Proc. Natl. Acad. Sci. USA
Vol. 78, No. 6, pp. 3911–3914, June 1981
Medical Sciences

Evidence for a lipoprotein carrier in human plasma catalyzing sterol efflux from cultured fibroblasts and its relationship to lecithin:cholesterol acyltransferase

(cholesterol efflux/cholesterol net transport/apolipoproteins/sterol carrier lipoprotein)

CHRISTOPHER J. FIELDING AND PHOEBE E. FIELDING

Cardiovascular Research Institute and Department of Physiology, University of California, San Francisco, California 94143

Communicated by John A. Clements, March 23, 1981

ABSTRACT **Immunoaffinity chromatography has been used to study the determinants of sterol efflux and net transport from cultured fibroblasts to human plasma medium. Sterol efflux was highly (≈80%) dependent upon a minor lipoprotein fraction containing apolipoprotein A-I unassociated with other apolipoproteins. The remaining activity was associated with the lipoprotein-free fraction of plasma and could be replaced by apoprotein-free albumin. Efflux was independent of lecithin:cholesterol acyltransferase (EC 2.3.1.43) activity. Net transport (i.e., the excess of efflux over influx) was completely inhibited by inhibition of lecithin:cholesterol acyltransferase or its removal by affinity chromatography on immobilized antibodies to apolipoprotein A-I or D (components of the transfer complex in human plasma). In uninhibited plasma, efflux and net transport rates had similar kinetics, suggesting that these were linked functions and that net transport was initiated by a carrier-dependent efflux step that, in the absence of lecithin:cholesterol acyltransferase activity, was associated with an equivalent influx of free sterol to the cells and that, in the presence of lecithin:cholesterol acyltransferase, was associated with esterification and transfer protein activity. The cholesterol carrier lipoprotein function (≈5% of plasma apolipoprotein A-I) appears to be the first step of lecithin:cholesterol acyltransferase-linked sterol transport from cells.**

Isolated plasma lipoproteins and various sterol-depleted media support sterol efflux* from cultured cells (1–3). This pathway is of major potential importance in regulating cell cholesterol content and reversing accumulation in sterol-loaded cells. The mechanism and regulation of efflux, however, are not well understood. In particular, it has remained unclear to what extent the rates and direction of sterol flux in plasma fractions represent those *in vivo* and whether sterol loss into plasma is necessarily correlated with esterification via the activity of lecithin:cholesterol acyltransferase (LCATase; EC 2.3.1.43) (4). Transferase activity recently has been shown to be coupled in plasma with the transfer of the sterol esters, formed by the activity of LCATase, to acceptors, primarily low and very low density lipoproteins. These reactions continue in plasma *in vitro* to modify plasma lipoprotein composition (5). The effects of individual classes of lipoproteins in plasma can be investigated rapidly under mild conditions by immunoaffinity chromatography using immobilized antibodies to individual lipoprotein antigens (6). We have used this approach to determine the roles of different lipoproteins in promoting sterol efflux and the relationship of this vector to the net transport of sterol and LCATase activity.

The publication costs of this article were defrayed in part by page charge payment. This article must therefore be hereby marked "*advertisement*" in accordance with 18 U. S. C. §1734 solely to indicate this fact.

MATERIALS AND METHODS

Culture of Human Skin Fibroblasts. Infant preputial skin fibroblasts were maintained at 37°C in the presence of 10% CO_2/90% air in Dulbecco's modified Eagle's medium supplemented with 10% fetal calf serum and gentamicin at 50 μg/ml. For individual experiments, cells were plated in 3.5- or 6-cm Falcon dishes at a density of 3–10 × 10^4 cells per plate. Fresh medium was added every third day until the cells were nearly confluent; under these conditions, doubling time was about 24 hr. Cells used for determination of sterol efflux were labeled with medium complexed with [1,2-^{3}H]cholesterol (New England Nuclear) (7). Incubation was for 2–4 days at 37°C. Final cell sterol specific activity was 3.5–5.6 × 10^4 dpm/μg of sterol. Free and ester sterol were measured with cholesterol oxidase and cholesterol esterase (8) in samples of medium or cells that had been dissociated with trypsin/EDTA or 0.1 M NaOH.

