glenner[2], liberates N-terminal glycyl-L-proline from either glycyl-L-proline-2-naphtylamide or peptides. It was purified from the rat liver[3], pig kidney[4-7], human submaxillary gland[8] and lamb kidney[9]. The tissue distribution of the enzyme in the rat was examined histochemically[10]. The clinical study of the enzyme showed that the enzyme activities in the human sera were increased in patients with hepatitis and decreased in patients with gastric cancer[11] and rheumatoid arthritis[12]. In our previous report[7], we showed that the enzyme in the kidney was a glycoprotein containing 18.3% of carbohydrates and had a serine residue at the N-terminal position. In order to compare the chemical nature of the enzyme in several organs of the pig, an immunochemical study has been performed using the antibodies prepared against the enzyme purified from the pig kidney in the rabbit.

Materials and methods. Fresh pig kidney, liver, submaxillary gland, and blood were obtained from a slaughterhouse. They were stored at − 80 °C. The enzyme of the kidney was purified according to the method described in our previous report[7] and used as antigen. Antibodies against the enzyme were prepared in a male rabbit (2 kg) by injecting the antigen (2.0 mg of protein dissolved in 1.0 ml of saline) intracutaneously as a mixture with an equal volume of Freund's complete adjuvant 4 times at weekly intervals. The rabbit was completely bled and the serum was collected 7 days after the last injection. The equivalent point between the antiserum and the highly purified enzyme was determinded by the method of Kabat and Meyer[13]. The homogenates of the kidney, liver and submaxillary gland were prepared in 9 vol. of 0.25 M sucrose using an Ultra Turrax homogenizer, and the microsomal fraction of each homogenate was prepared by the method of Hogeboom[14]. Solubilization of the enzyme from each microsomal fraction was effected by autolysis[4], and the solubilized enzymes from these 3 organs were separated by ammonium sulfate fractionation (55–85%). The enzyme in the serum was also separated by ammonium sulfate fractionation (55–85%) and was further purified by DEAE-cellulose and sephadex G-200 column chromatographies by the previously reported procedures[7]. The enzyme activity was assayed by the photometric method of Nagatsu et al.[15], using Gly-Pro-p-nitroanilide tosylate (Gly-Pro-pNA) as substrate. 1 unit of enzyme activity was defined as the amount of enzyme catalyzing the formation of 1 µmole of p-nitroaniline per min at 37 °C.

The effect of antiserum on the enzyme activity was studied. 30–100 ng of the enzyme protein were mixed with 0.25 ml of antiserum and incubated for 45 min at 37 °C, then the enzyme activities were measured. The antiserum was also checked for enzyme activity, and the value was substracted from the total activities as a blank. Double immunodiffusion analysis and immunoelectrophoresis were performed by the method of Ouchterlony[16] and Scheidegger[17], respectively. Isoelectric focusing in polyacrylamide gel was carried out by the thin-layer slab gel technique[18]. Equal volumes of 2 kinds of Ampholine with different pH ranges

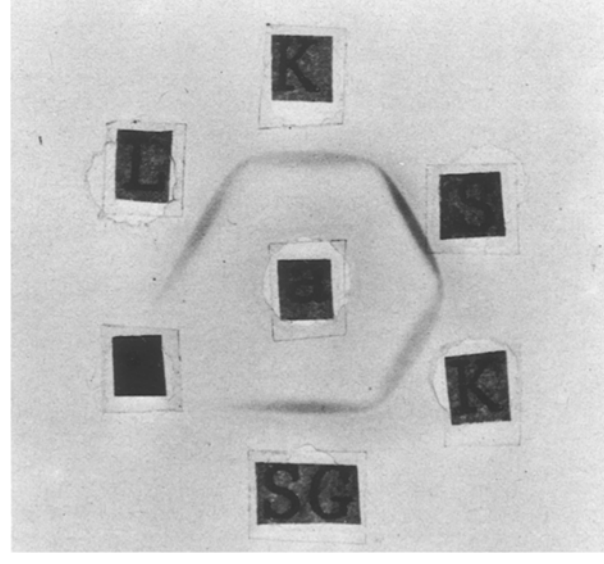

Fig. 1. Double immunodiffusion analysis of partially purified dipeptidyl aminopeptidase IV from the pig several organs. The center well (a) contained 20 µl of the antiserum to the kidney enzyme. The outer wells contained 20 µl of the partially purified enzyme. K: the kidney enzyme (10 µg); L: the liver enzyme (15 µg); SG: the submaxillary enzyme (15 µg); S: the serum enzyme (150 µg); B: blank, 0.01 M Tris-HCl buffer, pH 7.4.