contrast, in untreated squid (graph B), a large portion of Cd in the liver supernatant was bound to high molecular weight species (mol. wt > 70,000). Only a small Cd peak was observed in the 11,000–16,000 molecular weight region close to the position of rat liver metallothionein. In addition, a small portion of the Cd was found to be associated with lower molecular weight species (mol. wt < 3000). The chromatographic distribution of Zn was very similar to that of Cd. However, only very little Zn was detected in the region close to the metallothionein position. The distribution of Ag differed appreciably from that of Cd. A major portion of the Ag was bound to low molecular weight species (mol. wt < 20,000). To sum up, it has been shown in this study that the subcellular and Sephadex G-75 chromatographic distributions of the heavy metals in normal squid liver are strikingly different from those in the livers of Cd and Ag-exposed rats. These results could indicate that the physiological and nutritional significance of the heavy metals in squid are quite different from those in rat.

Subcellular distribution of cadmium, zinc, copper, iron and silver in normal squid *(Todarodes pacificus)* liver homogenates

Subcellular fraction	Metal contents (% of reconstituted total)				
	Cd	Zn	Cu	Fe	Ag
Nuclei and cell debris	28±2.7	31±4.3	20±4.1	23±2.8	15±2.4
Mitochondria	33±5.1	24±3.3	12±1.5	20±2.3	14±2.4
Microsomes	14±1.1	9.0±0.9	4.8±1.0	42±2.3	6.6±1.6
Cytosol	26±2.7	35±2.1	63±4.9	14±0.9	64±4.3

Values represent means ± SEM for 5 liver samples. Ranges of the concentrations of the heavy metals in the liver samples are as follows: Cd, 15–33; Zn, 31–89; Cu, 111–267; Fe, 81–126; Ag, 1.3–2.2 (expressed as µg/g wet wt liver tissue).

Accepting this point of view, and knowing that Cd and Ag are either toxic or nonessential for mammals, further investigations on the Cd or Ag-binding species in untreated squid liver are needed.

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Free cholesterol not carried by lipoproteins in human serum

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Summary. Considerable amounts of nonesterified cholesterol were found in human serum freed from lipoproteins. This cholesterol, when incubated with synaptosomal plasma membranes of dog brain, evokes changes of the ouabain-sensitive ATPase activity, as the exogenously added cholesterol does.

The ability of exogenous cholesterol to become incorporated into the phospholipid bilayer of biomembranes in vitro, causing marked functional changes in integral proteins, has been reported previously[2–5]. Such phenomena have recently been verified in in vivo experiments as well[6]. Monomeric cholesterol, in very dilute aqueous solutions (up to 5×10^{-6} M) is a very active compound, affecting integral proteins of biomembranes. For example, the specific activity of adenylate cyclase decreases to 50% after preincubation of aqueous cholesterol solutions with synaptosomal plasma membranes (SPM); the ouabain-sensitive ATPase shows a definite increase in activity after 1–3 h preincubation.

In subsequent studies, we used the ouabain-sensitive ATPase as a measure of the functional changes resulting from the binding of cholesterol into biomembranes. The availability of approximately ideal conditions for kinetic work, and the introduction of the water soluble glucoside of cholesterol, synthesized on purpose in our laboratory, permitted the discovery of the synergistic nature of this binding[2]. Cholesterol glucoside preincubated in monomeric aqueous solutions at concentrations higher than 5×10^{-6} M, which was employed in our studies so far, evokes a biphasic curve of changes in the ouabain-sensitive ATPase activity. Up to the concentration of 15×10^{-6} M, the glucoside behaves in an identical manner to cholesterol, i.e., it evokes an equally intense increase in the specific activity of the ouabain-sensitive ATPase. Above this concentration, however, the specific activity of the enzyme starts dropping exponentially[5].

These in vitro phemonema suggested a search for an in vivo free fraction of cholesterol, hitherto unsuspected, which would act in a similar manner to aqueous solutions of exogenous cholesterol. The detection of such a fraction was performed and the results are presented and discussed in the present communication.

Materials and methods. Serum (2 ml) from freshly drawn human blood was made up to 12 ml with a saturated solution of KBr to achieve a density of d = 1.25 g/ml. This mixture was exposed to 48-h ultracentrifugation, at 321,500 × g (60,000 rpm) in the L5-75 Spinco Ultracentri-

Analytical data for the cholesterol content in whole human sera and fractions obtained by ultracentrifugation at a density d = 1.25 (see text)