Affinity Chromatography of Plasma Antigens. Human plasma lipoprotein apolipoproteins (apo) were isolated by procedures published from this and other laboratories. In brief, apo A-I and apo A-II were prepared from high density lipoprotein ($1.063\ g/cm^3 < \rho < 1.21\ g/cm^3$) (9) by delipidation with ethanol/ether at −20°C, and molecular sieve and DEAE-cellulose chromatography in 6-M urea (10). apo B was isolated from low density lipoprotein ($1.02\ g/cm^3 < \rho < 1.05\ g/cm^3$) from which other detectable antigens, particularly apo E and apo D, had been removed with a column of the respective immobilized antibody (6). apo C-I, apo C-II, and apo C-III were isolated from very low density lipoprotein ($\rho < 1.006\ g/cm^3$) delipidated and fractionated as for the apoproteins of high density lipoprotein (11). apo D was isolated as described (12) and had a molecular weight of 35,000 by $NaDodSO_4$ gel electrophoresis. apo E was prepared from delipidated very low density lipoprotein by molecular sieve and ion-exchange chromatography (13). All apoproteins showed a single component by anionic or cationic electrophoresis and by $NaDodSO_4$ electrophoresis (14, 15). Chemical compositions did not differ from those previously described. Antibodies prepared in rabbits against each apoprotein gave a single line of identity between human plasma and the corresponding isolated antigen and gave no reaction with the other apoproteins.

To prepare immunoaffinity columns, antiserum (15–20 ml) was fractionated with DEAE-cellulose and the purified IgG

Abbreviations: LCATase, lecithin:cholesterol acyltransferase; apo, apolipoprotein.

* Efflux is defined as the flux from cells to medium, influx is the flux from medium to cells, and net transport is the difference efflux−influx.

fraction was coupled with CNBr-activated Sepharose (16). Columns of immobilized antibodies (0.9 × 10 cm) removed all detectable antigen (>98% of plasma content) from 0.75–1.0 ml of human plasma when the original plasma and the column eluate were analyzed at equivalent protein concentrations by radial immunodiffusion assay (17).

Immobilized antibody against human fibrinogen (Sigma; >95% clottable) was prepared by similar procedures. The antibody was unreactive with any of the apoproteins, had no effect on the level of LCATase activity, and removed no detectable cholesterol from plasma. To prepare antigen-deficient plasma for efflux or net transport studies, freshly drawn blood, collected into 1/20 vol of cold 0.2 M sodium citrate, was cooled in ice water and centrifuged (200 × g, 20 min) to remove the cells, and the plasma was passed rapidly through immobilized anti-fibrinogen antibody to prevent coagulation during later incubation. The eluate was then chromatographed on immunoaffinity columns containing the immobilized antibody to individual lipoprotein antigens. Fractions containing detectable protein were pooled. The complete removal of the corresponding antigen was confirmed on portions of eluate concentrated by reverse dialysis under conditions such that 1–2% of the plasma antigen concentration would have been detected. In some experiments, the proportionate removal of other apoproteins complexed in plasma with the antigen assayed was also determined as described (6).

Assay of Efflux and Net Transport Rates. Efflux rates were assayed in terms of the rate of appearance of ^3H radioactivity in the medium when cells were preincubated with [^3H]cholesterol, washed, incubated with minimal essential medium containing human serum albumin at 3 mg/ml (pH 7.4) for 1–2 hr, and then transferred to medium containing complete or fractionated human plasma. Multiple portions of medium were either assayed directly in scintillation counting medium (Aquasol, New England Nuclear) or extracted with chloroform and methanol (18) for analysis of sterol mass and radioactivity. In some experiments, the free and ester fractions were analyzed individually after these components had been separated by thin-layer chromatography on silica gel plates developed in hexane/diethyl ether/acetic acid, 83/16/1 (vol/vol).

Net mass transport of cholesterol from cells to medium was assayed as the difference between decrease in free sterol in the medium in the absence of cells and the decrease in free sterol in the medium in the presence of cells, when the rate of synthesis of medium sterol ester was unchanged, during incubation of dishes of fibroblasts or of empty dishes in the human plasma media described above. In a typical experiment, 6-cm dishes (with and without cells) were filled with 3 ml of cold medium containing 1–2 mg of plasma protein per ml in minimal essential medium; a 1-ml aliquot was immediately taken for analysis. After incubation for 30–60 min at 37°C, a second sample was taken from the dish. Experiments consisted of five or six identical dishes at each point and set of conditions. Standard deviations for the means of pentuplicate determinations were <1% and <1.5% for free and ester cholesterol, respectively, for samples containing 1–10 μg of sterol.

