Purification and Characterization of Lipoprotein Lipase from Pig Myocardium

By CHRISTIAN EHNHOLM, PAAVO K. J. KINNUNEN, JUSSI K. HUTTUNEN,
ESKO A. NIKKILÄ and MASARU OHTA*

Department of Serology and Bacteriology and Third Department of Medicine,
University of Helsinki, Helsinki, Finland

(Received 5 February 1975)

1. Lipoprotein lipase was purified from pig myocardium by a two-step purification procedure involving (a) the formation of an enzyme–substrate complex and (b) affinity chromatography on Sepharose which contained covalently linked heparin. The purified enzyme gave in sodium dodecyl sulphate–polyacrylamide-gel electrophoresis one main band with an apparent molecular weight of 73000. The enzyme, which was purified 70000-fold, had a specific activity of 860 μmol of unesterified fatty acid liberated/h per mg of protein. 2. The purified enzyme hydrolysed [^{14}C]triolein emulsions in the absence of added cofactors but its activity was increased fivefold by adding normal human serum. Of the low-density lipoprotein apoproteins only apolipoprotein CII could be substituted for serum in activating the enzyme. This lipase had maximum activity at 0.05–0.15M-NaCl. Heparin increased the activity of the purified enzyme twofold at low concentrations, but high concentrations inhibited. The triglyceride lipase of pig myocardium thus resembles lipoprotein lipase purified from adipose tissue and from plasma, but is clearly different from pig hepatic triglyceride lipase.

The uptake and utilization of lipoprotein triglyceride by myocardium has been demonstrated both in man (Carlson *et al.*, 1970) and in rat (Regan *et al.*, 1966; Enser *et al.*, 1967). By analogy with adipose tissue (Bezman *et al.*, 1962) and mammary gland (Otway & Robinson, 1968), the uptake is thought to involve lipoprotein lipase, which catalyses the hydrolysis of lipoprotein triglyceride (Korn, 1955; Hollenberg, 1960). However, the successful purification of the enzyme from myocardium has not been reported thus far.

The pig has proved a good model for experimental atherosclerosis (Thomas *et al.*, 1970). Two triglyceride lipases have been characterized after their partial purification from pig plasma obtained after intravenous administration of heparin (Ehnholm *et al.*, 1974b). One of the enzymes is similar to the lipoprotein lipase isolated from pig adipose tissue (Bensadoun *et al.*, 1974). The other enzyme resembles a triglyceride lipase, which is present in liver plasma membranes (Spitzer & Spitzer, 1956; Hamilton, 1964; Assman *et al.*, 1973; Greten *et al.*, 1974). The present work was undertaken to purify triglyceride lipase from myocardium and to compare the properties of the enzyme with triglyceride lipases from other tissues.

* Present address: First Department of Medicine, Sapporo Medical School, Sapporo, Japan

Materials and Methods

Enzyme assay

Lipoprotein lipase was assayed in duplicate by using as substrate synthetic [^{14}C]triolein emulsified in the presence of gum arabic (Bensadoun *et al.*, 1974). The assay system contained in a total volume of 0.5 ml: 2.4 nmol of glycerol tri[1- ^{14}C]oleate (54.9 $\mu\text{Ci}/\mu\text{mol}$; The Radiochemical Centre, Amersham, Bucks., U.K.); 1.51 μmol of non-radioactive triolein (Sigma Chemical Co., St. Louis, Mo., U.S.A.); 5 mg of gum arabic (Sigma Chemical Co.); 10 mg of fatty acid-poor bovine serum albumin (bovine albumin powder; Armour Pharmaceutical Co., Eastbourne, Sussex, U.K.); 0.05 ml of normal dialysed human serum; 0.1 mmol of Tris-HCl buffer, pH 8.4; 5 units of heparin (Medica, Helsinki, Finland); 0–0.1 ml of the enzyme preparation. The final concentration of NaCl in the assay system was always adjusted to 0.1 M. Because of the high activity of the purified enzyme preparations, the enzyme could be diluted to 0.05 M-NaCl with salt-free buffers, if required. The mixture was incubated for 1 h at 28°C. Radioactive unesterified fatty acids were separated from the assay mixture in the liquid-liquid phase-partitioning system of Belfrage & Vaughan (1969). In the experiments with [^{35}S]heparin, the unesterified fatty acids were isolated by a modification (Huttunen

et al., 1970) of the ion-exchange resin method of Kelley (1968). The enzyme was not dialysed in any of the experiments described in the present paper.

