gen immunologically related to *R. americana* protein 10, nor did the other insect species tested.

The *R. americana* protein 10 is monomeric and when submitted to electrophoresis in the presence of SDS, it migrates as a polypeptide called V_6 (fig. 4)[2,3]. The *R. americana* V_6 polypeptides obtained from larval hemolymph and from ovary (and eggs) are immunologically and structurally identical[3]. Since in the hemolymph of *R. hollanderi* and of *R. milleri* antigens occur which give reactions showing total or partial identity with *R. americana* protein 10, we made an electrophoretic analysis of the ovary and hemolymph proteins of these species. The results obtained (fig. 2A) show the presence of polypeptides with similar migrations to *R. americana* V_6 polypetide in the hemolymph and ovary of *R. milleri* and *R. hollanderi*. The estimated mol.wts of the V_6 polypeptides are: *R. americana:* 43,000; *R. hollanderi:* 44,300 and *R. milleri:* 45,500.

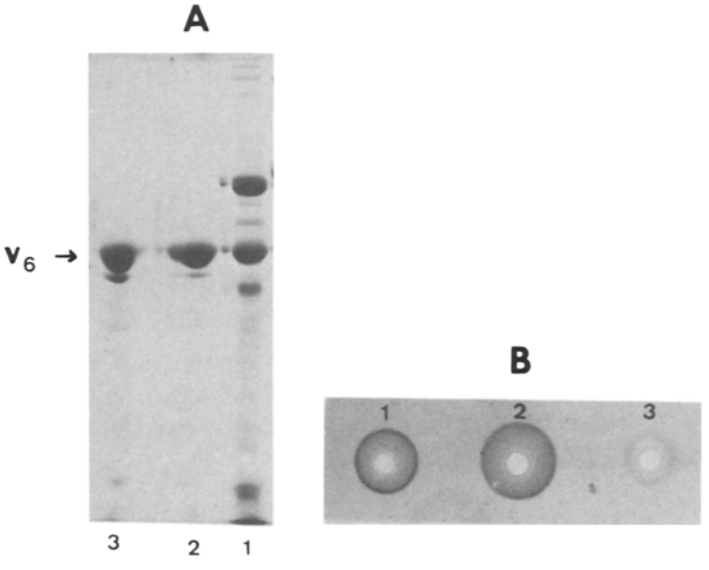

Figure 4. SDS polyacrylamide gel electrophoresis and radial immunodiffusion of protein 10 obtained from *Rhynchosciara* species. Protein 10 was obtained by cutting it out of a polyacrylamide cylinder, in which hemolymph proteins were fractionated by electrophoresis (similar to the ones in fig. 3). The protein 10 was eluted and analyzed by electrophoresis in the presence of SDS (A) and by radial immunodiffusion as described by Marinotti and Bianchi[3] (B). *A* Comparative SDS electrophoresis of *Rhynchosciara americana* hemolymph (1); *Rhynchosciara americana* protein 10 (2) and *Rhynchosciara hollanderi* protein 10 (3). *B* Radial immunodiffusion of protein 10 of *Rhynchosciara americana* (1), *Rhynchosciara hollanderi* (2) and *Rhynchosciara milleri* (3) against the serum anti-protein 10 of *Rhynchosciara americana*.

For the *R. hollanderi* protein 10 a disc electrophoresis analysis (fig. 3) shows a different migration of this native protein when compared to *R. americana* native protein 10. However, the protein 10 from *R. hollanderi* (and also from *R. milleri)* reacts with the serum anti-protein 10 (fig. 4).

When *Trichosia pubescens* hemolymph and ovary proteins were analyzed by electrophoresis (fig. 2B) no major polypeptides were present in the migration region of the V_6 polypeptide. It is also important to notice the nonexistence of any major polypeptide shared by *T. pubescens* larval hemolymph and ovaries, as occurs with the *Rhynchosciara* V_6 polypeptide.

Discussion. For the majority of the insect species studied the vitellogenins make up the bulk of the protein yolk and other hemolymph proteins make only a minor contribution[11]. In the case of *R. americana*[3] and, as our present results suggest, in all the analyzed species of the genus *Rhynchosciara,* the larval protein 10 makes an important contribution to the egg protein pool. In the larval hemolymph of *Bombyx mori* a group of four proteins, designated 30 K by Izumi et al.[7] occurs, which is transferred to the eggs. However, in spite of similar function, these proteins do not show any immunological relation to Rhynchosciara protein 10 when tested by double immunodiffusion.