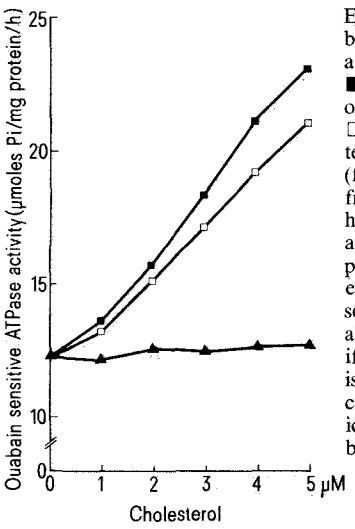
I Patient	II Total serum cholesterol		III Nonesterified serum cholesterol		IV Nonesterified lipoprotein bound cholesterol		V Nonesterified cholesterol in lipoprotein-free serum (fraction B)		VI Ratio of nonesterified cholesterol in fraction B to nonesterified cholesterol bound to lipoproteins
	mg/100 ml	$\times 10^{-3}$ M	mg/100 ml	$\times 10^{-3}$ M	mg/100 ml	$\times 10^{-3}$ M	mg/100 ml	$\times 10^{-3}$ M	mole/mole
1 (♂)	270	6.976	65.52	1.693	51.24	1.324	11.40	0.294	0.174
2 (♂)	278	7.183	79.72	2.059	49.45	1.277	28.84	0.745	0.362
3 (♀)	217	5.607	43.40	1.121	36.04	0.931	7.23	0.186	0.166
4 (♀)	264	6.821	57.12	1.474	49.88	1.288	8.76	0.226	0.153
5 (♂)	258	6.666	51.80	1.501	43.54	1.125	9.23	0.238	0.159
6 (♀)	238	6.149	62.02	1.602	46.41	1.199	17.82	0.460	0.287
7 (♂)	264	6.821	52.50	1.356	45.39	1.172	11.64	0.300	0.221
8 (♀)	240	6.201	55.27	1.428	50.86	1.312	10.56	0.272	0.133

fuge, with a Ti-75 fixed angle rotor at 4 °C. This treatment forces the lipoproteins upwards, towards the surface[7]. The volume of this upper layer is approximately 4–5 ml. Lipoproteins in the upper layer were assayed electrophoretically[8]. The same methodology was also used for the layer immediately below the lipoprotein fraction (B) and this was found to be free of electrophoretically detectable lipoproteins. The total cholesterol content was measured[9] and, for comparison with the untreated serum, the final volume of both layers was adjusted to equal that of the starting serum. The results were expressed in mg of cholesterol per 100 ml of serum and in moles/l. The lower fraction (B) containing a considerable quantity of nonesterified cholesterol, was extracted according to Folch[10] and assayed by gas-liquid chromatography (Perkin Elmer, 3920B)[11]. No esterified cholesterol was found in it. TLC of cholesterol, occasionally necessary prior to the application of gas chromatography, was performed in a cyclohexane:ethyl acetate 60:40 solvent system. Exhaustive dialysis of the lower fraction B was made for 48 h at 4 °C, against 5 changes of 5 l of buffer (0.15 M NaCl/30 mM Tris · HCl pH 7.4). Results indicated that this fraction is dialyzable and more than 90% of the cholesterol content came out of the dialysis bags. A part of the lipoprotein fraction (A) was also dialyzed under the same conditions and no appreciably quantity of cholesterol was removed within that period of time. KBr was removed by dialysis of the samples for 12 h at 4 °C against 2 changes of 1 l of 0.15 M NaCl/30 mM Tris · HCl pH 7.4.

Synaptosomal plasma membranes (SPM) were prepared from the brains of young dogs (puppies), as described previously[4]. Protein was measured according to Lowry et al.[12] using bovine serum albumin (Sigma) as standard.

Preincubations of SPM (0.35 mg/ml) with concentrations of nonesterified cholesterol (aqueous solutions of exogenous cholesterol, lipoprotein cholesterol, or nonesterified cholesterol present in the fraction B of the lipoprotein free serum) specified in the figure, were for 3 h at 37 °C in a final volume of 3 ml. The mixtures were 5 mM with respect to Tris · HCl (pH 7.4) and 0.15 M with respect to NaCl. They were stirred magnetically.

Determination of the ouabain-sensitive ATPase activity as a functional parameter, indicating the binding of cholesterol onto the SPM, was performed as before[2].



Evoked changes of the ouabain-sensitive ATPase activity at different concentrations of: ■—■, aqueous solutions of exogenous cholesterol: □—□, nonesterified cholesterol present in the lower layer (fraction B) of the lipoprotein free serum (residual proteins had no effect on the enzymatic activity; these cholesterol free proteins were obtained by exhaustive dialysis as described in the text); ▲—▲ absence of effect of nonesterified cholesterol bound on the isolated lipoproteins. The curves drawn represent a typical experiment which has been repeated 3 times.

Results and discussion. Preincubation of synaptosomal plasma membranes (SPM) from brains of young dogs, with monomeric cholesterol in aqueous solutions, resulted in the incorporation of cholesterol into the SPM, evoking drastic changes of all integral proteins studied so far in our laboratory. Scrapings of dog aorta, myocardial anuclear homogenates, and myometrial plasma membranes gave, qualitatively, identical results[2–5]. These experiments require only 1–3 h of preincubation in toto and, thus, they differ from those described with lipoproteins[13,14] or liposomes[14–16] as a source of cholesterol in which 1–3 h are not enough to give discernible results.