Sources of enzyme

Fresh pig hearts were obtained from a slaughterhouse. For enzyme purification the left ventricle was dissected out and rinsed free from blood with ice-cold 0.9% (w/v) NaCl. After a careful removal of fat and fascias, the muscle tissue was weighed and minced with scissors. The temperature was maintained at 0–4°C throughout the tissue preparation and the enzyme purification. In some experiments fresh hearts were frozen and stored at –20°C for 1–14 days before being processed. No significant loss of enzyme activity was observed under these conditions.

Enzyme purification

Heart muscle (100g wet wt.) was homogenized for 5 min in an Ultra-Turrax homogenizer in 0.9% (w/v) NaCl containing 200 units of heparin/ml and 80 ml of normal human serum. The final volume of the homogenate was 315 ml. The homogenate was incubated at 37°C for 15 min. It was then centrifuged at 4°C at 27000g_{av.} for 30 min and the supernatant was then decanted (extract I). The pellet was resuspended in 200 ml of 0.9% (w/v) NaCl and the procedures for homogenization, incubation at 37°C and centrifugation were repeated (extract II).

The two supernatants were combined. Triglyceride emulsion (0.125 vol.; 20%, w/v) (Intralipid; Vitrum, Stockholm, Sweden; an emulsion containing 0.13 M-triglycerides, 16 mM-egg lecithin, 0.85 mM-unesterified cholesterol and 0.27 M-glycerol) was added and the mixture was incubated at 37°C for 15 min by using a magnetic stirrer. After centrifugation at 76000g_{av.} for 60 min, the floating fat layer was collected. The clear infranatant was again mixed with Intralipid and the incubation and the centrifugation procedures were repeated. The fat layers from the two centrifugations were combined and added to 150 ml of cold (0–4°C) acetone. The mixture was stirred and then centrifuged at 20000g_{av.} for 10 min. The sediment was washed with 150 ml of ice-cold acetone and then with 2 × 70 ml of ice-cold diethyl ether, and finally dried under N₂ at room temperature (20°C). This preparation could be stored at –20°C for several days without significant loss of activity. The dried powder was dissolved in 5 mM-sodium barbital buffer, pH 7.4, containing 20% (v/v) glycerol and 1 M-NaCl. The addition of glycerol greatly increased the stability of the enzyme at this stage of the purification. The solution was centrifuged at 27000g_{av.} for 15 min. The pellet contained no triglyceride lipase activity.

The clear supernatant was applied to a column (3 cm × 27 cm) of Bio-Gel P-30 (Bio-Rad Laboratories, Richmond, Calif., U.S.A.), equilibrated with

5 mM-sodium barbital buffer, pH 7.4, containing 20% (v/v) glycerol and 1 M-NaCl. Fractions (10 ml) were collected and those containing lipase activity were pooled. The NaCl concentration of the preparation was adjusted to 0.5 M with 5 mM-sodium barbital buffer, pH 7.4, containing 20% (v/v) glycerol, and it was applied to a column (3 cm × 8 cm) of Sepharose 4B (Pharmacia, Uppsala, Sweden) containing covalently bound heparin (Iverius, 1971). The column was equilibrated with 0.15 M-sodium barbital buffer, pH 7.4, containing 20% (v/v) glycerol. The column was washed with 0.4 M-NaCl in 5 mM-sodium barbital buffer, pH 7.4, containing 20% (v/v) glycerol, then the enzyme was eluted with a linear gradient (flow rate 0.4 ml/min) from 0.4 to 1.5 M-NaCl in 5 mM-sodium barbital buffer, pH 7.4, containing 20% (v/v) glycerol. The total volume of the gradient was 300 ml and fractions (5.5 ml) were collected.

