BBA 66077

HUMAN LIPOPROTEIN LIPASE

I. PURIFICATION AND SUBSTRATE SPECIFICITY

C. J. FIELDING*
Department of Biochemistry, University of Oxford, Oxford (Great Britain)

(Received October 13th, 1969)

SUMMARY

1. Human lipoprotein lipase has been purified 16 000-fold from post-heparin plasma. The product shows no activity against triglyceride in the absence of added lipoprotein.

2. The enzyme is active against both diglyceride and monoglyceride emulsions in the absence of lipoprotein, but monoglyceride hydrolase activity requires the presence of either deoxycholate or unesterified fatty acid.

3. The rate of enzyme activity is strongly dependent upon the identity of the lipoprotein present.

4. The temperature optimum of enzyme activity is also significantly dependent upon the nature of the lipoprotein co-factor.

INTRODUCTION

The circulating triglyceride of mammalian plasma is degraded by lipoprotein lipase, but this enzyme is without activity against artificial triglyceride emulsions in the absence of added plasma lipoproteins[1,2]. Although the structure of the lipoproteins of human plasma has recently become better understood[3] little information is available on the detailed role of these factors in the activity of lipoprotein lipase, or of the possible effects of variations in the lipoprotein composition of whole plasma upon the rate of enzymatic hydrolysis.

In the present publication, a method is given for the preparation of highly purified human lipoprotein lipase from post-heparin plasma completely free from contaminating lipoproteins. Using this preparation, the substrate specificity of the enzyme, and the role of the lipoproteins in its activity, have been investigated.

MATERIALS AND METHODS

Triolein, diolein, monolein, oleic acid, linolenic acid and α-L-lecithin (all more

* Present address: Department of Medicine, The University of Chicago, Chicago, Ill. U.S.A.

than 99.5% pure, confirmed by thin-layer chromatography in the laboratory[4,5]) were obtained from the Sigma Chemical Company, London. Glyceryl tri([1-^{14}C]oleate), specific activity 34.1 mC/mmole, was obtained from the Radiochemical Centre, Amersham. Purity from contaminating unesterified fatty acid or partial glycerides was found to be greater than 99.7% (ref. 4). High-molecular-weight dextran (nominal mol. wt. $5 \cdot 10^5$) was purchased from Pharmacia, Sweden. Butyl-PBD scintillant was obtained from Ciba, Cambridge.

Highly purified chylomicrons, very low density, low density, and high density lipoproteins were prepared from the pooled plasma of morning blood samples from male donors, aged 20–30 years[6]. The purity of each fraction was confirmed by electrophoresis on cellulose acetate strips (Sephraphore III, Gelman Instrument Company, Michigan), followed by staining with 0.002% nigrosine in 5% acetic acid, or with Oil Red O in 70% methanol–water. Each fraction was pure from detectable amounts of contaminating proteins. Lipoprotein concentrations were calculated from protein content, by use of the appropriate factor to allow for lipid content[3]. Glycerol was assayed by the method of LAMBERT AND NEISH[7]. Lipoprotein lipase was assayed as previously described[8] except that in some experiments isolated lipoproteins were used to activate the substrate triglyceride emulsion ("Intralipid 20%", Vitrum, Sweden) in place of whole serum.

Post-heparin plasma was taken from male donors as above. 10 000 I.U. of sodium heparin (Evans Medical Company, Liverpool) was injected intravenously, and after 10 min, 500 ml of blood was taken from a brachial vein into cooled polythene tubes containing 1/20 vol. of 0.25 M sodium citrate. After centrifugation at $2000 \times g$ for 20 min at 0°, the supernatant plasma was removed and stored at −25° until required for purification. Lipoprotein lipase activity was stable for at least 6 months under these conditions. An enzyme content of 6–10 enzyme units*/ml was obtained in the post-heparin plasma.

All other materials and methods were as previously described[9].

RESULTS

Preparation of purified lipoprotein lipase

The purification method was developed from that reported for the enzyme from rat post-heparin plasma, which can be obtained more than 96% pure by a combination of ultracentrifuge and adsorption techniques[9].

