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FATE OF INTRAVENOUSLY ADMINISTERED HIGH-DENSITY LIPOPROTEIN LABELED WITH RADIOIODINATED CHOLESTERYL OLEATE IN NORMAL AND HYPOLIPIDEMIC RATS

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Radioiodinated cholesteryl oleate ($^{125}\text{I-CO}$) was found to associate rapidly with plasma lipoproteins following intravenous administration to rats. The high-density lipoprotein (HDL) fraction was observed to contain the highest amount of radioiodinated ester. Isolation and purification of this HDL fraction ($^{125}\text{I-CO-HDL}$) and subsequent administration to rats demonstrated a plasma clearance similar to that previously observed for HDL labeled by direct iodination. Moreover, the concentration of radioactivity appearing in the adrenal cortex and ovary 0.5 h after intravenous administration of $^{125}\text{I-CO-HDL}$ was greater than that observed after administration of $^{125}\text{I-CO}$, and the uptake of radioactivity by these tissues was considerably greater in hypolipidemic rats. These findings are consistent with existing knowledge relating to the metabolic fate of HDL and radioiodinated cholesterol derivatives in the rat, and suggest that radioiodinated cholesteryl esters may become useful probes for labeling lipoproteins.

Introduction

The association of plasma lipoproteins with the genesis of atherosclerosis has generated considerable interest in their structure, biosynthesis, and metabolic fate. Current evidence indicate that these macromolecular complexes consist of a core of cholesteryl esters and triacylglycerols encased by a phospholipid monolayer. Within this outer phospholipid membrane are embedded free cholesterol and distinct proteins (apolipoproteins). Cholesterol contributes stability to the membrane and the

apolipoproteins confer specific physiologic function, i.e., binding to specific tissue receptors [1].

To date, studies dealing with receptor binding and metabolism of lipoproteins have been performed largely with radioiodinated preparations wherein the label is introduced by treating the specific lipoprotein fractions with iodine monochloride [2–4]. This has been an extremely useful procedure, but it has certain disadvantages for kinetic studies. First of all, the iodination procedure is not specific for the protein component and as much as 20% of the radioactivity can become associated with lipids [5,6]. This labeling of the lipid components can be reduced by prefeeding animals with a diet of saturated fats, but it is not eliminated entirely [6].

Direct iodination of lipoproteins also introduces the risk of causing denaturation. Despite the fact that no marked alteration in the immunologi-

Abbreviations: VLDL, very-low-density lipoproteins; LDL, low-density lipoproteins; HDL, high-density lipoproteins; $^{125}\text{I-CO}$, [19- ^{125}I]iodocholesterol oleate; $^{125}\text{I-CO-HDL}$, HDL fraction labeled with [19- ^{125}I]iodocholesteryl oleate; 4-APP, 4-aminopyrazolo-(3,4-d)-pyrimidine.

cal and electrophoretic properties of lipoproteins have been observed following radioiodination [2,5], most investigators prefer to screen the product biologically in animals to obviate the effects of any denaturation [2,6].

Finally, those components which make up the surface of the plasma lipoproteins, namely phospholipid, cholesterol and the apolipoproteins, are known to exchange readily from one lipoprotein to another [7]. This, coupled with the fact that each of the various apolipoproteins within each of the lipoproteins can display different plasma half-lives [5], complicates the interpretation of kinetic studies with radioiodinated lipoproteins.

In contrast, the lipids comprising the core of lipoproteins, such as cholesteryl esters and triacylglycerols, do not freely exchange. Instead, these lipids require transfer proteins found in plasma in order to accomplish such an exchange. A cholesteryl ester transfer protein has been identified in rabbit [8] and human [9,10] plasma, but is low or nondetectable in rat plasma [10].

The recent synthesis of cholesteryl esters labeled with radioiodine at the C-19 position [11,12] suggested the use of these γ -emitting tracers as probes in lipoprotein studies. These tracers were attractive as lipoprotein probes, not only because of the greater ease in counting and quantitating γ -emitting nuclides, but also because of the ease in synthesis of these tracers from stable iodinated precursors by isotope exchange with ^{125}I .

