# Subfractionation of 1.006–1.063 g/ml components of badger plasma lipoproteins by using heparin-Sepharose affinity chromatography: relevance to the endocrine regulation of lipoprotein metabolism

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**Abstract** Badger plasma lipoproteins with density 1.006–1.063 g/ml have been subfractionated by means of affinity chromatography on a heparin-Sepharose column, using a modification of the method reported by Weisgraber and Mahley (1980. *J. Lipid Res.* **21:** 316–325). These experiments have provided evidence for the presence of three lipoprotein subfractions hereinafter termed fractions I, II, and III. Fraction I was cholesteryl ester- and phospholipid-rich (ca. 35% and 30% of lipoprotein mass, respectively), and contained apoA-I as its prominent apolipoprotein constituent. In contrast, triglyceride-rich fractions II and III both exhibited a complex apolipoprotein pattern, including apoB-100, apoA-I, and apoE whose amino acid composition and $NH_2$-terminal sequence in the badger are reported. However, fraction III appeared markedly enriched in apoE when compared to fraction II. On polyacrylamide gel electrophoresis, fraction I presented as a spectrum of particles with diameters in the 140–190 Å range. In contrast, fraction II migrated as a single band with a diameter of approximately 200 Å, and fraction III presented as a single band or a doublet with a diameter of 195–200 Å. The respective plasma concentrations and chemical compositions of the three chromatographic fractions were determined at four different dates of the year (i.e., April, August, November, and January), each of which corresponded to a different endocrine status in the badger. Thus hypothyroidism appeared to be associated with an increase in the concentration of fraction I, while the lowering in summer of the plasma level of testosterone correlated well with an increase in the concentration of fraction II. At the same time, the respective proportions of hydrophobic lipids in this latter material modified with an increase of triglycerides. Finally, both the apolipoprotein pattern of fraction III, and the chronologic profile of the successive variations of its concentration, suggest that it could represent a metabolic precursor of fraction II. These results suggest that the respective metabolism of the lipoproteins constituting the three chromatographic fractions could be under control by thyroid and testis secretions, operating via a complex combined regulation of the activities of the enzymes and receptors involved in these metabolic processes.—**Laplaud, P. M., L. Beaubatie, S. C. Rall, and D. Maurel.** Subfractionation of 1.006–1.063 g/ml components of badger plasma lipoproteins by using heparin-Sepharose affinity chromatography: relevance to the endocrine regulation of lipoprotein metabolism. *J. Lipid Res.* 1987. **28:** 900–912.

**Supplementary key words** gradient slab-gel electrophoresis • density gradient ultracentrifugation • apolipoprotein A-I • apolipoprotein B • apolipoprotein E • thyroid hormones • male sex hormones

In man, it is well established that certain endocrine disorders can promote premature atherogenesis. Such observations have emphasized the importance of studies aimed at the elucidation of the interrelationships between endocrine activities on the one hand, and lipid metabolism and transport by plasma lipoproteins on the other. In this context, we demonstrated in 1977 that the male European badger exhibited spontaneous seasonal variations of large amplitude in its plasma thyroxine and testosterone concentrations (1). These results suggested that this animal species could be used as an original and valuable model for studies dealing with the endocrine regulation of plasma lipoprotein metabolism. Indeed, since this date, successive studies in our laboratory have provided evidence for the presence, in

Abbreviations: VLDL, very low density lipoproteins, d < 1.006 g/ml; LDL, low density lipoproteins, d 1.006–1.063 g/ml; IDL, intermediate density lipoproteins, a part of LDL with d 1.006–1.019 g/ml; HDL, high density lipoproteins, d 1.063–1.21 g/ml; apo, apolipoprotein; EDTA, ethylene diamine tetraacetic acid; SDS, sodium dodecyl sulfate; HL, hepatic lipase.

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the European badger, of a rather complex plasma lipid transport system which undergoes considerable seasonal modification (2–4). We have unequivocally related such alterations to cyclical changes in the respective activities of the thyroid (5) and testis (6). Of special interest is the lipoprotein content of the 1.006–1.063 g/ml density interval which, on the basis of data from analytical ultracentrifugation (2) and density gradient ultracentrifugation (3), appears highly heterogeneous. The protein moieties of lipoproteins belonging to this density range contain elevated amounts of both apoA-I and an equivalent to human apoB-100 (7). In addition, a protein component with $M_r \cong 45{,}000$, and thus comparable with that of apoE, is also present (7). Furthermore, badger lipoproteins with d 1.006–1.063 g/ml are particularly affected by modifications in the endocrine status of the animal. Indeed, in this density range both seasonal spontaneous hypothyroidism (3) and thyroidectomy (5) induce the appearance of elevated concentrations of cholesteryl ester-rich lipoproteins (d ≅ 1.015–1.027 g/ml), with a concomitant decrease in the proportion of apoB in their protein moiety. It is of note that, in our experience, neither intact animals nor thyroidectomized badgers exhibited any sign of atherosclerotic lesions when morphological studies were performed on their aortas, coronary vessels, and heart tissues (5). On the contrary, castration and, to a lesser degree, the seasonal spontaneous reduction in testis activity, result in a moderate increase in plasma triglyceride concentration. These lipids were essentially transported by lipoproteins with d ≅ 1.027–1.065 g/ml, in which, in castrated badgers, the proportion of apoB was higher than that observed in their counterparts in intact animals (6). The questions then arise as to *1)* how many different lipoprotein populations are present within the 1.006–1.063 g/ml density range, and *2)* how may a methodology be devised which allows isolation of each of these lipoprotein components, and enables study of their composition, metabolism, and the endocrine regulation of their metabolism.

In 1972, Iverius (8) demonstrated the ability of heparin-Sepharose to bind β-lipoproteins at low salt concentrations, and the role of their apolipoproteins in this interaction. Subsequently, both apoB and apoE were shown to interact with heparin, through a process that can be counteracted by neutralization of the positive charges of arginine and lysine residues (9). As other apolipoproteins, and especially apoA-I, do not interact with heparin under usual experimental conditions, this affinity chromatography technique provides a means for separating lipoproteins of similar hydrated density but differing in their apolipoprotein content. Such a methodology has been employed in studies of both human (10–12) and animal (13–17) lipoproteins. For their part, Weisgraber and Mahley (18) subfractionated human HDL into four molecular species, namely a quantitatively prominent "HDL without E" fraction, "HDL with E", and two "β-subclasses", each containing apoB. This prompted us to perform heparin-Sepharose affinity chromatography of badger lipoproteins, with d 1.006–1.063 g/ml, in an attempt to fractionate these lipoproteins into several subfractions differing in their apoprotein content and thus, most probably, in their in vivo metabolism.

## MATERIALS AND METHODS

### Animals and diets

The adult male badgers, bred in the Centre d'Etudes Biologiques des Animaux Sauvages, were approximately 2–6 years old. They were kept individually in 6 $m^2$ parks under natural conditions of light, temperature, and rainfall. The animals were offered a diet consisting of a commercial food for dogs (Canina, Duquesne-Purina) and containing the following proportions by weight of the major constituents: protein, 20%: animal fat, 6 %; carbohydrate, 5%; vitamin A, 15,000 I.U./kg; vitamin $D_3$, 1,500 I.U./kg. Water was provided ad libitum. With the exception of a few cold days in December or January, food consumption by our animals was regular during the entire year.

