Detection of Haptoglobin in the High-Density Lipoprotein and the Very High-Density Lipoprotein Fractions from Sera of Calves with Experimental Pneumonia and Cows with Naturally Occurring Fatty Liver

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ABSTRACT. In addition to the lipoprotein-deficient d >1.25 fraction, haptoglobin was detected in the high-density lipoprotein (HDL) and the very high-density lipoprotein (VHDL) fractions from sera of calves with experimental pneumonia and cows with naturally occurring fatty liver. It was not found in the chylomicrons, very low-density lipoprotein and low-density lipoprotein fractions. Washing of the HDL fraction did not decrease the haptoglobin concentration. Transferrin and immunoglobulin G were immunoblotted to examine the possibility of contamination of the lipoprotein fractions by the d >1.25 fraction. The two serum proteins were detected only in the d>1.25 fraction, not in any lipoprotein fractions. The distribution pattern of haptoglobin in the lipoprotein fractions was distinct from that of serum albumin. Concentrations of haptoglobin in the HDL fractions from pneumonic sera were largely proportional to those in whole sera. Cholesteryl ester concentrations were decreased in sera from calves with pneumonia, as in cows with fatty liver. A protein immunologically related to hemoglobin was also detected in particular in the VHDL fractions from sera of both groups. These results suggest that haptoglobin or a complex with the hemoglobin-like protein may have a role or roles related to the lipid metabolism.—KEY WORDS: bovine, cholesteryl ester, haptoglobin, hemoglobin, high-density lipoprotein.

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Haptoglobin (Hp) is a hemoglobin (Hb)-binding acute phase protein. The main function of Hp was initially believed to be the prevention of loss of iron through the kidney by binding with Hb. After a report contradictive of the conjecture concerning the main Hp function [33], many biological roles of Hp, including bacteriostatic action [6], regulation of prostaglandin synthesis [11], angiogenic action [5] and induction of apoptosis [12] have been suggested. The liver is the major site of synthesis of Hp. In addition to the liver, Hp is synthesied by the lung [37], adipocytes [8] and the uterus [10].

Unlike in humans, Hp is undetectable in serum in healthy cattle. It is induced by cytokines [20], and its serum concentrations increase in cattle with inflammatory diseases [15, 16, 28]. Other than cytokines, Hp is induced by the anti-inflammatory glucocorticoids [9, 39] and ethionine (a fatty liver-inducing agent) [31], and is detected in the sera of cows with naturally occurring fatty liver [19, 24, 38], suggesting the involvement of Hp in the regulation of lipid metabolism. Acute phase proteins such as C-reactive protein and serum amyloid A (apoSAA) bind to lipoproteins and alter the concentration and density distribution of plasma lipids and apolipoproteins [2]. It is conceivable that Hp also is associated with lipoproteins. We show here that Hp was detected in the high-density lipoprotein (HDL) and very high-density lipoprotein (VHDL) fractions from sera of cows with fatty liver and calves with pneumonia.

MATERIALS AND METHODS

Induction of pneumonia: Twenty 2.5-month-old male Holstein calves weighing 72 to 96 kg were used. A suspension of *Pasteurella haemolytica* (serotype 1, I29 strain; 1×10^9 colony forming units) was separately administered to right lungs of 10 calves using a fiber-optic bronchoscope [34]. Ten other calves received the vehicle (phosphate-buffered saline; PBS) alone and were used as controls. Serum was collected at 0 (1 hr before administration) and 0.25 (6 hr after), and at 1, 2, 3, 4 and 7 days after treatment. Calves were exsanguinated at day 1 (3 control and 3 inoculated calves), day 2 (3 control and 3 inoculated), day 4 (2 control and 2 inoculated) and day 7 (2 control and 2 inoculated), to examine pathologic changes and also to collect bronchoalveolar lavage fluids.

