plasma, the release of radioactivity is very small. The release of radioactivity is rapid at the beginning but after 5–10 min, notwithstanding the concentrations of conjugate, it slows down and practically stops when the protein-bound radioactivity is about 50% of the initial value. If after 60 min of incubation of L_{30}-HSA-ara-[2,8-^{3}H]$AMP_{6.4}$ in mouse plasma (750 µg/ml), new labeled conjugate is added, the release of radioactivity from the conjugate starts again, following the same curve as in the first 60 min (experiment not shown in fig. 1).

These in vitro experiments indicate that some (40–50%) of the conjugated ara-AMP molecules remain linked to L-HSA in mouse plasma. The inhibition of DNA synthesis, produced by L-SA-ara-AMP conjugates selectively in the liver of mice with *Ectromelia* virus hepatitis, is very probably caused by these drug molecules which are not released freely in plasma and can be transported by L-SA selectively into the hepatocytes.

It is probable that the ara-AMP molecules which remain linked to L-HSA are protected by a steric hindrance from the enzymes which cleave the linkage between the drug and the carrier protein. However, it is not possible to exclude the possibility that different drug-protein bonds are formed during the conjugation and that the ara-AMP molecules which remain linked to L-HSA are coupled by a bond which is not enzymatically cleaved.

Experiments in vivo. In contrast to previous results[6] the values of protein-bound radioactivity in mouse plasma after injection of the conjugates labeled in the ara-AMP moiety are lower than those after administration of an equal dose of L_{30}-[^{3}H]HSA-ara-$AMP_{6.2}$ (fig. 2). In the latter the radioactive label is linked to protein by a bond which is very strong and not enzymatically cleaved[14]. A splitting of the bond between the drug and the protein (or between ara-A and the phosphate) accounts for this finding as has been demonstrated by the experiments in vitro (fig. 1).

Figure 3 shows the curve of disappearance from plasma of conjugated ara-AMP as identified by RIA. Deoxycoformycin, a drug which when administered i.p. to mice at the dose of 2 µg/g completely inhibits ara-A deamination for more than 5 h[21], does not increase the plasma levels of coupled ara-AMP (fig. 3). This suggests that ara-AMP conjugated to L-HSA is not deaminated in mouse plasma in contrast to free ara-A and ara-AMP which are very rapidly metabolized to the hypoxanthine derivative[18–20]. Coupled ara-AMP, detected by RIA, is present in plasma in percentages of the administered dose (fig. 3) which are lower than the percentages of protein-bound radioactivity after injection of the conjugates labeled in the drug moiety (fig. 2). This may occur because those ara-AMP molecules which are protected by steric hindrance from the enzymes which cleave the bond with L-HSA (as was suggested above) may be the same which are not accessible to antibodies and consequently are not detected by RIA (see 'materials and methods').

In conclusion, the present experiments show that in plasma of mice some ara-AMP molecules are enzymatically released from L-HSA conjugates whereas some of them remain linked to the carrier, probably because they are protected by steric hindrance from the activity of enzymes. Evidence has been obtained that ara-AMP is not deaminated when it is conjugated to L-HSA.

1 Acknowledgments. This work was supported by Consiglio Nazionale delle Ricerche, Progetto Finalizzato per il Controllo delle Malattie da Infezione, grant 830065052; ara-AMP was a gift from Dr M. L. Black, Warner-Lambert Co (Ann Arbor, MI). ara-HxMP was a gift from Dr R. W. Sidwell, ICN (Irvine, California). The excellent technical assistance of Mr Goffredo Nanetti is gratefully acknowledged.

2 The abbreviations used are: ara-AMP, adenine-9-β-D-arabinofuranoside 5-monophosphate; ara-HxMP, hypoxanthine-9-β-D-arabinofuranoside 5-monophosphate; AF, asialofetuin; L-HSA, human lactosaminated serum albumin; L-HSA-ara-AMP, conjugates of L-HSA with ara-AMP; L-[^{3}H]HSA-ara-AMP, conjugates tritiated in the protein moiety; L-HSA-ara-[2,8^{3}H]AMP and L-HSA-ara-[2^{3}H]AMP, conjugates tritiated in the adenine moiety of ara-AMP. The molar ratios lactose/HSA and ara-AMP/HSA are indicated by subscripts; for example L_{30}-HSA-ara-$AMP_{6.2}$ is a conjugate with a molar ratio of lactose/HSA of 30 and of ara-AMP/HSA of 6.2.

