LEVIN L. WATERS*

Department of Pathology, Yale University School of Medicine

THE EXTRACTION OF LIPIDS FROM HUMAN PLASMA OR SERUM BY 2-OCTANOL**

The lipoprotein complexes of blood plasma are so bound that very little lipid is extracted by ethyl ether at room temperature. The addition of alcohol[1] or the use of low temperatures[2] improves the extraction of lipids by ether. In all such extractions cholesterol is more readily removed than phospholipids, indicating possible differences in the intramolecular forces that bind these components.

Since the structure of plasma lipoproteins and their stability may be of fundamental importance in disease states involving lipid transport or lipid deposition in tissues, it has been thought of interest to give a brief report of the lipid extractant effect on human blood plasma or serum of caprylic alcohol (2-octanol). Two-octanol, (methyl hexylcarbinol, sec-n-octyl alcohol) is an eight-carbon alcohol with the formula: $CH_3(CH_2)_5$-CHOH-CH_3. It has surface-active properties, finds limited use as an antifoaming agent and is a solvent for lipoids. Its solubility in water is low and is given[3] as 0.096 gm/100 ml. at 25° C. These properties suggested its trial as an agent for the extraction of lipids from human plasma and serum.

MATERIALS AND METHODS

The materials used were 2-octanol (Mallinkrodt, reagent grade) b.p. 176°-179° C. and recently outdated human plasma, (acid-citrate-dextrose [ACD] plasma from the American Red Cross Blood Bank Program in Connecticut). Pooled samples of human serum from patients in a general hospital were used.

The extraction of the plasma or serum sample was carried out as follows: 2-octanol was added to a volume of plasma (or serum) in a centrifuge tube of convenient size so that the caprylic alcohol-plasma ratio was 1 : 10. The test tube was stoppered and actively shaken for one hour. At the end of this time three layers were present in the tube, (1) an orange-yellow layer of caprylic alcohol at the top containing much lipid

---

* Professor of Pathology.

** This work was carried out under a Grant-in-Aid from the American Heart Association, and supported in part by the Norwalk and Stamford-Darien-New Canaan (Connecticut) Heart Associations. It was also aided by a grant (H-1794) of the U.S. Public Health Service.

Received for publication 11 June 1964.

in solution, (2) an emulsified layer in the center of the tube, and (3) an aqueous serum layer below. The tube then remained at 4° C. overnight. On standing, the emulsion largely cleared, leaving a well-defined lipid-rich caprylic alcohol layer overlying the aqueous plasma or serum layer beneath. If the central emulsion does not clear, or if shortening the procedure is desired at the end of the shaking period, this can readily be accomplished by centrifugation. Regularly there appears between the caprylic alcohol layer and the aqueous layer a small but definite precipitate. The nature of this precipitate will be described in the section on results. In an effort to ascertain optimal conditions, a few extractions were run with proportions of caprylic alcohol to plasma as 1:1, 1:2, 1:20, 1:100.

Following extraction and centrifugation, the bottom aqueous plasma or serum layer was removed without contamination from the upper caprylic alcohol layer. It was then analyzed for lipids by standard methods in use in this laboratory. These include: for cholesterol and cholesterol esters—a modified Schoenheimer-Sperry method,[4] for lipid phosphorus—a modified Youngburg procedure,[5] for fatty acids—a modified Stoddard and Drury technique.[6] Total proteins were also determined by the usual micro-Kjeldahl procedure. It should be indicated that in our hands the above listed Sperry-Webb method for total cholesterol gives highly repeatable values, but these values are consistently ten per cent lower than those obtained by nondigitonin methods on the same materials.

For the biological experiments with lipid-extracted plasma or serum, traces of caprylic alcohol were removed by shaking with ether, removal of the ether layer, and finally removal of residual ether at low pressure at 37° C.

RESULTS

Thirty samples of recently outdated ACD blood bank plasma were extracted with 2-octanol, the proportion of extractant to plasma being 1 to 10. Blood lipid values and total protein levels were determined on the plasma before and after the extractions. Treatment with 2-octanol in this series of plasmas removed an average of 99.3 per cent (S.D. = .38) of the total cholesterol fraction, all of the measurable free cholesterol, an average of 77.3 per cent (S.D. = 8.3) of the fatty acids, and an average of 50.9 per cent (S.D. = 7.5) of the lipid phosphorus. The extractant action on plasma cholesterol was remarkably uniform, while that on fatty acids and lipid phosphorus was more variable. Total plasma proteins were little altered and varied within the limits of error of this determination.