Preparation of apolipoproteins

Very-low-density lipoproteins were purified from the plasma of human donors by ultracentrifugation (Havel *et al.*, 1955). The removal of lipid and the purification of the polypeptides were performed as described by Brown *et al.* (1969). The polypeptides were characterized by polyacrylamide-gel electrophoresis (Herbert *et al.*, 1971) and by amino acid analysis (Shore & Shore, 1951).

Other procedures

Protein was measured by the method of Lowry *et al.* (1951), either directly or after precipitation with 5% (w/v) trichloroacetic acid, with dried bovine serum albumin as a standard.

Polyacrylamide-gel electrophoresis was performed in 5 and 10% (w/v) gels in the presence of sodium dodecyl sulphate by the method of Weber & Osborn (1969). NaCl concentrations were determined in a conductivity meter (Radiometer, Copenhagen, Denmark). Amino acid analyses were performed in a Beckman amino acid analyser, model 120C. [³⁵S]-Heparin was purchased from The Radiochemical Centre, Amersham, Bucks., U.K.

Results

Extraction and purification of lipoprotein lipase from pig heart homogenate

In agreement with the results of Borensztajn *et al.* (1970) and Iselin & Schuler (1957), heparin facilitated the extraction of triglyceride lipase from heart homogenates. The addition of heparin (200 units/ml) to the extraction medium increased the triglyceride lipase activity in the solution from less than 30% to 50–60% of the total activity in the homogenate (Table 1). If heparin was added to the extraction

Table 1. *Purification of pig heart lipoprotein lipase*

Values in parentheses give the range in three separate purifications.

Step	Protein (mg)	Vol. (ml)	Total activity (μ mol of unesterified fatty acids/h)	Specific activity (μ mol of unesterified fatty acids/h per mg of protein)	Purification (fold)
Homogenate	20800	315	250	0.012 (0.012–0.013)	1
Extract I	9660	210	110	0.012 (0.010–0.013)	1 (0.8–1)
Extract II	2050	186	25	0.012 (0.010–0.013)	1 (0.8–1)
Lipid-free enzyme substrate complex	100	20	140	1.4 (1.2–1.5)	115 (100–115)
Gel filtration	79.2	88	120	1.5 (1.3–1.6)	120 (110–120)
Affinity chromatography					
Applied	79.2	88	120	1.5 (1.3–1.6)	120 (110–120)
Unabsorbed	60.8	152	11	0.2 (0.2–0.3)	15 (15–25)
Peak fraction	0.008	5.5	19	2400 (2000–2500)	200 000 (154 000–208 000)
Peak total	0.100	60.5	86	860 (530–860)	72 000 (44 000–72 000)

medium, the addition of heparin (10 units/ml) to the assay mixture caused an 18% decrease in the triglyceride lipase activity.

The next step in the purification procedure was the formation of an enzyme–substrate complex by using an artificial triglyceride emulsion, Intralipid. The best recovery of the complex was obtained by using a mixture of the 20% (w/v) emulsion of Intralipid and heart extract (1:8, v/v). With this amount of triglyceride, 17–23% of the activity remained in the infranatant. The addition of serum was necessary for the formation of the complex. After the removal of lipid from the enzyme–substrate complex with acetone and diethyl ether, the residue was dissolved in 5 mM-sodium barbital buffer containing 20% (w/v) glycerol. The enzyme activity in this preparation was 54% of that in the original homogenate and 99% of the activity in the soluble extracts of the homogenate. Thus an absolute gain in the activity was observed in this step (20% of the activity in the infranatant, 100% in the enzyme–substrate complex). This gain is probably due to inhibition of the activity by the heparin which is present in the extracts of the homogenate (see below).

Preliminary experiments with this preparation indicated that only 5–25% of the triglyceride lipase activity in the solution was bound to a heparin–Sephacrose column. The enzyme retained by the column could be eluted by increasing the NaCl concentration of the eluent to 0.85 M. The binding of triglyceride lipase to heparin–Sephacrose could substantially be improved if the preparation was first subjected to gel filtration on Bio-Gel P-30. The change in the behaviour of the enzyme after gel filtration was in all probability due to the removal of heparin remaining in the preparation at this stage. Experiments with [^{35} S]heparin indicated that more than 50% of the remaining heparin was separated from the fractions containing triglyceride lipase when

the preparation was chromatographed on Bio-Gel P-30 with buffers containing 1 M-NaCl and 20% (v/v) glycerol (Fig. 1).