The present research was undertaken in order to evaluate the potential of employing radioiodinated analogs of cholesteryl esters as markers for metabolic studies with lipoproteins. Since others [13,14] have shown that HDL is the principal carrier of cholesteryl ester in the rat and is taken up by cells via a receptor-mediated process [15,16], rat HDL was selected for radioactive labeling. Accordingly, rat HDL was labeled *in vivo* by intravenous administration of [19- ^{125}I]iodocholesteryl oleate (^{125}I -CO) and the HDL fraction was subsequently isolated by sequential ultracentrifugation. This radioiodinated HDL fraction (^{125}I -CO-HDL) was then administered to normal and hypolipidemic rats and its fate compared with that obtained for ^{125}I -CO administered in physiological saline.

Materials and Methods

Animals

Female Sprague-Dawley rats (Spartan Research Animals, Inc.) weighing 200–260 g were used in all experiments. The rats were housed in temperature- and light-controlled quarters and had free access to food (Teklad 4% Rat and Mouse Diet) and water.

Radioiodinated cholesteryl oleate

[19- ^{125}I]iodocholesteryl oleate (^{125}I -CO) was prepared as previously described [11,12] and stored in benzene at 4°C until used. Radiochemical purity was determined by thin-layer chromatography (TLC) using silica gel plates (Eastman Kodak) developed in either benzene/ethyl acetate (9:1, v/v), or carbon tetrachloride (2 ×). The plates were developed in iodine vapor and scanned for radioactivity using a Berthold Radiochromatogram Scanner model LB 2723. In all analyses, the single radioactive peak was coincident with the standard visualized with iodine vapor.

^{125}I -CO was formulated for injection immediately prior to use by the addition of Tween vehicle. This vehicle consisted of saline containing 10% ethanol and 1.6% Tween 80 (poly(oxyethylene sorbitan monooleate) obtained from Sigma) and was added to ^{125}I -CO immediately after removal of the benzene under nitrogen. Prior to injection, aliquots of the formulated compounds were assayed for radioactivity in a Searle 1185 Automatic Gamma System. Aliquots were also analyzed by TLC as described above.

Preparation of HDL fraction labeled with ^{125}I -CO (^{125}I -CO-HDL)

Donor rats received intravenous injections of ^{125}I -CO (1.2–1.4 mCi; 2.30 μmol) via the tail vein in 2.0–2.5 ml of vehicle. At 0.5 h following injection, the rats were killed by exsanguination from the heart while under diethyl ether anesthesia. The blood was collected in heparinized vacutainer tubes and centrifuged at low speed for 10 min to obtain plasma. Plasma from five rats was pooled (total volume, 9.0 ml) mixed with NaCl and KBr solutions to a final density of 1.070 following the method of Havel et al. [13]. After ultracentrifugation at 40 000 rpm for 20 h at 15°C, the top 2.0 ml

(containing VLDL, LDL and chylomicrons) were removed as described by Lindgren [17]. The remaining infranate was used as ^{125}I -CO-HDL. In a preliminary tissue-distribution study, ^{125}I -CO-HDL obtained in this manner was compared to a preparation that was further purified by ultracentrifugation. This purified HDL fraction was prepared by adjusting the density of the infranate to 1.210 with NaCl and KBr solutions and centrifuging for an additional 40 h at 40000 rpm. The top 2.0 ml were removed. Since there was no major difference in the tissue distribution profiles of these two preparations (results not shown), the second centrifugation step was omitted in subsequent preparations.

Analysis of radioiodinated high-density lipoprotein

Polyacrylamide gel electrophoresis analysis of the ^{125}I -CO-HDL fraction was performed as described by Narayan [18]. Gels consisted of 3.5 cm of a 3.75% (w/v) main gel and 1.0 cm of a 2.5% (w/v) large-pore stacking gel. A 50 μl aliquot of the fraction was mixed with 25 μl of Sudan black dye (a lipoprotein prestain) for 1 h and 25 μl of the mixture was applied to the top of the stacking gel. The gels were run at 2.5 mA/tube until the

