STUDIES ON THE EFFECT OF GLYCOSAMINOGLYCANS ON THE RELEASE AND ACTIVITY OF PLASMA TRIGLYCERIDE LIPASES

Susan P. Williams

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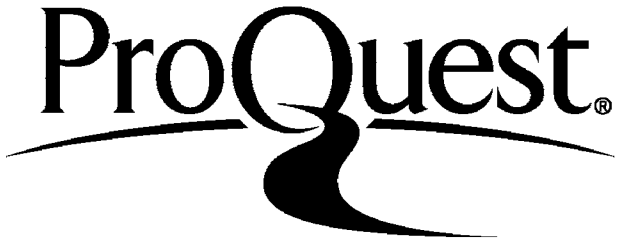
Registered at Bedford College

University of London



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ABSTRACT

The highly sulphated glycosaminoglycan (GAG) heparin is widely used clinically as an anticoagulant and antithrombotic. However, parenteral heparin, and also other anionic polysaccharides currently of interest as antithrombotics, release lipases from the capillary endothelium, resulting in clearing of circulating lipoproteins. These enzymes, lipoprotein lipase (LPL) and hepatic triglyceride lipase (HTGL) are released from the extra-hepatic and hepatic circulations respectively.

A number of recognised lipase assay systems were compared and shown to vary considerably in their effectiveness and selectivity in measuring LPL and HTGL in post-heparin plasma (PHP). In an effort to determine what differences in polysaccharide structure influence lipase release, heparin and other GAGs and sulphated polysaccharides have been compared for their ability to release lipase after intravenous injection into rats. Molecular weight and charge density (degree of sulphation) were shown to be important, as well as some unknown factor associated with the heparin-heparan family of GAGs. Heparin was able to release approximately 50% more LPL than the other polysaccharides tested, but was only moderately more effective at releasing HTGL. No major differences could be found in the time courses of lipase release and clearance for the different polysaccharides.

Purified LPL and HTGL were used to test the hypothesis that lipase release has a role in changing blood coagulability after injection of sulphated polysaccharides. HTGL was shown to have a significant effect on plasma anti-Xa clotting activities (by tests in vitro), to lengthen clotting times in the absence of heparin and

to shorten them in its presence. The change induced by HTGL in the absence of heparin could be related positively to the lipoprotein content of the test plasmas, which is consistent with the proposed action of HTGL via lipoproteins. LPL had only a minimal effect, increasing clotting times slightly in both the presence and absence of heparin, and this may be due to the presence of contaminatory antithrombin III.

CHAPTER 1

INTRODUCTION

The transport of lipids is of vital importance to the body, which relies largely on lipids for an energy source and as structural components for membranes and hormones. Due to their hydrophobic nature, only very small amounts of free lipids are present in the blood, but instead they exist in conjunction with specific proteins to form lipoproteins. Triglyceride-rich lipoproteins derived from the liver and intestine are acted upon in the peripheral circulation by the endothelial-bound enzyme lipoprotein lipase* to release free fatty acids and glycerol which can then be taken up by the surrounding tissue. Another triglyceride lipase, located on the hepatic endothelium, is thought to act further on the lipoproteins, and to also be involved in removing phospholipid and cholesterol. This enzyme is known as hepatic triglyceride lipase. When certain polyanions are introduced into the circulation, both these lipases are released into the blood stream.

1.1 THE PLASMA TRIGLYCERIDE LIPASES

1.1.1 Source of enzyme

Lipoprotein lipase (LPL) (E.C.3.1.1.34) is present on the capillary endothelium of many tissues and organs including the heart, lungs, adipose tissue, skeletal muscle (Krauss et al.,

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* In this thesis, LPL and HTGL will either be referred to specifically or as triglyceride lipase which will be used as a general term for both enzymes.

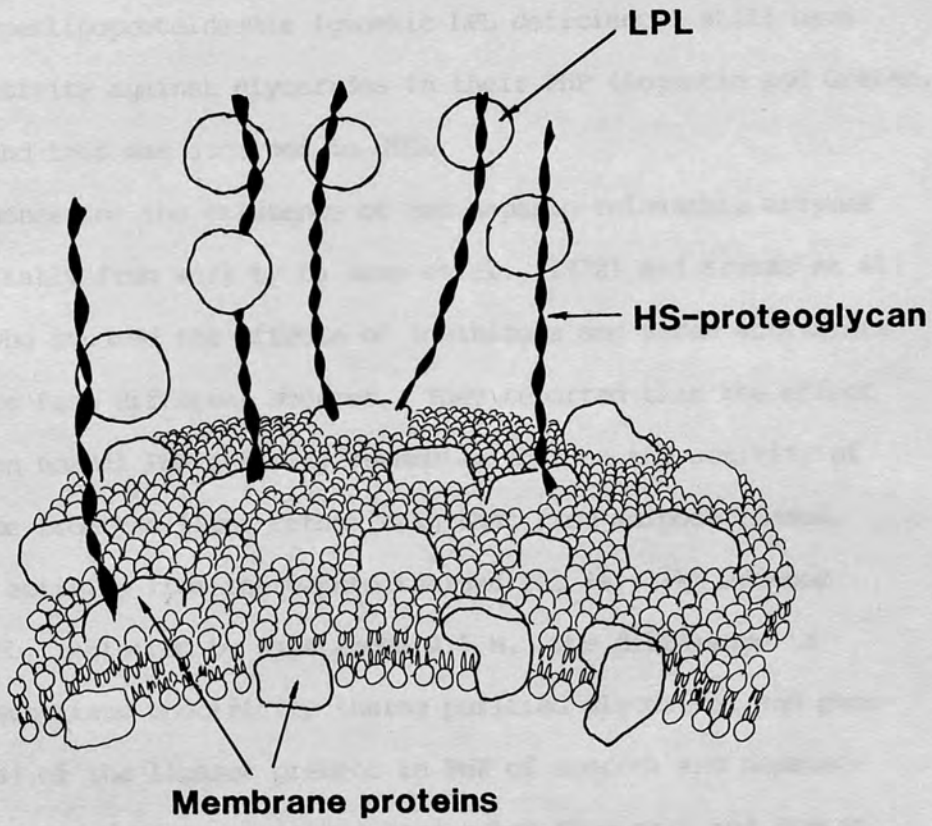
** This enzyme does not have a separate E.C. coding.

1973), kidney and spleen (Korn, 1955). The closely related enzyme hepatic triglyceride lipase (HTGL**) is similarly located on endothelial cells (Kuusi et al., 1979), but in the hepatic sinosoidal circulation (not on Kupffer cells). There have also been reports of HTGL-like activity in the adrenal glands and the ovaries (Cordle et al., 1983). For both cases, the enzyme is synthesized by the parenchymal cells of the various tissues and subjected to alteration there, possibly de-phosphorylation, before being transported by a vesicular system to the cell surface (Ghiselli and Catapano, 1979; Nilsson-Ehle et al., 1980). Evidence for this comes from comparing lipase activity of cultured endothelial and parenchyma cells, the activity being only produced and secreted by cells of the latter type (Wang-Iverson et al., 1980). It is thought that both enzymes are bound to the luminal surface via electrostatic interactions to glycosaminoglycans (GAGs). These anionic molecules are thought to be present at the endothelium as proteoglycans, where one or more GAG chains are bound to a protein core. The protein moiety may itself be a membrane protein (see Fig. 1) (Olivecrona et al., 1977) or be part of a discrete proteoglycan bound either covalently or ionically to the cell surface (Williams et al., 1983). The GAG heparan sulphate is found at the surface of endothelial cells (Olivecrona et al., 1977) and binding and other studies suggest that this is the molecule responsible for attachment of LPL (Williams et al., 1983; Shimada et al., 1981).