100 ml of post-heparin plasma was incubated with 1/40 vol. of triglyceride emulsion for 15 min at 37°, and the resulting enzyme–lipoprotein complex was purified in the ultracentrifuge, and by extraction with detergent, as previously described[9]. All purification steps were carried out at 0–2°. A purification of approx. 2500-fold was obtained in 20% yield. The enzyme solution from this step (1 ml) was extracted with acetone and ether[8] to remove the detergent, which in the case of the human enzyme was found to interfere with the later purification procedures. No enzyme activity was lost during this extraction. In some experiments dextran was added to the enzyme solution before extraction to a final concentration of 10 mg/ml to increase the bulk of the precipitate.

* 1 unit catalyses the release of 1 μmole unesterified fatty acid/h at 37°.

The precipitate, dried *in vacuo* was dissolved in 1 ml of 0.05 M NH_4OH–NH_4Cl buffer (pH 8.3) containing 0.5 mM potassium linolenate as stabilizing factor[8,9]. The use of polyunsaturated fatty acid in place of oleate as previously reported[9] has been found to prevent the gradual precipitation of fatty acid from this buffer during storage in the refrigerator.

Lipoprotein lipase is strongly adsorbed to calcium phosphate gel[10] and this property was used to provide a further purification. 0.25 ml of 0.2 M calcium phosphate gel was added to 1 ml of enzyme solution from the previous step, and after adsorption, the gel was washed with 1 ml of 0.1 M potassium oxalate, then 0.25 ml of 0.05 M sodium citrate[9]. The citrate eluate contained 60–65% of added enzyme activity, purified 4-fold at this step.

Unlike the rat preparation, the enzyme solution at this stage still probably contained significant contaminating lipoprotein, as shown by its residual activity against triglyceride emulsion in the absence of added lipoprotein. 0.75 ml of NH_4OH–NH_4Cl buffer (pH 8.3) containing 0.5 mM potassium linolenate, was added to each 0.25 ml of citrate eluate. A gelatinous precipitate formed, which after standing for 1–2 h at 0° was removed by centrifugation at $5000 \times g$ for 20 min. The clear supernatant contained 70–75% of the enzyme activity; it retained no detectable activity against triglyceride in the absence of added lipoprotein, and no activity against monolein when this partial glyceride was used in place of triglyceride in the assay medium. A further 2-fold purification was obtained at this step. The final specific activity was 2200 units/mg protein, representing a 16 000-fold purification from crude post-heparin plasma. The yield was about 10%. Unlike the preparation of the enzyme from rat plasma[9], the recovery of the human enzyme was not affected by the addition of heparin (1.0 I.U./ml) to the buffers used during the purification procedure.

Insufficient material has as yet been available to accurately estimate the purity of this preparation from contaminating non-enzyme proteins, although the final specific activity is similar to that of the pure rat enzyme. Further work on the characterization of the enzyme is in progress.

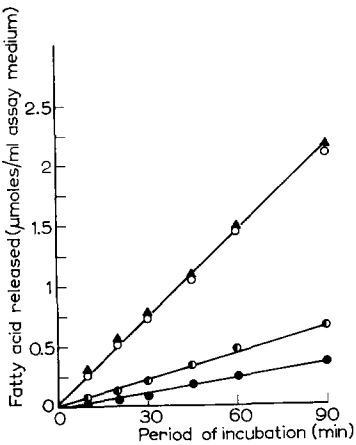
Effects of pure lipoproteins on enzyme activity

Whole human plasma contains significant monoglyceride hydrolase activity[11,12]. To avoid the contribution that this enzyme activity might make to the rate of fatty acid production from triglyceride emulsion activated with whole plasma, activation was carried out with pure lipoproteins. Lipoprotein and triglyceride were pre-incubated for 30 min at 37° before incorporation into the assay medium. The final lipoprotein content of these assays was 1.0 mg/ml in each case.

The rate of fatty acid release with all lipoproteins was linear at 37° for at least 90 min under the conditions of assay (Fig. 1). However, the rate of hydrolysis by the same quantity of enzyme was dependent upon the nature of the lipoprotein present. Significantly higher rates of hydrolysis were obtained with high density lipoprotein and chylomicrons, than with very low density or low density lipoproteins.

pH and temperature optima of the purified enzyme

Assay media were prepared with Tris–HCl buffer over the pH range 6.0–10.5. The rate of hydrolytic activity at 37° was determined over this range for each lipo-