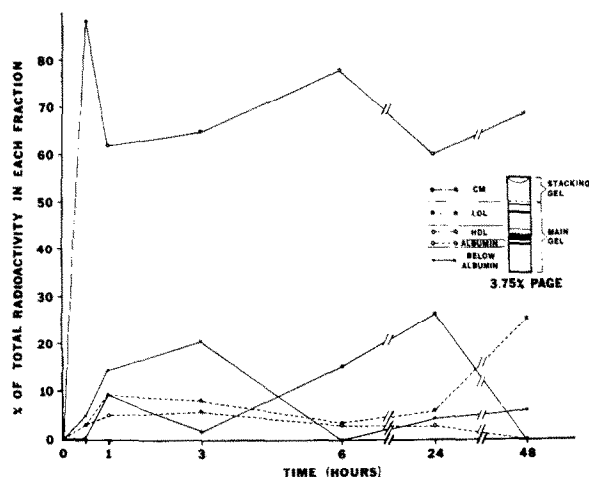


Fig. 1. Polyacrylamide gel electrophoresis (PAGE) of plasma following intravenous administration of ^{125}I -CO-HDL. Plasma samples are from one rat per time postinjection. Gels were sliced into the following regions corresponding to lipoprotein bands: region 1, stacking gel (2.5% w/v); regions 2-5, main gel (3.75% w/v); region 2, VLDL + LDL; region 3, HDL; region 4, albumin; and region 5, below albumin. CM, chylomicrons.

leading band migrated 1.7 cm into the main gel. The gels were sliced into regions corresponding to lipoprotein bands as described by Narayan [18]: region 1, stacking gel; region 2, main gel VLDL/LDL; region 3, main gel HDL; region 4, albumin, and region 5, main gel area below albumin (Fig. 1). The regions were assayed for radioactivity for 10 min or 1 million counts. Counting efficiency for ^{125}I was 85%.

Additionally, the ^{125}I -CO-HDL was analyzed by gel filtration using columns (1.6 \times 35 cm) packed with Sepharose 4B (Pharmacia). A 0.3 ml aliquot of the labeled HDL fraction was applied to the column and eluted with Tris-HCl buffer, 0.1 M, pH 8.0, at a flow rate of 0.06 ml/min. 60 1.5-ml fractions were collected and assayed for radioactivity and absorbance at 280 nm (Fig. 2).

In order to ascertain the stability of the ^{125}I -CO under the conditions of lipoprotein incorporation, a 0.1-ml aliquot of the ^{125}I -CO-HDL was mixed with 0.4 ml distilled water and extracted with chloroform/methanol (2:1, v/v) as described by Folch et al. [19]. The lipid extracts were then analyzed by TLC using the systems described above in order to assess the percentage of free versus esterified radioactively labeled cholesterol.

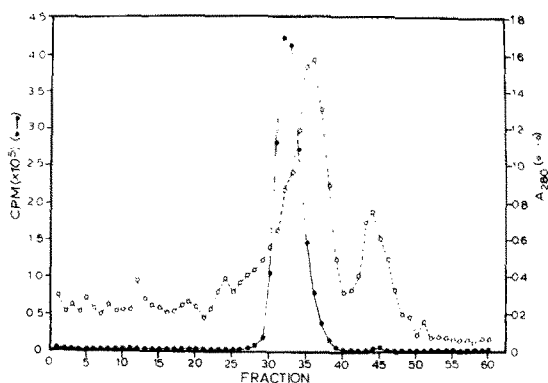


Fig. 2. Sepharose 4B gel filtration analysis of ^{125}I -CO-HDL prepared in vivo. A 0.3-ml aliquot of ^{125}I -CO-HDL was applied to a Sepharose 4B column (1.6 \times 35 cm) and eluted with Tris-HCl buffer, 0.1 M, pH 8.0; at a flow rate of 0.06 ml/min. 60 1.5-ml fractions were collected and assayed for radioactivity and absorbance at 280 nm. ●, Radioactivity (cpm); ○, absorbance at 280 nm.

Measurement of transfer of ^{125}I -CO from ^{125}I -CO-HDL following incubation with density < 1.070 lipoproteins

Rat lipoproteins of density < 1.070 (VLDL + LDL) were isolated as described above. A 2.0-ml aliquot of this fraction was incubated with 0.5 ml of the ^{125}I -CO-HDL preparation at 37°C in a water bath with agitation. At 0.5, 1, 3 and 24 h, 25- μl aliquots of the incubation medium were taken and analyzed by polyacrylamide gel electrophoresis as described above.

Preparation of hypolipidemic rats

Seven rats received a daily intraperitoneal injection of a solution of 4-aminopyrazolo-(3,4-d)-pyrimidine (4-APP, Sigma) in 10 mM sodium phosphate at pH 2.5 [20,21]. The dose was 40 mg/kg body weight per day for 3 days. A second group of eight rats received a daily subcutaneous injection of ethinyl estradiol in propylene glycol (1

mg/ml). The dose was 5 mg/kg per day for 5 days [22].