Fig. 2 shows the elution pattern of triglyceride lipase on heparin–Sephacrose after gel filtration. Less than 5% of the activity applied to the column was eluted in the void volume and the enzyme emerged at 0.85–0.95 M-NaCl. The asymmetry of the peak seen in Fig. 2 was observed in four of the six experiments performed. The reason for this asymmetry is not known.

The results of a typical purification of pig heart triglyceride lipase are shown in Table 1. The first purification step involving the formation of a substrate–enzyme complex resulted in 115-fold purification with 54% recovery. After heparin–Sephacrose chromatography the specific activity of the enzyme was 860 μ mol of unesterified fatty acid liberated/h per mg of protein, a 72 000-fold purification over that in the original homogenate. The overall recovery was 34%. The specific activity of the final preparation obtained in two other purifications was 530 and 790 μ mol of unesterified fatty acid liberated/h per mg of protein. One main band was found when 10 μ g of the purified protein was subjected to sodium dodecyl sulphate–polyacrylamide-gel electrophoresis. Its mobility corresponded to an apparent molecular weight of 73 000. Since the amount of protein loaded on the gel was small and the lipolytic activity was destroyed by sodium dodecyl treatment, the identity of the band with the purified enzyme remains uncertain.

Properties of lipoprotein lipase from porcine myocardium

The maximum velocity of the purified heart triglyceride lipase was obtained by using a triolein concentration greater than 1 mM. The activity was

Fig. 1. Gel filtration of myocardial lipoprotein lipase on Bio-Gel P-30

The lipid-free triglyceride lipase preparation obtained after the enzyme-substrate complex step was dissolved in 5 mM-sodium barbital buffer, pH 7.4, containing 20% (v/v) glycerol and 1 M-NaCl. [^{35}S]Heparin (80 units; specific radioactivity 0.03 $\mu\text{Ci}/\text{unit}$ of heparin) was added and the preparation was applied to a column (3 cm $\times$ 27 cm) of Bio-Gel P-30, equilibrated with 5 mM-sodium barbital buffer, pH 7.4, containing 20% glycerol and 1 M-NaCl. Fractions (10 ml) were collected. $\circ$, Triglyceride lipase activity; $\blacksquare$, protein concentration; $\bullet$, ^{35}S radioactivity.

Fig. 2. Heparin-Sepharose chromatography of pig myocardial lipoprotein lipase

Pooled fractions from Bio-Gel P-30 gel chromatography which contained triglyceride lipase activity were applied to a column (3 cm $\times$ 8 cm) of heparin-Sepharose equilibrated with 0.15 M-NaCl-5 mM-sodium barbital buffer, pH 7.4, containing 20% (v/v) glycerol. The column was eluted with a linear NaCl gradient (0.4 ml/min) from 0.4 to 1.5 M-NaCl in 5 mM-sodium barbital buffer, pH 7.4, containing 20% glycerol. The total volume of the gradient was 300 ml. Fractions (5.5 ml) were collected. $\bullet$, Triglyceride lipase activity measured by the standard assay system; $\circ$, protein concentration; $\blacktriangle$, NaCl concentration.

directly proportional to the amount of the enzyme added and there was a linear rate of release of fatty acids for at least 120 min at 28°C. Lipoprotein

lipase from pig heart had a single pH optimum at pH 8.4.

The effect of serum on the purified lipase is shown in

Fig. 3. Effect of serum (a) and apolipoprotein CII (b) on the activity of purified porcine myocardial lipoprotein lipase

The assays were performed under standard conditions by using various concentrations of serum and of apolipoprotein CII [human very-low-density lipoprotein apoprotein with C-terminal glutamic acid; apo Lp-Glu (Krauss *et al.*, 1973)]. No serum was added to the assay system in the experiments with apolipoprotein CII.