Preparation of lipoprotein fractions: This was done as described previously [30], with slight modifications. Briefly, 4 m*l* of serum was overlaid with 2 m*l* of a solution of d=1.006 and centrifuged at 114,000 × g for 16 hr. The resulting top layer (1 m*l*) was collected as a mixture of chylomicrons and the very low-density lipoprotein fraction (CM-VLDL; d<1.006), and the next 1-m*l* layer was discarded. To the bottom layer (4 m*l*), 2 m*l* of a solution of d=1.182 was added, mixed and centrifuged at 114,000 × g for 20 hr. The top 1-m*l* layer was saved as the low-density lipoprotein fraction (LDL; d<1.163) and the next 1-m*l* layer was again removed. The resulting bottom layer (4 m*l*) was mixed with 2 m*l* of a solution of d=1.478 and centrifuged at 114,000 × g for 40 hr to collect the top 1-m*l* layer as the HDL (d<1.21). After removal of the next 1-m*l* layer, 0.88 m*l* of the d=1.478 solution was added to the bottom 4-m*l* layer, and centrifuged at 114,000 × g for 40 hr. The top 1-m*l* layer was collected as VHDL (d<1.25). The next 1-m*l*

layer and the remaining bottom layer were saved as an intermediate layer and the lipoprotein-deficient d>1.25 fraction, respectively. The lipoprotein fractions were dialyzed against 0.9% NaCl solution containing 10 mM Tris-HCl (pH 7.5), 1 mM EDTA and 0.01% sodium azide.

Immunoblot analysis: Hp was purified from cow serum and anti-Hp was raised in rabbits [38]. Rabbit anti-bovine serum albumin (BSA), anti-bovine immunoglobulin G (IgG) and anti-bovine transferrin sera were purchased from Bethyl Laboratories (Montgomery, Tex, U.S.A.), and rabbit anti-human Hb serum was from Sigma Chemical Co. (St Louis, Mo, U.S.A.). Immunoblot analysis was done as described previously [34]. The 2nd antibodies used were goat anti-rabbit IgG serum conjugated to horseradish peroxidase (Cappel Products, Organon Teknika, West Chester, Pa, U.S.A.). The relative amounts of immunoreactive proteins detected by use of a chemiluminescence reagent (ECL, Amersham International, Little Chafont, Buckinghamshire, UK) were determined by densitometry. Purified Hp at different concentrations was similarly immunoblotted to obtain a standard curve. The detection limit was approximately 10 *n*g of Hp under the conditions used. The percentage distribution of Hp in the lipoprotein fraction was calculated by the following equation:

$$\frac{\text{density of Hp in lipoprotein fraction} \times 1/4^{*}}{\text{density of Hp in lipoprotein fraction} \times 1/4^{*} + \text{density of Hp in the } d\text{>}1.25 \text{ fraction} \times 20^{**}} \times 100$$

(*1 m*l* of each lipoprotein fraction was prepared from 4 m*l* of serum; **dilution factor).

Other methods: The serum Hp concentration was evaluated by enzyme-linked immunosorbent assay (ELISA) [19]. The serum concentration of apolipoprotein A-I (apoA-I) was measured as described previously [21]. Protein concentration was determined by the method of Lowry *et al.* [13]. Serum concentrations of triglycerides (TG), total cholesterol (TC), free cholesterol (FC), phospholipids (PL) and nonesterified fatty acids (NEFA) were measured enzymatically. The cholesteryl ester (CE) concentration was calculated by subtracting the FC concentration from that for TC. Significance was analyzed using a paired *t*-test.

RESULTS

Hp was detected in the HDL and the VHDL fractions from sera of calves with pneumonia and of cows with fatty liver (Fig. 1). The distribution of Hp in the lipoprotein fractions was restricted to the HDL and VHDL fractions, and bands for Hp were not found in the CM-VLDL and the LDL fractions. In both sera from calves with pneumonia and cows with fatty liver, the Hp bands (the 23 kDa α-chain was more distinct than the 35 kDa β-chain) in the VHDL fractions were denser than those in the intermediate layers, suggesting that Hp detected in the lipoprotein fractions was not attributable to contamination by the d>1.25 fraction. The ratios of the concentration of Hp in the lipoprotein fractions to that of Hp in whole serum were calculated to be 0.288% for sera of calves with pneumonia (0.146% in HDL and 0.143% in VHDL) and 0.255% for sera of cows with fatty liver (0.140% in HDL and 0.115% in VHDL). When another 4 HDL fractions from sera of different calves with pneumonia at day 2 were analyzed as in Fig. 1, values for the percentage distribution showed individual variation (0.03 to 0.2%), but Hp was always detected in all HDL fractions examined.