It was first noted by Hahn in 1943 that injection of heparin led to the release of a 'factor' which cleared lipaemic plasma (in vivo and in vitro). Other workers established this factor as an enzyme which acts in vivo and in vitro against triglycerides. Much of the early work was done using heart and adipose tissue extracts

Fig 1 Proposed model for the binding of LPL at the endothelium

(From Olivecrona et al., 1977)



(Korn, 1955; Korn and Quigley, 1957), post-heparin plasma (Korn and Quigley, 1957) and milk which is also a good source of a possibly identical enzyme (Olivecrona et al., 1982; Hernall et al., 1975). It was not at the time realized that at least two enzymes are present in post-heparin plasma (PHP), so varying results were obtained (La Rosa et al., 1972; Augustin and Greten, 1979a) in testing the effect of certain inhibitors such as sodium chloride and protamine (which inhibit LPL but not HTGL). It was also observed that patients with type 1 hyperlipoproteinaemia (genetic LPL deficiency) still have lipase activity against glycerides in their PHP (Augustin and Greten, 1979a), and this was ascribed to HTGL.

Evidence for the existence of two heparin-releasable enzymes came initially from work by La Rosa et al. (1972) and Krauss et al. (1973), who studied the effects of inhibitors and serum activators on lipases from different sources. They reported that the effect of salt on normal PHP activity resembles that on the activity of the enzyme from the liver rather than that from adipose tissue. Also the activity from the hepatectomized rat is, like adipose tissue LPL, inhibited by salt above 0.5 M. The difference in overall substrate specificity (using purified glycerides and phospholipids) of the lipases present in PHP of control and hepatectomized rats had also previously been noted (Augustin and Greten, 1979a). Also, heparin-Sepharose chromatography of the latter PHP gave only one peak on elution, whereas the PHP from the control animal gave two peaks, corresponding to the enzymes of hepatic and extra-hepatic origins. The enzymes have different antigenic determinants to which specific antisera can be raised (Augustin and Greten, 1979a; Huttenen et al., 1975).

1.1.2 Molecular properties

The active form of LPL is believed to be a dimer (Iverius and Östlund-Lindqvist, 1976) and that of HTGL to be a monomer (Ehnholm et al., 1974) and possibly also a tetramer (Twu et al., 1984). Published values for the molecular weight (MW*) of the monomer LPL from different sources have ranged between 34,000-73,000 daltons (Smith et al., 1978; Augustin and Greten, 1979b), although most have been within 50,000-60,000. Among the latest figures available for the monomer of bovine milk LPL is 42,000 determined by gel filtration in 6 M guandinium chloride and sedimentation equilibrium (Olivecrona et al., 1982). Results from SDS-PAGE (sodium dodecyl sulphate-polyacrylamide gel electrophoresis) give 55,000-63,000 for milk LPL (Olivecrona et al., 1982) and 56,000 for the rat adipose tissue enzyme (Parkin et al., 1982). LPL is a glycoprotein, and it is thought that the high carbohydrate content affects its electrophoretic mobility, which would explain why generally higher figures are obtained by this means than by other methods (Nilsson-Ehle et al., 1980). The active enzyme is believed to be a dimer (Iverius and Östlund-Lindqvist, 1976).

It has been suggested (Fielding et al., 1977) that there are two LPL enzymes in PHP: one from adipose tissue which has a monomer MW of 69,000 and low affinity for triglyceride (TG) and a separate enzyme from heart tissue with a lower MW of 37,500, but with high TG affinity. This has not been verified by other workers.

The MW of HTGL purified from human PHP has been determined by

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* MW in this text corresponds to the relative molecular mass (M_r) of the monomers which combine to form the active enzyme dimer.

SDS-PAGE and gel filtration as 69,000 (~~Ehnholm et al., 1975~~ Augustin + Greten, 1979a). The value for the enzyme from other species appears to be lower (Frost et al., 1982; Twu et al., 1984), the latest figure obtained by SDS-PAGE analyses of the enzyme purified from rat liver homogenate being 53,000 (Twu et al., 1984).

1.1.3 Amino acid analysis

Early work suggested that the amino acid composition of LPL, and HTGL are very similar, if not identical, and led to the suggestion that the enzymes could be interconverted by cleavage of short peptide sequences or by glycosylation (Augustin and Greten, 1979a). However, these results were criticized by Östlund-Lindqvist (1979), who attributed the observed similarities to heavy contamination of both preparations with antithrombin III (At III). At III is a glycoprotein of similar MW to LPL and HTGL, which also binds strongly to the heparin-Sepharose columns used in purification of the enzymes from PHP. Indeed, it was found that removal of this contaminant using a second heparin-Sepharose column modified so as to have low affinity for At III, resulted in significant differences being found in the amino acid composition of the two enzymes (Östlund-Lindqvist, 1979). In particular, LPL was shown to contain proportionally more threonine, methionine, isoleucine and leucine, but less tyrosine. Minor differences between species (bovine and human) were also noted.

Both enzyme monomers consist of a single polypeptide chain, with identical glycine N-terminals and serine C-terminals (Augustin et al., 1978). Amino acid analysis showed that each chain contains an equal proportion of hydrophilic and hydrophobic amino acid residues (Augustin et al., 1978). Evidence suggests that the polar

residues serine and histidine are present in the active site of both enzymes (Twu et al., 1984; Vainio et al., 1982).

1.1.4 Carbohydrate analysis

It has been suggested (Augustin et al., 1976) that there exists microheterogeneity of the enzymes (especially LPL) via the carbohydrate chain, and this may explain in part the wide variation in results obtained by different workers for the amount of carbohydrate present. For example the carbohydrate value for LPL varies from 3.3% for the enzyme from rat heart (Chung and Scanu, 1977) to 18% for the human PHP enzyme (Augustin and Greten, 1979b). The figures generally quoted are given in Table 1.

The absence of fucose is surprising since it is usually a component of circulating plasma glycoproteins (Augustin et al., 1976). It has been suggested that heparin is part of the enzyme structure but since neither iduronic acid nor glucuronic acid can be detected, this is not likely (Augustin et al., 1976). It has been suggested that the greater carbohydrate content of HTGL stabilizes its tertiary structure (Augustin et al., 1976), since it is detectable in the circulation for longer than is LPL after they are both released by heparin.

1.1.5 Lipid analysis

Although early reports indicated small amounts (1.8% w/w) of phospholipid present in LPL, (Fielding et al., 1974), no lipid is detectable in the more highly purified LPL or HTGL (Augustin et al., 1976).

1.2 LIPOPROTEIN METABOLISM

All the major plasma lipids are insoluble in water, so exist as lipoproteins - 'macromolecular complexes of lipids bound to a

TABLE 1: Carbohydrate analysis of LPL and HTGL

	Carbohydrate Content (w/w)					
	Total	Amino sugars		Neutral sugars		
		Glucosamine	Galactosamine	Mannose	Galactose	Sialic acid
LPL*	6.9%	1.9%	0	3.2%	0	1.8%
HTGL**	8.0%	3.0%	traces	2.0%	2.0%	1.0%

* Fielding et al., 1974

** Augustin et al., 1978

variety of polypeptides' (Owen and McIntyre, 1982) - to allow transportation to cells and tissues. All lipoproteins are basically composed of a hydrophobic core containing neutral lipids (mainly triglycerides and cholesterol esters), enclosed in a monomolecular surface film of apoproteins and polar lipid (phospholipid and cholesterol) (Nilsson-Ehle et al., 1980). They exist in the circulation as distinct, spherical particles with diameters ranging from 4-600 nm, depending on the lipoprotein class; the classes differ in lipid and apoprotein content, physical properties and function.

1.2.1 Classification of lipoproteins

There are four major classes of lipoprotein defined by the density range at which they float after density gradient ultracentrifugation, dependent on the proportions of lipid and protein (although there is heterogeneity within each group). The lipoprotein groups can also be separated by electrophoresis, gel filtration and affinity chromatography (using differences in the major protein content). The four groups in order of decreasing particle size are chylomicrons (CMs), very low density lipoproteins (VLDL), low density lipoproteins (LDL) and high density lipoproteins (HDL). A summary of their composition and physical characteristics is given in Table 2.