### Collection and treatment of blood

For each of the successive series of manipulations, blood samples were taken from animals that had been fasted overnight for approximately 18 hr. Blood was collected on EDTA (final concentration 1 mM), at approximately 11 AM, by puncture of the radial vein. Plasma was then separated by low speed centrifugation and brought to the laboratory on ice.

### Chemical analysis

The enzymatic techniques used for measurement of the concentrations of the different classes of lipids, both in plasmas and in lipoprotein fractions, have beeen described elsewhere (4). The methodology of Lowry et al. (19) was employed for the assay of protein concentrations, using bovine serum albumin as standard.

### Ultracentrifugal methods

In each of the manipulations described below, all the NaCl and/or NaBr solutions used for adjustment of densities, as well as for dialysis of the lipoprotein fractions, contained EDTA (0.4 g/l), sodium azide (0.1 g/l), and merthiolate, (1 mg/l).

*Isolation of 1.006–1.063 g/ml lipoproteins.* Lipoproteins were isolated using an MSE Prepspin 50 ultracentrifuge (MSE, Crawley, UK) in an aluminum fixed-angle rotor (capacity 8 × 14 ml), at 17°C and 40,000 rpm (100,000 $g_{avg}$). Under these conditions, VLDL were first floated by centrifugation for 20 hr in a 1.006 g/ml NaCl medium. Density 1.006–1.063 g/ml lipoproteins were then isolated in a 1.063 g/ml NaCl medium by centrifugation for 24 hr.

Prior to further analysis, LDL were dialyzed for 3 × 12 hr

against an NaCl solution of d 1.006 g/ml at 4°C, and concentrated to the desired volume using an Amicon 8 MC micro-ultrafiltration system fitted with XM 50 ultrafiltration membranes, exclusion limit 50,000 (Amicon, Lexington, MA).

*Determination of the hydrated density range of each d 1.006–1.063 g/ml lipoprotein subfraction isolated by affinity chromatography.* For this purpose, we made use of a density gradient adapted from that proposed by Shen et al. (20) for the subfractionation of human d 1.019–1.063 g/ml lipoproteins. Prior to these manipulations, badger plasma d 1.006–1.063 g/ml lipoproteins isolated as reported above were exhaustively dialyzed for 3 × 12 hr against an NaBr solution of d 1.040 g/ml at 4°C. Discontinuous gradients were then constructed in polycarbonate tubes (nominal capacity 14 ml) by layering successively, using a Buchler Auto Densiflow (Buchler Instruments, Fort Lee, NJ) 3.2 ml of NaBr solution, d 1.054 g/ml; 2.6 ml of lipoprotein solution, d 1.040 g/ml (protein concentration: 0.5–2.0 mg/ml); 3.2 ml of NaBr solution, d 1.0275 g/ml; and 3.2 ml of NaCl solution, d 1.006 g/ml. For determination of the shape of the gradient after centrifugation, identical control gradients were constructed using 2.6 ml of NaBr solution, d 1.040 g/ml, in place of the lipoprotein sample. In all cases, ultracentrifugation was performed in an MSE 6 ×14 ml titanium swing-out rotor, for 48 hr at 40,000 rpm (196,000 $g_{avg}$) at 17°C. Successive 1.0-ml fractions were then aspirated and subjected to further chemical and electrophoretic analysis after dialysis for 3 × 12 hr at 4°C against an NaCl solution of d 1.006 g/ml.

### Affinity chromatography

Our experiments were based on the methodology reported by Weisgraber and Mahley (18) for subfractionation of human HDL. Preliminary experiments were conducted using the elution scheme originally described by these authors, i.e., a five-step gradient using a series of NaCl–Tris buffers, pH 7.4, with progressive increases in NaCl concentration from 0.05 M to 0.07 M, 0.115 M, 0.29 M, and finally 0.6 M. This gradient resulted in effective separation of lipoprotein subfractions with different apolipoprotein content. However, experience showed that a similarly effective separation could be obtained using a NaCl gradient consisting of only four successive steps. Thus the methodology used in the experiments reported hereinafter was as follows. The concentrate of d 1.006–1.063 g/ml lipoproteins (15–25 mg of lipoprotein protein) in 2–3 ml of NaCl–Tris buffer (0.115 M NaCl, 0.005 M Tris, pH 7.4) to which $MnCl_2$ (final concentration 0.025 M) was added immediately before applying the sample to the column, was subjected to affinity chromatography in a 12 × 300 mm glass column (LKB, Bromma, Sweden), operated at 4°C. The column had been filled with heparin-Sepharose prepared from heparin (Hynson, Westcott, Dunning, Inc., Baltimore, MD) and Sepharose CL 6 B (Pharmacia Fine Chemicals), according to Weisgraber and Mahley (18). Elution with the manganese-containing buffer (application buffer described above) was continued until lipoprotein fraction I (see results) had been collected. At this point, the manganese was deleted but the NaCl concentration in eluting buffer remained unchanged. This resulted in the elution of lipoprotein fraction II. After the elution of this latter fraction was completed, the NaCl concentration in the buffer applied to the top of the column was increased to 0.29 M, which allowed elution of lipoprotein fraction III. A final increase in the NaCl concentration in the eluant to 0.6 M was then made.

The elution was monitored at 280 nm using an LKB Uvicord detector and recorder. After collection of the fractions, those corresponding to each peak were pooled, concentrated using the same apparatus described above, and dialyzed against 0.15 M NaCl for 3 × 12 hr at 4°C.

### Electrophoretic methods

Polyacrylamide gel electrophoresis of plasma lipoproteins and of lipoprotein fractions was performed using *1)* the three-step gradient method of Fruchart (21) and *2)* commercially available polyacrylamide gel slabs (Lipofilm, Sebia, Issy-les-Moulineaux, France). In both cases, no attempt was made to quantify the various components observed.

Continuous-gradient slab-gel electrophoresis was performed on a Pharmacia electrophoresis apparatus GE-2/4 loaded with gradient gels PAA 2/16 (Pharmacia Fine Chemicals, Uppsala, Sweden), according to conditions already reported (5). The Stokes diameters of the particles were calculated using the Stokes-Einstein equation as described by Anderson et al. (22). The molecular weights of apolipoproteins were estimated by electrophoresis in SDS-polyacrylamide gels of either 10% monomer concentration as described by Weber and Osborn (23), or 3% monomer concentration according to the modification of Weisgraber et al. (24) of the methodology of Stephens (25). Calibration curves for estimation of molecular weights were constructed from a series of molecular weight markers ranging in size from 14,000 to 94,000 (Low Molecular Weight electrophoresis calibration kit, Pharmacia Fine Chemicals), and from 56,000 to 280,000 (BDH Biochemicals, Poole, U.K.). Staining of the gels was performed using either Coomassie brilliant blue R250 (10% monomer gels) or the technique of Karlson et al. (26) (3% monomer gels).