3 Fiume, L., Busi, C., Mattioli, A., Balboni, P. G., and Barbanti-Brodano, G., FEBS Lett. *129* (1981) 261.
4 Fiume, L., Busi, C., and Mattioli, A., FEBS Lett. *146* (1982) 42.
5 Fiume, L., Busi, C., and Mattioli, A., FEBS Lett. *153* (1983) 6.
6 Fiume, L., Mattioli, A., Busi, C., and Accorsi, C., Gut *25* (1984) 1392.
7 Krantz, M., Holtzman, N. A., Stowell, C. P., and Lee, Y. C., Biochemistry *15* (1976) 3963.
8 Wilson, G., J. biol. Chem. *253* (1978) 2070.
9 Ashwell, G., and Harford, J., A. Rev. Biochem. *51* (1982) 531.
10 Fiume, L., Mattioli, A., Busi, C., Spinosa, G., and Wieland, Th., Experientia *38* (1982) 1087.
11 Gray, G. R., Archs Biochem. Biophys. *163* (1974) 426.
12 Schwartz, B. A., and Gray, G. R., Biochem. Biophys. *181* (1977) 542.
13 Fiume, L., Mattioli, A., Busi, C., Balboni, P. G., Barbanti-Brodano, G., De Vries, J., Altmann, R., and Wieland, Th., FEBS Lett. *116* (1980) 185.
14 Jentof, N., and Dearborn, D. G., Meth. Enzym. *91* (1983) 570.
15 Fiume, L., Busi, C., Campadelli-Fiume, G., and Franceschi, C., Experientia *31* (1975) 1233.
16 Morell, A. G., Van der Hamer, C. J. A., Scheinberg, I. H., and Ashwell, G., J. biol. Chem. *241* (1966) 3745.
17 Minden, P., and Farr, R. S., in: Handbook of Experimental Immunology, p. 151. Ed. D. M. Weir. Blackwell, Oxford, London, Edinburgh, Melbourne 1973.
18 Whitley, R. J., Ch'ien, L. T., Buchanan, R. A., and Alford, C. A. Jr, Perspect. Virol. *9* (1975) 315.
19 Whitley, R. J., Tucker, B. C., Kinkel, A. W., Barton, N. H., Pass, R. F., Whelchel, J. D., Cobbs, C. G., Diethelm, A. G., and Buchanan, R. A., Antimicrob. Agents Chemother. *18* (1980) 709.
20 Preiksaitis, J. K., Lank, B., Ng, P. K., Brox, L., Le Page, G. A., and Tyrrel, D. L. J., J. infect. Dis. *144* (1981) 358.
21 Plunkett, W., Alexander, L., and Loo, L. T., Biochem. Pharmac. *28* (1979) 201.

0014-4754/85/101326-03$1.50 + 0.20/0

Calcium accelerates cholesterol phase transitions in analog bile

M. M. Berenson and J. R. Cardinal

University of Utah School of Medicine and College of Pharmacy, Salt Lake City (Utah 84132, USA), 25 April 1984

Summary. Analog bile supersaturated with cholesterol was constituted, filtered and divided into equal portions containing no calcium or calcium, 2.5–15 mM. Aliquots were removed over the next 48 h and filtrates analyzed for cholesterol, bile acid and lecithin. Calcium accelerated cholesterol loss from solution in a dose-related fashion.

Key words. Calcium; cholesterol supersaturation; lithogenicity.

Cholesterol supersaturation of bile is always present in patients with cholesterol gallstones[1]. However, since cholesterol supersaturation also occurs in healthy man, lithogenicity is probably best defined as an inability to maintain cholesterol in solution in a supersaturated state[2–3]. In spite of supersaturation in both groups, cholesterol microcrystals are consistently present in bile

of subjects with cholesterol gallstones, but absent in subjects without gallstones[4]. The processes responsible for maintenance of cholesterol supersaturation are poorly understood. Supersaturation is not maintained in in vitro bile analogs with total lipid concentrations similar to gallbladder bile, nor in most subjects with gallstones. It is, however, maintained in bile of normal subjects. Both inhibitors and accelerators of cholesterol have been reported to be present in human bile[5-8].

Studies have shown that calcium interacts with components of the cholesterol-solubilizing system and it has been suggested that this provides a mechanism to prevent precipitation of insoluble calcium salts and the subsequent formation of gallstones[9-11]. We speculated that the interaction of calcium with the cholesterol-solubilizing system might perturb maintenance of cholesterol supersaturation and previously reported preliminary findings suggesting that this occurred[12].