RESULTS

Rates of Efflux and Net Transport. When fibroblasts were incubated under the conditions described with medium containing [^3H]cholesterol, the cells became labeled to an extent such that, after 3 days, their specific activity was >90% that of the labeled medium, compatible with reported fibroblast membrane sterol exchange rates (19). Specific activities of free cholesterol and of cholesteryl ester (2–8% of total sterol in these experiments) were equivalent. In these experiments, cell sterol specific activities were 3.6–5.6 × 10^4 dpm/μg. When the labeled cells were transferred to unlabeled lipoprotein-deficient serum (centrifugal $\rho > 1.21$ g/cm^3 infranatant solution), net transport of sterol mass from the cells into the medium was measured and the specific activity of this component was compared to that of the cells. Medium specific activity was 99 ± 6% (mean ± SD, three experiments) that of the cells in the corresponding experiments, when corrected for the content of initial, unlabeled sterol. Similar results were obtained with medium containing 1–2% (vol/vol) plasma in place of lipoprotein-deficient serum, because of the slow exchange of sterol in this system ($t_{1/2}$, 10–12 hr). Efflux of sterol radioactivity into either medium was linear for at least 1.5 hr under either condition and brief (1 min) exposure of the cells to the unlabeled medium released <2% of activity present after 60-min incubation. Efflux rates into 1–2% plasma under these conditions were 35–45 ng of sterol per μg of cell sterol per hr (Table 1).

When plasma medium was incubated in the absence of cells, loss of free cholesterol from the medium, mediated by the action of LCATase, was associated with an equivalent increase in sterol

Table 1. Sterol efflux and net transport rates in plasma depleted of individual apoproteins

Medium*	Efflux rate, μg/hr	Net sterol transport, μg/hr	Δ
Whole plasma	0.34 ± 0.03	0.26 ± 0.03	0.08
− apo A-I	0.08 ± 0.01	0.00 ± 0.02	0.08
− apo A-II	0.28 ± 0.04	0.15 ± 0.02	0.13
− apo B	0.31 ± 0.04	0.19 ± 0.03	0.12
− apo D	0.32 ± 0.03	0.02 ± 0.02	0.30
− apo E	0.37 ± 0.03	0.30 ± 0.03	0.13
+ DTNB (1.4 mM)	0.45 ± 0.05	0.02 ± 0.02	0.43

Values are means ± SD of five experiments performed as described in the legend to Fig. 1. Efflux rates were determined from cells labeled with [^3H]cholesterol (35–55 × 10^4 dpm/μg of cell cholesterol) and the equivalence of the specific activities of cellular and effluxed sterols was determined with lipoprotein-deficient plasma. Net sterol transport was determined chemically (as shown in Fig. 1) as the difference in free sterol consumption by LCATase in the presence and absence of fibroblasts in a 60-min incubation at 37°C. All assays were carried out at a plasma concentration of 2.0% (vol/vol). Values are normalized as rates per 10 μg of cell sterol. Plasma depleted of apo C-I, apo C-II, or apo C-III showed no significant decrease in efflux-promoting capacity. The difference between net transport and efflux rates estimates the rate of sterol influx to the cells. The cholesterol content of the whole plasma medium was 29–35 μg/ml, and affinity chromatography on anti-apo A-I, A-II, B, D, and E removed 38%, 35%, 63%, 11%, and 22% of plasma sterol, respectively.

* DTNB, dithiobis(2-nitrobenzoic acid).

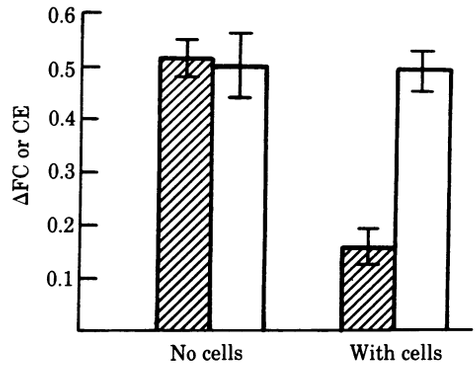

FIG. 1. Cholesteryl ester synthesis in medium and free cholesterol decrement in the presence and absence of cultured fibroblasts. Falcon dishes, empty or containing fibroblasts (350 μg of cell protein; 13 μg of cell-free cholesterol), were incubated with medium containing 1.4% plasma (vol/vol) in phosphate-buffered saline (initial volume, 3 ml). A 1-ml sample was taken and then the dishes were incubated for 60 min at 37°C. A second 1-ml sample was then collected and the differences between initial and final free and ester cholesterol contents of the medium were determined chemically (8). Values are expressed in terms of the change in free (FC) or ester (CE) sterol (as cholesterol) relative to the 2-ml incubation volume. Hatched bars, loss of free cholesterol from medium; open bars, gain in esterified cholesterol in medium. Values are means ± SD for quintuplicate dishes.