It is not apparent from the facts known about the vitellogenesis and the biology of *Rhynchosciara* why a larval protein contributes massively to the formation of the eggs in this group of insects. The results obtained support the conclusion that protein 10 is specific for the genus *Rhynchosciara.*

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Nonspecific reaction of a thiol:protein disulfide oxidoreductase with the disulfide bonds of insulin

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Summary. A thiol:protein disulfide oxidoreductase from bovine liver was isolated after separation from protein disulfide isomerase. The enzyme, after activation (reduction) with glutathione, was reacted with stoichiometric amounts of insulin and the sulfhydryl groups of the partially reduced hormone were labeled with iodo (l-^{14}C)acetamide. After separation of the insulin chains, the radioactivity was found in both the peptides, with a ratio A-chain/B-chain equal to 2/1.

Key words. Thiol:protein disulfide oxidoreductase (TPOR); insulin; disulfides; high performance liquid chromatography (HPLC).

Thiol:protein disulfide oxidoreductase (TPOR, EC 1.8.4.2) catalyzes the reduction of disulfide bonds in numerous proteins by thiols. The reaction was mainly studied in the inactivation of insulin by glutathione[1-3] and for this reason the enzyme was once known as glutathione insulin transhydrogenase.

During the past two decades several authors reported the char-

acteristics of preparations of TPOR more or less purified. The mol.wts ranged particularly widely, between 40,000 and 60,000[4-8]. Moreover the presence of a second activity of protein disulfide isomerase (PDI, EC 5.3.4.1), able to reactivate 'scramble' ribonuclease, was discussed by some authors[9-12].

In our laboratory the purification of TPOR from bovine liver led to different results starting from distinct livers, and, while two livers yielded a single protein of 60,000 mol.wt, showing both the activities of TPOR and PDI, eight other livers gave, at the final step, two proteins of mol.wts respectively of 34,000 and 44,000, the first enzyme representing only a TPOR and the second one a PDI. These two enzymes could be separated by covalent chromatography according to the procedure of Hillson and Freedman[10].

In the present paper we study the reaction of TPOR, separated by PDI and previously activated (reduced) with glutathione, with stoichiometric amounts of insulin in order to establish whether the three disulfide bonds in the hormone react similarly with the enzyme or whether the reduction of insulin follows an ordered sequence.

Materials and methods. Iodo(1-^{14}C)acetamine (53 mCi/mmole) and ^{125}I-monoiodoinsulin (50 μCi/μg) were obtained from the Radiochemical Centre (Amersham, England). ^{125}I-monoiodoinsulin was diluted before use with zinc-free insulin, prepared according to the method already described[13]. Glutathione, cytidine 2′,3′-cyclic monophosphate, yeast glutathione reductase, bovine pancreas ribonuclease A, bovine serum albumin and NADPH were purchased from Sigma (St Louis, MO). All types of Sephadex were products of Pharmacia (Uppsala, Sweden).

TPOR was isolated according to the procedure of Carmichael et al.[8] and eventually it was purified from PDI by covalent chromatography[10]. Ribonuclease was randomly oxidized with the method of Anfinsen and Haber[14], dialyzed against distilled water and freeze-dried.

TPOR activity was assayed with 0.1 mg ^{125}I-monoiodoinsulin (0.5 μCi/mg) 2 mg bovine serum albumin, 1 mM glutathione and