Fig. 3. The enzyme was stimulated fivefold by the normal human serum (100 µl/ml) but showed definite activity in the absence of serum. A similar activation was observed with apolipoprotein CII (human very-low-density lipoprotein with C-terminal glutamic acid; apo Lp-Glu) (Krauss *et al.*, 1973), but not by other low-molecular-weight apolipoproteins from human very-low-density lipoproteins. This effect is similar to that obtained for lipoprotein lipase from pig adipose tissue (Bensadoun *et al.*, 1974). The amount of apolipoprotein CII required for half-maximum activation was 5 µg/ml (Fig. 3). Maximum stimulation occurred at 30–40 µg/ml, whereas at higher concentrations (>40 µg/ml) the activation declined.

The enzyme showed maximum activity with 0.05–0.15M-NaCl. Higher salt concentrations caused a progressive decrease in the activity and at 0.6M-NaCl an 80% inhibition was observed. These experiments were carried out without prior dialysis by diluting the enzyme preparation to the appropriate NaCl concentration with NaCl-free buffers. Both the partially purified enzyme (before affinity chromatography) and the final preparation were activated by protamine sulphate, and even at the highest concentration tested (1mg/ml) no inhibition was seen. The effect of heparin on the enzyme depended on the concentration of heparin used. At low concentrations (5–10 units/ml) the activity increased twofold, but concentrations exceeding 20 units/ml caused a distinct inhibition. This observation agrees with the behaviour of the enzyme at earlier stages of the purification. Inhibition by heparin was seen in the crude preparation when heparin was added to the extraction medium, and heparin only stimulated activity after gel filtration on Bio-Gel P-30.

All the studies on the characteristics of the purified lipase were repeated with at least two separate preparations with essentially similar results.

Discussion

The method described here for the purification of pig myocardial lipoprotein lipase is based on the sequential use of two steps, which are highly specific for triglyceride lipases that are released by heparin. Complex-formation between triglyceride lipase and its substrates was first used to purify chicken adipose-tissue lipoprotein lipase (Korn & Quigley, 1957). It has since been applied to the purification of lipoprotein lipase from several other tissues (Fielding, 1969, 1970; Ganesan *et al.*, 1971). The formation of the complex has an absolute requirement for a factor present in serum, presumably apolipoprotein CII (Miller & Smith, 1973), and hence serum must be added to the homogenate before further processing. Affinity chromatography, which was introduced by Iverius (1971), has been used to purify triglyceride lipase from chicken, rat and pig adipose tissue (Egelrud, 1973; Greten & Walter, 1973; Bensadoun *et al.*, 1974), rat liver (Assman *et al.*, 1973), human and pig plasma obtained after heparin administration (Greten *et al.*, 1972; Ehnholm *et al.*, 1973, 1975) and bovine and human milk (Egelrud & Olivecrona, 1972; Hernell & Olivecrona, 1974). With myocardium this step alone does not give sufficient purification when applied directly to extracts of defatted homogenates (Ehnholm *et al.*, 1974a; Dolphin & Rubinstein, 1974). Chromatography of acetone-dried-powder extracts gave a 300–650-fold purifi-

cation, whereas the method now reported yields 70000–100000-fold purification over that in the crude homogenate. The specific activity obtained, 530–860 μmol of unesterified fatty acids/h per mg of protein, is very close to that reported for lipoprotein lipase from pig adipose tissue (Bensadoun *et al.*, 1974). The degree of purification and the yield of the enzyme observed in this study may, however, be overestimates because of the variable effects of heparin on lipase activity during the purification procedures.

The extraction of acetone-dried powders or heart homogenates with heparin instead of aq. $\text{NH}_3\text{--NH}_4\text{Cl}$ (Korn, 1955) considerably improves the yield of lipoprotein lipase. The presence of excess of heparin, however, decreased the binding of the enzyme to heparin–Sepharose. Similar findings have also been reported from a triglyceride lipase from rat liver plasma membranes (Assman *et al.*, 1973). The problems caused by an excess of heparin in the preparation were circumvented in the present study by subjecting the enzyme preparation to gel filtration under conditions (1M-NaCl) that dissociate the reversible binding between the lipoprotein lipase and heparin. This step removed more than 50% of the heparin present and a quantitative binding of the enzyme on heparin–Sepharose was attained.