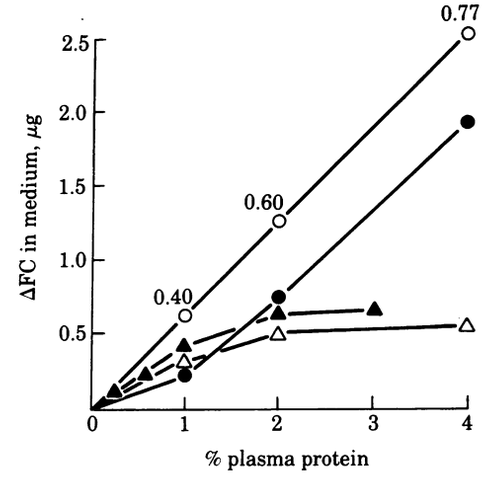

FIG. 2. Dependence of sterol efflux and net transport on plasma concentration in the medium. Cells were labeled by preincubation with [^{3}H]cholesterol and then incubated with fibrinogen-depleted plasma in phosphate-buffered saline at the indicated dilutions. Initial and final samples were taken for analysis of sterol mass and radioactivity as in Fig. 1. Efflux rates were calculated from the specific activity of cell sterol; net transport rates were calculated from the difference in initial and final free cholesterol mass in the medium between empty dishes and dishes containing fibroblasts. ○, decrease in free cholesterol in the absence of cells; ●, decrease in free cholesterol mass in the presence of fibroblasts under the same conditions; △, net cholesterol mass transport; ▲, sterol efflux rates. Proportions indicated are of the contribution of medium free sterols to the total utilized for LCATase activity at each medium concentration point.

mass in the ester fraction (Fig. 1). When fibroblasts were present, the net increase in cholesteryl ester was unchanged but the net decrease in free cholesterol was significantly decreased. This decrease represents the contribution of cellular free cholesterol to the total sterol utilized for the LCATase reaction; in six experiments, the mean (±SD) net rate of sterol transport from cells to medium (determined as the difference between the decrease in loss of cholesterol mass in the presence and absence of the cells) was 30 ± 4 ng/μg of cell sterol in a 60-min incubation period.

Both efflux and net sterol transport were saturable processes, as shown by the determination of both rates as a function of medium plasma composition (Fig. 2). The rate of efflux, assayed with [^{3}H]cholesterol, was 1.2- to 1.4-fold greater than the rate of net sterol transport, measured by the change in free sterol mass in the medium in response to esterification. However, the shape of the saturation curves was highly similar and the apparent K_m for each process was the same.

Effects of Inhibition of LCATase Activity on Efflux and Net Transport. When LCATase was removed from plasma by immunoaffinity chromatography on immobilized antibody to apo D, the rate of sterol efflux from cells to medium was not decreased (Table 1). On the other hand, the net transport of sterol was almost completely inhibited and there was no increment of free or ester sterol mass in the medium.

Esterification in the plasma medium was also inhibited (>98%) by 1.4 mM dithiobis(2-nitrobenzoic acid). This inhibitor had no effect on cell sterol content or sterol synthesis. When the cells were incubated for 24 hr with 1.04 mM [^{14}C]acetate in the presence of inhibitor, there was as great an incorporation of radioactivity into cholesterol (109 ± 7% of control values) as in the absence of inhibitor. This inhibition of LCATase resulted in an almost complete block of net sterol transport into the medium (Table 1) but, again, efflux was not decreased under these same conditions.

Plasma was incubated (60 min, 37°C) to decrease its free sterol content (5). LCATase was then inhibited with 1.4 mM dithiobis(2-nitrobenzoic acid), and this plasma was incubated with fibroblasts under the conditions shown in Table 1. Efflux (0.32 ± 0.02 μg of sterol per hr per 10 μg of cell sterol) was not significantly decreased from control values (Table 1). However, there was no increase in net transport of sterol from cells to medium. Net sterol transport under the conditions described in these studies therefore was not generated as such by a cell-to-medium gradient of free sterol but was coupled directly to esterification. In summary, when LCATase was inhibited or removed, efflux of sterol was unchanged but was associated with an equivalent influx of sterol to the cells, resulting in no net sterol transport. However, when LCATase was fully active, the bulk of cholesterol efflux was associated with net sterol transport to the medium, with subsequent esterification.