The HDL fractions prepared from sera of different calves with pneumonia were washed once or twice by mixing with a d=1.21 solution and centrifuging at 114,000 × g for 40 hr. The washing of HDL had little effect on the Hp concentrations (arbitrary unit; means of 3 experiments: 225

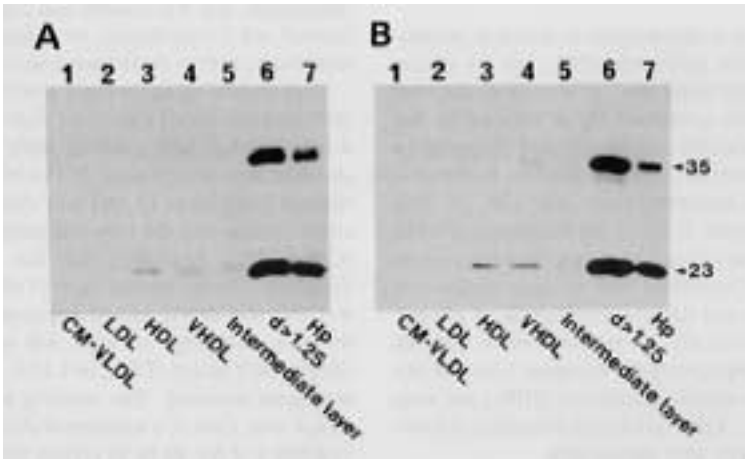

Fig. 1. Immunoblot analysis of Hp in the lipoprotein fractions from sera of a calf with pneumonia (2 days after inoculation, A) and of a cow with fatty liver (B). A 6.67 μ*l* aliquot of each lipoprotein fraction and of the d>1.25 fraction which had been diluted 20-fold with PBS was applied per well. Purified Hp (0.165 μg) was run in lane 7. 35, 35 kDa β-chain; 23, 23 kDa α-chain.

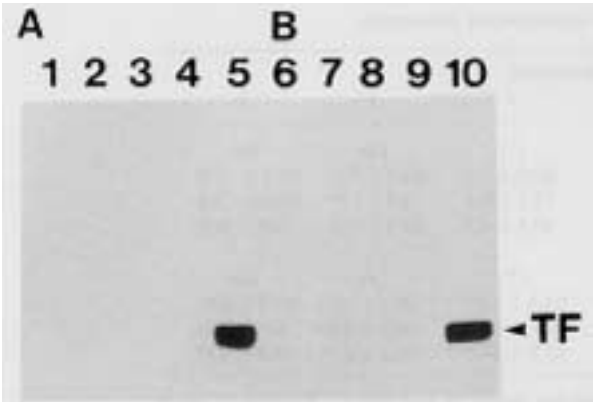

Fig. 2. Immunoblot analysis of transferrin (TF) in the lipoprotein fractions from sera of a calf with pneumonia (A) and a cow with fatty liver (B). Lanes are: 1 and 6, CM-VLDL; 2 and 7, LDL; 3 and 8, HDL; 4 and 9, VHDL; and 5 and 10, *d*>1.25 fraction. Other conditions were as described in Fig. 1.

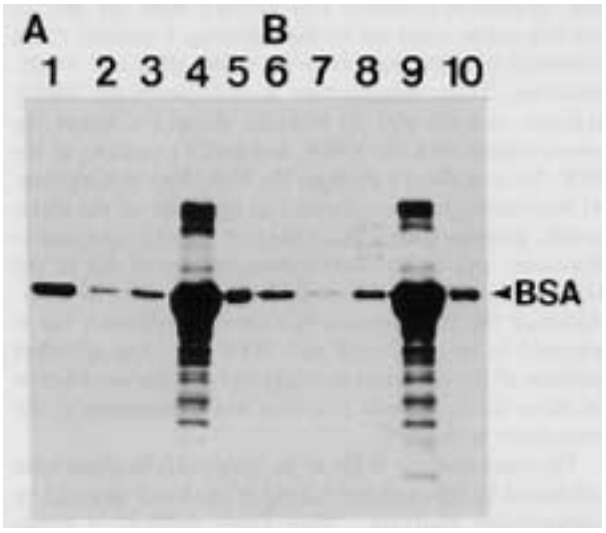

Fig. 3. Immunoblot analysis of bovine serum albumin (BSA) in the lipoprotein fractions from sera of a calf with pneumonia (A) and a cow with fatty liver (B). Lanes are: 1 and 6, CM-VLDL; 2 and 7, LDL; 3 and 8, HDL; 4 and 9, VHDL; and 5 and 10, *d*>1.25 fraction. Other conditions were as in Fig. 1.