1.2.2 Apoproteins

There are eight major polypeptides/proteins present in lipoproteins of the different classes (see Table 3) which, as well as playing a structural role, may act as enzyme co-factors in lipoprotein metabolism. They also interact with liver receptors with consequent removal from the circulation (Owen and McIntyre, 1982). They are present in varying amounts in the different classes, bound

TABLE 2: Composition and physical characteristics of normal human plasma lipoproteins (Ghiselli and Catapano, 1979; Lewis, 1977; Morrisett et al., 1975)

% Composition	CMs	VLDL	LDL	HDL	
Protein	0.5-2.0	3- 8	17-22	40-55	
Cholesterol	1- 2	7-10	10-15	10-12	
Cholesterol esters	1- 2	8-14	25-40	15-25	
Phospholipids	8-10	16-20	18-24	18-22	
Triglycerides	85-90	50-65	8-14	3-6	
Diameter (nm)	80-600	25-80	18-25	7-10	4-7
Density range in preparative ultra-centrifuge	< 0.950	0.950-1.006	1.006-1.063	1.063-1.120	1.120-1.210
Electrophoretic mobility in agarose*	(no mobility)	pre-β	β	α	

* Lipoproteins were previously known by these terms.

† The two major HDL subclasses. HDL_3 has a higher % of protein and less phospholipid, cholesterol, cholesterol esters and triglycerides (Morrisett et al., 1975). There is also a minor sub-group, HDL_1, within the HDL_2 density range, which is rich in cholesterol esters (Owen and McIntyre, 1982).

TABLE 3: The apoproteins

Apoprotein	Major component	Minor component
A-I	HDL	CM
A-II	HDL	CM
B	LDL, VLDL, CM	
C-I	VLDL, CM	HDL
C-II	VLDL, CM	HDL
C-III	VLDL, CM	HDL
D		HDL, VLDL
E		CM, VLDL, HDL

(Owen and McIntyre, 1982)

non-covalently to the lipid which allows their exchange and transfer during metabolism (Owen and McIntyre, 1982).

1.2.3 Origin of lipoproteins

The liver and intestinal mucosa are the sites of synthesis of VLDL and HDL. VLDL carries endogenous triglycerides into the circulation, along with dietary and newly-synthesized cholesterol (Smith et al., 1978; Lewis, 1977). HDL transports most of the cholesterol (HDL is thought to be important here (Owen and McIntyre, 1982)). The mucosal cells of the small intestine also produce CMs, and LDL is formed from enzymic degradation of VLDL as shown in Figure 2.

Lecithin cholesterol acyltransferase (LCAT) is another important enzyme in lipoprotein metabolism. It is present on the HDL surface where it catalyses the following reaction in HDL:-

lecithin + cholesterol → cholesteryl ester + lysolecithin

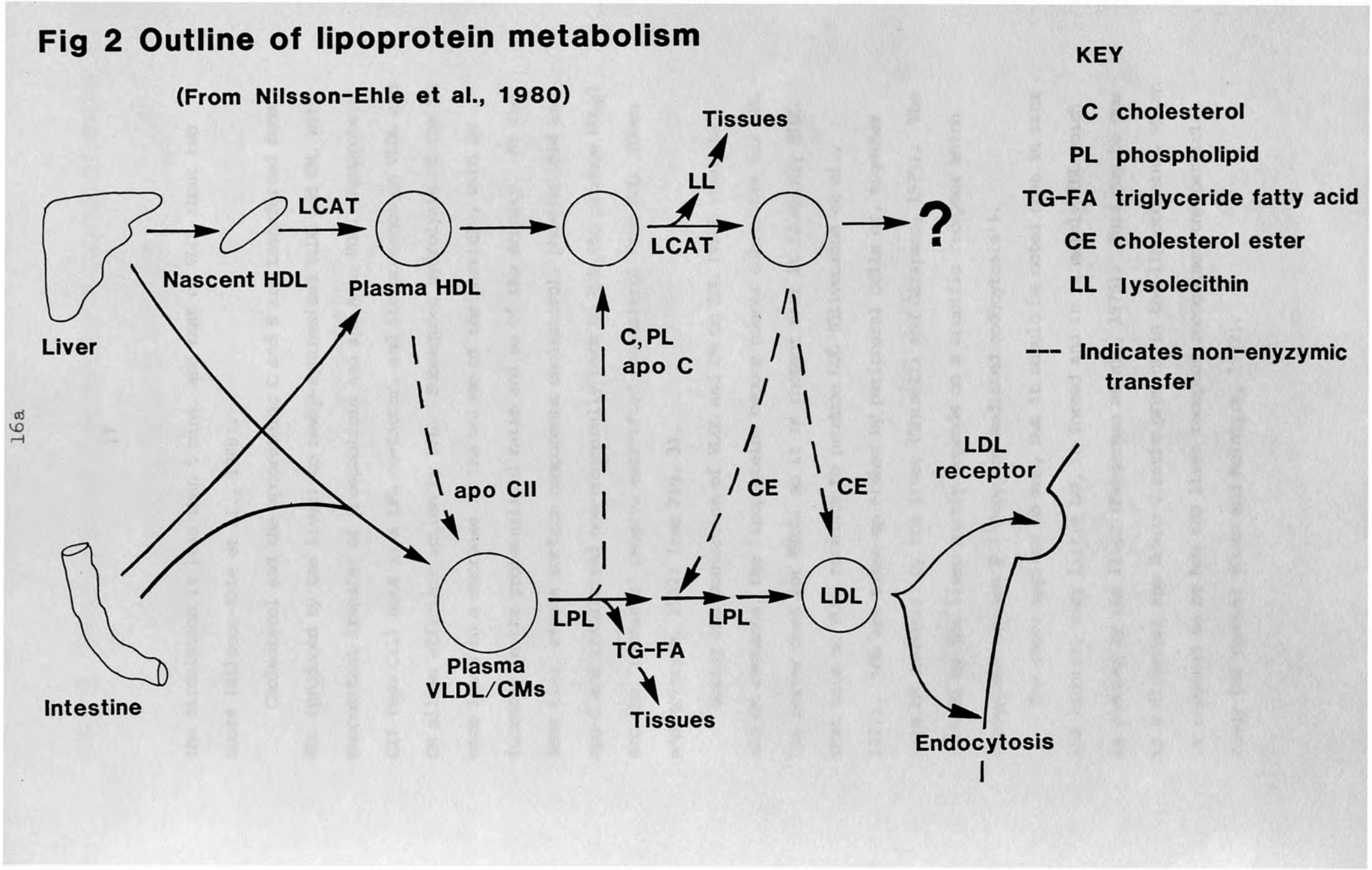
Fatty acids are transferred from lecithin to the hydroxy group of cholesterol (Nilsson-Ehle et al., 1980). Cholesterol is passed to HDL from VLDL and CMs, esterified by LCAT in HDL, then either re-transferred back to CM- or to ~~and~~ VLDL-remnants via the cholesterol ester transfer protein (CETP) (Owen and McIntyre, 1982). LPL has been reported to enhance the CETP-mediated transfer of cholesterol ester from HDL to VLDL during its action against TG of VLDL in vitro (Tall et al., 1984). The physiological significance of this finding is not known.