The differences between hepatic triglyceride lipase, purified from rat and canine liver (Assman *et al.*, 1973; Greten *et al.*, 1974) and from human and pig plasma obtained after heparin administration (Ehnholm *et al.*, 1973; Greten *et al.*, 1974; Ehnholm *et al.*, 1974b, 1975), and lipoprotein lipase from different tissues (Hernell & Olivecrona, 1972; Egelrud, 1973; Bensadoun *et al.*, 1974; Ehnholm *et al.*, 1974b, 1975) are obvious. The hepatic lipase is not inactivated at high ionic strength and does not require factors present in serum. It is immunologically different from lipoprotein lipase. Antibodies produced towards human or rat hepatic lipase do not cross-react with lipoprotein lipase in human plasma obtained after heparin administration (Huttunen *et al.*, 1975) or in heparin perfusate of rat heart (Jansen *et al.*, 1973).

Myocardial triglyceride lipase purified by the present procedure differs, however, in one respect from lipoprotein lipases described thus far. These lipoprotein lipases are eluted from heparin–Sepharose at 1.1–1.25M-NaCl concentration, whereas the myocardial enzyme emerged at 0.85–0.95M-NaCl. This is probably due to different preliminary purification steps. Thus preliminary experiments indicate that if lipoprotein lipase from porcine adipose tissue is purified by the procedure reported here, the enzyme emerges from heparin–Sepharose like the myocardial enzyme (C. Ehnholm & P. K. J. Kinnunen, unpublished work). The reason for the change in the

chromatographic behaviour induced by the initial purification steps is unknown.

We acknowledge the skilful technical assistance of Mrs. Ritva Timonen, Mrs. Ulla Toiviainen and Miss Seija Puomilahti. This work was aided by grants from the National Council for Medical Sciences, Finland, from the Orion Foundation and from the Finnish Cultural Foundation.

References

- Assman, G., Krauss, R. M., Fredrickson, D. S. & Levy, R. I. (1973) *J. Biol. Chem.* **248**, 1992–1999
- Belfrage, P. & Vaughan, M. (1969) *J. Lipid Res.* **10**, 341–344
- Bensadoun, A., Ehnholm, C., Steinberg, D. & Brown, W. V. (1974) *J. Biol. Chem.* **249**, 2220–2227
- Beznan, A., Felts, J. M. & Havel, R. J. (1962) *J. Lipid Res.* **3**, 427–431
- Borensztajn, J., Otway, S. & Robinson, D. S. (1970) *J. Lipid Res.* **11**, 102–110
- Brown, W. V., Levy, R. I. & Fredrickson, D. S. (1969) *J. Biol. Chem.* **244**, 5687–5694
- Carlson, L. A., Kaijser, L. & Lassers, B. W. (1970) *J. Mol. Cell. Cardiol.* **1**, 467–475
- Dolphin, P. J. & Rubinstein, D. (1974) *Biochem. Biophys. Res. Commun.* **57**, 808–814
- Egelrud, T. (1973) *Biochim. Biophys. Acta* **296**, 124–129
- Egelrud, T. & Olivecrona, T. (1972) *J. Biol. Chem.* **247**, 6212–6217
- Ehnholm, C., Bensadoun, A. & Brown, W. V. (1973) *J. Clin. Invest.* **52**, 26a
- Ehnholm, C., Huttunen, J. K., Nikkilä, E. A. & Ohta, M. (1974a) *Abstr. Meet. Eur. Soc. Clin. Invest. 8th, Rotterdam*, p. 122
- Ehnholm, C., Shaw, W., Greten, H., Lengfelder, W. & Brown, W. V. (1974b) in *Proceedings of the Third International Symposium on Atherosclerosis, West Berlin*, (Schettler, G. & Weizel, A., eds.), pp. 557–560, Springer-Verlag, New York
- Ehnholm, C., Kinnunen, P. K. J. & Huttunen, J. K. (1975) *FEBS Lett.* **52**, 191–194
- Enser, M. B., Kunz, F., Borensztajn, J., Opie, L. H. & Robinson, D. S. (1967) *Biochem. J.* **104**, 306–317
- Fielding, C. J. (1969) *Biochim. Biophys. Acta* **178**, 499–507
- Fielding, C. J. (1970) *Biochim. Biophys. Acta* **206**, 109–117
- Ganesan, D., Bradford, R. H. & Bass, H. (1971) *Biochem. Biophys. Res. Commun.* **43**, 544–549
- Greten, H. & Walter, B. (1973) *FEBS Lett.* **35**, 36–40
- Greten, H., Walter, B. & Brown, W. V. (1972) *FEBS Lett.* **27**, 306–310
- Greten, H., Sniderman, A. D., Chandler, J. G., Steinberg, D. & Brown, W. V. (1974) *FEBS Lett.* **42**, 157–160
- Hamilton, R. L., Jr. (1964) *Post-Heparin Plasma Lipase from the Hepatic Circulation (Thesis)*, University Microfilms, Ann Arbor, Mich.
- Havel, R. J., Eder, H. A. & Bragdon, J. H. (1955) *J. Clin. Invest.* **34**, 1345–1353
- Herbert, P., Levy, R. I. & Fredrickson, D. S. (1971) *J. Biol. Chem.* **246**, 7068–7069

