ple, best-fit curves of the logarithms of CSI and cholesterol mole percentages were generated. Since the curves were not linear, areas beneath them were calculated by triangulation and used to compare differences between groups.

Results. Cholesterol supersaturation was present in all filtered bile analogs at 0.5 h: cholesterol concentration was 8.56 ± 0.14 mg/ml (mean ± SEM); cholesterol mole percentage was 12.1 ± 0.24; lecithin/bile acid + lecithin ratio was 0.198 ± 0.020; total lipid concentration was 9.4 ± 0.14 g/dl and CSI was 1.929 ± 0.053. Figure 1 compares the best-fit curves of the mean CSI and cholesterol mole percentages of bile analogs with and without calcium. Figure 2 compares best-fit curves of mean percentage change form basal for CSI and cholesterol mole percentages. Significant differences between control and calcium-treated samples were present at calcium concentrations of 0.010 or greater, and were almost achieved at the 0.005 calcium concentration for CSI. Observed differences of areas beneath the curves increased as the calcium concentration increased: $r = 0.93$ for percent change of cholesterol; $r = 0.76$ for percent change of CSI. Similarly, plots of the percentage change of CSI between 0 and 7 h revealed a progressive steepening of the slope with exception of the 0.015 calcium concentration which was more acute than the 0.02 concentration. The latter finding may be due to the fact that the initial CSI was higher in the 0.015 than in the 0.02 calcium-treated group. Differences between control and calcium-treated groups were most prominent within 24 h. Microscopic examination of bile samples following filtration at 0.5 h revealed neither cholesterol crystals nor liquid crystal mesophases. However, within 1–2 h liquid crystal mesophases were commonly seen. Cholesterol crystals were noted as early as 3 h, were more apparent at 5 and 7 h and thereafter steadily increased in number. It appeared that both the liquid crystal mesophases and cholesterol crystals were more prominent in calcium-treated samples than in their paired controls, but quantitation was not attempted.

Discussion. In the current study it was found that periodic filtration of highly supersaturated analog bile solutions removed cholesterol more rapidly in samples with calcium than in those without calcium. Filtration would be expected to remove all cholesterol crystals and most liquid crystalline material but not microaggregates below the filtration limits. If filtration had predominantly removed liquid crystalline material, the concentration of lecithin would have substantially declined and differences between the calcium-treated and untreated samples should have occurred. Over the 48-h period lecithin concentrations declined by 7.5% and significant differences between control and experimental groups were not present (Student's t-test). Thus, it is deduced that cholesterol removal resulted primarily from filtration of cholesterol crystals or possibly aggregated species of cholesterol below the limits of microscopic detection.

The mechanism by which calcium accelerated cholesterol loss from solution is not known and remains speculative. It is possible that it influenced phase transitions by 1) altering micelle formation and/or distribution of bile constituents, 2) promoting the development and/or growth of liquid crystal mesophases or multilamellar vesicles and 3) by facilitating nucleation and/or growth of cholesterol crystals. For example, calcium may have altered the aggregation of bile salts[14], influenced the species and microdomains of structures which stabilize cholesterol such that liquid crystal formation was augmented[15,16] and promoted liquid crystal mesophase or vesicle growth in a fashion similar to that reported for nonbirefringent aggregates (liposomes) in dilute model bile[17]. In turn, these effects may have diminished the cholesterol nucleation time.

To date, none of the clinically relevant studies of calcium in bile have demonstrated significant differences between subjects with and without cholesterol gallstones[3,8,9,19]. However, these studies have neither examined potential differences in lithogenicity between specimens with high and low calcium concentrations nor evaluated the effect of calcium on lithogenesis in a dynamic fashion. Thus, while it is recognized that the behavior of cholesterol in analog bile solutions is different from that in human bile, the current study suggests that further definition of the effects of calcium on cholesterol phase transitions in human bile is warranted.

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A specific protein in the genus *Rhynchosciara* (Diptera, Sciaridae)

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Summary. Proteins immunologically related to *Rhynchosciara americana* larval protein 10 occur in the hemolymph and ovaries of five different fly species of the genus *Rhynchosciara*. Electrophoretic analyses showed these proteins to have a mol.wt similar to that of the *R. americana* protein 10 (43,000), e.g. the *R. hollanderi* protein 44,300, the *R. milleri* protein 45,500.
Key words. Rhynchosciara; hemolymph proteins; ovary proteins; Sciaridae.