1.2.4 Lipoprotein catabolism

CM and VLDL carry TG from the liver and intestine into the circulation, and in man about 70-150 g TG would be given up to the tissues in a 24-hour period (Nilsson-Ehle et al., 1980). CM and VLDL TG are acted upon very quickly by LPL; the half-life of CM in

Fig 2 Outline of lipoprotein metabolism

(From Nilsson-Ehle et al., 1980)



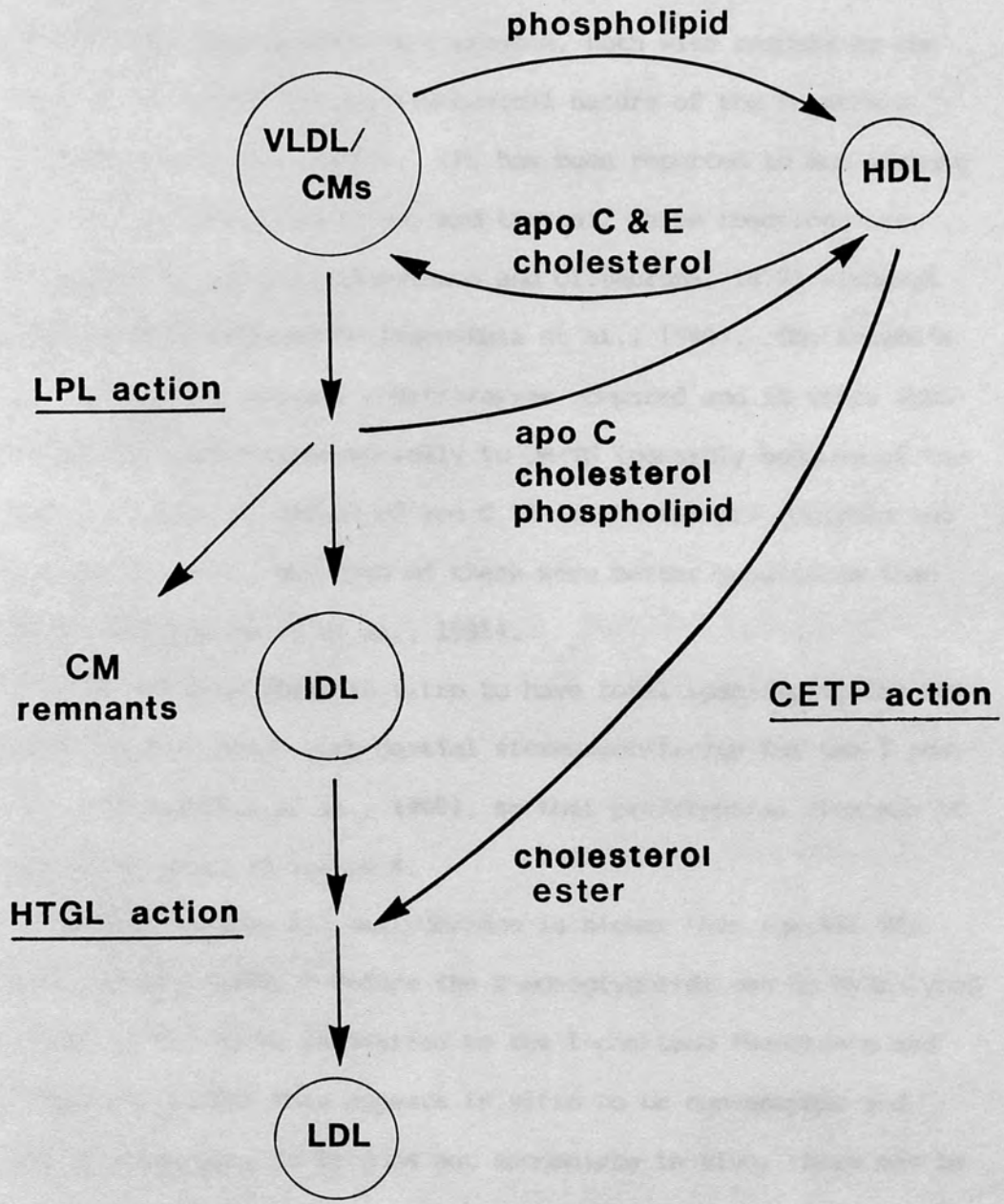
the circulation is less than 5 mins. and that of VLDL about two hours (Nilsson-Ehle et al., 1980).

Cholesterol and the apoproteins C and E are transferred from HDL (produced by the liver) to newly-synthesised VLDL and CM, with concomitant transfer of phospholipid and apo A to HDL. Apoprotein CII (apo CII) acts as a LPL co-factor, and its presence on VLDL and CM allows efficient action by LPL. Subsequent hydrolysis of the TG core leads to a decrease in the volume of the particle, with an increase in the protein:lipid ratio and so of the density. At the same time, excess surface components cholesterol, phospholipid and apo C are transferred non-enzymically back to HDL_3(to produce HDL_2) and the 'remnants' receive esterified cholesterol from HDL (Owen and McIntyre, 1982) (see Fig. 3).

During the conversion of VLDL and CM to IDL (VLDL remnants) and CM remnants, the lipoproteins become poorer substrates for LPL but better ones for HTGL, so it is thought that it is mainly HTGL that acts on VLDL remnants to produce LDL (Olivecrona et al., 1977). LDL are taken up either by peripheral cells or, together with CM remnants, by the liver (Ghiselli and Catapano, 1979). The removal by the liver partly depends on a specific receptor which recognises the apo E ('receptor mediated endocytosis').

The above applies to man, but it should be noted that in rats and rabbits, very little LDL is formed and it is mainly IDL which is removed by the liver (Faegerman et al., 1975). This may be due to a different apo E/apo C ratio present in the lipoproteins, which is reported to be how the liver receptor recognises the particles ready for removal (Owen and McIntyre, 1982).

Fig 3 Transfer of lipoprotein components during lipase action



1.2.5 Lipase specificity

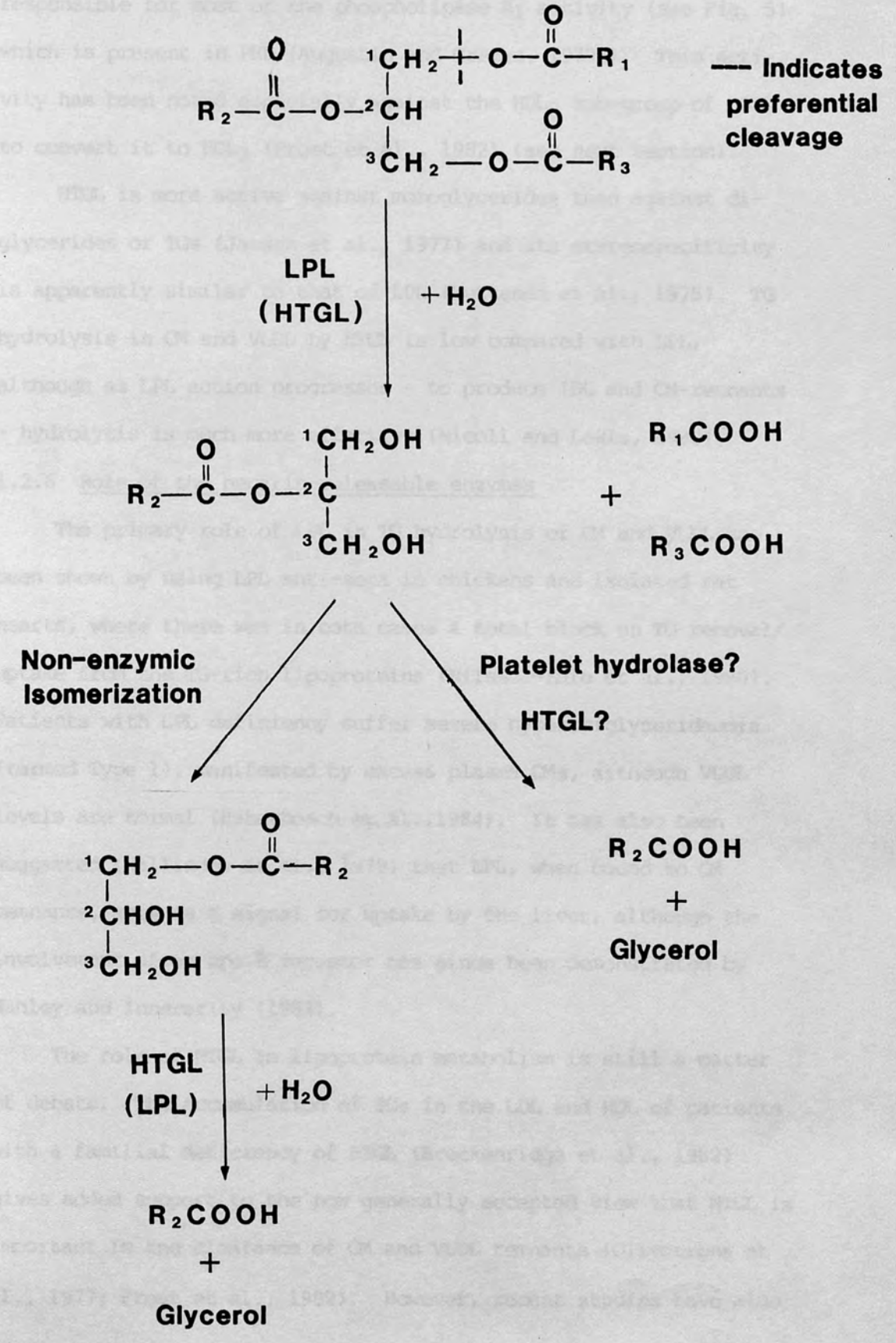
Both LPL and HTGL can hydrolyse acylglycerides, phospholipids and fatty acid Co.A esters (Olivecrona et al., 1977), but differ in their Kms towards the various substrates. LPL has a low substrate specificity compared with most enzymes, both with respect to the chemical substrate and physicochemical nature of the substrate (Nilsson-Ehle et al., 1980). LPL has been reported to act against tri-, di- and monoglycerides, and that all three reactions are stimulated by apo C II (Bengtsson and Olivecrona, 1979) although this has been disputed (Nilsson-Ehle et al., 1980). The enzyme's activity against natural substrates was compared and in vitro VLDL-TG was hydrolyzed preferentially to CM-TG (possibly because of the relatively greater amount of apo C II in the former) (Dolphin and Rubinstein, 1974), but both of these were better substrates than LDL or HDL (Eisenberg et al., 1981).