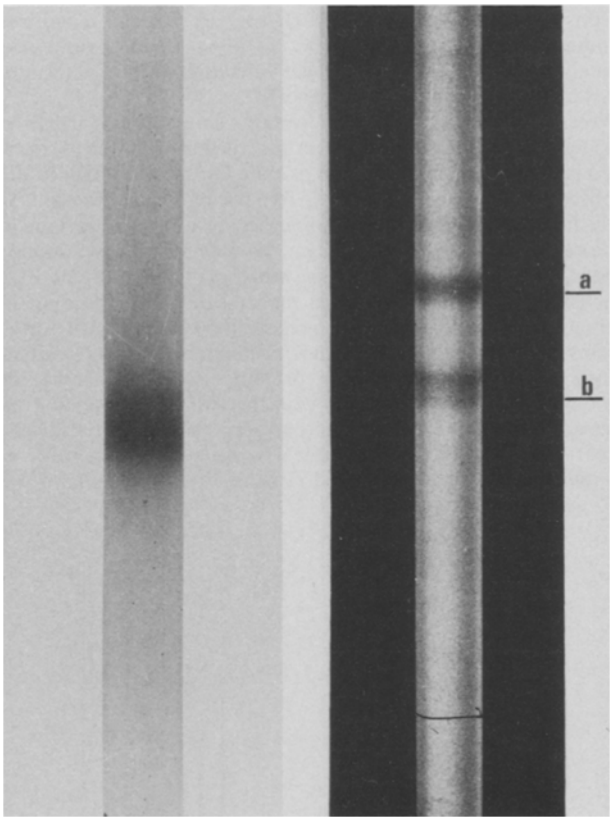

Figure 1. Two typical preparations of TPOR, from distinct bovine livers, analyzed by SDS-gel electrophoresis in polyacrylamide tubing. Left: one band of protein corresponding to an enzyme of 60,000 mol.wt showing both the TPOR and PDI activities. Right: two bands of proteins corresponding to *a* PDI, of 44,000 mol.wt and *b* TPOR, of 34,000 mol.wt. It is to note that TPOR *(b)* is split in two bands corresponding to the reduced (−SH) and the oxidized (−S−S−) forms of the enzyme. This effect, due to a partial reoxidation of the reduced enzyme in the gel, was demonstrated by running in disc-gel electrophoresis a partially reduced TPOR, then cutting the gel and assaying the enzyme activity in the slices.

Table 1

Sample	% of thiol groups labeled	Relative labeling of the peaks
A-chain	62.2	1.95
B-chain	31.8	1.00
Peak A-1	70.3	2.85
Peak A-2	24.7	1.00
Peak B-1	53.2	1.00
Peak B-2	46.8	0.95

The total C-14 recovered, due to the reacted iodo(1-^{14}C)acetamide, was about 95% of that expected on the basis of the μmoles of insulin used, likely for a small extent of reoxidation of the thiol groups during the reaction. After chymotryptic digestion of the separated chains, only peptides A-1, A-2, B-1 and B-2 were labeled by iodo(1-^{14})acetamide and this confirms a random reduction of the disulfide bonds of insulin by TPOR.

Table 2

Fraction	Amino acids and their relative amount
A-1	Gly(1.0) Ileu(0.95) Val(1.9) Glu(2.0) Cys(2.9) Ala(1.0) Ser(0.85)
A-2	Cys(0.95) Asp(0.98)
B-1	Val(2.0) Asp(0.95) Glu(1.98) His(1.95) Leu(3.0) Cys(0.85) Gly(0.97) Ser(0.98) Ala(1.0)
B-2	Leu(0.96) Val(1.0) Cys(0.90) Gly(1.98) Glu(0.94) Arg(0.98) Phe(0.96)

Amino acid analysis of the radioactive peptides obtained from the chymotryptic hydrolysis of A- and B-chains of insulin. The amino acid content of the peptides is in agreement with the results obtained by other authors (see text). Cystein was determined as carboxamidomethylated amino acid.

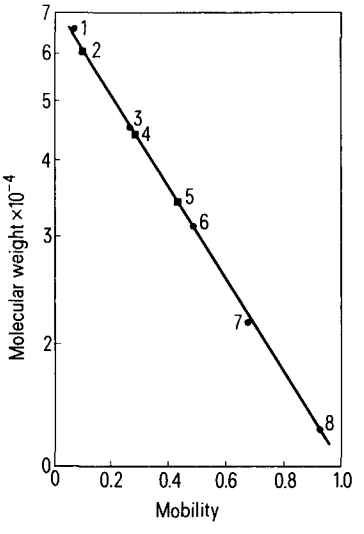

Figure 2. Mobility of the proteins obtained from the different preparations of thiol:protein disulfide oxidoreductase (■) and standard proteins (●) on 7.5% acrylamide gels in SDS. *1* Bovine serum albumin, mol.wt 66,200; *2* thiol: protein disulfide oxidoreductase, mol.wt 60,000; *3* ovalbumin, mol.wt 45,000; *4* protein disulfide isomerase, mol.wt 44,000; *5* thiol:protein disulfide oxidoreductase, mol.wt 34,000; *6* carbonic anhydrase, mol.wt 31,000; *7* soybean trypsin inhibitor, mol.wt 21,000; *8* lysozyme, mol.wt 14,500.