Several of the hemolymph proteins from *Rhynchosciara americana* were studied[2-6]. One of these, the larval protein 10, is transferred to the eggs during oogenesis[3]. The protein 10 is monomeric with a mol.wt of 43,000 and an isoelectric pH of 6.6[3]. Since the massive contribution of larval hemolymph proteins to the eggs is a characteristic which has so far only been shown in *R. americana* and in *Bombyx mori*[7], we undertook a study to investigate the distribution of this type of protein in other insect species.

Material and methods. The flies of the genus *Rhynchosciara* used in this work were collected in banana orchards and were maintained in the laboratory until they were used in the experiments. *Bradysia hygida* was generously provided by Dr H. Sauaia and Dr E.M. Laicine. *Musca domestica, Drosophila melanogaster, Bombyx mori, Trichosia pubescens* and *Sciara ocellaris* were a gift from the Biology Department (USP, São Paulo).

The monospecific serum anti-protein 10 used was the same as that obtained by Marinotti and Bianchi[3]. Immunodiffusion was carried out according to the method of Ouchterlony[8] using 1% noble agar in 25 mM Tris, 192 mM glycine buffer, pH 8.3 containing 0.1 mM phenylthiocarbamide and 0.01% (w/v) sodium azide (buffer 1). For the immunodiffusion tests, hemolymph, collected as described by Bianchi et al.[2], was diluted with buffer 1.

Disc electrophoresis was performed in 7% polyacrylamide gels according to the system described by Davis[9]. Electrophoretic separation was obtained with a constant current of 2.5 mA per column and the proteins were stained with amido black[2]. Slab 10% polyacrylamide gel electrophoresis, in the presence of sodium dodecylsulphate (SDS), and the preparation of samples for this electrophoresis, were carried out as previously described[2]. Mol.wt estimation was made by the method of Shapiro et al.[10], using as mol.wt markers the following proteins: phosphorylase B, bovine serum albumin, ovalbumin, carbonic anhydrase, soybean trypsin inhibitor and lysozyme. All these standards were obtained as a kit from Bio-Rad Laboratories.

Results. Using an antiserum specific for protein 10 from *R. americana*[3], double immunodiffusion analyses were made with the hemolymph obtained from several insect species. The results obtained are showed in figure 1 and the table. All five species of *Rhynchosciara* analyzed had antigens in the hemolymph with a reaction showing total identity (*R. hollanderi* and *R. sp* 3) or partial identity (*R. milleri, R. sp* 1 and *R. sp* 2) with the *R. americana* protein 10. Other species of Sciaridae (*Bradysia hygida, Sciara ocellaris* and *Trichosia pubescens*) did not have any anti-

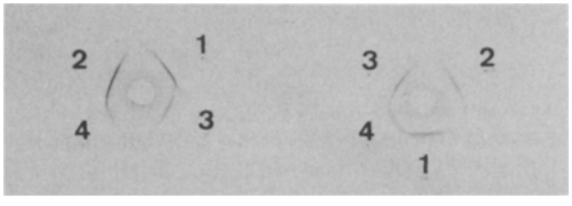

Figure 1. Ouchterlony immunodiffusion in 1% noble agar in 25 mM Tris, 192 mM glycine buffer, pH 8.3. Central well contained anti-protein 10 serum and the others contained larval hemolymph from *Rhynchosciara americana* (1); *Rhynchosciara hollanderi* (2); *Rhynchosciara milleri* (3) or *Rhynchosciara* sp. 3 (4).

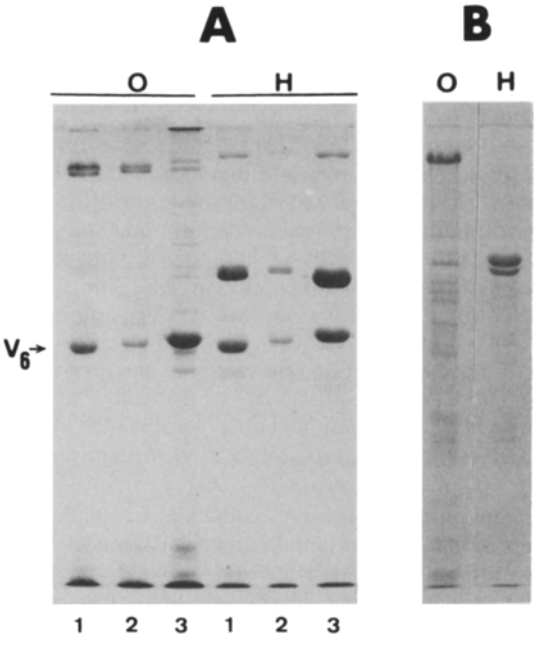

Figure 2. SDS polyacrylamide gel electrophoresis of larval hemolymph proteins (H) and ovary proteins (O). *A* Comparative electrophoresis of proteins from *Rhynchosciara americana* (1); *Rhynchosciara hollanderi* (2) and *Rhynchosciara milleri* (3). *B* Comparative electrophoresis of larval hemolymph and ovary proteins from *Trichosia pubescens*.