Isolation of apoA-I and apoE was performed using preparative electrophoresis according to Stephens (25). Details of our procedures have been previously published (7).

### Chromatographic isolation of apolipoprotein B

These procedures were performed using gel filtration chromatography on Sephadex G-200 in anionic detergent, according to the adaptation by Chapman and Goldstein (27) of the technique of Herbert et al. (28). The precise conditions used in our experiments have been reported (7).

### Amino acid and sequence analysis

Amino acid analyses of badger apoA-I, apoB, and apoE were carried out on a Beckman 121 MB analyzer (Beckman Instruments, Fullerton, CA).

Partial sequence analysis of apoE was carried out on a Beckman 890 C sequencer. Phenylthiohydantoin amino acids were identified and quantified on a Beckman 322 high performance liquid chromatograph. Details of these procedures have been already reported (7).

### Plasma hormones

Testosterone plasma levels were measured using commercially available radioimmunoassay kits (Biomérieux, France). Total plasma thyroxine was assayed by the isotopic competition technique according to Vigouroux (29).

## RESULTS

### Plasma lipids and electrophoresis of lipoproteins

On each series of samplings, plasma lipids (total cholesterol, esterified cholesterol/total cholesterol, triglycerides, and phospholipids) were analyzed in all animals used for our experiments. Results (see Table 3) were highly consistent with corresponding data obtained previously at similar dates of the year in male intact badgers, and described in our earlier reports (2–6).

A similar comment applies to the plasma lipoprotein profiles obtained by polyacrylamide gel electrophoresis according to the technique of Fruchart (21). For this reason, these data are not shown.

### Affinity chromatography

*Elution profile and determination of the apolipoprotein content of the lipoprotein fractions.* The affinity chromatography profile was qualitatively similar in the different lipoprotein pools assayed and exhibited three distinct fractions (**Fig. 1**). These components are hereinafter termed fractions I, II, and III, respectively. However, it is of note that, while a complete return to baseline was consistently achieved after eluting fraction I, such was not the case between fractions II and III, where a small amount of UV-absorbing material was present. Considering the fact that these latter fractions were representative of apoB-containing lipoproteins (see below), this phenomenon is similar to that originally reported by Weisgraber and Mahley (18) when eluting, within similar chromatographic conditions, fractions termed, respectively, the $\beta_1$- and $\beta_2$-subclasses of human HDL.

Electrophoresis in SDS-polyacrylamide gels with 10% monomer concentration provided evidence for the different apolipoprotein content of the three chromatographic fractions (Fig. 1). Fraction I was characterized by the prominent presence of an apolipoprotein with $M_r$ 27,000–29,000 and thus similar to that of human apoA-I. In addition, lower size peptides ($M_r$ in the 13,000–14,000 and 9,000–10,000 ranges, respectively) were also present. No high $M_r$ apolipoprotein material was detectable at the top of the gels, even when protein amounts in excess of 100 $\mu$g were electrophoresed. In contrast, gels representative of the protein content of fraction II clearly showed the presence of a high $M_r$ (approx. > 250,000) apolipoprotein material, while a detectable amount of the protein with $M_r$ in the 27,000–29,000 range was also present. In addition, traces of supplementary proteins with either higher (42,000–45,000) or lower (13,000–14,000 and 9,000–10,000) $M_r$ were also noted. Finally, the pattern representative of the protein content of fraction III was qualitatively similar to that of fraction II. However, the respective proportions of the components with respective $M_r$ 27,000–29,000 and 42,000–45,000 were markedly altered to the benefit of the latter.

Taken together, these results are consistent with previous results from this laboratory. Indeed, in a recent report (7), we provided unequivocal evidence for the presence, in badger d 1.006–1.063 g/ml lipoproteins, of high levels of apoA-I as well as of an apoprotein component ($apoB_H$) similar, in terms of $M_r$ and amino acid composition, to human apoB-100. We have further confirmed the consistency between our successive experiments using the techniques described below. First, as regards the component exhibiting an $M_r$ of 27,000–29,000, we have isolated it from the protein moiety of fraction I, using preparative electrophoresis. The resulting material, which stained as a single band in SDS-polyacrylamide gels of 10% monomer concentration, was submitted to amino acid analysis. Two different preparations were examined; in each case, results were indistinguishable from those previously reported by us for the amino acid content of badger apoA-I isolated either from d 1.006–1.063 g/ml or 1.063–1.21 g/ml lipoproteins (7). For this reason, these data are not presented.

The higher $M_r$ protein components contained in fractions II and III were electrophoretically examined using 3% monomer SDS gels (Fig. 1). Results were again comparable to those presented in our previous report dealing with the characterization of badger apoB proteins (7). Indeed, in both fractions II and III, the major apoB component exhibited an $M_r$ in the 550,000 range, and was thus typical of badger $apoB_H$. In addition, lower $M_r$ components of minor quantitative importance (possibly occurring as the result of partial proteolytic degradation of $apoB_H$, see ref. 7) were noted. The ratios of their respective $M_r$ values to that of $apoB_H$ were 85:100 and 37:100 relationships, respectively, and thus similar to those already reported by us (7). A single preparation of these apoB components was obtained by gel filtration chromatography on Sephadex G-200 in anionic detergent and submitted to amino acid analysis. Results were strictly similar to those presented for the same proteins in our recent report (7).

Supplementary proteins with respective approximate $M_r$ values of 80,000, 45,000, and > 30,000 were detected in the