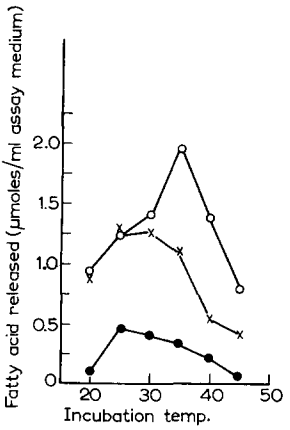


Fig. 1. Rate of enzyme activity in the presence of isolated lipoproteins. Incubations were carried out at 37°, pH 8.1, in assay medium[8] containing isolated lipoprotein fractions at a fixed concentration of 1.0 mg/ml, in place of whole serum. Initial triglyceride concentration was 2.0 μmoles/ml in each case, to include the concentration from the lipoprotein fraction[3]. The rate of hydrolysis was unchanged over triglyceride concentration from 1.0 to 5.0 μmoles/ml. In the absence of added triglyceride emulsion, high density and low density lipoproteins triglyceride was not hydrolyzed by lipoprotein lipase. Chylomicron triglyceride was hydrolyzed at a rate which was not increased by the addition of further triglyceride; very low density lipoprotein triglyceride was hydrolyzed at 75% of the rate given by addition of further triglyceride to a total initial concentration of 2 μmoles/ml. ▲———▲, chylomicrons; ◐———◐, very low density lipoprotein; ●———●, low density lipoprotein; ○———○, high density lipoprotein.

Fig. 2. Temperature optimum of lipoprotein lipase in the presence of triglyceride (initial concentration 2.0 μmoles/ml) emulsified with lecithin and activated with lipoprotein fractions. Incubations were at pH 8.1 for 1 h in assay medium[8] containing lipoproteins (1.0 mg/ml final concentration) or whole plasma (0.2 ml/ml assay medium). ○———○, high density lipoprotein; ●———●, low density lipoprotein; ×———×, whole serum.

protein fraction and for whole serum. An optimum of 8.1 was obtained in all experiments.

The temperature optimum of the rat enzyme was 37° (ref. 9). However, a higher enzyme activity at 27 than at 37° has been reported for the human plasma enzyme activated with whole serum[13]. In the present experiments, the purified enzyme activated with lipoprotein fractions or with whole serum was incubated at the pH optimum over the temperature range 20–45° (Fig. 2). When high density lipoprotein or chylomicrons were the activating agent, the temperature optimum was 35°. For low density and very low density lipoproteins the optimum was 25°, and a marked decrease in activity was found at higher temperatures. With whole plasma a broad maximum was obtained between 25 and 30°.

This pattern of optimal temperatures was unchanged over the pH range 7.2–8.5.

Lipid products of enzyme activity

Experiments by CARLSON AND WADSTROM[14] on the lipid products of enzyme activity, using post-heparin plasma as a source of enzyme and chylomicrons as substrate, showed that loss of triglyceride was accompanied by an accumulation of partial glycerides in the early stages of the assay. Free glycerol was also produced.

Because of the possibility of monoglyceride hydrolase activity introduced by

the presence of whole plasma, these experiments were repeated in the present research with purified enzyme activated with isolated lipoproteins.

Glyceryl tri([1-^{14}C]oleate) solution in diethyl ether was diluted with unlabelled triolein to a specific activity of 1.0 mC/mmole. α-L-lecithin was added to a proportion of 0.05 μM lecithin/μM triglyceride. The mixture in ether solution was dried down under N_2, and emulsified in 0.145 M NaCl in a Vortex mixer for 5 min. The emulsion produced was stable for several hours. High density lipoprotein was added to a final concentration of 1.0 mg/ml in the assay medium, and the mixture was preincubated for 30 min at 37°. Albumin solution and Tris–HCl buffer (pH 8.1) were added as usual to complete the assay medium. The final triglyceride concentration was 1.0 μmole/ml.

Sufficient purified enzyme in 0.05 M NH_4OH–NH_4Cl buffer (pH 8.3)–0.5 mM potassium linolenate, was added to give a final concentration of 1–5 units/ml in the assay medium. At the same time linolenate buffer only was added to an otherwise identical control incubation mixture. Duplicate 0.5-ml samples were taken into 10 ml of chloroform–methanol (2:1, by vol.) at zero time and at intervals thereafter, during incubation at 37°. After addition of 1.5 ml of water, the lower phase containing lipid was dried down under N_2, and carrier triolein, diolein, monolein, and oleic acid in 0.5 ml of chloroform–methanol (2:1, by vol.) were added. Thin-layer chromatography was carried out using 1/10 vol. of each sample. The separated lipid spots were visualized with iodine vapour, then scraped off and counted directly in glass vials in a Beckman scintillation counter, using as scintillant 0.7% butyl-PBD, 8% naphthalene in toluene–methoxyethanol (3:2, by vol.). Less than 1% of further unesterified fatty acid was obtained by reextraction of the aqueous phase from the above procedure with acidified heptane–isopropanol. No detectable residual amounts of monoglyceride or other neutral lipids were found in this second extraction. No radioactivity could be detected in the phospholipid or cholesterol ester spots[4]. 0.2 ml of the aqueous phase was also assayed for free glycerol.