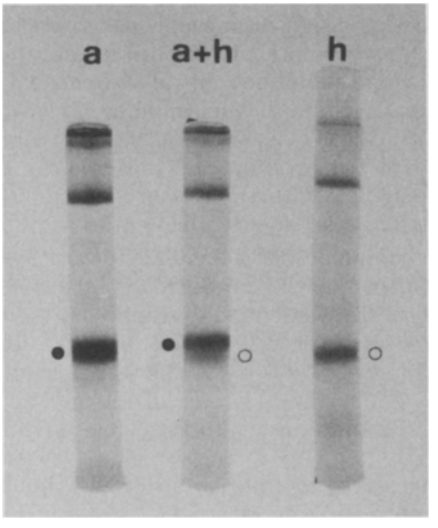

Figure 3. Disc electrophoresis of *Rhynchosciara americana* and *Rhynchosciara hollanderi* larval hemolymph proteins. Larval hemolymph was diluted in 25 mM Tris; 192 mM glycine buffer, pH 8.3 and submitted to electrophoresis in a 7% polyacrylamide cylinder. The running time was 4 h. a, *Rhynchosciara americana* hemolymph; h, *Rhynchosciara hollanderi* hemolymph; a+h, *Rhynchosciara americana* hemolymph plus *Rhynchosciara hollanderi* hemolymph. ●, *Rhynchosciara americana* protein 10; ○, *Rhynchosciara hollanderi* protein 10.

Occurrence of proteins immunologically related to *Rhynchosciara americana* protein 10*

Insect species	Immunologic identity Total	Partial	Absence of related antigens
Rhynchosciara hollanderi	+		
Rhynchosciara milleri		+	
Rhynchosciara sp. 1**		+	
Rhynchosciara sp. 2		+	
Rhynchosciara sp. 3	+		
Bradysia hygida			+
Sciara ocellaris			+
Trichosia pubescens			+
Musca domestica			+
Drosophila melanogaster			+
Bombyx mori			+

* Hemolymph from several insect species was tested by double immunodiffusion with serum anti-protein 10. ** *Rhynchosciara* sp. 1, sp. 2 and sp. 3 are species clearly distinct from *Rhynchosciara americana, Rhynchosciara hollanderi* and *Rhynchosciara milleri*. It was not possible to classify these flies at the specific level.

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.

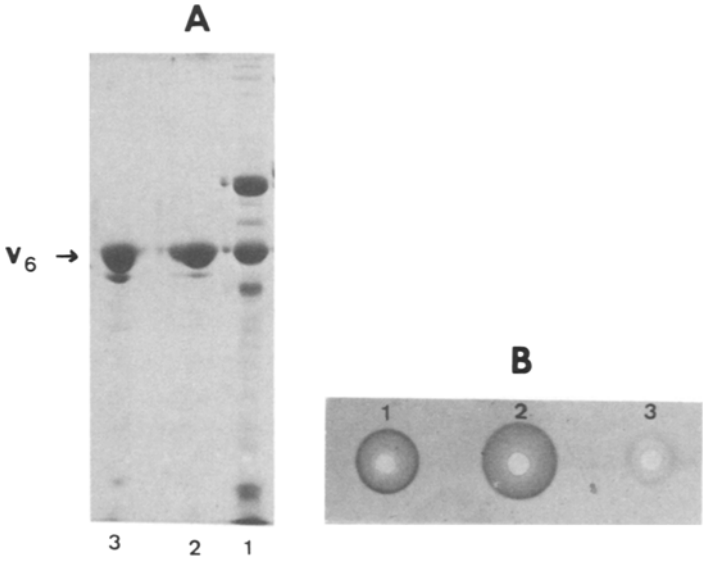

Figure 4. SDS polyacrylamide gel electrophoresis and radial immunodiffusion of protein 10 obtained from *Rhynchosciara* species. Protein 10 was obtained by cutting it out of a polyacrylamide cylinder, in which hemolymph proteins were fractionated by electrophoresis (similar to the ones in fig. 3). The 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-