Lipid extraction by 2-octanol was carried out on ten samples of pooled human serum derived from patients in a general hospital. The procedure followed was the same as for ACD plasma and the results are given in Table 1. It is evident that 2-octanol removed nearly all the total cholesterol fraction (99.2 per cent), all of the free cholesterol, 69.2 per cent of the fatty acids and 41.4 per cent of the phospholipids of serum.

Extractions with varying proportions of 2-octanol to blood bank plasma were also carried out. It was found that larger proportions of 2-octanol

increase the extraction of fatty acids and of lipid phosphorus, but also grossly denature more plasma proteins. When the proportion of caprylic alcohol to plasma was reduced to 1 to 100 the extraction of cholesterol, fatty acids, and lipid phosphorus was less complete.

TABLE 1. LIPIDS AND TOTAL PROTEINS OF POOLED HUMAN SERUM BEFORE AND AFTER EXTRACTION WITH 2-OCTANOL

Serum	*Total cholesterol mg/100 ml.*	*Free cholesterol mg/100 ml.*	*Fatty acids mEq./l.*	*Lipid phosphorus mg/100 ml.*	*Total protein g/100 ml.*
1. Before extraction	122.8	39.8	13.6	6.8	4.39
1. After extraction	1.2	0.0	3.75	3.4	4.39
2. Before extraction	116.2	43.3	18.3	7.5	4.39
2. After extraction	1.2	0.0	3.75	3.3	4.39
3. Before extraction	132.1	45.6	15.6	7.2	6.37
3. After extraction	1.2	0.0	4.75	3.9	6.25
4. Before extraction	139.4	36.5	15.3	6.5	6.37
4. After extraction	0.7	0.0	3.75	3.9	6.25
5. Before extraction	144.4	43.1	15.3	7.1	5.25
5. After extraction	0.7	0.0	4.0	4.1	5.32
6. Before extraction	156.0	47.8	18.0	7.6	6.31
6. After extraction	2.2	0.0	4.7	4.2	6.17
7. Before extraction	139.4	51.2	13.3	7.6	6.50
7. After extraction	1.2	0.0	4.9	4.9	6.30
8. Before extraction	141.1	40.6	13.3	7.6	6.37
8. After extraction	0.7	0.0	6.6	5.8	6.43
9. Before extraction	139.4	40.1	14.0	7.6	6.25
9. After extraction	0.7	0.0	5.6	5.2	6.25
10. Before extraction	160.2	52.2	16.0	8.5	6.87
10. After extraction	0.7	0.0	7.3	5.6	6.68
Average extraction (per cent)	99.2 (S.D. = .051)	100	69.2 (S.D. = 9.45)	41.4 (S.D. = 9.14)	

It was of interest, in addition to the above extractions, to ascertain whether or not 2-octanol was effective in extracting lipids from the major serum lipoprotein classes. Lipoproteins of two different sera were separated by the method of Havel *et al.*[7] into low (d.<1.063) and high (d.>1.063) density fractions. The extractions of cholesterol, fatty acid, and lipid phosphorus from these serum subfractions were comparable to the extractions of these substances from whole serum or plasma.

To prepare lipid-extracted plasma or serum for biological experimentation, it was necessary to remove traces of caprylic alcohol. This was done

by repeatedly shaking the sample with ether, separating the ether layer, and subsequently by removing residual ether under reduced pressure at 37° C. Three such carefully prepared dog sera were injected intravenously into other anesthetized dogs in the amount of 10 ml/kg. without observable signs of immediate toxicity. There were no significant changes in blood pressure, heart rate, or respirations. Also hypercholesterolemic rabbit serum was delipidized by 2-octanol and was injected intracorneally into rabbits. Histologic examination of six corneas revealed the lipid deposits to be enormously reduced when compared with deposits in control corneas injected with the nonextracted sera, and stromal tissue reactions were likewise greatly reduced or nonexistent. It should be emphasized, however, that great care in the removal of the caprylic alcohol and ether was necessary to achieve this result.