0.1 M phosphate buffer pH 7.5, containing 5 mM EDTA, in 1.9 ml total volume. Reactions were initiated by the addition of 0.1 ml of diluted TPOR and carried on for 5 min at 37 °C. The reaction was stopped with 1 ml trichloroacetic acid (20%, w/v) and after centrifugation, 1 ml of the supernatant, containing the iodinated B-chain of insulin, was counted for radioactivity using a sealed gamma-vial[15]. Alternatively, a spectrophotometric assay was used with 1 mM glutathione, 0.1 mM NADPH, 3 µg glutathione reductase, 0.1 mg insulin and 0.1 M phosphate buffer pH 7.5, containing 5 mM EDTA, in 2.0 ml total volume. The reaction was started by adding TPOR (10–20 µl) at 37 °C and the oxidation of NADPH was recorded at 340 nm.

Protein disulfide isomerase activity was assayed by measuring the extent of reactivation of scrambled ribonuclease at 37 °C in 0.5 ml of solution containing 1 mM glutathione, 70 µg of randomly oxidized ribonuclease, 0.1 M phosphate buffer and enzyme. At fixed times 20 µl of solution were assayed for ribonuclease activity against a blank. Ribonuclease activity was measured by incubation at 37 °C of aliquots of enzyme in a total volume of 1.0 ml containing 0.1 M Tris-HCl (pH 7.0) and 0.1 mg cytidine 2′,3′-cyclic monophosphate. A unit of ribonuclease activity was defined as a change of 1.0 absorbance unit per min at 288 nm.

Reaction of reduced TPOR with insulin. Known amounts of TPOR devoid of PDI activity (0.13–0.16 µmoles) were completely reduced by incubation with a 10-fold excess of glutathione at 37 °C for 10 min in a nitrogen atmosphere. Excess of glutathione and glutathione disulfide were removed through a column of Sephadex G-25 fine (2.5 × 25 cm) equilibrated with 0.1 M phosphate buffer pH 7.5 in the absence of oxygen. The reduced enzyme was collected into an anaerobic vial containing a stoichiometric amount of insulin under nitrogen, and the mixture was allowed to react at room temperature for 20 min. Then a six-fold excess of iodo(1-^{14}C) acetamide was added to the mixture of TPOR and insulin and then allowed to react for 3 h at 37 °C. Dithiothreitol (10 µmoles) was then added and after 20 min at 37 °C all thiol groups were alkylated with an excess of nonradioactive iodoacetamide (15 µmoles). The reaction mixture (10 ml) was applied to a Sephadex G-10 column (2.5 × 30 cm) to remove unreacted iodoacetamide and then concentrated to 5 ml.

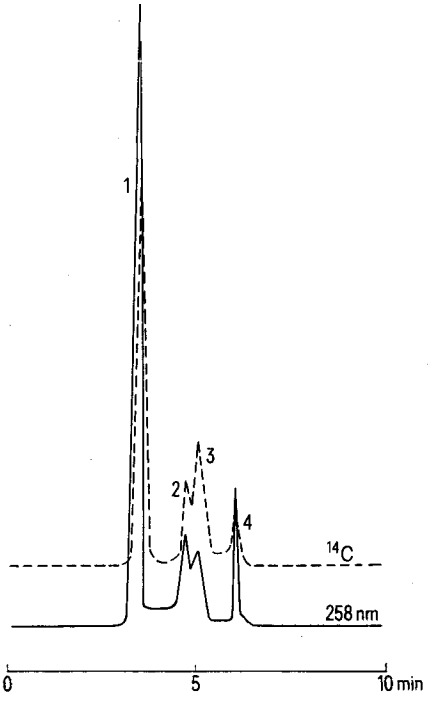

Figure 3. HPLC analysis of the reaction mixture, after gel-filtration on Sephadex G-10 and concentration, showing the absorbance at 258 nm and the radioactivity of the peaks. Peak 1 is TPOR, peaks 2 and 3 correspond to B-chain and A-chain respectively, peak 4 is a small amount of products of iodo(1-^{14}C) acetamide unresolved by gel-filtration. C-14 ratio between A and B chains is 2/1. 10 µl of solution were injected in the gel permeation column.