The time-course of lipid hydrolysis is shown in Fig. 3. Fatty acid production

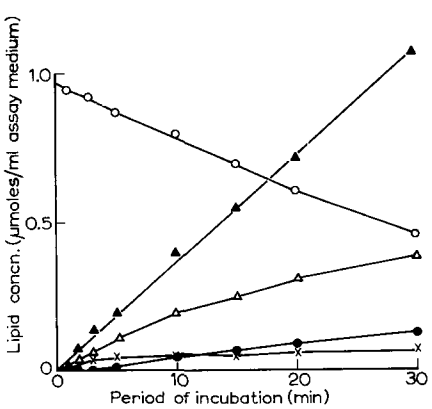


Fig. 3. Lipid products of hydrolysis of ^{14}C-labelled triglyceride by lipoprotein lipase. Initial triglyceride content was 2.0 μmoles (1.0 μC/μmole triglyceride)/ml assay medium. Assay conditions were as in Fig. 1. Radioactivity in counts/min was converted to μmoles lipid by assuming a relative specific radioactivity for triglyceride:diglyceride:monoglyceride:unesterified fatty acid of 3:2:1:1. ○———○, triglyceride; ×———×, diglyceride; △———△, monoglyceride; ▲———▲, unesterified fatty acid; ●———●, glycerol.

was maintained over the period of assay. Diglyceride was present at a constant low level throughout the incubation. There was a rapid accumulation of monoglyceride, and, after a delay of about 5 min, of free glycerol. The production of free glycerol was also calculated from the excess of unesterified fatty acid over the quantity of monoglyceride produced. This estimate was within 5% of the value obtained by the direct colorimetric estimation of glycerol, for each assay taken during the incubation. Similar procedures were carried out at the same time intervals with samples taken from the control flask not containing enzyme. No detectable amount of fatty acid was released during this assay. Unlabelled oleic acid was added to control assays (1.0 μmole/ml) and the incubation repeated. After incubation, the oleic acid spot was assayed for radioactivity and fatty acid content. The radioactivity was less than 2% of that found when enzyme was present. This result suggests that non-enzymatic exchange between the free and esterified fatty acids was not a significant factor under the conditions of assay.

Experiments were also carried out using radioactive triglyceride emulsions activated with very low density and low density lipoproteins. The same pattern of accumulation of lipid products was obtained in each case.

Substrate specificity of lipoprotein lipase

In view of the significant delay before the production of free glycerol during the incubation of lipoprotein lipase with triglyceride emulsion (Fig. 3) the activity of this enzyme against partial glycerides in the presence and absence of lipoproteins was investigated. Activity was determined as the rate of unesterified fatty acid production in each case.

Experiments were carried out with emulsions stabilized either with sodium deoxycholate[12] or with lecithin as in the preceding experiments. Emulsions of triglyceride, diglyceride and monoglyceride were prepared to give an initial concentration of 2.0 μmoles/ml in each case in the complete assay medium. Emulsions of lecithin were also prepared at the same concentration, alone or with deoxycholate.

All emulsions were stable during the period of assay even in the absence of added lipoprotein. Monoglyceride emulsions were optically clear; those of diglyceride and triglyceride were opaque but cleared when enzyme activity was present. Control flasks not containing enzyme were included in all experiments. No hydrolysis occurred in the absence of added enzyme. The results are shown in Table I. Monoglyceride was an effective substrate in the presence of sodium deoxycholate, but not when lecithin was used to emulsify this lipid. Sodium deoxycholate was inhibitory at 0.02% (w/v) to triglyceride and diglyceride hydrolase activity, but monoglyceride hydrolase activity was significantly stimulated. Lecithin was not a substrate for the purified enzyme, as shown by the absence of detectable fatty acid production when this lipid alone was present in the emulsion (Table I).