Plasma clearance of ^{125}I -CO and ^{125}I -CO-HDL

Rats were injected intravenously by the tail vein with 0.2 ml of ^{125}I -CO (11.9 μCi ; 0.037 μmol) or 0.2 ml of ^{125}I -CO-HDL (7.5–7.7 μCi). The animals were killed at 0.5, 1, 3, 6, 24 and 48 h after injection, the plasma separated from each animal, and an aliquot of plasma from each time period assayed for radioactivity as described above. The plasma disappearance for the two preparations is graphed in Fig. 3. Aliquots of plasma were analyzed by polyacrylamide gel electrophoresis as described above.

Tissue distribution of radioactivity after administration of ^{125}I -CO or ^{125}I -CO-HDL to normal and hypolipidemic rats

Groups of normal, 4-APP-treated and ethinyl estradiol-treated rats each received 0.2 ml of ^{125}I -CO or ^{125}I -CO-HDL intravenously by the tail vein. The amount of radioactivity administered to each group was as follows: ^{125}I -CO, normal 13.6 μCi , 0.182 μmol ; 4-APP-treated, 11.9 μCi , 0.037 μmol ; ethinyl estradiol-treated, 25.4 μCi , 0.64 μmol . ^{125}I -CO-HDL, normal, 7.5 μCi ; 4-APP-treated, 0.9 μCi ; ethinyl estradiol-treated, 0.9 μCi . At least three animals were used in each group and all were killed at 0.5 h after injection by exsanguination from the heart. The blood was collected in heparinized vacutainer tubes. Samples of whole blood were taken for counting and the remaining blood centrifuged at low speed for 10 min to obtain plasma. The following organs were removed, rinsed of blood and blotted dry: adrenal cortex, liver, lung, ovary, spleen and thyroid. Large organs were minced with scissors. Samples were transferred into tared cellulose acetate capsules, weighed and placed in polystyrene gamma tubes. The uptake of radioactivity for adrenal cortex, blood, liver and ovary is shown in Table I. Aliquots of plasma from all rats were analyzed by polyacrylamide gel electrophoresis as described above.

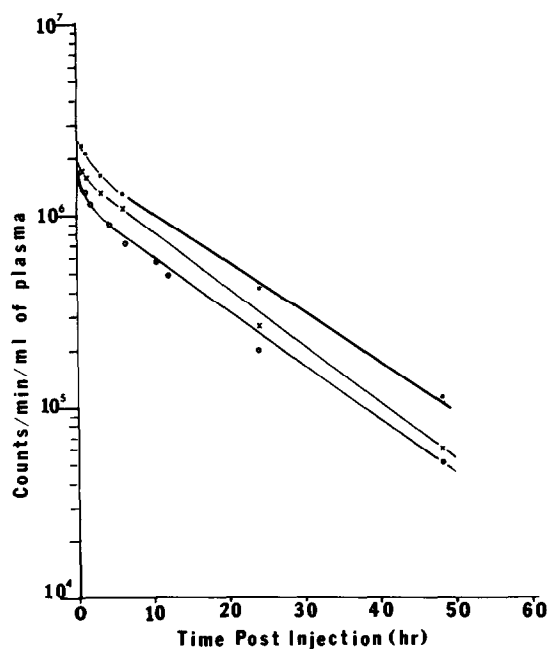


Fig. 3. Disappearance of radioactivity from plasma of rats after injection of ^{125}I -labeled HDL, ^{125}I -CO-HDL and ^{125}I -CO. Dose for ^{125}I -labeled HDL (O) was $7 \cdot 10^6$ cpm/rat, $t_{1/2}$ 10.5 h (data from Ref. 2). Values represent means of three rats per time period. Dose for ^{125}I -CO-HDL (X) was $(14.0-14.5) \cdot 10^6$ cpm/rat, $t_{1/2} = 10.0$ h. Values represent mean of 3–5 rats per time period. Dose for ^{125}I -CO (■) was $22.4 \cdot 10^6$ cpm/rat, $t_{1/2} = 11.6$ h. Values represent one rat per time period.

Results and Discussion

Administration of ^{125}I -CO to donor rats resulted in the appearance of approximately 5% of

TABLE I

TISSUE UPTAKE OF $^{125}\text{I-CO}$ AND $^{125}\text{I-CO-HDL}$ AT 0.5 h POSTINJECTION IN NORMAL AND HYPOLIPIDEMIC RATS.