The amount of plasma or serum proteins grossly rendered insoluble by treatment with 2-octanol was low. Insoluble material appearing at the caprylic alcohol-plasma interface was collected at the end of extraction of 100 ml. lots of ACD plasma. Five such precipitates were collected, extracted with ether, dried, and weighed. Total proteins were determined on the original plasmas. The mean weight of the precipitates was 15.6 mg. (range 6.5 mg. to 31.0 mg.) or 0.27 per cent of the mean of the total plasma proteins. On the basis of nitrogen these pooled precipitates contained 69.1 per cent protein, the remainder representing in part an insoluble substance that appears at the alcohol-water interface whenever caprylic alcohol is mixed with aqueous solutions. With serum, the amount of protein rendered insoluble by treatment with caprylic alcohol was markedly reduced, suggesting that in the extractions of citrated plasma, the solubility of small amounts of fibrinogen may have been affected.

DISCUSSION

The results of these experiments reemphasize the weakness of the forces that bind lipids to proteins in the lipoprotein complexes of human plasma or serum. This fact is important in considering the structure of lipoproteins and their behavior in biological systems.

It has long been known that alcohols promote the ether extraction of lipids from tissues or blood serum, and this effect was believed due to the gross dehydration of the protein moieties of the complex.[1] Macheboeuf and Sandor,[8] however, were able to remove large quantities of lipids (about 50 per cent) by adding concentrations of ethyl alcohol to horse serum in the range of 6 to 12 per cent and then shaking with ether and allowing the mixture to stand for six hours. The proteins were not rendered in-

soluble. These observations led to the suggestion that the hydrophobic or lipid moieties of the lipoprotein complexes interact weakly as lipid-lipid associations and are protected outwardly by ionization and hydration phenomena of the hydrophilic protein moieties. Ethyl alcohol was assumed to affect the permeability of the protective outer zone, allowing ether to reach the lipids and remove them. Certain other alcohols, subsequently, were also shown to promote the extractant effect of ether on the lipids of horse serum.[9]

Macheboeuf and Tayeau[10,11] also theorized that the lipids in serum lipoproteins might be displaced or substituted by substances with similar hydrocarbon chains, providing they were sufficiently soluble in water. It was found that the addition of various salts of long-chain fatty acids (soaps) to horse serum released considerable quantities of lipids. The solubility of these detergent compounds in water was important as was the presence of a hydrocarbon end group. The binding of the soap to the lipoprotein molecules was suggested but not proven.

Two-octanol would seem to combine in one substance some of the properties of the ether-alcohol system and of the ether-soap systems just described. As an alcohol it may alter the permeability of the hydrophilic lipoprotein shell, although its low solubility might argue against this as would the dependency of the extraction on thorough shaking. As a long-chain hydrocarbon and as a good solvent for lipids, 2-octanol may remove or displace lipids from the hydrophobic component of lipoproteins. There is no evidence in the reported experiments that caprylic alcohol is bound to the extracted protein residue. Finally its low solubility provides a non-aqueous phase for the collection of released lipids.

Recently Avigan[12] has succeeded in extracting large quantities of lipids from purified lipoproteins of human serum. This was accomplished by shaking with ether for 16 hours at 4° C. Denaturation of the extracted proteins, tested by electrophoretic, physical, and immunological methods, was not observed. In Avigan's experiments high density serum lipoproteins (d. 1.063-1.21) were relatively resistant to extraction by ether alone. Their lipids, however, were largely removed by extraction by ether-alcohol (3:2), leaving a water-soluble protein. Two-octanol, in our preliminary experiments, extracted the high density fraction ($d.>1.063$) as well as the low density one ($d.<1.063$). Further work on this subject and on the physico-chemical properties of the extracted proteins after treatment with caprylic alcohol is in progress.

No sure explanation for the gross denaturation (insolubility) of a small quantity of protein during extraction with 2-octanol is apparent.