Because net transport, like efflux, was saturable and because LCATase activity was linear with plasma concentration in the medium (Fig. 2), the proportion of free sterol required for esterification that was supplied by the cells also varied. When the efflux pathway was half saturated, the cells and medium each provided an approximately equal mass of sterol for esterification; at higher concentrations, the proportion of cellular contribution was greatly decreased.

Effects of apo Antigens on Efflux and Mass Transport. LCATase is associated with apo A-I, its protein cofactor, in whole plasma (6). When this antigen was removed, together with LCATase activity, by affinity chromatography on anti-apo A-I, the efflux of sterol from the cells was greatly (≈80%) decreased (Table 1). The residual efflux rate into plasma after the removal of apo A-I (8 ± 1 ng/μg of sterol per hr) was similar to the rate obtained with human serum albumin (7 ± 1 ng/μg of sterol per hr, three experiments) at its plasma concentration under the same conditions. The rate with albumin was not decreased by passing it over immobilized anti-apo A-I. Anti-apo A-II removed ≈95% of apo A-I because of the predominance in plasma of the corresponding mixed apoprotein complex (6). Efflux rates were only slightly decreased under these conditions. The residual component of efflux was not further decreased by removal of

the other apoprotein antigens shown in Table 1 or by antibodies to apo C-I, apo C-II, or apo C-III and therefore reflects an activity of apo A-I unassociated with these other apoproteins. On a protein weight basis, the activity of the unassociated apo A-I in promoting efflux was therefore manyfold greater than that of the major fraction containing both apo A-I and apo A-II, and lipoproteins containing the other apoproteins shown had no efflux-promoting activity in whole plasma.

Sterol mass transport was dependent upon the presence of the antigens (apo A-I, apo D) shown earlier (6) to be associated with LCATase activity in unfractionated plasma.

The level of unassociated apo A-I in plasma was determined by sequential affinity chromatography on antibodies to apo B, A-II, C, D, and E proteins to remove bulk-complexed A-I. Complete removal of these antigens was confirmed in each case with the appropriate immunoassay. The level of the residual unassociated apo A-I was then determined. When this fraction was passed over immobilized anti-A-I, residual sterol in the eluate was <2 μg/ml of original plasma. The concentration of unassociated apo A-I was 95 ± 10 μg/ml plasma and the concentration of the cholesterol associated with it was 34.2 ± 2 μg/ml. This finding indicates the lipoprotein nature of the unassociated apo A-I in uncentrifuged plasma.

DISCUSSION

These studies indicate that the efflux of sterol into plasma medium from cultured cells is largely mediated by the activity of a minor lipoprotein component containing apo A-I unassociated with other major lipoprotein apoproteins. Although its association with unreported apoproteins is not ruled out, this fraction is clearly much more active in promoting efflux than is the major high density lipoprotein fraction containing apo A-I in complex with apo A-II. The sterol/protein ratio of the sterol carrier lipoprotein fraction is most similar to the minor centrifugal component HDL_{2b} (20), but no direct evidence links the two.

Plasma also contains a second mechanism of efflux, independent of apo A-I, whose rate is similar to that mediated by albumin at its plasma concentration. This (unlike apo A-I-dependent efflux) is independent of LCATase because the albumin did not contain detectable enzyme activity. It is noteworthy that complete removal of the very low density and low density lipoprotein fractions of plasma by immunoaffinity chromatography on anti-apo B did not decrease efflux rates in whole plasma, whereas centrifugally isolated low density lipoprotein was highly active in promoting efflux (21, 22). Other differences in the reactivity of plasma lipoproteins in sterol metabolism in plasma or after centrifugation have been described (6). It seems most likely that, in this case as well, the activity of low density lipoprotein in promoting efflux is generated during isolation.

Efflux and net transport of sterol in plasma in the presence of LCATase activity occur at similar rates and have similar saturation plots. It therefore is likely that the efflux component measured with [^{3}H]cholesterol represents the same pathway as assayed chemically in terms of sterol mass transport. It follows that apo A-I-dependent efflux is normally tightly coupled to net transport—i.e., that influx relative to these other rates is low. However, efflux did not determine the ability of the cells to lose cholesterol irreversibly because when LCATase was removed from the plasma or inhibited, efflux was not decreased but net transport was blocked. This means that in the absence of esterification there was no net loss of sterol from the cells to the plasma medium and efflux was balanced by a corresponding rate of influx. Accumulation of labeled sterol in the medium under these conditions was not decreased and remained linear. The pathway for efflux may be distinct from that of influx. However, because the pool of sterol carrier lipoprotein is large relative to that of LCATase, and sterol exchange rates between lipoproteins is rapid relative to those between plasma and cells (3, 22), this cannot be determined unambiguously at the present time.