LPL has been shown in vitro to have total specificity for the 1 (or 3) ester bonds with partial stereospecificity for the 1 position (Nilsson-Ehle et al., 1980), so that preferential cleavage of a TG is as shown in Figure 4.

Action against 2,3-diglycerides is slower than against TGs (Smith et al., 1978). Before the 2-monoglyceride can be hydrolysed by LPL, it has to be isomerized to the 1-position (Bengtsson and Olivecrona, 1980a) This appears in vitro to be non-enzymic and rate-limiting but, as it does not accumulate in vivo, there may be an alternative mechanism for 2-MG hydrolysis - a platelet monoglyceride hydrolase has been implicated here, as has HTGL (Nilsson-Ehle et al., 1980).

HTGL has high phospholipase activity and is thought to be

Fig 4 Hydrolysis of triglycerides by LPL and HTGL



responsible for most of the phospholipase A_1 activity (see Fig. 5) which is present in PHP (Augustin and Greten, 1979a). This activity has been noted especially against the HDL_2 sub-group of HDL, to convert it to HDL_3 (Frost et al., 1982) (see next section).

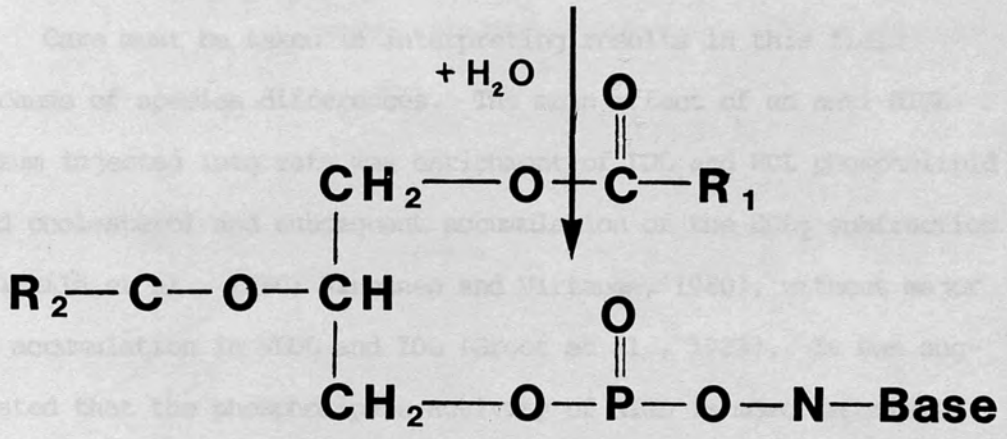
HTGL is more active against monoglycerides than against diglycerides or TGs (Jansen et al., 1977) and its stereospecificity is apparently similar to that of LPL (Huttenen et al., 1975). TG hydrolysis in CM and VLDL by HTGL is low compared with LPL, although as LPL action progresses - to produce IDL and CM-remnants - hydrolysis is much more effective (Nicoll and Lewis, 1980).

1.2.6 Role of the heparin-releasable enzymes

The primary role of LPL in TG hydrolysis of CM and VLDL has been shown by using LPL anti-sera in chickens and isolated rat hearts, where there was in both cases a total block on TG removal/uptake from the TG-rich lipoproteins (Nilsson-Ehle et al., 1980). Patients with LPL deficiency suffer severe hypertriglyceridaemia (termed Type 1), manifested by excess plasma CMs, although VLDL levels are normal (Haberbosch et al.,1984). It has also been suggested (Wallinder et al., 1979) that LPL, when bound to CM remnants, acts as a signal for uptake by the liver, although the involvement of an apo E receptor has since been demonstrated by Mahley and Innerarity (1983).

The role of HTGL in lipoprotein metabolism is still a matter of debate. The accumulation of TGs in the LDL and HDL of patients with a familial deficiency of HTGL (Breckenridge et al., 1982) gives added support to the now generally accepted view that HTGL is important in the clearance of CM and VLDL remnants (Olivecrona et al., 1977; Frost et al., 1982). However, recent studies have also

