Use of constant composition polyvinylpyrrolidone columns to study the interaction of fat particles with plasma

DAVID D. O'HARA, DANIEL PORTE, JR., and ROBERT H. WILLIAMS

Department of Medicine, University of Washington, Seattle, Washington

ABSTRACT Fat particles (lipoproteins of $S_f > 400$) have been obtained from dog and human lymph and from human post-alimentary plasma. They were flocculated by a solution of 5% polyvinylpyrrolidone (PVP) in 10% NaCl and layered at the bottom of tubes of PVP of various concentrations between 2% and 5% (w/v). As the concentration of PVP increased, a greater proportion of the particles accumulated at the top of the tube ("primary" particles). When the concentration of PVP in the tube was held constant at 3% PVP, dilution of the sample with plasma was found to produce an increased proportion of bottom particles ("secondary" particles). This observation suggests that bottom particles result from an interaction of top particles with plasma.

KEY WORDS dog · man · lymph · plasma · primary · secondary · fat particles · interconversion · separation · polyvinylpyrrolidone column · constant composition · gradient · very low density lipoproteins

PLASMA LIPOPROTEINS may be differentially flocculated by solutions of the nonionic polymer, polyvinylpyrrolidone (PVP). The large lipoprotein aggregates ($S_f > 400$, fat particles) responsible for plasma turbidity may be specifically flocculated in 5% PVP–10% NaCl solution (1, 2) and further separated into top (primary) and bottom (secondary) particles in PVP concentration gradient columns (3). The ability of such columns to separate particles has provided a tool for the study of lymph (4) and hyperlipemic plasma (5–7).

The purpose of this study was to characterize further the behavior of these particles on PVP columns. We have determined the effect of varying the PVP concentration and the particle concentration on the distribution of the particles in the column. Results indicate that the PVP distribution of particles is altered by the concentration of PVP and by interactions between the particles and plasma in vitro.

METHODS

Plasma and Lymph Samples

Samples of turbid plasma were obtained from 10 normal subjects 4–6 hours after the ingestion of fat loads containing 2 g of corn oil per kg body weight. Turbid plasma was also obtained from eight hyperlipemic subjects after an overnight fast. EDTA (1.0 mg/ml of blood) was used as anticoagulant. A patient with filariasis and chyluria ingested 250 μc of palmitic acid-1-^{14}C in 10 ml of corn oil to provide radioactive human lymph. Dogs were fed a similar quantity of radioactive palmitic acid and radioactive dog lymph was collected through a cannula inserted through the neck into the thoracic duct.[1] The fibrin clot was removed and the lymph centrifuged briefly to remove cellular debris. For PVP and ultracentrifugal determinations, the samples were always kept at room temperature and analyzed the same day.

PVP Particle Separation

Particle separation by PVP utilized the method of Gordis (3), except that the concentration gradient column was replaced by one of uniform concentration (3%, w/v) when it was observed that 3% PVP gave results comparable to those with the gradient. For purposes of comparison, uniform columns of 2 and 5% PVP were also used.

Abbreviations: PVP, polyvinylpyrrolidone; TG, triglyceride; S.A., specific activity.

[1] This lymph was kindly provided by D. E. Strandness and E. L. Bierman.

To make these solutions, PVP was dissolved in 10% NaCl, containing 0.01% Na EDTA, and brought to pH 7.4 by the addition of 0.1 N NaOH. Lusteroid tubes were filled with 15 ml of the PVP solution containing either a concentration gradient or a uniform concentration of PVP. The fat particles were flocculated in a solution of 5% PVP–10% NaCl by mixing 1 ml of the plasma or lymph sample with 100 mg of NaCl and then adding 0.25 ml of 25% PVP–10% NaCl solution. One milliliter of this latter solution, now containing 5% PVP and 10% NaCl, was immediately layered at the bottom of the previously prepared PVP columns through a long 21 gauge needle from a calibrated tuberculin syringe. In the particle dilution experiments, the plasma and lymph samples were serially diluted with 0.85% NaCl or plasma made particle-free by centrifugation at 10^6 *g*-min in a fixed angle rotor. The diluted samples were then immediately flocculated in the 5% PVP–10% NaCl solution, and a 1 ml aliquot of this solution was layered at the bottom of the PVP columns. Smaller aliquots of undiluted sample, containing particle loads identical with those of the diluted samples, were also analyzed on PVP columns.