The findings from such experiments led to a search for a fraction in human serum that would act in a similar manner to aqueous solutions of exogenous monomeric cholesterol. For this purpose the density of samples from serum of different patients was increased up to d = 1.25 with the aid of a saturated solution of KBr[7]. Ultracentrifugation at 321,000 × g for 48 h separates the serum in 2 layers. Lipoproteins in the upper layer (fraction A) were assayed electrophoretically. The lower layer (fraction B) was found free of electrophoretically detectable lipoproteins. As shown in table (column V) up to 25% of the cholesterol, behaving as if it were in monomeric solution, was perhaps loosely bound by hydrophobic, etc., forces to proteins or peptides with densities close to 1.25 (residual proteins). An experiment with material obtained from the 2 separated fractions of a 1.25 density sample gave the results depicted in the figure. Parts of these 2 fractions were assayed for their effect on the ouabain-sensitive ATPase activity of synaptosomal plasma membranes. The specific activity of

the enzyme was estimated by preincubation of the membranes for 3 h with samples of the 2 fractions in quantities corresponding to 1×10^{-6} M–5×10^{-6} M cholesterol. It is of interest that the changes are produced only by the monomeric cholesterol existing in the fraction B. Control experiments, using cholesterol-free residual proteins of the fraction B, had no effect on the enzymatic activity. These proteins free of cholesterol were obtained by exhaustive dialysis of the fraction B as described in the experimental section. Lipoproteins fail to produce any changes within that period of preincubation time. The results agree with those obtainable with the exogenously added aqueous solutions of cholesterol at concentrations up to 5×10^{-6} M (see fig.).

A test of the state of this nonesterified cholesterol was applied by detecting it gas-chromatographically after extraction, etc., in the urine (up to 1.7×10^{-6} M). The free (monomeric) material may, probably, partly be filtered at the glomeruli of the kidneys and then reabsorbed at subsequent portions of the nephron. The data are limited at present to 16 cases, of which 8, more thoroughly studied, are presented here. Six of these patients, known not to suffer from any vascular disease, were used for the derivation of a mean value of the ratio of the nonesterified cholesterol in fraction (B) over that of the nonesterified cholesterol remaining in lipoprotein ($x=0.17\pm0.03$). Patient No. 2 had a history of coronary disease and patient No. 6 had well recognized symptoms of vascular disease. Although, at this point, high ratios may be a coincidence, the problem warrants further studies in normal subjects and patients. It is early yet to make pronouncements on the factor(s) influencing the ratio (table, column VI). Triglycerides, fatty acids, etc., which all show a certain statistical relation with atherosclerosis, may be responsible.

Such a study is now in progress. The fact remains that 'free' monomeric nonesterified cholesterol, not carried by lipoproteins, may evoke changes of cellular membrane proteins, capable ultimately of harming those cells, and similar material is present is serum, often at concentrations very much higher than those permitted by the 'solubility' of the free compound in aqueous media. At such concentrations, cholesterol continuously present in the circulation would eventually incorporate into the phospholipid bilayer and produce changes similar to those shown in the figure, where the ouabain-sensitive ATPase was selected as a parameter. Such an increase of the nonesterifed cholesterol in the membranes of aortic endothelial cells could be involved as an initiator, in association with other factors (mechanical, etc.) of preatherotic lesions[17].

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Superoxide dismutase activity in the skin of rats irradiated by He-Ne laser[1]

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Summary. The activity of the enzyme superoxide dismutase has been measured in the skin of rats irradiated by a low-power He-Ne laser. The irradiation was performed at the doses of 4, 8 and 20 J/cm^2 in a single or repeated treatment. The increase in activity of superoxide dismutase in the skin of irradiated animals was not statistically significant.

In the present work the activity of the enzyme superoxide dismutase (SOD) was measured in the skin of rats irradiated by a He-Ne laser. The choice of this enzyme was suggested by the following considerations: a) the SOD is responsible for the neutralization of the toxic O_2^- radicals which are a product of oxidative processes which normally occur in the cell[3,4]; b) these O_2^- radicals increase notably in inflammatory processes[5,6]; c) the copper zinc SOD (Cu, Zn-SOD) has 2 cupric ions in the active site which are responsible for the peak of absorbance at 680 nm[7,8]; at 632.8 nm, which is the wavelength of the He-Ne laser, the Cu, Zn-SOD shows 80% of the maximum absorbance[7].

Materials and methods. Male Sprague-Dawley rats (250–300 g) were used for the experiment. Before and during the treatment they were allowed water and food ad libitum. The day before laser irradiation 2 areas (1.5×1.5 cm) of the lumbar region, symmetrically located on the right and on the left of the dorsal midline were depilated, keeping the animals under ether anesthesia. Of the 2 depilated areas, the one on the right side was irradiated and the other on the left side served as contralateral control. The irradiation was performed with a continuous wave He-Ne laser (Valfivre, Italy, 25 mW nominal output, wavelength 632.8 nm). A spot of about 1 cm in diameter with a minimum scanning