Under the incubation conditions described here, net transport of sterol from cells to medium is therefore linked to esterification of sterol. Other studies (5, 6) have shown that esterification in unfractionated plasma is also linked to the ability of LCATase to transport away its cholesteryl ester product to suitable acceptor lipoproteins, primarily very low density and low density lipoproteins, through the activity of cholesteryl ester transfer protein (6, 12, 23). Together with our previous studies, this research then suggests the existence in plasma of a series of linked reactions by which cellular cholesterol is modified and transported to the acceptor lipoproteins reacting with hepatic receptors which mediate its uptake and irreversible catabolism (24). It is a major finding of the present research that the first step of this reaction series is catalyzed by a specific minor carrier lipoprotein.

The excellent assistance of Kathy Moser, Mary Fisher, and Elaine Sargent is acknowledged. The research was supported by grants from the National Institutes of Health (Arteriosclerosis SCOR HL 14237 and HL 23738).

1. Weinstein, D. B., Carew, T. E. & Steinberg, D. (1976) *Biochim. Biophys. Acta* **424**, 404–421.
2. Stein, O., Goren, R. & Stein, Y. (1978) *Biochim. Biophys. Acta* **529**, 309–318.
3. Ray, E., Bellini, F., Stoudt, G., Hemperly, S. & Rothblat, S. (1980) *Biochim. Biophys. Acta* **617**, 318–334.
4. Aron, L., Jones, S. J. & Fielding, C. J. (1978) *J. Biol. Chem.* **253**, 7220–7226.
5. Fielding, C. J. & Fielding, P. E. (1981) *J. Biol. Chem.* **256**, 2102–2104.
6. Fielding, P. E. & Fielding, C. J. (1980) *Proc. Natl. Acad. Sci. USA* **77**, 3327–3330.
7. Bates, S. R. & Wissler, R. W. (1976) *Biochim. Biophys. Acta* **450**, 78–88.
8. Heider, J. G. & Boyett, R. L. (1978) *J. Lipid Res.* **19**, 514–518.
9. Havel, R. J., Eder, H. A. & Bragdon, J. H. (1954) *J. Clin. Invest.* **34**, 1345–1354.
10. Shore, B. & Shore, V. (1968) *Biochemistry* **7**, 2773–2777.
11. Brown, V. W., Levy, R. I. & Fredrickson, D. S. (1969) *J. Biol. Chem.* **244**, 5687–5694.
12. Chajek, T. & Fielding, C. J. (1978) *Proc. Natl. Acad. Sci. USA* **75**, 3445–3449.
13. Havel, R. J., Kotite, L., Vigne, J.-L., Kane, J. P., Tun, P., Phillips, N. & Chen, G. C. (1980) *J. Clin. Invest.* **66**, 1351–1362.
14. Davis, B. J. (1964) *Ann. N.Y. Acad. Sci.* **121**, 404–427.
15. Laemmli, U. K. (1970) *Nature (London)* **227**, 680–685.
16. Porath, J. R., Axen, R. & Ernback, S. (1967) *Nature (London)* **215**, 1491–1492.
17. Mancini, G., Carbonara, A. O. & Heremans, J. F. (1965) *Immunochemistry* **2**, 235–254.
18. Bligh, E. J. & Dyer, W. J. (1959) *Can. J. Biochem. Physiol.* **37**, 911–917.
19. Phillips, M. C., McLean, L. R., Stoudt, G. W. & Rothblat, G. H. (1980) *Atherosclerosis* **36**, 409–422.
20. Anderson, D. W., Nichols, A. V., Pan, S. S. & Lingren, F. T. (1979) *Atherosclerosis* **29**, 161–179.
21. Wu, J.-D. W. & Bailey, J. M. (1980) *Arch. Biochem. Biophys.* **202**, 467–473.
22. Bell, F. P. (1976) in *Low Density Lipoproteins*, eds. Day, C. E. & Levy, R. S. (Plenum, New York), pp. 111–133.
23. Chajek, T., Aron, L. & Fielding, C. J. (1980) *Biochemistry* **19**, 3673–3677.
24. Windler, E., Chao, Y.-S. & Havel, R. J. (1980) *J. Biol. Chem.* **255**, 5475–5480.