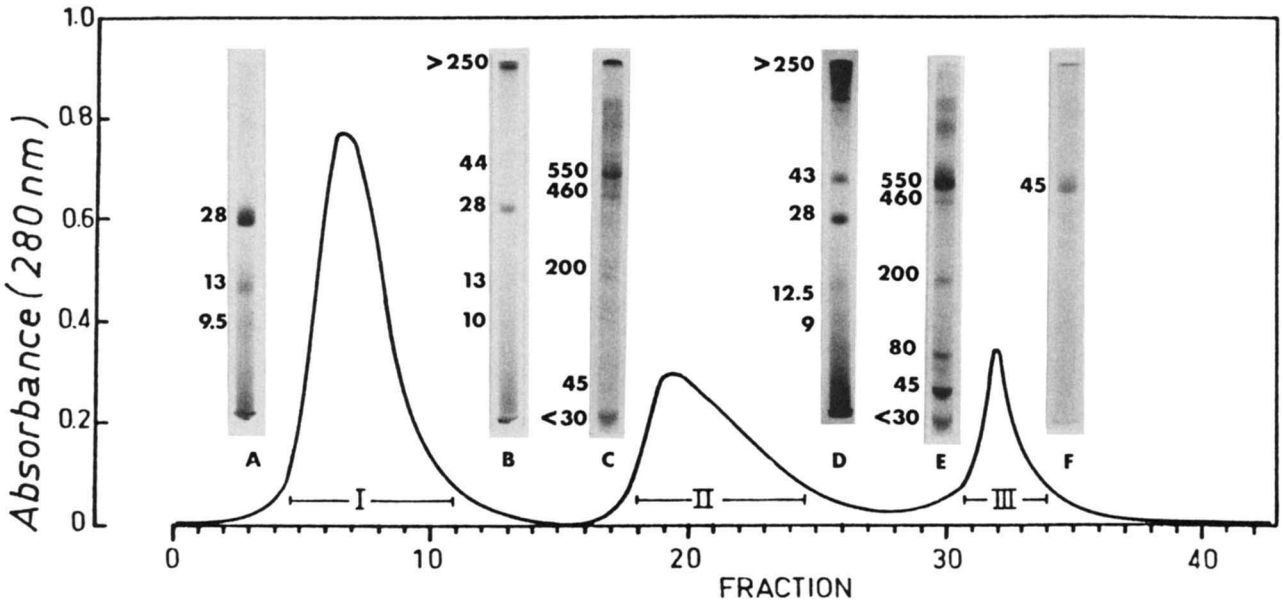


**Fig. 1.** Heparin-Sepharose affinity chromatography of badger plasma d 1.006–1.063 g/ml lipoproteins. The elution profile shown on this figure is representative of a lipoprotein sample originating from a pool of badger plasmas obtained in January. However, the affinity chromatography patterns corresponding to samples obtained at the different dates of the year were qualitatively similar (see Results, Affinity chromatography). In each case, 15–20 mg of lipoprotein protein in 0.005 M Tris buffer, pH 7.4, containing 0.115 M NaCl and 0.025 M $MnCl_2$, was applied to the 12 × 300 mm column. The column was operated at a rate of 24 ml/hr and 4-ml fractions were collected. Gels A, B, and D: electrophoresis of the apolipoproteins of fractions I, II, and III, respectively, in SDS-polyacrylamide gels of 10% monomer concentration. Gels C and E: electrophoresis of the apolipoproteins of fractions II and III, respectively, in SDS-polyacrylamide gels of 3% monomer concentration. Gel F is representative of badger apoE after its purification from the apoprotein moiety of fraction III. Seventy–100 μg protein was applied to each gel. Molecular weights are expressed in kD.

lower part of the 3% monomer gels. Among these components, that exhibiting an $M_r \cong 80{,}000$ was observable only in gels representative of the protein content of fraction III and could correspond to a protein with similar $M_r$ whose presence was already noted in our early studies (2). As in 10% monomer gels, the component with $M_r \cong 45{,}000$ was present in much greater proportion among the protein moiety of fraction III than in that from fraction II.

This apolipoprotein component was isolated from the protein moiety of fraction III, using preparative SDS-polyacrylamide gel electrophoresis under experimental conditions similar to those used for purification of apoA-I. The resulting material, which consistently stained as a single band in 10% monomer analytical gels (Fig. 1), was submitted to amino acid analysis and partial sequence determination. Thus, in accordance with its molecular weight, the amino acid content of this apolipoprotein was shown to be very close to that of both human and canine apoE (30), this latter species being chosen for its close phylogenetic vicinity with the badger (**Table 1**). Indeed, modifications in the respective proportions of the different amino acids between the two animals were only apparent in the cases of glutamic acid (badger, 23.3%; dog, 26.7%) and glycine (badger, 7.3%; dog, 4.8%). It is however of note that, in both cases, values in the badger were identical to those in the corresponding human protein.

The amino acid sequence of the amino-terminal portion of badger apoE was determined to the 18th residue; results are presented in **Table 2** and compared to those for the homologous protein from dog (30) and man (31). ApoE from different species are known to be non-homologous at both the $NH_2$- and COOH-termini except for closely related species; indeed, our data show that a relatively high degree of homology was retained between amino-terminal sequences in badger and dog apoE since identical residues were identified in the two animal proteins at nine positions in the amino acid chain.

*Electrophoretic studies on the lipoprotein fractions.* The electrophoretic behavior of the lipoprotein content of the three successive affinity chromatography fractions was assayed in three different systems. Firstly, we performed electrophoresis of both the starting lipoprotein material and the resulting chromatographic fractions on the three-step gradient gels used in all our previous studies dealing with badger lipoproteins (2–6) (**Fig. 2**). Total d 1.006–1.063 g/ml lipoproteins exhibited the heterogeneous appearance repeatedly reported by us when studying badger lipoproteins, especially during the cold months. Fraction I appeared to consist of a broad spectrum of particles, with mobilities extending to the position typical of human HDL. In contrast, fraction II presented as an intensely stained and unique band, migrating slightly farther in gel 3 than human LDL. For its part, fraction III presented as a band with mobility comparable to, or slightly lower than, that of the lipoprotein

TABLE 1. Amino acid composition of apolipoprotein E from badger, dog, and man[a]

| | Badger | Canine | Human |
|---|---|---|---|
| | | *mol%* | |
| Lys | 3.4 ± 0.3 | 4.5 | 4.2 |
| His | 1.0 ± 0.1 | 0.6 | 1.0 |
| Arg | 9.9 ± 0.1 | 10.5 | 9.6 |
| Asp | 5.4 ± 0.1 | 6.5 | 5.2 |
| Thr | 4.7 ± 0.1 | 4.1 | 4.1 |
| Ser | 5.0 ± 0.5 | 4.2 | 6.8 |
| Glu | 23.3 ± 1.2 | 26.7 | 23.4 |
| Pro | 4.7 ± 0.4 | 3.8 | 3.0 |
| Gly | 7.3 ± 0.8 | 4.8 | 7.3 |
| Ala | 11.7 ± 0.5 | 11.8 | 11.5 |
| Val | 6.6 ± 0.2 | 5.3 | 6.8 |
| Met | 2.5 ± 0.1 | 1.9 | 0.7 |
| Ile | 1.3 ± 0.2 | 0.9 | 0.7 |
| Leu | 10.5 ± 0.2 | 12.3 | 13.1 |
| Tyr | 1.3 ± 0.1 | 0.8 | 1.2 |
| Phe | 1.6 ± 0.2 | 1.1 | 1.1 |
| Cys | 0 | 0 | 0.4 |
| Tryptophan | n.d.[b] | n.d. | n.d. |

[a]In the case of badger apolipoprotein E, the results are means ± SD of three different preparations; each sample was analyzed in duplicate or triplicate. Values for canine and human apolipoprotein E are taken from ref. 30.
[b]Not determined, n.d.

constituting fraction II; in addition, a certain amount of stained material remained at the limits separating the 1st and 2nd and 2nd and 3rd gels, respectively. Finally, a faint supplementary band, entering gel 3 for a small distance, was occasionally seen.

The same lipoprotein samples were also examined using commercially available polyacrylamide gel slabs (Fig. 2); results were generally consistent with those reported in the preceding paragraph. However, as regards fractions II and III, it is of note that, while most of the lipoprotein components appeared as a single band, some stained material was seen at the limit between the 1st and 2nd gels.