Methods and materials. Analog bile was composed by coprecipitation of 135 mM sodium glycodeoxycholic acid (Calbiochem, San Diego, Calif.), 35 mM purified crude egg lecithin (Sigma Chemical Co., St. Louis, Mo.) and 10 mg/ml cholesterol (Steraloids Inc., Pauling, N.Y.) and reconstitution with deionized water at pH 7 and 37°C[13]. 30 min after reconstitution, 10-ml samples of analog bile were filtered (0.45 µm Millipore, Millipore Corp., Bedford, Mass.) and divided. One half of the samples were added to vials containing $CaCl_2$. The $CaCl_2$ had been placed in the vials in liquid form to provide concentrations varying from 0.005 to 0.030 mEq/ml (2.5–15 mM) upon addition of the bile, and had then been dried. It appeared as a thin film on the bottom of the vials and upon addition of bile immediately entered solution. The remaining half of the solution served as controls. All samples were flushed with nitrogen, tightly sealed and continuously rotated in the dark at 37°C. At 1, 2, 3, 5, 7, 24 and 48 h, 0.5-ml aliquots were removed, filtered, and analyzed by high pressure liquid chromatography. The HPLC instrument (Model ALC 200, Waters Associates, Milford, Mass.) was equipped with a UGK Loop Injector, a Model 6000 A Pump and a Model R-400 Differential Refractometer. The chromatography employed a µBONDAPAK Phenyl Column (Waters Associates, Milford, Mass.), 30 cm long, with an internal diameter of 0.33 mm and a solvent system of methanol-water in a ratio of 98.2 (v/v) at pH 2 and a flow rate of ~ 1.5 ml/min. Standard curves of peak response vs concentration were linear. Peak areas were determined by the triangulation technique. Reproducibility was ± 10% for bile salt and ± 15% for both lecithin and cholesterol. Additionally, unfiltered aliquots of bile were periodically examined by light and polarizing microscopy prior to filtration.

Cholesterol supersaturation varied modestly among original (0.5 h) bile filtrates, but not between paired samples. Therefore, lipid concentrations and cholesterol saturation indices (CSI) present in the 0.5-h solutions were assigned values of 100% and subsequent compositional changes related to it. For each sam-

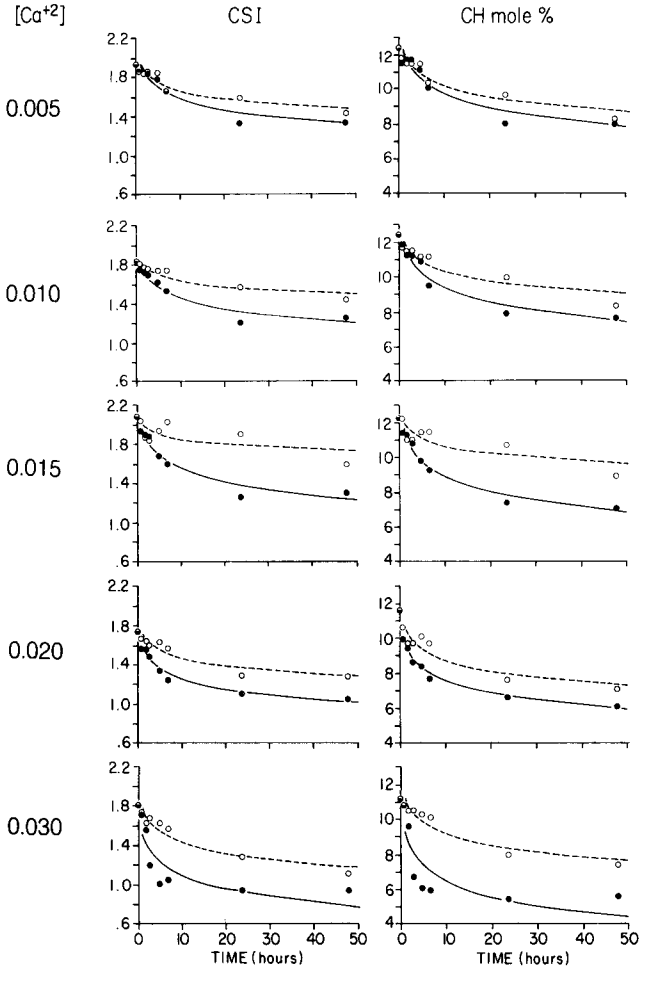

Figure 1. Comparison of mean logarithmic cholesterol saturation indices (CSI) and cholesterol mole percentages (CH mole%) best-fit curves for calcium-treated (solid line, closed circles) and control (dashed line, open circles) bile analogs. n = 6 separate studies for 0.005, 0.010, 0.020 and 0.030 mEq/ml Ca^{+2} concentrations; n = 8 for 0.015 mEq/ml Ca^{+2}.