After overnight incubation at 37°C the tubes containing 16 ml of PVP solution were sliced 40 mm from the bottom. The top fraction, "top particles," was brought to a known volume and stored at −19°C. The bottom fraction was quantitatively transferred to a cellulose tube, a layer of 0.85% NaCl was added, and the tube was centrifuged for 10^6 *g*-min. The "bottom particles" were thereby packed into a *surface* layer, which was sliced off and stored at −19°C.

Fat particles ($S_f > 400$) were also separated from plasma and lymph samples into a layer of 0.85% NaCl by centrifugation for 10^6 *g*-min (8). The infranatant solution from lymph fractions and unusually turbid plasma was centrifuged again in the same way. The supernatant fractions were pooled and all fractions stored at −19°C.

Lipid Extraction and Analysis

Suitable aliquots of the stored samples were extracted in 20 volumes of chloroform–methanol 2:1 (v/v) (9). It was necessary to remove PVP from the sample before performing any lipid analyses (except for the phospholipid determination). The chloroform phase was therefore evaporated to dryness and the lipid was reextracted in 12 ml of absolute ethanol, 6 ml of water, and 9 ml of petroleum ether (bp 30–60°C). The lower ethanol–water phase was washed twice with petroleum ether and the combined petroleum ether fractions, containing lipid free from PVP, were brought to a final volume for analysis. Preliminary studies demonstrated no loss of lipid other than lipid P with this technique. Aliquots from the chloroform or petroleum ether extracts were analyzed for total cholesterol by the ferric chloride method (10) after saponification and digitonide precipitation (11); for triglyceride glycerol by modifications (12, 13) of the method of Carlson (14); and for lipid phosphorus by the Bartlett method (15). Triglyceride (TG) was calculated in milligram equivalents of triolein, and phospholipid as phosphorus × 25. The percentage composition, by weight, of the lipoprotein fractions was calculated by assuming $^2/_3$ of total cholesterol to be esterified, and the average mol wt of fatty acid esters to be 280 (i.e., sterol × 1.5) (16).

Aliquots from the petroleum ether extract were assayed for radioactivity in a liquid scintillation counter with Liquifluor (Pilot Chemicals, Watertown, Mass.). For determination of the specific activity of particle triglycerides, the triglyceride was isolated by thin-layer chromatography on silica gel, the triglyceride spot was scraped off and eluted in ethyl ether, and aliquots were taken for TG-glycerol analysis and ^{14}C counting in the liquid scintillation counter.

Lipid Recovery

The recovery of TG-^{14}C radioactivity from the PVP columns was 86 ± 7% (SD, n = 52), and from direct centrifugation of lymph 97 ± 7% (n = 4) when compared with whole lymph. The recovery of unlabeled triglyceride from the PVP columns was 91 ± 9% (n = 13) and from direct centrifugation of plasma 94 ± 10% (n = 33) when compared with whole plasma. Recovery of cholesterol by direct centrifugation of plasma was 91 ± 9% (n = 16) and of phospholipid 91 ± 10% (n = 12). Separation of fat particles ($S_f > 400$) from lymph or plasma with PVP columns, as judged from determinations of either radioactivity of triglyceride, gave a recovery of 93 ± 14% (n = 108) when compared with the separation of the fat particle fraction by direct centrifugation.