A and B chains of insulin were separated by gel-permeation HPLC on an I-60 column (Waters Ass., Milford, MA; separation range of the column: 1000–20,000 mol.wt) using 0.05 M phosphate buffer pH 7.5 as eluent at flow rate of 1.8 ml/min (10–20 µl injected). The eluate was monitored at 258 nm (Model Lambda Max 480, Waters Ass.) and counted by a radioactivity flow detector Flo-One HP (Radiomatic Instruments, Tampa, FLA).

Alternatively, the reaction mixture was loaded on a Sephadex G-50 fine column (3 × 54 cm) and eluted with 0.2 M ammonium acetate-ammonia buffer pH 8.3, recording the absorbance at 280 nm. The fractions containing A and B chains were hydrolyzed with 20 µg of chymotrypsin for 20 h at 37 °C. After exhaustive freeze-drying, the chymotryptic peptides obtained from each A and B chains were dissolved in 0.5 ml of bidistilled water and samples of 10 µl were assayed by HPLC. The column (Aminex A-9, cationic exchanger, Bio-Rad Lab., Richmond, CA) was equilibrated with 0.05 M perchloric acid pH 1.5 and samples were eluted with 0.05 M perchloric acid-acetonitrile (95:5) at a flow rate of 0.6 ml/min. The eluate was read at 210 nm and the peaks were separately collected and counted at the C-14 channel in a Beckman Liquid Scintillation Counter. Radioactive fractions were hydrolyzed with 6 N HCl at 110 °C for 24 h and free amino acids were determined by a Waters HPLC amino acid analysis system (post-column derivatization with o-phtalaldehyde and detection by fluorescence).

Results and discussion. Bovine livers, freshly obtained from the slaughterhouse, gave different results when used for the purifications of thiol:protein disulfide oxidoreductase according to the procedure of Carmichael et al.[8]. Two out of 10 livers yielded, at the final step, a protein, judged homogeneous by polyacrylamide disc-gel electrophoresis and polyacrylamide SDS-gel electrophoresis, with 60,000 mol.wt and showing both the TPOR and PDI activities. On the other hand, the same purification procedure on eight other livers produced a preparation containing two proteins (fig. 1) recognized as a thiol:protein disulfide oxidoreductase of mol.wt 34,000 and a protein disulfide isomerase of mol.wt 44,000 (fig. 2). In this case the two activities could be separated by covalent chromatography on thiopropyl Sepharose, and TPOR obtained by this way was used for the reaction with insulin, in order to avoid possible rearrangements of the

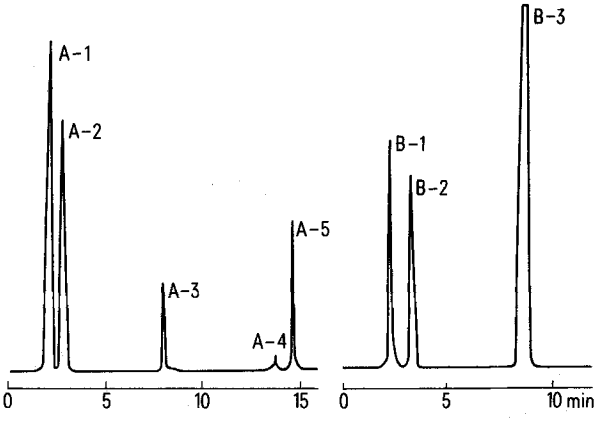

Figure 4. HPLC analysis of peptides obtained by hydrolysis of A and B chains of insulin with chymotrypsin. Radioactivity (C-14) was found in peaks A-1 and A-2 and in peaks B-1 and B-2.

disulfide groups in the hormone structure due to the PDI activity.