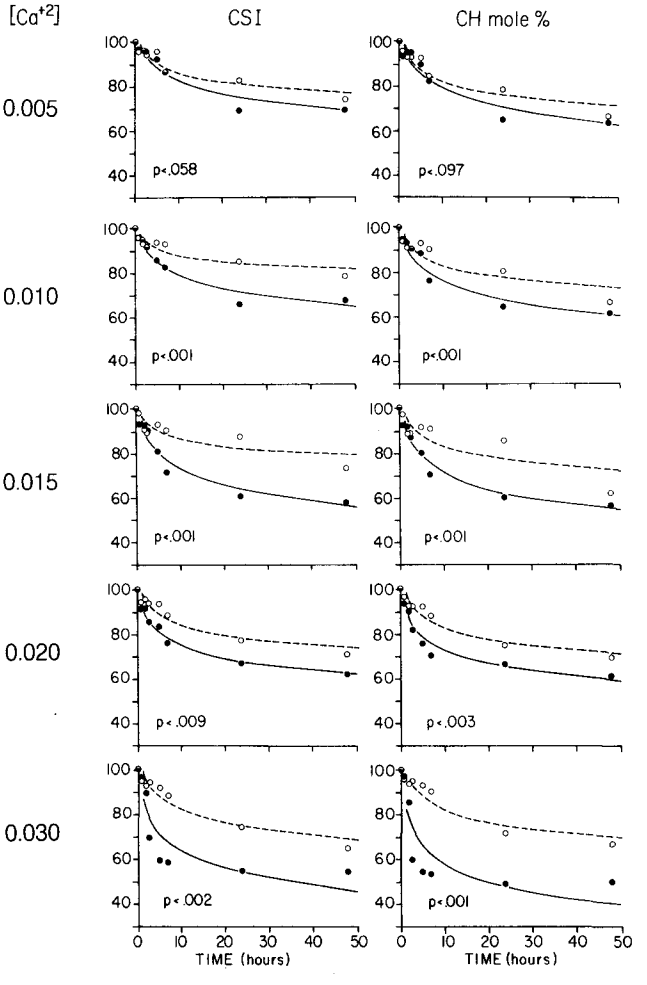

Figure 2. Comparison of mean logarithmic percentage change from basal (0.5 h) cholesterol saturation indices (CSI) and cholesterol mole percentage (CH mole%) best-fit curves for calcium-treated (solid lines, closed circles) bile analogs. p values indicate significance of differences between areas under the curves.

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.

Acknowledgment. The authors are grateful for the technical assistance of Susan Coffin. This work was supported, in part, by NIH Grant 5 RO1 AM 25221.

1 Carey, M.C., and Small, D.M., J. clin. Invest. *61* (1978) 998.
2 Holzbach, R.T., Marsh, M., Olszewski, M., and Holan, K.R., J. clin. Invest. *52* (1973) 1467.
3 Holan, K.R., Holzbach, R.T., Hermann, R.E., Cooperman, A.M., and Claffey, W.J., Gastroenterology *77* (1979) 611.
4 Sedaghat, A., and Grundy, S.M., New Engl. J. Med. *302* (1980) 1274.
5 Holzbach, R.T., Kibe, A., Thiel, E., Howell, J.H., Marsh, M., and Hermann, R.E., J. clin. Invest. *73* (1984) 34.
6 Kibe, A., Holzbach, R.T., LaRusso, N.F., and Mao, S.J.T., Science *225* (1984) 514.
7 Burnstein, M.J., Ilson, R.G., Petrunka, C.N., Taylor, R.N., and Strasberg, S.M., Gastroenterology *85* (1983) 801.
8 Gollish, S.H., Burnstein, M.J., Ilson, R.G., Petrunka, C.N., and Strasberg, S.M., Gut *24* (1983) 836.
9 Williamson, B.W.A., and Percy-Robb, I.W., Gastroenterology *78* (1980) 296.
10 Rajagopalan, N., and Lindenbaum, S., Biochim. biophys. Acta *711* (1982) 26.
11 More, E.W., Celic, L., and Ostrow, J.D., Gastroenterology *83* (1982) 1079.
12 Berenson, M.M., and Cardinal, J.R., Clin. Res. *30* (1982) 33A.
13 Mufson, D., Triyanad, K., Zarembo, J.F., and Ravin, L.J., J. pharm. Sci. *6* (1974) 327.
14 Moore, E.W., Hepatology *4* (1984) 228S.
15 Carey, M.C., Hepatology *4* (1984) 138S.
16 Muller, K., Hepatology *4* (1984) 134S.
17 Kibe, A., Marsh, M., Holzbach, R.T., and McMahon, J.T., Hepatology *3* (1983) 819.
18 Sutor, D.J., and Wilkie, L.I., Clinica chim. Acta *79* (1977) 119.
19 Nakayama, F., and van der Linden, W., Am. J. Surg. *122* (1971) 8.

0014-4754/85/101328-03$1.50 + 0.20/0

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

A.G. de Bianchi and O. Marinotti

Departamento de Bioquímica, Instituto de Química, Universidade de São Paulo, C.P. 20780, 01498 São Paulo, SP (Brasil),
21 May 1984

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.