The precise nature of such material, whether remaining at the limits between 1st and 2nd, and 2nd and 3rd gels, in the former electrophoretic system, or at the limit between 1st and 2nd gels in the latter, awaits identification. It is usually considered that, in man, the only lipoproteins with d < 1.063 g/ml and remaining at the limit between 1st and 2nd gels are VLDL, while lipoproteins remaining at the limit between 2nd and 3rd gels in the three-step gradient are IDL, including the abnormal lipoproteins present in type III hyperlipoproteinemias (32). These different types of particles were not observed in gels representative of total d 1.006–1.063 g/ml lipoproteins submitted to affinity chromatography, as attested to by the appearance of gel 1 and lane a on Fig. 2. Yet it remains possible that some lipoproteins with such migration characteristics existed in these samples, albeit in amounts too small to be detectable by our staining technique.

However, in our opinion, an artifactual origin is most likely, possibly due to an excess of non-lipoprotein-bound reagent. Indeed, variable binding of stains according to the chemical composition of lipoproteins is well known. Such a phenomenon was clearly demonstrated for Sudan Black (33, 34) which binds with greater affinity to cholesteryl esters than to triglycerides. This stain is the one used in the second technique reported above. Although, to our knowledge, no comparable data in the literature exist regarding the behavior of nitro blue tetrazolium as used in our first electrophoretic technique, it may be speculated that similar differences in lipid binding could exist when using this staining compound. A clear difference between the respective lipid compositions of the cholesteryl ester-rich fraction I on one hand, and of triglyceride-rich fractions II and III, on the other hand, is evident from our data (see below, Chemical composition). Therefore it is possible that, in gels used for examination of the electrophoretic behavior of these two latter fractions, non-bound nitro blue tetrazolium and Sudan black could create supplementary "bands" that do not represent authentic lipoprotein material.

Finally, the respective Stokes diameters (means ± SD, n = 6) of the lipoproteins contained in the different chromatographic fractions were determined by electrophoresis on 2–16% continuous gradient polyacrylamide gel slabs (**Fig. 3**). Fraction 1 again presented as a continuum of particles, with diameters ranging from 140.3 ± 4.9 to 190.0

TABLE 2. $NH_2$ - terminal sequence of apolipoprotein E from badger, dog, and man

| Species | Residue Number | | | | | | | | | | | | | | | | | |
|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|
| | . | . | . | . | 5 | . | . | . | . | 10 | . | . | . | . | 15 | . | . | 18 |
| Badger | D | V | E | P | E | S | P | L | E | G | E | P | E | P | E | P | K | L |
| Dog | D | V | Q | P | E | P | E | L | E | R | E | L | E | P | K | V | Q | Q |
| Man | K | V | E | Q | A | V | E | T | E | P | E | P | E | L | R | Q | Q | T |

The dog and human sequences are taken from references 30 and 31, respectively. Homologous regions in badger and canine apoE are indicated by rectangles.

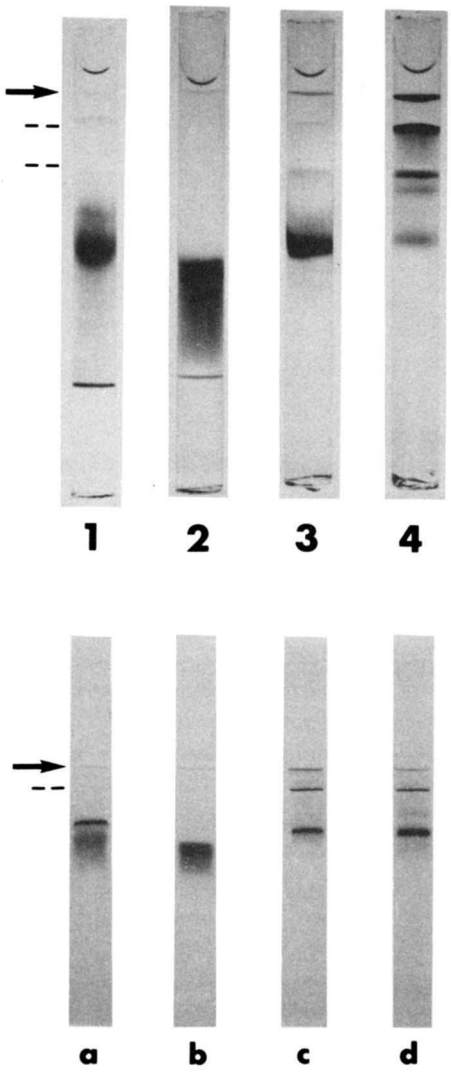


**Fig. 2.** Polyacrylamide gel electrophoresis of affinity chromatography fractions from badger d 1.006–1.063 g/ml lipoproteins. Top: electrophoresis in the three-step gradient system of Fruchart (21). Lipoproteins were pre-stained using nitro blue tetrazolium. The arrow indicates the point of application; upper and lower dotted lines correspond to the limits between 1st and 2nd, and 2nd and 3rd gels, respectively. Bottom: slab-gel electrophoresis using commercially available gels (Lipofilm, Sebia, France). Lipoproteins were pre-stained using Sudan black. The arrow indicates the point of application; the dotted line corresponds to the limit between the two successive gels (2% and 3% monomer concentration, respectively). Gel 1 and lane a: total 1.006–1.063 g/ml lipoproteins; gel 2 and lane b: fraction I; gel 3 and lane c: fraction II; gel 4 and lane d: fraction III.

± 5.4 Å. In contrast, fraction II showed a single band with diameter 201.2 ± 4.5 Å. The material contained in fraction III migrated either as a single band (diameter: 195.3 ± 3.2 Å, n = 4) or as a doublet (see Fig. 3) in which both components (respective diameters 195.3–196.2 and 198.8–200.1 Å) stained with comparable intensity.

*Seasonal changes in the respective concentrations and chemical composition of the fractions.* We have previously shown (2, 3) that the lipoprotein content of the 1.006–1.063 g/ml density interval in badger plasma varies widely, both qualitatively and quantitatively, according to the period of the year. Our more recent studies have clearly related these variations to seasonal modifications in the activity of the thyroid (5) and testis (6). It thus appeared of interest to investigate whether changes could occur, during the course of the year, in the respective plasma concentrations of the different fractions as well as in their chemical composition. For this purpose, affinity chromatography was performed on d 1.006–1.063 g/ml lipoproteins originating from pools of plasmas obtained in January, April, August and November. These dates were chosen so as to allow an estimation of the consequences of various endocrine conditions (see below, Plasma hormones) on these badger lipoproteins.

**Table 3** provides evidence for large seasonal changes in the respective concentrations of the three chromatographic fractions. Thus, plasma levels of fractions I and II reached simultaneous maxima in January (342.1 mg/100 ml and 213.7 mg/100 ml, respectively), and minima in April (101.8 mg/100 ml and 104.1 mg/100 ml, respectively), while a secondary maximum was evident for fraction II only in August. The concentration of fraction III showed a sixfold change from August (27.3 mg/100 ml) to November (158.7 mg/100 ml).