RESULTS

Effect of PVP Dilution on Fat Particle Separation by PVP Columns

The separation of fat particles at the top and bottom of PVP columns was found to occur whether or not the columns were made with a concentration gradient. However, the concentration of PVP in the nongradient columns greatly influenced the distribution of particles in the column. As the concentration of PVP in the nongradient column was decreased from 5 to 2%, a decreasing proportion of particle triglyceride and of radioactivity accumulated at the top (Table 1). The distribution ratio observed in 3% PVP was similar to that with the density gradient. Although the gradient column was more diffi-

TABLE 1 EFFECT OF PVP CONCENTRATION ON THE PERCENTAGE OF FAT PARTICLES AT THE TOP OF PVP COLUMNS

	5% PVP	3% PVP	Gradient	2% PVP
		%		
*Lymph**				
Dog A		99.8	99.9	
Dog B	99	92	92	
Dog C	99	94	87	71
Human (chyluria)	99	94	92	81
Plasma† (normal subjects)				
Dob		24	21	
Ken		58	64	
Nor	70	48		28
Hutch	86	72		35
Bee	83	68		45

* % of fat particles as indicated by cpm/ml of triglyceride-^{14}C.
† % of fat particles as indicated by mg/100 ml of triglyceride.

cult to prepare than the 3% PVP column, it did have the advantage of stabilizing the separated particles against displacement by convection currents in the column. With concentrations of PVP below 2%, the particles gave rise to a diffuse opalescence rather than aggregates at the top and bottom of the column, because, apparently, of "deflocculation" of the particles in the dilute PVP. This accounted for the broad hazy band at the top of the gradient column formed by the particles in dilute PVP. In contrast, the "top particles" in the 3% PVP formed a tightly packed surface band.

Effect of Initial Particle Concentration on PVP Column Separation

The relation between particle radioactivity or triglyceride concentration in vitro and the proportion of particles flocculated at the top of 3% PVP columns is shown in Table 2. Diluting the particles with plasma before flocculation in 5% PVP–10% NaCl clearly resulted in a progressive decrease in the percentage of "top particles" and a reciprocal increase in "bottom particles." Some reduction in "top particles" also resulted from dilution with 0.85% saline, but this was consistently less than with plasma and often required a dilution of more than 1:9. Decreasing the particle load on the PVP column without changing particle concentration by prior dilution had no significant effect on the distribution of particles in the column. The relationship of dilution with plasma, dilution with saline, and reduction in volume of undiluted aliquot is illustrated in Fig. 1.

There was also evidence that the concentration of fat particle in vivo affected the distribution of particles in the PVP column. In samples of native lymph, as the particle triglyceride concentration decreased, the percentage of particles at the top of the column decreased, and that at the bottom increased (Table 3). Although measurements of particle radioactivity confirmed the phenomenon, the absolute values differed because of the different specific activities in different samples of top and bottom particles. Lymph from Dog E, a dog that had been fasted for 3 days, resembled slightly turbid plasma more than creamy lymph. In the plasma of normal subjects who had ingested 2 g of fat per kg of body weight, top particles, if present at all, appeared only at the peak of lipemia when particle concentration usually exceeded 75 mg/100 ml (Table 3). Although the most turbid samples had the greatest percentage of top particles, a linear

TABLE 2 EFFECT OF LOAD AND CONCENTRATION OF FAT PARTICLES ON THE PERCENTAGE OF FAT PARTICLES AT THE TOP OF 3% PVP COLUMNS

Sample	1 ml Aliquot Undiluted	Particle-TG per ml	0.5 ml Aliquot Undiluted	1:1 Saline Diluted	1:1 Plasma Diluted	0.2 ml Aliquot Undiluted	1:4 Saline Diluted	1:4 Plasma Diluted	0.1 ml Aliquot Undiluted	1:9 Saline Diluted	1:9 Plasma Diluted	0.05 ml Aliquot Undiluted	1:19 Saline Diluted	1:19 Plasma Diluted
	%	*mg/100 ml*		%			%			%			%	
*Lymph**														
Dog A	99.8	6511	99.8	99.2	98.5	99.5	99.4	98.6	99.5	99.0	94.2	99.7	98.4	89.3
Dog B	92	4110							89	87	72			
Dog C	94	2432	94	91	80		86	65				94	90	38
Dog D	75	972	77	75	56	75	72	55	71	54	27			
Human (chyluria)	94	439	90	91	75	93	86	54	90	82	47			
Plasma† (normal subject)														
Ken	74	278	75	77	61									
Nor	48	86	55	39	18									
Plasma† (hyperlipemic subject)														
Sal (CI)‡	67	1538	58	60	26	n.d.§	41	18						
Guz (FI)‡	91	1280	85	87	85				83	85	60	86	69	38
Ran (CI)	19	535	21	13	13									
Wal (CI)	69	292				59	32	25						