Fig 5 Phospholipase A_1 action

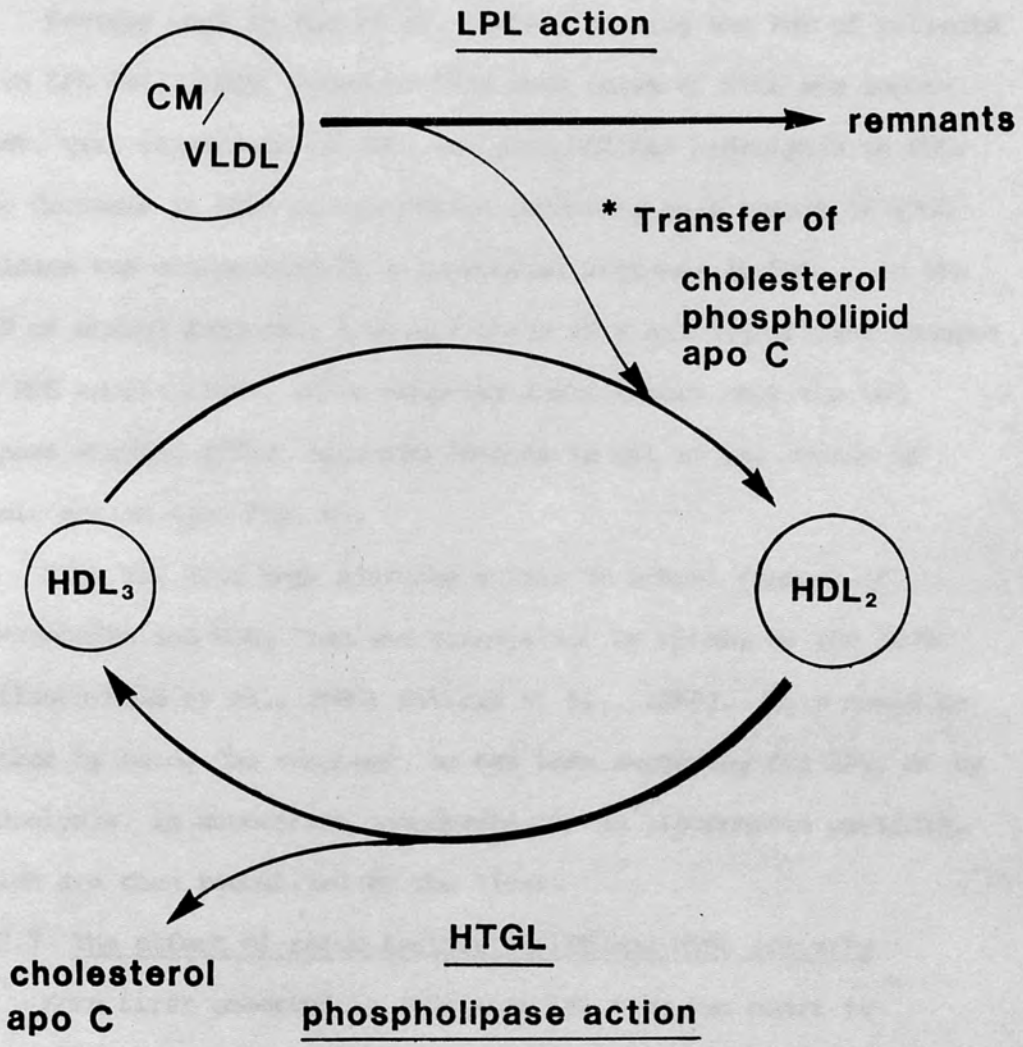


suggested a role in hydrolysis of phospholipids in LDL and HDL_2. This is followed in the latter by non-enzymic removal of cholesterol (to produce HDL_3) which is taken up by hepatocytes (Nilsson-Ehle et al., 1980) (see Fig. 6). Bengtsson and Olivecrona (1980b) have shown that HDL does bind strongly to HTGL and that this markedly inhibits the enzyme's action against TG emulsions.

Care must be taken in interpreting results in this field because of species differences. The main effect of an anti-HTGL serum injected into rats was enrichment of LDL and HDL phospholipid and cholesterol and subsequent accumulation of the HDL_2 subfraction (Nikkilä et al., 1980; Kinnunen and Virtanen, 1980), without major TG accumulation in VLDL and IDL (Groot et al., 1983). It was suggested that the phospholipase activity of HTGL is more important than its action against TGs (Groot et al., 1983). However, it has been disputed that this is true in man too (Goldberg et al., 1983) since rats (and rabbits) are unusual in that little of their VLDL is metabolized in the circulation further than IDL, so only a small amount of LDL is formed.

Goldberg and coworkers (1983) chose the cynomolgus monkey as a better human model (though there are doubts to its suitability (Jansen, 1984)), and showed that inhibition of HTGL by anti-serum in this species induced significant accumulation of an abnormally large, less dense form LDL sub-group (sometimes referred to as β-VLDL) and of IDL, as well as an increase in the HDL_2 concentration. They suggested the following: HTGL acts on TG-rich lipoproteins which are low in apo C II and following transfer of surface components, these lipoproteins are taken up directly by the liver. In other tissues, however, where LPL also degrades TG-rich lipo-

Fig 6 Involvement of HTGL and LPL in HDL metabolism



*=non-enzymic process

proteins, the surface components are transferred instead to HDL. This would mean that, on HTGL inhibition, proportionally more VLDL is hydrolysed by LPL, which would result in an increase in HDL_2, without it being due to the HTGL conversion of HDL_2 to HDL_3.

Further work by Rao et al. (1982), testing the PHP of patients with LPL deficiency, suggests that both roles of HTGL are important, viz. catabolism of VLDL and phospholipid hydrolysis in HDL. The decrease in HDL_2 concentration occurring as a result of HTGL release was accompanied by a reciprocal increase in HDL_3. In the PHP of normal subjects, however, there were not significant changes in HDL subfractions, which supports their theory that the two lipase enzymes effect opposite changes in HDL in the course of their action (see Fig. 6).

HTGL has also been assigned a role in actual removal of CM-remnants and HDL_2 from the circulation by uptake by the liver (Nilsson-Ehle et al., 1980; Nikkilä et al., 1980). This could be either by being the receptor, as has been suggested for LPL, or by hydrolysis, in uncovering components on the lipoprotein particle, which are then recognized by the liver.

1.2.7 The effect of serum factors on LPL and HTGL activity

Korn first observed in 1955 that LPL from rat heart is activated by serum and this has been found to be true for the enzyme from all major sources (Havel et al., 1973). This was shown in vitro to be due to apo C II (La Rosa et al., 1972), also called apo LP-glu to indicate its C-terminal amino acid. Hypertriglyceridaemia in humans due to its deficiency has been described (Haberbosch et al., 1984). The enzyme is reported to be inhibited by high apo C II concentrations (Haberbosch et al., 1984),

Early work on the other C-apoproteins suggested that they also stimulated LPL (La Rosa et al., 1972), but this was due probably to contamination by apo C II. Both apo CI (apo LP-ser) and apo C III (apo LP-Ala) inhibit LPL increasingly with concentration (Chung and Scanu, 1977; Owen and McIntyre, 1982), but the degree of response depends on the assay conditions used (Östlund-Lindqvist, 1979). This effect may be just an artefact of the in vitro system without any physiological significance (Nilsson-Ehle et al., 1980). The other apoproteins are generally reported not to affect the LPL reaction (Smith et al., 1978; Owen and McIntyre, 1982). However, it has been recently shown (Mackinnon and Cryer, 1984) that the rate of TG-VLDL hydrolysis by LPL may be affected by the apo E content of the lipoprotein.

There have been many reports on the inhibitory effect of serum on HTGL activity from many different species (~~Iverius and Östlund-Lindqvist, 1976;~~ Angervall and Hood, 1957 Huttenen et al., 1975; Feliste et al., 1982; Cordle et al., 1982) both as the purified enzyme and as PHP. HDL was found to be responsible for this inhibition in vitro (La Rosa et al., 1972; Bengtsson and Olivecrona, 1980b) due to the action of both apo A I and A II (Haberbosch et al., 1984) (the major components of HDLs). Recent work on the action of C-apoproteins on HTGL from rat liver showed no effect by C I and C II, but that apo C III produced a 90% inhibition of TG-hydrolysis, whilst having no effect on its action against monoglyceride or phospholipid (Twu et al., 1984). This suggests that C III interacts with the substrate, rather than by a direct effect on the enzyme.

Other workers, however, have found a threefold activation of HTGL by low serum concentrations (optimal at concentrations of 10%

(v/v) in the reaction mixture), with inhibition only occurring at high serum levels (Jahn et al., 1983). They further attributed this activation to the apo A II component of HDL and inhibition to the C-apoproteins. It was suggested that the wide variation in the effects found by different workers is due to the specific assay conditions used (Nilsson-Ehle et al., 1980).