Data on the chemical composition of the three chromatographic fractions are reported in **Table 4**. As regards fraction I, it is of note that the respective proportions of its different constituents remained essentially constant whatever the date of sampling. Thus this fraction was cholesteryl ester- and phospholipid-rich (approximately 35% and 30%, respectively), but deficient in triglyceride (< 1%, approximately). In contrast, fractions II and III both contained substantial amounts of triglycerides, although the proportion of this lipid class varied substantially during the course of the year. However, the increase noted in the percentage of triglycerides did not occur simultaneously in the two lipo-

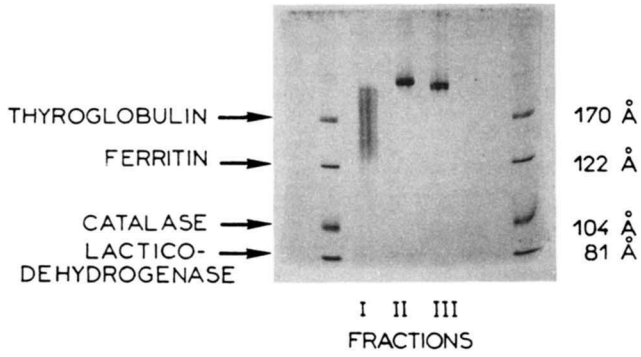


**Fig. 3.** Polyacrylamide slab-gel electrophoresis of affinity chromatography fractions from badger d 1.006–1.063 g/ml lipoproteins. Electrophoresis was performed on a PAA 2/16 polyacrylamide continuous gradient slab-gel (Pharmacia Fine Chemicals). The two outer lanes on electrophoresis slab contained a set of marker proteins whose names and Stokes diameters are indicated at the left and right of the photograph of the gel, respectively.

TABLE 3. Seasonal variations in the respective concentrations of plasma lipids, fractions isolated from d 1.006–1.063 g/ml lipoproteins using heparin-Sepharose affinity chromatography, and plasma hormones

| | January | April | August | November |
|---|---|---|---|---|
| Plasma lipids (mg/100 ml)[a] | | | | |
| Total cholesterol | 378 ± 23 | 213 ± 55 | 287 ± 51 | 383 ± 65 |
| Esterified cholesterol / Total cholesterol | 0.61 ± 0.04 | 0.72 ± 0.01 | 0.75 ± 0.02 | 0.62 ± 0.05 |
| Phospholipids | 490 ± 23 | 300 ± 75 | 360 ± 66 | 477 ± 46 |
| Triglycerides | 118 ± 26 | 89 ± 15 | 80 ± 11 | 135 ± 14 |
| Plasma concentration (mg/100 ml) | | | | |
| Fraction I | 342.1 | 101.8 | 148.8 | 286.3 |
| Fraction II | 213.7 | 104.1 | 169.9 | 119.7 |
| Fraction III | 76.5 | 55.1 | 27.3 | 158.7 |
| Plasma hormones (ng/ml)[b] | | | | |
| Thyroxine | 14.5 ± 0.8 | 19.4 ± 1.1 | 21.6 ± 2.2 | 15.7 ± 1.0 |
| Testosterone | 11.9 ± 2.7 | 22.3 ± 1.8 | 5.5 ± 1.8 | 3.0 ± 0.8 |

[a]Mean ± SD.
[b]Mean ± SEM.

protein components, being observed from April to August (from 17.4% to 26.1%) in fraction II and between August and November (from 17.7% to 30.9%) in fraction III.

Finally, it is of note that, in all samples assayed, an inverse relationship was observed between the respective proportions of triglycerides and cholesteryl esters in fractions II and III; however, in each of these fractions, the successive values of the ratio % triglyceride/% cholesteryl ester exhibited marked changes, i.e., from 0.73 in April to 1.47 in August (fraction II) and from 0.59 in August to 1.82 in November (fraction III).

*Determination of the respective density distributions of lipoproteins contained in the chromatographic fractions.* For this purpose, an additional experiment was performed on the fractions separated by affinity chromatography from the plasma samples obtained in late August. Using density gradient ultracentrifugation, the profile shown in **Fig. 4** was established. Fraction I presented as a broad spectrum of particles covering the 1.0137–1.0735 g/ml range; its distribution was bimodal, showing two successive maxima with respective approximate densities of 1.0350 and 1.0550 g/ml. Chemical analysis of the density gradient subfractions originating from fraction I demonstrated that the ratio of cholesterol/protein was subjected to a fivefold increase, from 0.42 to 2.15, between the denser (i.e., 1.0608–1.0735 g/ml) and the lighter (1.0172–1.0210 g/ml) subfractions in which measurable amounts of these lipoprotein components were present. In contrast, the density distributions of fractions II and III

TABLE 4. Seasonal variations in the respective chemical compositions of the fractions isolated from badger d 1.006–1.063 g/ml lipoproteins using heparin-Sepharose affinity chromatography

| | | Composition[a] | | | | | |
|---|---|---|---|---|---|---|---|
| Fraction | Date | Cholesteryl Esters | Unesterified Cholesterol | Esterified Cholesterol / Total Cholesterol | Triglycerides | Phospholipids | Protein |
| I | January | 35.3 | 8.5 | 0.71 | 0.4 | 30.6 | 25.2 |
| I | April | 33.5 | 8.1 | 0.71 | 1.4 | 30.6 | 26.5 |
| I | August | 37.2 | 9.4 | 0.70 | 0.7 | 28.5 | 24.1 |
| I | November | 34.2 | 8.5 | 0.71 | 0.8 | 32.5 | 24.0 |
| II | January | 23.1 | 8.3 | 0.62 | 19.7 | 25.0 | 23.9 |
| II | April | 24.0 | 8.3 | 0.63 | 17.4 | 26.1 | 24.2 |
| II | August | 17.8 | 7.3 | 0.60 | 26.1 | 24.9 | 23.9 |
| II | November | 21.0 | 6.8 | 0.65 | 22.7 | 25.8 | 23.6 |
| III | January | 20.8 | 6.8 | 0.64 | 24.9 | 21.5 | 26.0 |
| III | April | 26.6 | 6.9 | 0.70 | 19.6 | 22.5 | 24.4 |
| III | August | 29.8 | 8.2 | 0.68 | 17.7 | 21.6 | 22.7 |
| III | November | 17.0 | 5.9 | 0.63 | 30.9 | 20.6 | 25.6 |

[a]Each value is the mean of duplicate determinations and is expressed as a percentage of the whole lipoprotein.