In view of the unexpected lack of monoglyceride hydrolase activity in the presence of lecithin, when the conditions were similar to those in Fig. 3 where free glycerol was produced, a further investigation of the conditions required for effective monoglyceride hydrolase activity by lipoprotein lipase was made. During the hydrolysis of triglyceride, free glycerol was not produced until the level of fatty acid exceeded 0.1 μmole/ml. Potassium linolenate was added to the assay medium containing monoglyceride and lecithin, and after the addition of lipoprotein lipase

TABLE I

SUBSTRATE SPECIFICITY OF LIPOPROTEIN LIPASE

5.0 ml assays[8] contained neutral lipid (2.0 μmoles/ml) emulsified with lecithin (0.05 μmoles/ml) or sodium deoxycholate (0.02%, w/v) in the presence or absence of high density lipoprotein (1 mg/ml). After addition of enzyme (1.0 unit/ml assay) duplicate samples were taken at zero time, and after incubation for 1 h at 37°. Values are expressed as percent activity of that given by triglyceride emulsified with lecithin in the presence of high density lipoprotein.

	Sodium deoxycholate + high density lipoprotein	*Sodium deoxycholate alone*	*Lecithin + high density lipoprotein*	*Lecithin alone*
Triglyceride	45	0	100	0
Diglyceride	70	44	164	54
Monoglyceride	57	30	0	0
Monoglyceride + linolenate 0.1 μmole/ml	60	32	30	12
Lecithin	0	0	0	0

measurement of fatty acid production was carried out as before. A significant rate of hydrolysis was found in the presence or absence of lipoprotein (Table I).

Similar results were obtained in experiments with each of the purified lipoprotein fractions.

DISCUSSION

The method described has permitted the preparation of human lipoprotein lipase purified approx. 16 000-fold from post-heparin plasma. The product was free of detectable activity against triglyceride in the absence of lipoprotein.

The rate of enzymatic hydrolysis was strongly dependent upon the identity of the lipoprotein present, although linear release of fatty acid was obtained with all fractions (Fig. 1). The lower rate of fatty acid production from triglyceride activated with very low density or low density lipoproteins, than with chylomicrons or high density lipoproteins therefore does not seem to be the result of a decreased stability of the enzyme–substrate complex to incubation under the conditions of assay[15]. The greater effect of very low density lipoproteins than low density lipoproteins, at the same triglyceride concentration, suggests that the transformation of very low density lipoprotein to low density lipoprotein is unlikely to proceed through the action of lipoprotein lipase alone, in spite of the identity of the amino acid composition of the two fractions[6,16]. It may, however, involve the co-operative activities of lipoprotein lipase and lecithin: cholesterol acyltransferase[17], since this latter enzyme is present in significant amounts in whole plasma.

The pH optimum of lipoprotein lipase activated with pure lipoproteins or whole serum is similar to that previously reported for the human[13] and rat enzymes[2,9]. The temperature optimum of this enzyme under the same conditions is shown in Fig. 2. Whilst the maximum for high density lipoprotein was found to be 35°, which is similar to that for the rat enzyme[9], when the substrate was activated with very low density or low density lipoproteins a significantly lower maximum was found. Activation with

whole serum gave a maximum between 25 and 30°, as previously reported[13]. Below 30°, the rates of hydrolysis of triglyceride in the presence of high density lipoprotein and whole serum are identical, which may suggest that under these conditions high density lipoprotein is the most important lipoprotein in the activation of lipoprotein lipase. It has been previously shown that in both high density and low density lipoproteins marked conformational changes occur as the temperature is raised, in particular a decreased helical content, and an increased proportion of random coil[18–20]. It is an attractive possibility that such unfolding is involved in the changing activity with temperature of these cofactors.

As shown in Fig. 3, lipoprotein lipase preparations free of monoglyceride hydrolase activity, and free of triglyceride hydrolase activity in the absence of added lipoprotein, nevertheless catalyze the production of free glycerol from triglyceride emulsions in the presence of lecithin. An accumulation of monoglyceride throughout the assay period suggested that the hydrolysis of monoglyceride to free glycerol was rate limiting. Also, the production of free glycerol was delayed until the production of approx. 0.1 μmole fatty acid/ml assay medium had already occurred.