$^{125}\text{I-CO}$ or $^{125}\text{I-CO-HDL}$ was intravenously administered to either untreated, 4-APP-treated (40 mg/kg body wt. per day for 3 days intraperitoneally) or ethinyl estradiol-treated (5 mg/kg body wt. per day for 5 days subcutaneously) adult female Sprague Dawley rats. At 0.5 h postinjection, the rats were exsanguinated and the tissues removed, weighed and analyzed for radioactivity. All values are expressed as mean % administered dose/g tissue \pm S.E.

Tissue	$^{125}\text{I-CO}$			$^{125}\text{I-CO-HDL}$		
	Untreated (<i>n</i> = 4)	4-APP-treated (<i>n</i> = 3)	Ethinyl estradiol- treated (<i>n</i> = 4)	Untreated (<i>n</i> = 5)	4-APP-treated (<i>n</i> = 4)	Ethinyl estradiol- treated (<i>n</i> = 4)
Adrenal cortex	4.6 \pm 1.2	24.7 \pm 3.0	18.1 \pm 1.6	8.4 \pm 2.1	135.5 \pm 8.3	149.0 \pm 30.0
Blood	4.0 \pm 0.6	7.1 \pm 0.3	2.8 \pm 0.3	5.1 \pm 0.2	4.5 \pm 0.1	3.0 \pm 0.1
Liver	2.3 \pm 0.2	1.3 \pm 0.1	3.5 \pm 1.9	1.5 \pm 0.3	1.7 \pm 0.2	2.8 \pm 0.5
Lung	6.0 \pm 0.6	9.5 \pm 1.1	7.1 \pm 3.1	0.8 \pm 0.1	0.8 \pm 0.1	0.6 \pm 0.1
Ovary	2.1 \pm 0.4	10.1 \pm 2.7	2.9 \pm 0.4	3.8 \pm 0.6	14.0 \pm 1.5	24.0 \pm 3.9
Spleen	4.0 \pm 0.2	2.8 \pm 0.6	9.3 \pm 1.9	0.9 \pm 0.2	0.8 \pm 0.1	0.9 \pm 0.1
Thyroid	1.0 \pm 0.5	1.3 \pm 0.1	0.5 \pm 0.1	2.0 \pm 0.4	0.8 \pm 0.2	2.1 \pm 0.2

the dose in the HDL fraction within 0.5 h. Ultracentrifugal separation gave an HDL fraction which upon gel electrophoresis showed the majority of radioactivity (91.6%) to be associated with the HDL band. Moreover, lipid extraction of this HDL fraction and subsequent TLC analysis revealed that over 90% of the radioactivity remained associated with $^{125}\text{I-CO}$.

Gel filtration analysis of $^{125}\text{I-CO-HDL}$ (Fig. 2) revealed a single radioactive peak (fractions 29–38) corresponding to the leading shoulder (fractions 32–33) of the main protein peak. The main protein peak was shown in a subsequent experiment to correspond to that for rat serum albumin. This failure to resolve HDL completely from plasma albumin has been reported by others [23,24].

In order to examine the stability of the association of $^{125}\text{I-CO}$ with HDL, $^{125}\text{I-CO-HDL}$ was incubated *in vitro* with *d* < 1.070 fraction (VLDL + LDL). Polyacrylamide gel electrophoresis of incubated aliquots taken at various time periods up to 24 h revealed that 82–88% of the radioactivity remained in association with the HDL fraction with only 5% of the radioactivity redistributing to VLDL + LDL. These results further demonstrate that $^{125}\text{I-CO}$ is tightly associated with HDL and is, in all probability, confined to the core in a manner similar to that proposed for other cholesteryl esters. These results are also consistent with the

previously noted low levels of cholesteryl ester transfer protein in rat plasma [10], although there is no evidence available at this time to indicate that $^{125}\text{I-CO}$ would be a substrate for this carrier protein even if the latter were present in sufficient quantities.