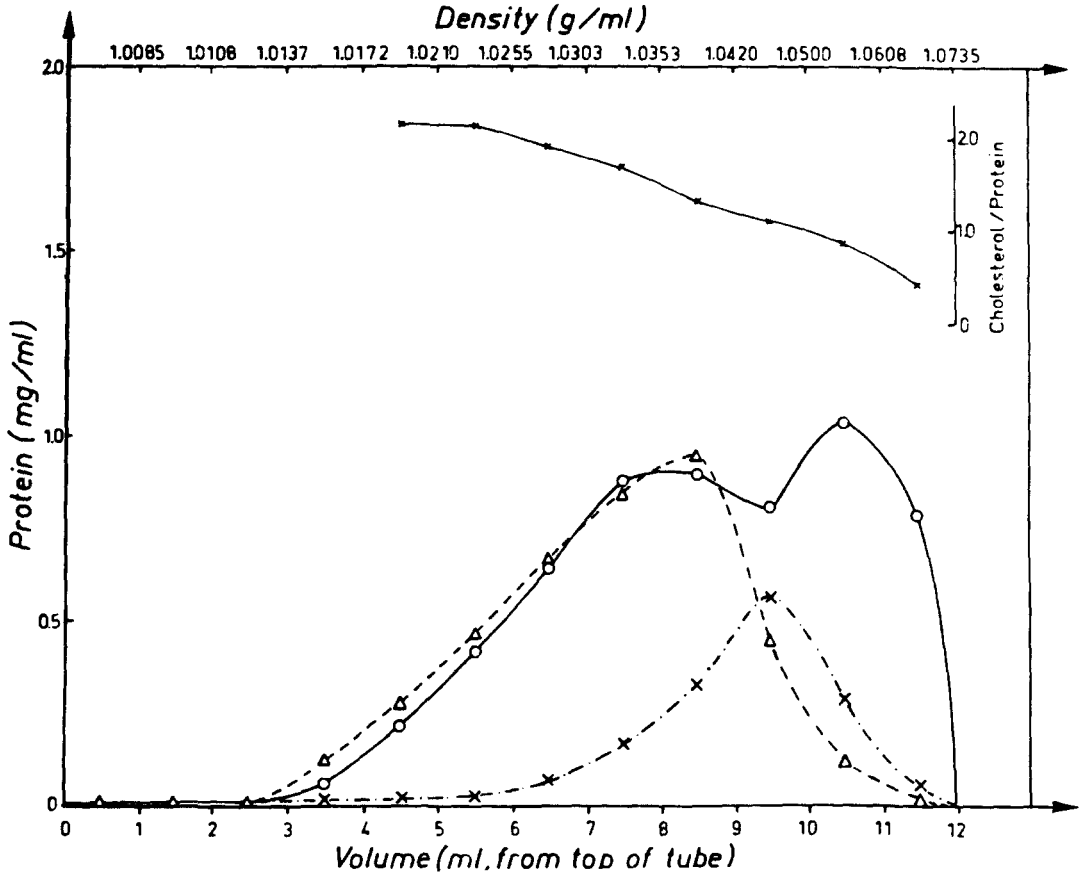


**Fig. 4.** Density distributions of the fractions separated from badgers d 1.006–1.063 g/ml lipoproteins by affinity chromatography. The density gradient ultracentrifugation procedure is a modification of that originally reported by Shen et al. (20) (see Materials and Methods). Fraction I, ( —○— ); Fraction II, (--△--); and Fraction III, (•—X—•). In the upper part of the diagram is presented the evolution of the cholesterol/protein mass ratio in density gradient subfractions from fraction I.

were both clearly unimodal, densities of the corresponding peaks of concentration being, respectively, 1.0385 g/ml and 1.0458 g/ml.

Polyacrylamide gradient gel electrophoresis was performed on peak subfractions from the density distributions described in the preceding paragraph (**Fig. 5**). Subfractions corresponding to the two successive maxima of the distribution of lipoproteins contained in chromatographic fraction I exhibited respective Stokes diameters of 165–181 and 139–153 Å. Peak subfractions of the density distributions of lipoproteins forming fractions II and III possessed Stokes diameters of 198 Å and 195 Å, respectively. Thus, these latter results are quite consistent with those presented in Fig. 3.

### Plasma hormones

Plasma concentrations of thyroxine and testosterone were measured in all badgers at each series of samplings. Results (Table 3) were consistent with our previous studies (1, 5, 6), both as regards hormone concentrations at a given date and their seasonal variations.

## DISCUSSION

In the present study, we have attempted to characterize discrete populations of lipoprotein particles, which are components of the 1.006–1.063 g/ml density interval in badger plasma. This particular density range was chosen for reasons presented both in the Introduction of the present report and in one of our preceding contributions to this Journal (7). Our data provide evidence for the presence of three different lipoprotein populations that differ in their apolipoprotein content, chemical composition, electrophoretic mobility, Stokes diameter, and density distribution. Furthermore, we have shown that these fractions exhibit distinct seasonal variations as regards both their respective concentrations and, in the case of fractions II and III, their content in the different lipid classes.

Fraction I isolated by affinity chromatography is representative of a broad spectrum of cholesteryl ester- and apoA-I-rich particles. Its chemical composition was nearly stable throughout the year, while its concentration in plasma

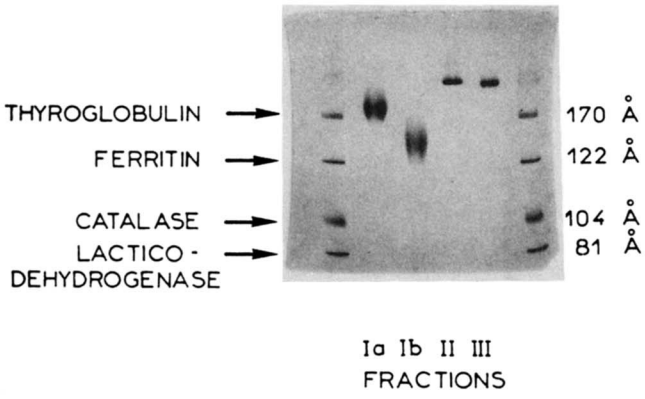


**Fig. 5.** Polyacrylamide slab-gel electrophoresis of peak subfractions from the density distributions of fractions separated from badger d 1.006–1.063 g/ml lipoproteins by affinity chromatography (see Fig. 4). Electrophoresis was performed on PAA 2/16 polyacrylamide continuous gradient slab-gel (Pharmacia Fine Chemicals). The two outer lanes on the electrophoresis slab contained a set of marker proteins whose names and Stokes diameters are indicated at the left and right of the photograph of the gel, respectively. Ia and Ib correspond to the two successive maxima in the density distribution of affinity chromatography fraction I, i.e., d 1.0303–1.0353 g/ml, and 1.0500–1.0608 g/ml, respectively (see Fig. 4). II corresponds to the maximum of the density distribution of fraction II (d 1.0353–1.0420 g/ml), and III to the maximum of the density distribution of fraction III (d 1.0420–1.0500 g/ml).

varied widely according to season, from a minimum in April to a maximum in late autumn/early winter. In the badger, seasonal variations in thyroid activity have been demonstrated by assaying different parameters, i.e., plasma thyroxine level, radioactive iodide glandular uptake, and biological half-life of $^{131}$I-labeled thyroxine (35). In this latter study, the authors have shown the chronological concordance existing between the respective variations of these various parameters. Thus, the spring (April) thyroxine maximum measured at the plasma level corresponds to a rise in thyroid uptake, in thyroxine excretion, and to a stimulation of peripheral metabolism (shortening of the half-life of thyroxine in plasma). Conversely, at the end of autumn or the beginning of winter (November to January), the observed fall in the plasma concentration of thyroxine accompanies the decrease of thyroid uptake and an important decrease in hormonal excretion while, at the same time, peripheral metabolism remains at a very low level.