for unwashed HDL; 265 for once-washed HDL; and 301 for twice-washed HDL), whereas it seemed to decrease their protein concentrations (mg/m*l*: 2.75 for unwashed HDL; 2.62 for once-washed HDL; and 2.49 for twice-washed HDL). Immunoblot analysis of transferrin (Fig. 2) and IgG (figure not shown) in the lipoprotein fractions from sera of calves with pneumonia and cows with fatty liver revealed that almost all signals of the two proteins were found only in the *d*>1.25 fractions, not in any lipoprotein fractions, again suggesting that the detection of Hp in the HDL and the VHDL fractions was not caused by contamination. BSA was abundantly distributed in the VHDL fraction as well as in the *d*>1.25 fraction (Fig. 3). The bands for BSA were

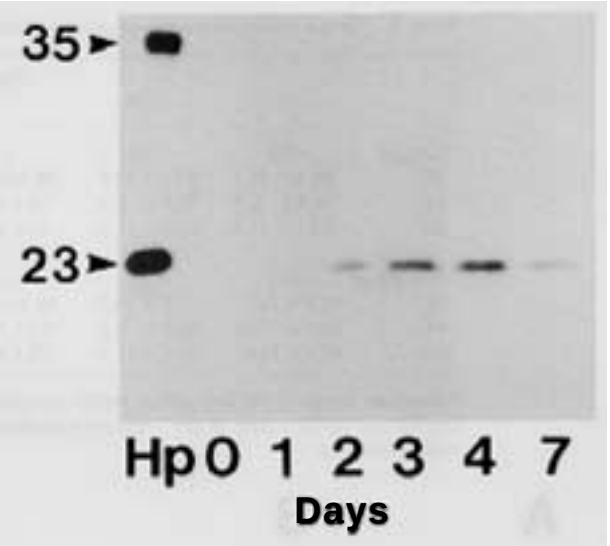

Fig. 4. Immunoblot analysis of Hp in the HDL fractions from pneumonic calf sera containing different Hp concentrations. The HDL fractions prepared from sera of calves with pneumonia at 0, 1, 2, 3, 4 and 7 days after inoculation were immunoblotted using anti-Hp. Concentrations of Hp in serum (μg/m*l*) and in HDL fractions (arbitrary unit) were: day 0 (<0.01 μg/m*l*; not detected), day 1 (194 μg/m*l*; 8), day 2 (717 μg/m*l*; 555), day 3 (940 μg/m*l*; 943), day 4 (722 μg/m*l*; 1,078), and day 7 (524 μg/m*l*; 295), respectively. Hp, purified Hp (0.165 μg).

also seen in the other fractions, the CM-VLDL fraction in particular, showing that the distribution of BSA in the lipoprotein fractions was distinct from that of Hp.

Immunoblot analysis of HDL fractions obtained from pneumonic sera containing different Hp concentrations indicated that the HDL Hp concentration concomitantly increased as the serum Hp concentration increased (Fig. 4). An increase of the Hp concentration was similarly observed in the VHDL fractions (figure not shown). Hp was not detected in any CM-VLDL or LDL fractions prepared from the 6 sera containing different Hp concentrations (not shown).

With the induction of pneumonia, serum cholesterol concentrations, CE in particular, were decreased (Table 1). The decrease of TC and CE concentrations at 1 day after treatment was observed in both control and inoculated groups, but their concentrations in controls were quickly restored at day 2, whereas those in the inoculated group remained decreased through 4 days after treatment. The serum FC concentration increased at day 1, but decreased at days 3 and 4 in the inoculated group. No significant change of FC concentration was observed in the control group. Changes of serum concentrations of TG, PL and NEFA were not specific to bacterial inoculation (data not shown). The serum concentration of apoA-I was not significantly altered during the course of the experiment.