Although the plasma clearance of $^{125}\text{I-CO}$ and $^{125}\text{I-CO-HDL}$ showed differences in the early distribution phase, the subsequent first-order elimination phase was similar for the two preparations (Fig. 3). This is not surprising since within 0.5 h after administration, $^{125}\text{I-CO}$ becomes associated with plasma lipoproteins, and the greatest percentage (67%) is associated with HDL. Moreover, at 1 h and later time periods following the administration of $^{125}\text{I-CO}$ or $^{125}\text{I-CO-HDL}$, there is little difference in the distribution of radioactivity among the various plasma lipoproteins. In addition, the plasma $t_{1/2}$ of 10.0 h for $^{125}\text{I-CO-HDL}$ corresponds to the value of 10.5 h found by Roheim et al. [2] (Fig. 3) and that of 10.6 h noted by Sigurdsson et al. [6] with ^{131}I - and ^{125}I -labeled HDL, respectively. The close agreement of these clearance values provides additional evidence for the intimate association of the $^{125}\text{I-CO}$ with the circulating HDL.

The most striking difference between $^{125}\text{I-CO}$ and $^{125}\text{I-CO-HDL}$ was in the amount of radioactivity taken up and retained by steroid-secreting

tissues, the adrenal cortex and ovaries. Several groups [15,25] have shown that lipoprotein-transported cholesterol is the primary substrate for steroidogenesis in these tissues. Moreover, it has been shown that adrenocortical [16] and luteal cells [26] of the rat utilize HDL cholesterol for this purpose. HDL is taken up into these cells by a receptor-mediated process which involves the binding of HDL to specific receptor sites on the cell membrane. The enhanced uptake of ^{125}I -CO by adrenal cortex and ovary (Table I) when it is administered as ^{125}I -CO-HDL is in agreement with these findings.

Another important consideration is that the binding of HDL for its cellular receptors is both reversible and saturable [16]. Thus, when ^{125}I -CO-HDL is administered to normal rats, its specific activity is immediately reduced by the endogenous plasma lipoproteins. This, in turn, explains the modest increase of uptake of radioactivity by target tissues when ^{125}I -CO-HDL was administered to normal rats. Treatment of rats with either 4-APP [20,21] or ethinyl estradiol [22], however, causes a profound lowering of circulating lipoprotein levels. When rats made hypolipidemic with these agents were given ^{125}I -CO or ^{125}I -CO-HDL, the appearance of radioactivity in the adrenal cortex and ovary at 0.5 h was dramatically increased over that observed for normal rats (Table I). Moreover, in the 4-APP-treated rats, the concentration of radioactivity in the adrenal cortex was more than 5-fold greater following administration of ^{125}I -CO-HDL than was observed for ^{125}I -CO. In contrast to the adrenal cortex, the ovaries were less responsive to hypolipidemia, but prior incorporation of ^{125}I -CO into HDL did cause a slight increase in uptake of radioactivity by the ovary following pretreatment with 4-APP, and this enhancement was even more pronounced in rats made hypolipidemic with ethinyl estradiol. These results are consistent with the work of Straus et al. [27] who showed that the uptake of rat ^{125}I -labeled HDL was greatest for the adrenals at 15 min following intravenous administration to immature 4-APP-treated rats. This uptake of radioactivity by the adrenals was markedly reduced by co-administration of human HDL.

At 24 h following the administration of ^{125}I -

CO-HDL to normal rats, the percent of administered dose per g of tissue was 9.5 and 16.1 for the adrenal cortex and ovary, respectively. This retention of radioactivity in the steroid-secreting tissues following administration of ^{125}I -CO-HDL represents the major distinction from results obtained with radioiodinated HDL (^{125}I -labeled HDL) preparations. Radioactivity localizing in the adrenal gland was found to decline rapidly within the first hour following intravenous administration of ^{125}I -labeled HDL to mice [28]. This lack of retention of radioactivity by the adrenal is explained by the rapid degradation of ^{125}I -labeled HDL by proteolytic enzymes. Radioiodinated cholesteryl esters, on the other hand, are not subject to the same rapid metabolic fate and are stored in steroid-secreting tissues. The latter is particularly true for esters of 19-iodocholesterol since they have been shown to be poor substrates for rat adrenal cholesteryl ester hydrolase [12]. Consequently, it is reasonable for radioactivity to persist in adrenal and ovarian tissue for a prolonged period of time following administration of ^{125}I -CO-HDL.

Various methods have been employed to label lipoproteins with [^{14}C]- and [^3H]cholesterol derivatives, and recently a non-hydrolyzable cholesteryl alkyl ether was described as a possible lipoprotein marker [29-31]. Our results with ^{125}I -CO indicate that lipoproteins can now be labeled in the lipid core with a readily detectable γ -emitting nuclide. These results have encouraged us to synthesize other radioiodinated lipids as possible lipoprotein markers.

Acknowledgements

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