We therefore suggest that lipoproteins contained in fraction I could represent a population of particles whose metabolism is specifically regulated by thyroid hormones, the latter possibly acting through two different mechanisms that have been already demonstrated in man and/or different animal species. Firstly, in addition to more moderate actions on PHLA (postheparin lipase activity) (36) and LPL (lipoprotein lipase) (37), it has been shown that the most significant effect of hypothyroidism on lipolytic enzymes lies in a strong decrease in HL (hepatic lipase) activity (38). According to current hypotheses, HL is partially responsible for the elimination of cholesterol from the circulation through its action as a phospholipase acting on $HDL_2$ particles (d 1.063–1.125 g/ml) during their passage through the liver (39). Thus, in the badger, seasonal hypothyroidism could first lead to a decrease in HL activity, itself responsible for increased concentrations of lipoprotein particles in the $HDL_2$ density range. Such increased amounts of $HDL_2$ were actually reported by us, both in late autumn in intact badgers (2, 3) and throughout the year in thyroidectomized badgers (5). These apoA-I-containing particles would therefore become available for a progressive lipidation process, primarily involving cholesteryl esters through the action of LCAT (lecithin:cholesterol acyltransferase). Although this enzyme has been recently shown in the rat to exhibit decreased activity in hypothyroidism (40), such a process would progressively lead to the appearance of increasing amounts of apoA-I-, cholesteryl ester-rich lipoproteins, with hydrated densities lower than that of their parent particles and thus similar to those found in fraction I.

On the other hand, the existence of high affinity, saturable binding sites for HDL containing apoA-I but not apoE has been reported, under a variety of experimental conditions, in man and several animal species. Indeed, numerous reports dealing with the cellular binding of HDL have appeared during the last few years (for recent examples and list of relevant papers, see refs. 41 and 42). At present, the exact nature and mode of function of these binding sites have not been fully characterized, nor has a possible regulation of their efficiency by thyroid hormones been shown. However, in so far as these binding sites would be protein in nature, a hypothyroidism-induced decrease in their synthesis is conceivable, as has been demonstrated for apoB, E receptors (43, 44). Such a mechanism could account for impaired cellular catabolism of lipoproteins constituting fraction I, thereby promoting their accumulation in plasma. These accumulating lipoproteins could be subjected to an even greater enrichment in cholesteryl esters, resulting in a supplementary decrease in their density. This is consistent *1)* with our present data regarding the cholesterol/protein ratio in subfractions obtained from fraction I using density gradient ultracentrifugation, and *2)* with our previous results showing the progressive accumulation, during late fall/early winter, in density gradient subfractions of badger plasma with densities in the 1.015–1.065 g/ml range, of lipoproteins with respective electrophoretic mobilities decreasing with density and intermediary between those of human LDL and HDL, and thus similar to that of the material constituting fraction I (3).

Fractions II and III shared the presence of apoB in their apolipoprotein moiety. In addition, we have demonstrated the presence of apoE in the lipoproteins constituting fraction III. The observation, in SDS gels representative of the apolipoprotein content of fraction II, of a faintly stained band with similar $M_r$ (see Fig. 1), makes it reasonable to assume the presence of low amounts of apoE in this latter material. In accordance with our previous results on apoB

isolated from the apolipoproteins of total d 1.006–1.063 g/ml badger lipoproteins (7), apoB isolated from fractions II and III exhibited an $M_r$ and an amino acid composition comparable to that of human apoB-100. These findings suggest that lipoproteins constituting both fractions II and III originate from hepatocyte-secreted VLDL. In contrast, their chemical composition differed markedly from that of human LDL, in that their triglyceride content was much higher, primarily to the detriment of cholesteryl esters. This holds true irrespective of the date of year considered, although pronounced variations in the ratio % triglycerides/% cholesteryl esters were apparent. It is, however, of note that the sum of the respective percentages of triglycerides and cholesteryl esters, i.e., the most hydrophobic lipid classes usually defined as the "core components" of a lipoprotein particle, remained constant for each fraction considered independently of the date of sampling (approximately 41–44% in fraction II and 45–48% in fraction III).

Lipoproteins constituting fraction III differed from those in fraction II in the greater proportion of apoE. Their lipid composition also changed with the period of the year but, contrary to fraction II, their triglyceride content was maximal in November, occurring simultaneously with the greatest recorded value for their plasma concentration. The combination of these two findings makes fraction III a likely candidate for the moderate increase in triglyceridemia repeatedly observed by us in intact badgers at this period of the year (2, 5, 6). Although no corresponding evidence is available at present, it is tempting to speculate on a possible precursor–product relationship between fractions II and III, which would be in accordance with their qualitatively similar apolipoprotein patterns. It is known that the later steps in the conversion of IDL to LDL involve loss of apoE by the lipoprotein particle. Therefore, the final product of such a metabolic pathway in the badger may be represented by fraction II, the more apoE-rich fraction III being its precursor. The respective apolipoprotein patterns of fractions II and III comprised a component with $M_r \cong 28{,}000$ thereby resembling apoA-I, whose presence was demonstrated in fraction I. It is therefore reasonable to assume that badger apoA-I was actually present in fractions II and III. In these latter lipoprotein components however, and especially in fraction II, we cannot exclude that apoA-I could be a contaminant resulting from incomplete or delayed elution of lipoproteins constituting fraction I. However, the fact that fraction II migrated as a single band upon polyacrylamide gel electrophoresis argues against this possibility.

In a recent year-long study (6), we have shown that castration of the badger leads to the appearance of a moderate hypertriglyceridemia, corresponding essentially to an increase in the amount of glycerides transported by lipoproteins with density in the d 1.027–1.065 g/ml region, but not by VLDL. At the same time, the proportion of apoB in these d 1.027–1.065 g/ml lipoproteins was enhanced. In addition, it is of note that the main consequence of castration on the electrophoretic profile of badger lipoproteins, either in whole plasma or in density gradient fractions, was a marked increase in the intensity of the band with mobility similar to those of fraction II and of the prominent band from fraction III. To explain these facts, we had suggested that male sex steroids could possess, with respect to the synthesis of apoB, E receptors, a regulatory action similar to that demonstrated for thyroid hormones (43, 44). Also, in addition to its role in the metabolism of $HDL_2$ (see above), HL has been shown to act in the final stages of the catabolism of VLDL to LDL (45–47), and especially by hydrolyzing glyceride substrates on the lipoprotein particle and thus rendering certain apoprotein sites accessible to hepatic receptors (48). On the other hand, studies using stanozolol have shown that HL activity is stimulated by androgenic steroids (49, 50). Thus, the low levels of plasma testosterone observed in the badger from August to December could lead to decreased efficiency of the cellular binding pathway specific for apoB-containing lipoproteins both because of an inadequate configuration of the particle and of decreased availability of the corresponding receptors. Therefore, it seems reasonable to propose that male sex hormones could exert a specific regulatory action on the metabolism of fractions II and III isolated in the present experiments, although the consequences of this action could appear intermixed with those of seasonal variations in thyroid activity.

Testing the hypotheses presented in this discussion will be the subject of further work. Indeed, both in vivo studies in intact and operated animals and experiments involving binding to membrane receptors of the different lipoprotein fractions isolated using affinity chromatography as described in the present report are now in progress in our laboratory.

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