A 64.5 kDa protein immunologically related to Hb was detected in the lipoprotein fractions from sera of calves

Table 1. Serum cholesterol concentrations of calves with experimental pneumonia

	Days after treatment					
	0	0.25	1	2	3	4
Control	(10)	(10)	(10)	(7)	(4)	(4)
TC	64.4 ± 13.3	63.2 ± 13.9	58.3 ± 11.8**	60.6 ± 5.1	60.3 ± 9.7	60.5 ± 13.0
FC	10.3 ± 2.4	10.4 ± 2.4	10.4 ± 2.4	10.1 ± 0.8	9.9 ± 1.9	10.4 ± 3.2
CE	54.1 ± 11.0	52.8 ± 11.5	47.9 ± 9.5**	50.5 ± 4.5	50.3 ± 7.8	50.1 ±9.9
Inoculated	(10)	(10)	(10)	(7)	(4)	(4)
TC	72.7 ± 14.2	71.6 ± 14.2	65.3 ± 11.6**	64.5 ± 13.7**	60.3 ± 9.1*	59.0 ± 6.9*
FC	11.6 ± 2.7	12.0 ± 2.8	13.1 ± 2.3**	11.1 ± 2.7	9.5 ± 0.9*	9.7 ± 0.7*
CE	61.1 ± 11.6	59.6 ± 11.6	52.1 ± 9.5**	53.5 ± 11.2**	50.7 ± 8.4*	49.3 ± 6.3*

Values are means ± SD and unit is mg/d*l*. Sample numbers are shown in parentheses. Because of small sample size (n=2), values at day 7 were not included. *, $P<0.05$; **, $P<0.01$, compared with the respective values for day 0.

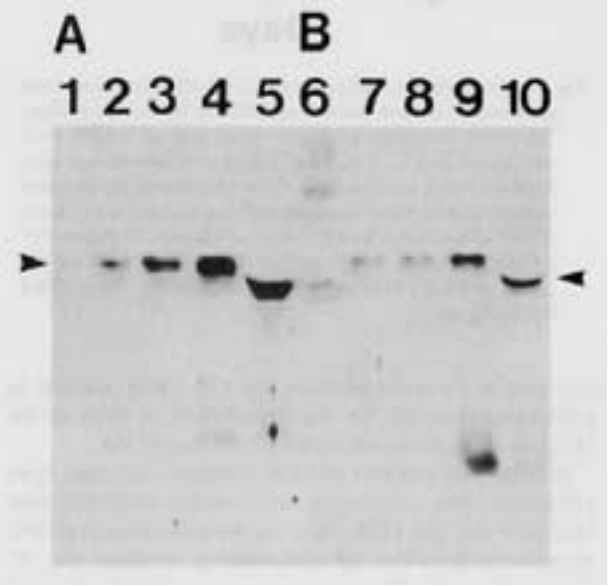

Fig. 5. Immunoblot analysis of Hb in the lipoprotein fractions from sera of a calf with pneumonia (A) and a cow with fatty liver (B). Lanes are: 1 and 6, CM-VLDL; 2 and 7, LDL; 3 and 8, HDL; 4 and 9, VHDL; and 5 and 10, *d* >1.25 fractions. The low molecular mass proteins seen in lanes 4 and 9 were identified as the 15 kDa Hb-like protein [35]. The 64.5 kDa Hb-like protein is indicated by arrows. Other conditions were as in Fig. 1.

with pneumonia and cows with fatty liver (Fig. 5). The signals for the Hb-like protein were most distinct in VHDL, moderate in HDL and LDL, and faint in the CM-VLDL fraction. The 15 kDa Hb-like protein previously shown in the HDL fraction [35] was found to be more abundant in the VHDL fraction than in the HDL fraction.

DISCUSSION

The present study showed that, in addition to the lipoprotein-deficient *d*>1.25 fraction, Hp is distributed in the HDL and VHDL fractions from sera of calves with experimentally induced pneumonia and from cows with naturally occurring fatty liver. The possibility that Hp in the lipoprotein fractions was derived from the *d*>1.25 fraction can be ruled out for the following 5 reasons: 1) the distribution of Hp was restricted to the HDL and VHDL fractions; 2) the intermediate layer between the VHDL fraction and the *d*>1.25 fraction showed a lower Hp concentration than the VHDL fraction; 3) washing of the HDL fraction did not decrease the HDL Hp concentration; 4) transferrin (of liver origin) and IgG (one of the major serum proteins) were not detected in any lipoprotein fractions; and 5) the distribution pattern of Hp in the lipoprotein fractions was distinct from that of BSA. Although the physiological relevance is unknown, Hp is reported to be associated with BSA [7]. The different patterns of the two proteins suggested that the distribution of Hp in the lipoprotein fractions was independent of the association with BSA.