* % of fat particles as indicated by cpm/ml of triglyceride-^{14}C.
† % of fat particles as indicated by mg/100 ml of triglyceride.
‡ CI, carbohydrate-induced hyperlipemia; FI, fat-induced hyperlipemia.
§ Not done.

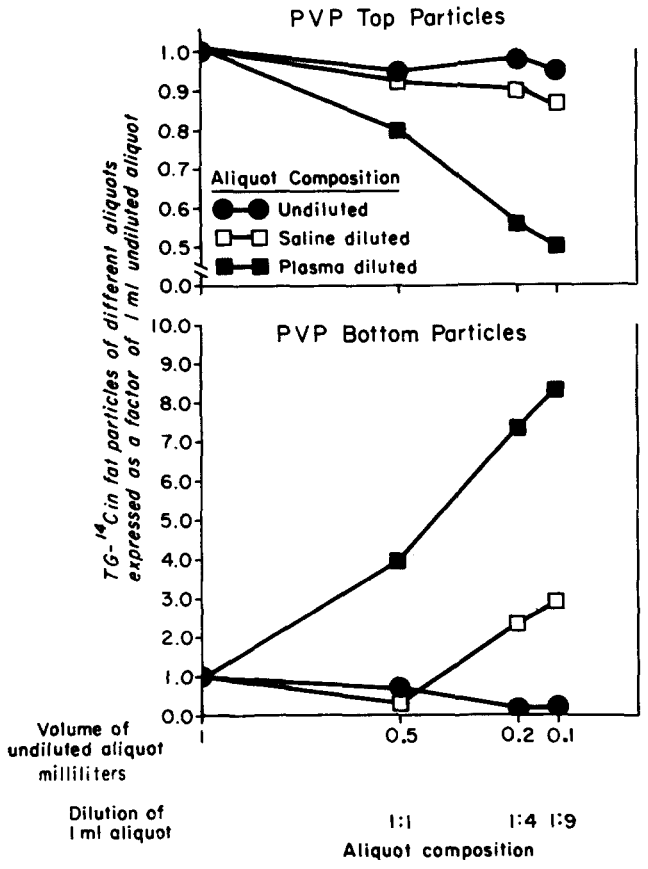

FIG. 1. Effect of dilution of fat particles on the distribution of fat particles in 3% PVP columns. Fat particles were obtained from human chyluria and diluted with 0.85% NaCl or particle-free plasma (infranate of 10^6 *g*-min centrifugation). All values corrected to 100% recovery.

relation between particle concentration and particle PVP distribution was not evident at particle triglyceride concentrations lower than 100 mg/100 ml.

The specific activity (S.A.) of the triglyceride in fat particles was determined for three samples of lymph (Table 4). In the undiluted aliquot, the S.A. was higher for the top particles than for the bottom particles. When the concentration of bottom particles was increased by 1:9 dilution with plasma, the S.A. of this fraction also increased while the S.A. of the top particle did not change. At this dilution, there was a smaller but similar effect of 0.85% saline on both particle distribution and S.A.

DISCUSSION

The separation of lipoproteins by PVP columns depends upon different rates of flotation of lipoproteins in the system. Differential flocculation in PVP occurs because certain lipoproteins apparently are flocculated into aggregates large enough to float to the top of the column overnight at a gravitational force of 1 × *g* (1000 *g*-min). Substantial flotation under these conditions would require a minimum aggregate diameter of about 3 μ (8). This aggregation by PVP appears to be reversible in view of the deflocculation that occurred in dilute (2%) PVP. Until the differences between primary and secondary particles (17) are more fully understood, the optimal PVP concentration for separating them cannot be established. Since the definition at present remains operational, and since 3% PVP gave the same results as the original gradient columns (3), this concentration was chosen for most of our studies. This similarity was probably related to the maximum PVP concentration of 3–4% attained by the gradient technique used (18). Because 5% PVP flocculated 99% of the particles to the top of the tube in four of the five lymph samples tested, it is possible that a better separation could have been achieved with this higher concentration of PVP. We have observed that plasma samples containing only bottom particles in 3% PVP also had only bottom particles in 5% PVP, but before 5% PVP can be used generally, this observation requires further substantiation with known mixtures of primary and secondary particles.