After reduction of TPOR with glutathione the reduced enzyme was separated by an excess of thiol to avoid a turnover reaction of the enzyme. In fact the stoichiometric ratio between reduced-TPOR and insulin allows the enzyme to split only one of the three disulfide bonds in the hormone before being transformed into the oxidized (inactive) form.

The reaction of partially reduced insulin with iodo (1-^{14}C)acetamide shows that both the polypeptide chains of the hormone are alkylated by the radioactive reagent (fig. 3) with a ratio of approximately 2/1 for the A/B chains. The chymotrypsin digestion of the separated chains led to two radioactive fragments in the A-chain and two labeled peptides in the B-chain, as shown in figure 4. A summary of these results is reported in table 1.

Further evidence that the TPOR used in this work does not act sequentially on the disulfide bonds of insulin was obtained from the analysis of chymotryptic peptides A-1, A-2, B-1 and B-2, as shown in table 2. These results are in agreement with experimental C-14 ratios previously reported (A-1/A-2 = 3/1 and B-1/B-2 = 1/1) as well as with the reports of various authors[16-18] on the action of chymotrypsin on A and B chains of insulin.

It is not yet clear, from these results or those of various authors[8,10-12,19], whether different preparations of TPOR consist of a single enzyme, sometimes containing traces of proteases, or if there are distinct enzymes with TPOR and PDI activities. However, the results reported in this paper, obtained with a preparation of TPOR deprived of PDI activity, lead to the conclusion that this enzyme reduces aspecifically the disulfide bonds of insulin.

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Observations on the toxicity and metabolic relationships of polygodial, the chemical defense of the nudibranch *Dendrodoris limbata*[1]

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Summary. Polygodial (**1**), the defense metabolite stored in the skin of the nudibranch *Dendrodoris limbata,* is toxic for the mollusc itself when injected into the hepatopancreas. Biosynthetic experiments using labeled mevalonic acid were devised to investigate a possible metabolic relationship between **1** and the mixture of sesquiterpenoidic esters **2**, stored in the hepatopancreas. The results suggest that **1** and **2** are biosynthesized by independent pathways.

Key words. Polygodial; chemical defense; fish antifeedant; nudibranchs; *Dendrodoris limbata;* biosynthesis; marine natural products.

The recognized ability of many dorid nudibranchs to defend themselves by using secondary metabolites toxic or repellent to predators[2] raises questions about a) how the animal acquires its chemical defense; b) where the animal stores the noxious chemicals and c) how the animal manages to avoid suffering from the effects of its own chemical weapons.

Recently accumulated evidence showed that the majority of the defense metabolites are of dietary origin[3-5] (sponges) and suggested[2] that these metabolites are stored in skin glands from which they are delivered in response to a predator's attack. Dorid nudibranchs which extract defensive allomones from the diet have conceivably developed adaptations which allow the harmful sponge metabolites to pass through their digestive tracts.

However, the discovery of the ability of the dorid nudibranch *Dendrodoris limbata* to synthesize its chemical defensive weapon[6], polygodial (**1**), followed by other reports[7,8] in which the synthetic capacity of other nudibranchs is described, provides a unique opportunity to follow the metabolic fate of the defense metabolites by radiolabeling methods.

Polygodial (**1**) is contained only in the skin extracts of *D. limbata,* while from the digestive gland (hepatopancreas) of the same animal the biogenetically related and biologically inactive sesquiterpenoid esters (**2**) have been isolated. Since **1** and **2** are sesquiterpenes based on a common drimane skeleton it seems probable either that **1** is biosynthesized from the biologically inactive mixture **2** and then stored in skin glands or, vice versa, that the mixture **2** could represent the result of a detoxification process of **1** for its excretion since **1** could be toxic for the animal itself in view of its high chemical reactivity[9,10]. A third possibility is that **1** and **2** are biosynthesized in different sites by two independent pathways.

We report here results of experiments devised to test the above hypotheses as well as evidence that **1** is toxic for *D. limbata* when injected into the hepatopancreas.

Materials and methods. Two sets of experiments were performed in March and April 1984, in which labeled mevalonic acid was given to specimens of *D. limbata;* 9 and 14 animals were used respectively. 0.5 μCi of [2-^{14}C]-mevalonic acid-dibenzylethylenediamine salt (Amersham; 31 mCi/mmole) were injected into the