An explanation of this effect is shown in Table I. When partial glycerides were used as substrate, whilst diglyceride was more effective than triglyceride under all conditions, monoglyceride was a substrate only in the presence of sodium deoxycholate or of added fatty acid. Significant diglyceride and monoglyceride hydrolase activities were obtained in the complete absence of added lipoprotein.

Previous demonstrations of monoglyceride hydrolase activity in serum were carried out in the presence of lipoprotein lipase activity[11] or of sodium deoxycholate[12], that is, under conditions where lipoprotein lipase itself would show significant monoglyceride hydrolase activity (Table I). It appears likely that the monoglyceride hydrolase activity of whole plasma may be due, at least in part, to the activity of lipoprotein lipase. The release by heparin of monoglyceride hydrolase activity concomitantly with lipoprotein lipase is also suggestive of common origin. However, considerable evidence exists that the properties of the hydrolytic activities against triglyceride and monoglyceride are different. The results obtained from studies of the inhibition of these activities by NaCl, protamine and pyrophosphate showed the significantly greater sensitivity of triglyceride hydrolase activity to these agents. Individuals showing a familial lowering of triglyceride hydrolase activity retained monoglyceride hydrolase activity at the normal level[12]. Finally, whilst hydrolysis of triglyceride and diglyceride is inhibited by sodium deoxycholate, hydrolysis of monoglyceride is stimulated (Table I).

No evidence is available of the detailed structure of lipoprotein lipase. However, the evidence above suggests that lipoprotein lipase may be a multifunctional complex, in which separate hydrolytic sites binding triglyceride and monoglyceride, together effect the complete degradation of lipoprotein triglyceride. However, it is also possible that the co-purification of lipoprotein lipase and monoglyceride hydrolase activities is due to a similar ability to associate with lipid during the preparative process. Further research on the structure of lipoprotein lipase is needed before these alternatives can be distinguished.

ACKNOWLEDGEMENTS

Samples of human plasma were provided through the kindness of Dr. B. E. Juel-Jensen, the Radcliffe Infirmary, Oxford. This investigation was supported by a personal grant to the author from the Medical Research Council, London.

REFERENCES

1 E. D. KORN, *J. Biol. Chem.*, 215 (1955) 15.
2 E. D. KORN AND T. W. QUIGLEY, *J. Biol. Chem.*, 226 (1957) 833.
3 A. M. SCANU, *Advan. Lipid Res.*, 3 (1965) 63.
4 J. L. BROWN AND J. M. JOHNSTON, *J. Lipid Res.*, 3 (1962) 480.
5 V. P. SKIPSKI, R. F. PETERSON AND M. BARCLAY, *J. Lipid Res.*, 3 (1962) 467.
6 R. S. LEVY, A. C. LYNCH, E. D. MCGEE AND J. W. MEHL, *J. Lipid Res.*, 8 (1967) 463.
7 M. LAMBERT AND A. C. NEISH, *Can. J. Res.*, 23B (1950) 83.
8 C. J. FIELDING, *Biochim. Biophys. Acta*, 159 (1968) 94.
9 C. J. FIELDING, *Biochim. Biophys. Acta*, 178 (1969) 499.
10 E. A. NIKKILA, *Biochim. Biophys. Acta*, 27 (1958) 612.
11 B. SHORE AND V. SHORE, *Am. J. Physiol.*, 201 (1961) 915.
12 H. GRETEN, R. I. LEVY AND D. S. FREDRICKSON, *J. Lipid Res.*, 10 (1969) 326.
13 H. GRETEN, R. I. LEVY AND D. S. FREDRICKSON, *Biochim. Biophys. Acta*, 164 (1968) 185.
14 L. A. CARLSON AND L. B. WADSTROM, *Clin. Chim. Acta*, 2 (1957) 9.
15 C. J. FIELDING, *Biochim. Biophys. Acta*, 206 (1970) 118.
16 B. SHORE AND V. SHORE, *Plasma (Milan)*, 2 (1954) 621.
17 J. A. GLOMSET, *J. Lipid Res.*, 9 (1968) 155.
18 A. M. SCANU, *Biochim. Biophys. Acta*, 181 (1969) 268.
19 A. M. SCANU, H. POLLARD, R. HIRZ AND K. KOTHARY, *Proc. Natl. Acad. Sci. U.S.*, 62 (1969) 171.
20 D. M. G. DEARBORN AND D. B. WETLAUFER, *Proc. Natl. Acad. Sci. U.S.*, 62 (1969) 179.