The concentrations of Hp in the lipoprotein fractions were calculated by densitometer tracing of Hp bands detected by immunoblot analysis. This assay method is semi-quantitative and has disadvantages in terms of simplicity and rapidity. The ELISA method developed for Hp [19] can be used to measure the serum Hp concentration but is not applicable to lipoprotein Hp, because values obtained by this method are influenced by the concentration of BSA. Single radial immunodiffusion, another method for determination of the Hp concentration [16, 39], is too insensitive to evaluate lipoprotein Hp concentrations. Development of an ELISA method that is sensitive and, moreover, is not influenced by BSA and other factors, is required to precisely determine Hp concentrations in lipoprotein fractions.

It is unknown whether the Hp species in the lipoprotein fractions and the *d*>1.25 fraction are different. Human Hp consists of 3 subtypes; Hp 1–1, Hp 2–1 and Hp 2–2 [27]. Haptoglobin-related protein (Hpr), which shows structural homology to Hp, has been isolated [14]. Human and rat Hp are reported to be glycosylated [3, 4, 29]. The presence in bovines of the subtypes or Hpr has not yet been demonstrated. There are only a few fragmentary reports

concerning the glycosylation of bovine Hp [17].

ApoSAA is induced during the acute phase response and binds to HDL. In a rabbit model, the association of apoSAA resulted in depletion of apoA-I (the major apoprotein in the HDL) and lipids from apoSAA-enriched HDL particles [2]. HDL cholesterol redistributed to other lipoprotein fractions, while apoA-I disappeared from the circulation. We have previously shown that the apoSAA concentration is increased in the HDL fractions from calves with pneumonia [34]. In this study, the serum apoA-I concentration was found to be unaltered. Unlike the apoSAA binding to rabbit HDL, the association of Hp to HDL is suggested to be governed by a mechanism independent of the displacement of apoA-I.

The increase of the Hp concentration in the HDL fraction and the decrease of that of serum CE were simultaneously observed in calves with pneumonia. In bovines, more than half of CE is distributed in the HDL fraction [30]. A decrease in the CE concentration is also observed in cows with fatty liver [18, 19, 30]. We previously found that serum activity of lecithin:cholesterol acyltransferase (LCAT), which is distributed in the HDL and is responsible for esterification of cholesterol, was suppressed in cows with fatty liver [18, 32] and in calves with pneumonia (Nakagawa and Katoh, manuscript in preparation). The decrease of the CE concentration in sera from calves with pneumonia is suggested to be attributable to the suppressed LCAT activity. The Hp locus in human chromosome 16 is located close to the loci of LCAT and cholesteryl ester transfer protein [22], suggesting an intimate association of Hp and the cholesterol metabolism. Indeed, the human Hp subtype patterns significantly correlate with serum cholesterol concentrations [1, 23]. Induction of Hp and its association with the HDL may be linked to the decrease of CE concentration by reducing hepatic synthesis of LCAT, or by inhibiting the enzyme activity directly or indirectly through its interaction with the substrates (FC and phosphatidylcholine) or the activator (apoA-I). Biliary Hp is known to be a potent promoter of cholesterol crystallization [36].

We have recently shown the presence in the cow HDL fraction of the 15 kDa protein which has an N-terminal amino acid sequence nearly identical to that of bovine Hb α [35]. Other than the 15 kDa protein, the protein having molecular mass similar to that of native Hb (64.5 kDa) was detected by immunoblot analysis using anti-Hb. Like the 15 kDa protein, the 64.5 kDa Hb-like protein was most distinct in the VHDL fraction. It is conceivable that VHDL is a reservoir of Hb-like proteins and also of Hp for HDL. In human serum, Hpr and Hb are associated with a subset of HDL fractions, and the complex of Hpr-Hb is responsible for trypanocidal activity [25, 26]. The complex of Hp with Hb-like proteins in the HDL may act, for example, as a bactericidal factor, as in human trypanosomiasis. The functional relevance of the association of Hp with the lipoprotein fractions remains unanswered. Studies along these lines should open many new avenues of knowledge concerning the role of the multifunctional protein Hp in the lipid metabolism.

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