A Preparation of C^{14}-labeled Triglyceride in Plasma as a Tracer for Plasma Particulate Fat.* (27326)

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Recent interest in disorders associated with an increased concentration of triglyceride in plasma has focused attention on the dynamics of triglyceride metabolism. In most instances of hypertriglyceridemia, the increase is accounted for by elevated levels of the triglyceride-rich, very low density lipoprotein fraction of plasma containing fat particles large enough to scatter light. The rate of removal of these lipoproteins from the circulation can be studied by determining the clearance of radioactive triglyceride administered in the form of very low density lipoprotein.

The need for a preparation suitable for administration to human subjects has led to development of a method for dispersing triglyceride in plasma to form a combination of lipid and plasma protein with the physical characteristics of native particulate fat. In the present study, the method of preparation of a labeled triglyceride-plasma complex, its physical properties, and its removal rate in man are described.

Methods. The radioactive triglyceride-plasma complex is prepared as follows: Tripalmitin-1-C^{14}, specific activity approximately 13 mc/mM (Nuclear-Chicago), is dissolved in petroleum ether (B.P., 30°-60°C), and extracted twice with 3 volumes of methanol-0.1 N NaOH (2:1 v:v). The radioactive triglyceride recovered in the petroleum ether upper phase is virtually free of contamination with labeled fatty acids (counter current distribution) and partial glycerides (thin layer silicic acid chromatography). An aliquot of the purified labeled tripalmitin is evaporated to dryness and dissolved in a minimal volume of warmed acetone. The solution is taken up in a micro-syringe (Hamilton) and slowly added to an aliquot of plasma at room temperature during continuous mechanical stirring in a Vortex mixer (Scientific Industries, Inc., Springfield, Mass.). The triglyceride-plasma mixture is layered under a 1 to 10 dilution of plasma in isotonic saline, centrifuged at approximately 20,000 *g* for 2 hours, and the top fraction harvested. Ten to twenty per cent of the radioactivity present in the original C^{14}-labeled tripalmitin can be recovered in the low density radioactive triglyceride-protein complex obtained in this manner.

Zone electrophoresis on a starch granule medium was performed according to the method of Kunkel(1) using barbital buffer pH 8.6, ionic strength 0.1 μ, and run overnight at 4°C.

For determination of particle size, aliquots of the C^{14}-labeled triglyceride-plasma complex were passed through a series of Millipore filters, 125 mμ-1200 mμ (Millipore Filter Corp., Bedford, Mass.) and aliquots of the filtrates assayed for radioactivity.

For studies in human subjects, the previously described procedures were carried out under sterile conditions. The plasma used in preparation of the radioactive dose was obtained from the subjects in the post absorptive state one day prior to the test. The syringe used for harvesting the radioactive material and injecting the dose was pre-rinsed with an aliquot of this plasma to decrease losses of radioactivity due to adsorption on the glass.

Diabetic patients served as recipients. The labeled triglyceride preparation (5-10 μc) was administered intravenously within 10-20 seconds. Venous blood was obtained *via* an indwelling needle every minute for the first 10 minutes and at longer intervals thereafter. Blood samples were immediately placed into Versene Vacutainer tubes (Becton, Dickinson and Co.), kept in ice, centrifuged at 4°C and the plasma separated. Aliquots of plasma were extracted in chloroform-methanol (2:1 v:v), filtered, dried, and re-extracted in 5%

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ethanol-95% ethyl ether. Free fatty acids were removed from each sample by passage through a column of a freshly charged strongly basic ion exchange resin (Amberlite IRA 400) using a modification of the method of Carlson(2). Radioactivity in the eluates was assayed with a Packard Tricarb automatic scintillation detector using 2,5 diphenyloxazole and p-bis 2-(5-phenyloxazolyl)-benzene as phosphors.

In a single study, C^{14}-labeled human lymph chylomicrons were obtained from a patient with metastatic carcinoma *via* an indwelling thoracic duct catheter.‡ Lymph was collected for 6 hours after ingestion of 20 μc of palmitic acid-1-C^{14} in 40 ml of corn oil. With sterile precautions, the lymph was centrifuged at 3,000 *g* for 10 minutes at 4°C to remove cellular debris, then was injected intravenously into a recipient. A total of 2.3 g of particulate triglyceride (120 ml) with a specific activity of approximately 0.5 μc/mM was administered within 2 minutes.