This may be associated with the finely emulsified state of the plasma or serum produced by shaking it with the alcohol. It has been suggested[18] that bubbling or foaming may result in such denaturation. Also in the extraction of plasma, as mentioned, the fibrinogen-fibrin transformation may be activated.

The fact that practically all of the cholesterol and large amounts of the fatty acids and phospholipids can readily be removed from human plasma and serum by organic solvents in mild conditions poses certain questions of biologic interest. How readily are these complexes dissociated in the living tissue environment and by what means? Is serum lipoprotein instability *in vivo* a matter of the denaturation of the whole molecule or of the separation of its lipid moiety? The demonstrated looseness of the lipid-protein bond invites experimentation in the prevention of this bonding *in vivo* and more basically on the role of the molecularly bound serum lipids in the body economy.

SUMMARY

Brief *in vitro* treatment of human plasma with 2-octanol (secondary caprylic alcohol) removes nearly all the ester and free cholesterol, about three quarters of the fatty acids, and approximately one half of the lipid phosphorus. Comparable amounts of cholesterol are removed from human serum, and slightly less fatty acids and lipid phosphorus. Less than one per cent of the plasma or serum proteins are rendered insoluble. The extraction process is apparently a physical one, dependent upon the affinity of the solvent for the lipid moiety of lipoproteins after thorough admixture of the alcohol and aqueous phases. The extracted plasma or serum has potential value for studies of lipoproteins in biological systems.

ACKNOWLEDGMENT

The technical assistance of Edward Iannucci, Peter Integlia, and Helen Cavallaro is gratefully acknowledged.

REFERENCES

1. Bloor, W. R.: A method for the determination of fat in small amounts of blood. *J. biol. Chem.*, 1914, *17*, 377-384.
2. McFarlane, A. S.: Behavior of lipoids in human serum. *Nature*, 1942, *149*, 439.
3. Lange, N. A. and Forker, G. M. (Eds.): *Handbook of Chemistry*. 10th ed. McGraw-Hill, New York, 1961, p. 642.
4. Sperry, W. M. and Webb, Merrill: A revision of the Schoenheimer-Sperry method for cholesterol determination. *J. biol. Chem.*, 1950, *187*, 97-106.
5. Hawk, P. B., Oser, B. L., and Summerson, W. H.: *Practical Physiological Chemistry*. 12th ed. Philadelphia, The Blakiston Co., 1947, p. 541.

6. Man, E. B. and Gildea, E. F.: A modification of the Stoddard and Drury titrimetric method for the determination of fatty acids in blood serum. *J. biol. Chem.*, 1932, *99*, 43-60.
7. Havel, R. J., Eder, H. A., and Bragdon, J. H.: The distribution and chemical composition of ultracentrifugally separated lipoproteins in human serum. *J. Clin. Invest.*, 1955, *34*, 1345-1353.
8. Macheboeuf, M. A. and Sandor, Georges: Recherches sur la nature et la stabilité des liaisons protéides-lipides du sérum sanguin. Etude de l'extraction des lipides par l'éther en présence d'alcool. *Bull. Soc. Chim. biol. (Paris)*, 1932, *14*, 1168-1190.
9. Delage, B.: Contribution à l'étude de la stabilité des liaisons lipoprotéidiques dans le sérum sanguin. *Bull. Soc. Chim. biol. (Paris)*, 1935, *17*, 927-937.
10. Macheboeuf, M. A. and Tayeau, Francis: Libération des lipides de certaines cénapses lipidoprotéidiques du sérum sanguin par divers savons. *C. R. Soc. Biol. (Paris)*, 1938, *129*, 1181-1184.
11. ————: Sur l'état des lipides et du cholestérol dans le sérum sanguin; destruction de certaines cénapses lipidoprotéidiques et libération de leurs substances lipoidiques par un savon. *C. R. Acad. Sci. (Paris)*, 1938, *206*, 860-862.
12. Avigan, Joel: Modification of human serum lipoprotein fractions by lipide extraction. *J. biol. Chem.*, 1957, *226*, 957-964.
13. Putnam, F. W.: In *The Proteins: Chemistry, Biological Activity, and Methods.* Neurath, Hans and Bailey, Kenneth (Eds.), The Academic Press, Inc., New York, 1953, p. 817.