TABLE 3 RELATIONSHIP BETWEEN ENDOGENOUS FAT PARTICLE CONCENTRATION AND PERCENTAGE OF FAT PARTICLES AT THE TOP OF 3% PVP COLUMNS

	Time after Fat Load	Fat Particle TG	Fat Particles at Top
	hr	*mg/100 ml*	%
Lymph			
Dog A		6,511	99.7*
Dog B		4,110	96
Dog D		972	77
Human (chyluria)		439	74
Dog E		29	14
Plasma			
Ken	6	278	74*
Bee	4	174	69
Ken	8	121	62
Love	4	97	37
Nor	4	86	48
Hutch	6	76	60
Dob	4	75	29
Harb	4	75	0
Hark	4	65	0
Uye	4	44	17
Love	6	31	0

* % of fat particles as indicated by mg/100 ml of triglyceride.

When samples of turbid plasma or lymph were diluted with plasma in vitro before being flocculated by 5% PVP–10% NaCl, an increasing proportion of particles remained at the bottom of the column. This did not occur when smaller aliquots of the undiluted samples were placed on the column. The effect did not seem to be related only to the salts in plasma, since dilution with

TABLE 4 SPECIFIC ACTIVITY OF TRIGLYCERIDES IN FAT PARTICLES SEPARATED BY 3% PVP COLUMN

Lymph	Top Particles			Bottom Particles		
	0.1 ml Aliquot Undiluted	1:9 Saline Diluted	1:9 Plasma Diluted	0.1 ml Aliquot Undiluted	1:9 Saline Diluted	1:9 Plasma Diluted
		cpm/mg			*cpm/mg*	
Dog A	11,400	11,300	11,900	6,800	7,550	9,600
Dog B	1,500	1,450	1,550	1,300	1,300	1,500
Human (chyluria)	15,000	13,000	13,000	7,000	8,500	12,000

0.85% NaCl caused much less "bottom particle" formation than dilution with plasma. Dilution of particles in vitro with plasma has also been found to affect the electrophoretic mobility of particles on starch blocks, so that there is an increased proportion of particles in the region where secondary particles migrate (19). It appears, therefore, that the primary particles in lymph or plasma are altered, partly by simple dilution, but even more by interactions with plasma constituents, to form secondary particles. These secondary particles, when subsequently mixed with 5% PVP–10% NaCl, no longer form aggregates able to float in the PVP column after 1000 *g*-min.

A similar interaction between primary particles and plasma may occur in vivo to form secondary particles. In our study, the less concentrated lymph samples had a greater percentage of bottom particles (Table 3). Bierman and Strandness (19) found that the percentage of primary particles in lymph increased during the early phases of fat absorption as the concentration of fat particles increased. In addition, the mixing of infused lymph in the circulation of dogs resulted in secondary particle formation without the participation of the liver (19). Although a consistent relationship between particle-triglyceride concentration and particle distribution was found for lymph, it was not evident for plasma containing concentrations below 100 mg/100 ml. Perhaps the two types of particles are removed at different rates or the amount of soluble lipoprotein "acceptor" for primary particles may differ among individual subjects, or may fluctuate during alimentary lipemia.