Results. The C^{14}-labeled triglyceride-plasma protein complex formed in the manner described shares several of the properties attributed to native chylomicrons. The fraction tested in these studies was harvested after flotation in a saline medium following centrifugation at 20,000 *g* for 2 hours. Thus, the particles collected had an estimated minimum diameter of 40 mμ (S_f 100). Approximately two-thirds of these radioactive fat particles were less than 800 mμ and about half were smaller than 400 mμ (Millipore filtration), a size distribution similar to that of native particles present in plasma during alimentary lipemia.

To compare the electrophoretic mobility of the labeled particulate fat with native fat particles, a plasma sample obtained during alimentary lipemia and the radioactive plasma-triglyceride complex were run concurrently on a starch granule bed (Fig. 1). The migration of radioactivity paralleled that of the turbidity; a large fraction of each showed little migration while approximately 20% of the radioactivity migrated in the α_2 globulin region. In contrast, the grossly visible, insoluble particles produced by mixing plasma with a dried thin film of labeled tripalmitin did not migrate (Fig. 2). A cottonseed oil emulsion (Lipomul I.V., Upjohn Co.) mixed with plasma also showed no electrophoretic mobility.

Approximately 50 μg of triglyceride can be complexed with the lipoproteins contained in 1 ml clear fasting plasma and the resulting mixture will remain free of visible particulate material. Larger quantities of triglyceride

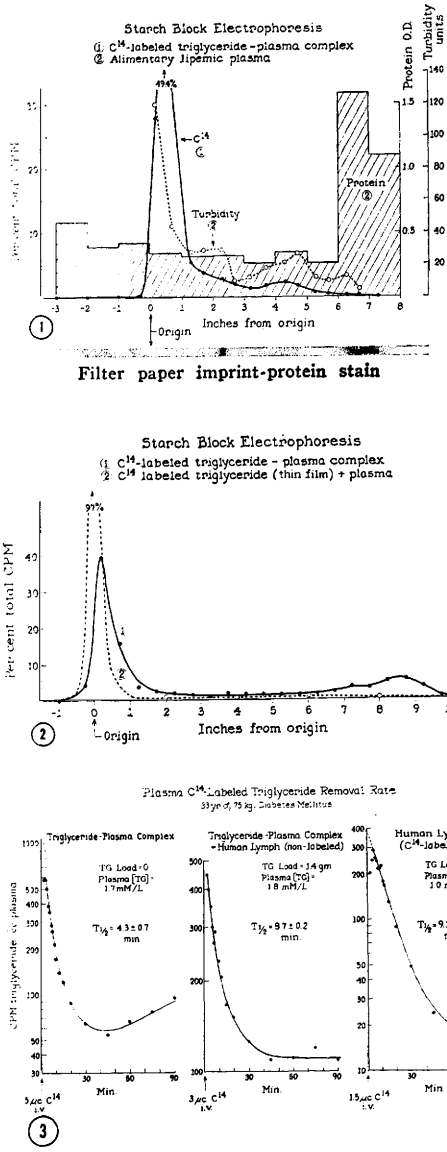

FIG. 1-3

‡ Courtesy of Dr. Allan E. Dumont, Dept. of Surgery, New York Univ., Coll. of Med.

will cause some denaturation of protein, but will also increase the yield of labeled very low density material. Lipemic plasmas bind the added triglyceride less efficiently and give considerably poorer yields. At least .05 ml acetone can be added to 1.0 ml plasma without obvious alterations in plasma proteins or in the nature of the lipid-protein complex. The addition of cholesterol, phospholipid or both to the triglyceride solution in acetone prior to complexing with plasma did not increase the yield of radioactive particulate fat.

To test the capacity of plasma fractions to bind triglyceride, the major protein classes were separated by zone electrophoresis on a starch granule medium and eluted with isotonic saline. Addition of labeled triglyceride in acetone to the fractions resulted in formation of a low density triglyceride-protein complex in each case.

Trace doses (less than 1 mg) of C^{14}-labeled particulate fat, prepared from whole plasma, given intravenously to fasting human subjects, were rapidly removed from circulation (Fig. 3). Initial half times of disappearance (T ½) of label during this rapid exponential removal phase averaged 4.2 $\pm$ 1.0 minutes in 6 studies.

In contrast, the initial removal rate was significantly prolonged when a small fat load (2 g; unlabeled human lymph or Lipomul, I.V.) was given intravenously along with the tracer (Fig. 3). The removal rate of a comparable load of labeled human lymph was of the same order of magnitude (Fig. 3).

Discussion. To be suitable for study of removal rate of triglyceride from the circulation in man, the radioactive triglyceride must be administered in a form that shares the physical properties of native particulate fat. The triglyceride-plasma complex described here has similar flotation characteristics, size, and electrophoretic mobility as the circulating particulate lipid during fat absorption.