- Hernell, O. & Olivecrona, T. (1974) *J. Lipid Res.* **15**, 367-374
- Hollenberg, C. H. (1960) *J. Clin. Invest.* **39**, 1282-1287
- Huttunen, J. K., Ellinboe, J., Pittman, R. C. & Steinberg, D. (1970) *Biochim. Biophys. Acta* **218**, 333-346
- Huttunen, J. K., Ehnholm, C., Nikkilä, E. A. & Ohta, M. (1975) *Eur. J. Clin. Invest.* **5**, in the press
- Iselin, B. & Schuler, W. (1957) *Helv. Physiol. Pharmacol. Acta* **15**, 14-24
- Iverius, P.-H. (1971) *Biochem. J.* **124**, 677-683
- Jansen, H., van Zuylen-van Wiggen, A. & Hülsman, W. C. (1973) *Biochem. Biophys. Res. Commun.* **55**, 30-37
- Kelley, T. F. (1968) *J. Lipid Res.* **9**, 799-800
- Korn, E. D. (1955) *J. Biol. Chem.* **215**, 1-14
- Korn, E. D. & Quigley, T. W., Jr. (1957) *J. Biol. Chem.* **226**, 833-839
- Krauss, R. M., Herbert, P. N., Levy, R. I. & Fredrickson, D. S. (1973) *Circulation Res.* **33**, 403-411
- Lowry, O. H., Rosebrough, N. J., Farr, A. L. & Randall, R. J. (1951) *J. Biol. Chem.* **193**, 265-275
- Miller, A. L. & Smith, L. C. (1973) *J. Biol. Chem.* **248**, 3359-3362
- Regan, T. J., Moschos, C. B., Lehan, P. H., Oldewurtel, H. A. & Hellems, H. K. (1966) *Circulation Res.* **19**, 307-316
- Otway, S. & Robinson, D. S. (1968) *Biochem. J.* **106**, 677-682
- Shore, V. G. & Shore, B. (1951) in *Blood Lipids and Lipoproteins: Quantification, Composition and Metabolism* (Nelson, G. S., ed.), chapter 15, Wiley-Interscience, New York
- Spitzer, J. A. & Spitzer, J. J. (1956) *Am. J. Physiol.* **185**, 18-22
- Thomas, W. A., Florentin, R. A., Nam, S. C., Daoud, A. S. & Tiamson, E. (1970) in *Atherosclerosis* (Jones, R. J., ed.), pp. 414-420, Springer-Verlag, New York
- Weber, K. & Osborn, M. (1969) *J. Biol. Chem.* **244**, 4406-4412