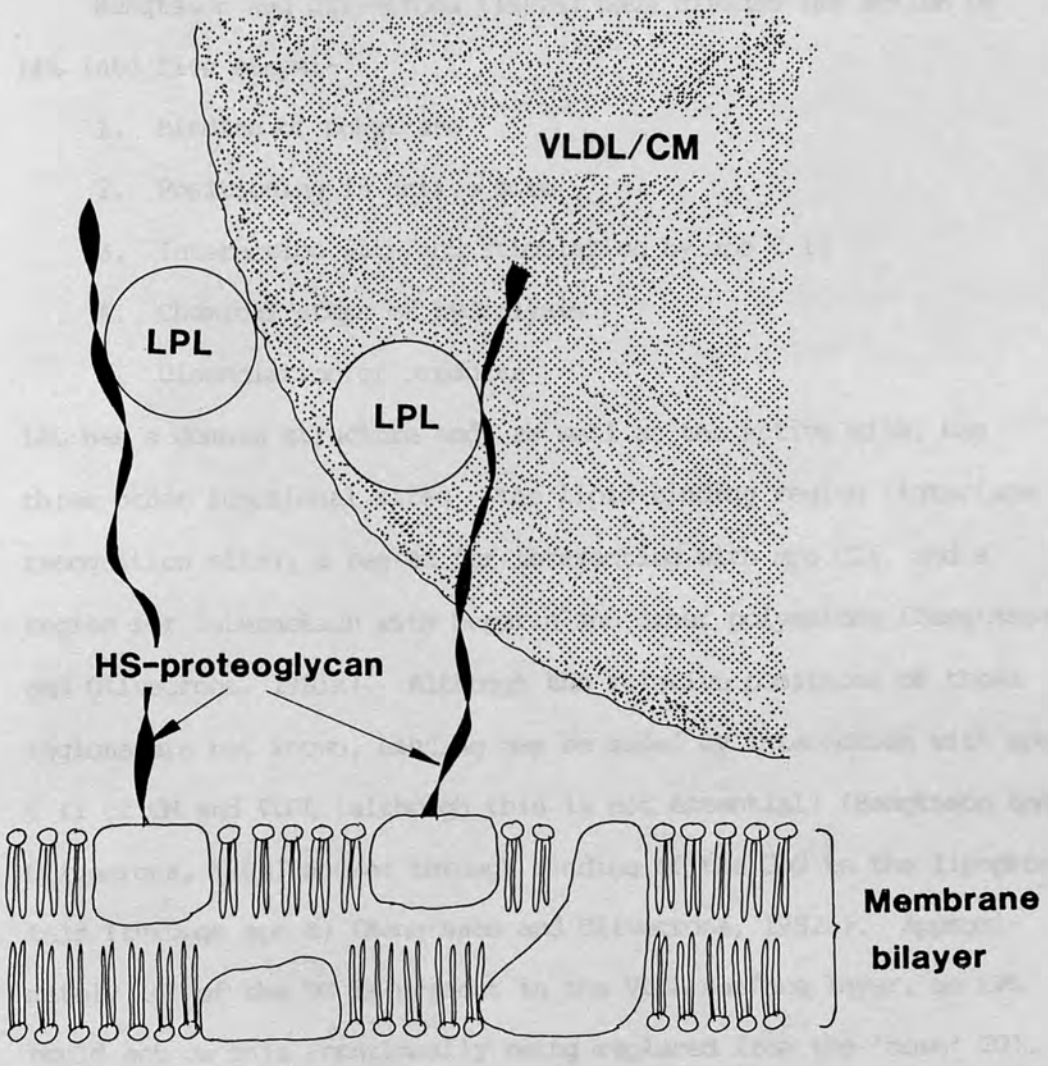
1.3 ENZYMIC ACTIVITY - Mode of action in vivo

1.3.1 LPL

The conversion of CM and VLDL to remnants and IDL is accomplished by LPL in its position at the capillary endothelium. As mentioned earlier, it is bound to the membrane via heparan sulphate proteoglycans which extend 20-50 nm from the surface and so would allow interaction of the lipoprotein substrates with the enzyme molecule, as shown in Figure 7 (Olivecrona et al., 1977). This is supported by electron microscope studies (Nilsson-Ehle et al., 1980).

Certain conclusions have been reached on the mode of action of LPL against CM and VLDL, based on clearance times and turnover numbers of the enzyme against these substrates. A 'typical' CM of diameter 150 nm would contain approximately 3×10^6 TG molecules, so 6×10^6 hydrolytic events would have to occur within five minutes (its normal clearance time) (Olivecrona et al., 1977). Working on a turnover number of 650 molecules second $^{-1}$ (40,000 minute^{-1}) approximately 30 LPL molecules would be needed to act simultaneously to accomplish this. According to the fluid mosaic model of membrane structure, LPL molecules anchored by a membrane protein would be able to move to bind the substrate. The VLDL particle, however, has approximately 15,000 TG molecules which could be hydrolysed by a single LPL molecule in one minute (Olivecrona,

Fig 7 Proposed model allowing the action of endothelial-bound LPL molecules against lipoproteins



(From Olivecrona et al., 1977)

1983), whereas they are present in the circulation for at least 2 hours. This suggests that VLDL is bound and released during the course of degradation at different sites in the circulation (Nilsson-Ehle et al., 1980).

Bengtsson and Olivecrona (1982a) have divided the action of LPL into five steps:-

1. Binding of substrate
2. Positioning in active site
3. Interaction with and stimulation by apo C II
4. Chemical steps of hydrolysis
5. Dissociation of products

LPL has a domain structure and, as well as the active site, has three other functional sites - the lipid-binding region (interface recognition site), a region for interacting with apo CII, and a region for interaction with heparin and other polyanions (Bengtsson and Olivecrona, 1981a). Although the relative positions of these regions are not known, binding may be aided by interaction with apo C II of CM and VLDL (although this is not essential) (Bengtsson and Olivecrona, 1983) and/or through binding of the GAG to the lipoprotein (through apo B) (Bengtsson and Olivecrona, 1982a). Approximately 10% of the TG is present in the VLDL surface layer, so LPL could act on this (continually being replaced from the 'core' TG). It has also been suggested that LPL may use its phospholipase activity to 'eat its way' through the membrane to reach the TG core (Nilsson-Ehle et al., 1980).

The amino acid sequence of apo C II has been worked out and, using fragments and synthetic sequences, the relative efficiency of varying lengths of the activator has been investigated (Smith et

al., 1978). Initially it was thought that the lipid-binding region (N-terminal residues 1-49) mediated the activation effect, but full activation may be achieved by residues 55-78 (Bengtsson and Olivecrona, 1980c). Residues 55-78 have been reported to contain a ß-pleated sheet structure which for activation interacts with a ß-pleated sheet section of LPL and the importance of an arginine-49 residue, which in the native molecule lies adjacent to this area, has recently been implicated (Holdsworth et al., 1984).

It has now been claimed that as good binding of substrate can (under optimal conditions) be achieved in the absence as in the presence of apo C II (Bengtsson and Olivecrona, 1983; Haberbosch et al., 1984) and that under some conditions there may even be competitive binding for CM and VLDL sites between LPL and apo C II (Haberbosch et al., 1984). One theory of apo C II action is that, by interacting with the lipid, it aids either the actual entry of the substrate molecule into the active site or the removal of products (Bengtsson and Olivecrona, 1980c). This is unlikely since activation is seen with many different substrates and so is unspecific. It is generally accepted that apo C II does not modify the stereospecificity of the enzyme (Ghiselli and Catapano, 1979; Vainio et al., 1982). A current view is that apo C II binds preferentially to a more catalytically effective conformation of the enzyme, so pushing the equilibrium in its favour (Bengtsson and Olivecrona, 1980c).

$$E \rightleftharpoons E^*$$

less effective — more effective (removed by apo C II binding)

Since LPL assay conditions can be adjusted to give very high hydrolysis rates in the absence of apo C II (Bengtsson and Olivecrona, 1980c), apo C II is not a true cofactor by the definition of being 'directly involved in catalysis'. By the above theory it acts more like a positive effector on an allosteric enzyme (Olivecrona and Bengtsson, 1980). Some workers have suggested that apo C II actually effects a conformational change in the enzyme which aids binding (Shirai et al., 1982). Kinetic studies show that a 1:1 molecular complex of apo C II and LPL forms during activation, which supports either of the last two theories.

The detailed mechanism of LPL action is as yet unresolved, although, in common with other enzymes with histidine and serine at the active site, it is likely that a tetrahedral intermediate is formed (Vainio et al., 1982).

The dissociation of products from the active site is often the rate-limiting step of enzyme action, and apo C II has also been implicated, since it has been shown to aid displacement of benzene boronic acid, an inhibitor of LPL, possibly by esterase-type activity which has been observed in apo C II (Vainio et al., 1982). Once the products of hydrolysis are removed from the active site, some FFA goes into the circulation, but most will be taken up by surrounding tissue cells, where it is either oxidized or re-esterified for storage (Nilsson-Ehle et al., 1980). In vitro studies (Nilsson-Ehle et al., 1980) have shown that lipolytic products (FFA and glycerol) would be able to move via lateral diffusion in the cell membrane and so get across the capillary endothelium.