Differences in the sites of removal of artificial, finely dispersed, lipid emulsions and natural chylomicrons have been demonstrated in animal experiments(3). Presumably these differences are due to the larger size of the emulsion particles, and the abnormal detergents used to stabilize the emulsions. The use of plasma to form a triglyceride-rich particle without artificial surfactants increases the likelihood that this lipid-protein complex will be handled normally.

The liver may be responsible for removal of most of these particles from circulation. The rapid removal rate of trace doses approaches that of dyes such as indocyanine green which are removed exclusively by hepatic parenchymal cells(4). It has been observed in animal studies that a large proportion of intravenously administered labeled chylomicrons is removed by the liver(5); however, extrahepatic clearance of chylomicrons can also be demonstrated(6).

The half-time of removal of the C^{14}-labeled triglyceride-plasma complex after intravenous administration is faster than that reported for homologous lymph chylomicrons in rats(7) and dogs(8,9). Presumably, this is due to the amount of lipid injected, since it has been shown in animal studies that rate of removal of lymph chylomicrons and artificial fat emulsions is inversely related to the load of fat administered(7,10).

In this study, the half-time of disappearance was significantly prolonged following administration of a physiological fat load of either labeled human lymph chylomicrons or the radioactive triglyceride-plasma complex added to lymph or Lipomul. These results emphasize the importance of the size of the administered dose of fat in evaluating data concerned with removal of labeled particulate lipid from the circulation. The relatively rapid clearance of the plasma-triglyceride complex was observed in fasting subjects given traces of lipid which produced no alteration in size of the plasma triglyceride pool. Under these conditions, removal would be expected to proceed at a rate influenced only by the size of the endogenous particulate fat pool and the capacity of the removal mechanism.

It must be emphasized that the disappearance of labeled triglyceride in this form measures only the removal rate of triglyceride in the particulate fraction of the plasma lipoproteins and does not reflect total triglyceride turnover, since the removal of that portion of plasma triglyceride associated with higher density lipoproteins is not determined.

Summary. A method is described for dispersing C^{14}-labeled triglyceride in plasma to form low density fat particles which are similar in size, flotation and electrophoretic mobility to the particulate fat present in plasma during fat absorption. The removal rate in man of a trace dose (less than 1 mg) of this preparation of radioactive particulate fat is rapid. Small loads (2 g) of unlabeled lymph or emulsified fat given simultaneously significantly prolong the removal rate. This rate is comparable to that observed after administration of a similar load of labeled human chylomicrons.

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Nephelometric Determination of Glycogen in the Rat Liver Glycogen Deposition Assay of Adrenal Steroids. (27327)

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In liver glycogen deposition assays for endocrine principles of the adrenal cortex and synthetic adrenal steroids, glycogen is precipitated from alkaline liver digests with ethanol and subsequently determined by somewhat lengthy, indirect, quantitative procedures(1-7). Previously reported nephelometric methods require preliminary precipitation of glycogen with an ethanol-sodium chloride solution(8) or preparation of a protein-free filtrate(9). During routine use of the NF XI method(3), one that does not involve deproteination, it was observed that glycogen formed a mildly stable suspension before its precipitation and hence suggested the possibility of a nephelometric procedure. The studies done to determine the proper experimental conditions and practicability of such an analysis are the subject of this report.

Procedure. Experimental rat liver digests (diluted digests) are prepared as follows: Each liver is freed of surface blood with 0.9% NaCl and digested in 12 ml of hot 30% KOH. The digest is diluted to 100 ml with water after storage at room temperature overnight. Simultaneously, livers of several adrenalectomized, starved, uninjected rats are also obtained for each series of glycogen determinations. They are treated as above, then pooled (blank pool) for use in preparation of an instrument blank and as a diluent for the glycogen standard used in positioning the galvanometer index.

In separate stoppered glass vessels, 2 ml of blank pool and 2 ml of standard glycogen solution consisting of 0.5 mg of rat liver glycogen per ml of blank pool are added to 42 ml of 57.5% alcohol. Two ml of each diluted digest are treated in the same manner as the blank pool and glycogen standard. The vessels are closed, shaken, and allowed to stand 35 min. The samples are thoroughly mixed again and transferred to 20 $\times$ 40 mm cuvettes. Nephelometric readings are obtained against the blank using a Coleman Model 14 Universal Spectrophotometer at a wave length of 575 mμ and at a galvanometer sensitivity determined by the glycogen standard.