The mechanism of the interaction between primary particles and plasma to form secondary particles is not known. If the bottom particles are produced by this interaction in vivo, the lower TG specific activity of the bottom particles with respect to the top in undiluted samples could mean (*a*) that primary particle TG is diluted with endogenous TG in the formation of secondary particles, (*b*) that the secondary particles represent a greater proportion of chylomicrons synthesized earlier in the intestine when the S.A. of the TG pool would be less, or (*c*) that different lipid transfer properties may exist between primary and secondary particles. The increase in S.A. of the bottom particles with plasma dilution indicates that the new secondary particle triglyceride must have originated from the primary particle, but our data do not indicate whether this was by lipid transfer or complex formation between primary particles and other plasma macromolecules. Both mechanisms may be involved since lipid is transferred in vitro between dog chylomicrons and plasma lipoproteins (20) and between very low and high density lipoproteins (21), and the addition of protein from plasma to lymph particles has also been reported (22).

We would like to thank Miss Dorothy Coltrin, Mrs. Maghy Stern, Mrs. Susan Page, and Miss Marlene Zuti for excellent technical assistance. We are especially appreciative of the interest and support given by Dr. E. L. Bierman.

This work was supported in part by PHS Research Grants AM-02456 and T1-AM-5020 from the National Institute of Arthritis and Metabolic Diseases. A portion of the work was conducted through the Clinical Research Center facility of the University of Washington (National Institutes of Health Grant FR-37).

The studies were carried out during the tenure (by D. P.) of an Advanced Research Fellowship of the American Heart Association and supported in part by the Idaho Heart Association.

Manuscript received 25 August 1965; accepted 27 November 1965.

REFERENCES

1. Burstein, M., and A. Prawerman. *Pathol. Biol.* **7:** 1035, 1959.
2. Cornwell, D. G., and F. A. Kruger. *J. Lipid Res.* **2:** 110, 1961.
3. Gordis, E. *Proc. Soc. Exptl. Biol. Med.* **110:** 657, 1962.
4. Zilversmit, D. B. In *Biochemical Problems of Lipids*, edited by A. C. Frazer. Elsevier Publishing Co., New York, 1963, pp. 257–262.
5. Ahrens, E. H., Jr., and N. Spritz. In *Biochemical Problems of Lipids*, edited by A. C. Frazer. Elsevier Publishing Co., New York, 1963, pp. 304–312.
6. Nye, W. H. *Proc. Soc. Exptl. Biol. Med.* **116:** 350, 1964.
7. Spritz, N. *New Engl. J. Med.* **271:** 291, 1964.
8. Dole, V. P., and J. T. Hamlin. *Physiol. Rev.* **42:** 678, 1962.
9. Folch, J., M. Lees, and G. H. Sloane Stanley. *J. Biol. Chem.* **22:** 497, 1957.
10. Zlatkis, A., B. Zak, and A. J. Boyle. *J. Lab. Clin. Med.* **41:** 486, 1953.
11. Sperry, W. M., and M. Webb. *J. Biol. Chem.* **187:** 97, 1950.
12. Bierman, E. L., and J. T. Hamlin, III. *Diabetes* **10:** 432, 1961.

13. Mendelsohn, E., and A. Antonis. *J. Lipid Res.* **2:** 45, 1961.
14. Carlson, L. A. *Acta Soc. Med. Upsalien.* **64:** 208, 1959.
15. Bartlett, G. R. *J. Biol. Chem.* **234:** 466, 1959.
16. Bierman, E. L., D. Porte, Jr., D. D. O'Hara, M. Schwartz, and F. C. Wood, Jr. *J. Clin. Invest.* **44:** 261, 1965.
17. Bierman, E. L., E. Gordis, and J. T. Hamlin, III. *J. Clin. Invest.* **41:** 2254, 1962.
18. *Biochemists' Handbook*, edited by Cyril Long. D. Van Nostrand Co., Princeton, 1961, pp. 110–112.
19. Bierman, E. L., and D. E. Strandness, Jr. *Am. J. Physiol.*, in press (1966).
20. Minari, O., and D. B. Zilversmit. *J. Lipid Res.* **4:** 424, 1963.
21. Nichols, A. V., and L. Smith. *J. Lipid Res.* **6:** 206, 1965.
22. Hofmann, A. F. *Am. J. Physiol.* **199:** 433, 1960.