The mechanism for removal of TGs from the plasma is not saturated under normal conditions, so that when TG plasma concentra-

tions (in CM and VLDL) increase after a meal, this can be easily dealt with by the LPL (Nilsson-Ehle et al., 1980). Fielding et al. (1977) thought that LPL enzymes differ according to their tissue of origin in their affinity for triglyceride. The heart enzyme was reported to have a higher affinity for TG than the enzyme from adipose tissue, to ensure that it was saturated even when plasma TG levels are low. However, the validity of this finding seems doubtful as it could not be reproduced by other workers (Olivecrona, personal communication).

1.3.2 HTGL

HTGL is generally assumed to have an extra-cellular location in the liver, similar to that of LPL in other tissues (Nilsson-Ehle et al., 1980). Most of the general aspects of action described for LPL will probably apply also to HTGL, but different lipoprotein classes are involved and there is no activating effect by apo C II. As mentioned earlier, there have been suggestions that apo A II present on HDL is an activator for HTGL (Jahn et al., 1983).

Kinnunen and Virtunen (1980) have proposed that HTGL works, at least against some of its substrates (possibly LDL and HDL_2) by receptor-mediated endocytosis via coated pits. In this model, endothelial-bound HTGL enzymes(s) bind the substrate, the cell membrane invaginates and both are internalized to form coated vesicles. The enzyme could then act on the lipoprotein in the cell, with or without the aid of lysosomes, and the modified lipoprotein re-released into the circulation.

1.3.3 Hormonal regulation of lipolytic activity

As well as the passive regulation of enzymic activity described earlier, both enzymes are under hormonal control. There are

significant, longer term effects on activity brought about by the nutritional state - for example, LPL activity in adipose tissue is low in fasting but high in fed animals, whereas the reverse is true for the enzyme in heart and skeletal muscle (Nilsson-Ehle et al., 1980). These changes are thought to be controlled by hormones such as adrenaline, insulin, ACTH* and prolactin in the case of LPL (Nilsson-Ehle et al., 1980), by oestrogens in the case of HTGL (Augustin and Greten, 1979a). HTGL and LPL are both made in cells different from those in which they are 'displayed' and it seems that as well as regulation at the transcription level (via rate of mRNA formation), there is control on transport from parenchymal cells and uptake by the endothelial cells (Schoonderwoerd et al., 1983). Peptide hormones such as ACTH, for example appear to decrease both the amount of enzyme secreted from the synthesising cells and the amount bound by the non-parenchymal cells, by decreasing the number of enzyme-binding sites on the membrane (Schoonderwoerd et al., 1983).

1.4 RELEASE OF TRIGLYCERIDE LIPASES BY HEPARIN AND OTHER GLYCOSAMINOGLYCANS

The release of LPL and HTGL into the circulation on injection of heparin is now a well-established phenomenon. However, under normal conditions some chondroitin sulphate circulates, but there is little or no heparin in the blood (< 2 ng/ml (Lindahl and Höök, 1978). The presence of $\geq$ 1 µg/ml as is usual in anticoagulant and antithrombotic therapy, should be seen therefore as a highly artificial situation.

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* ACTH - adrenocorticotrophic hormone

1.4.1 The GAG group

Glycosaminoglycans (GAGs, formerly known as acid mucopolysaccharides) are widespread in animal tissues. They consist of chains of alternating monosaccharide units or uronic acids (L-iduronic acid or D-glucuronic acid) and amino sugars, the latter being either D-glucosamine (in the case of heparin, heparan sulphate (HS) and hyaluronic acid) or D-galactosamine (in chondroitin, chondroitin sulphate and dermatan sulphate (DeS)). Their acid components enable them to interact with proteins and other biomolecules, to perform several important structural and controlling functions (Lindahl and Höök, 1978). Only heparin, HS and DeS have, as far as is known, biological relevance to this project.

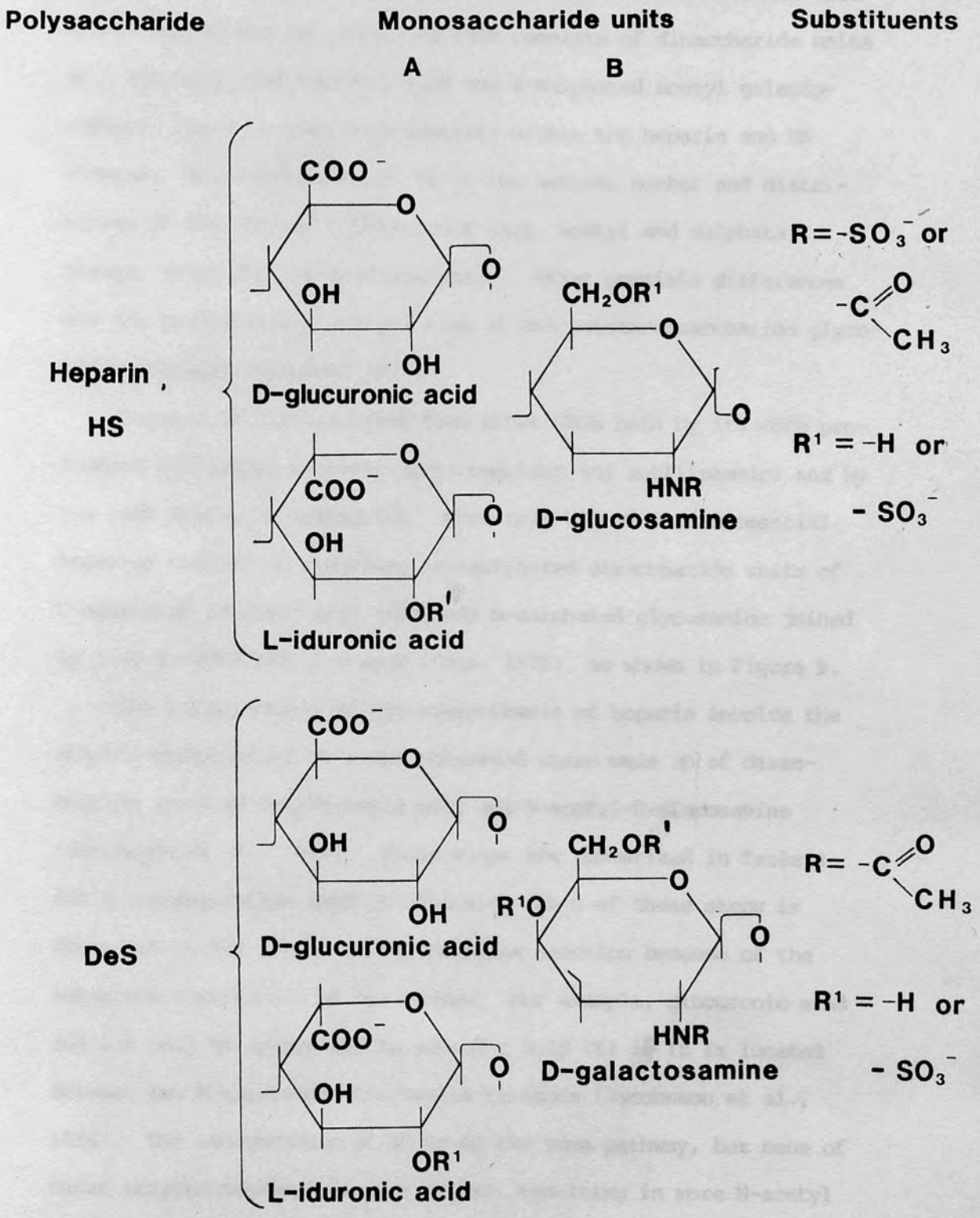
Heparin, which is a salt of the strongest organic acid isolated from mammalian tissues, is synthesized and stored in mast cell granules, from which it is released under certain physiological conditions. Mast cells are present in connective tissue, such as that in mucosa, around venules and in the bronchial lumen. HS is a common cell surface membrane component of arterial walls, organ cell surfaces and connective tissue, and is also present in the cytoplasm and to a lesser extent in the nucleus (Comper, 1981). DeS is also a ubiquitous GAG, present in skin, tendons and heart valves, but its function is not known. It has previously been called β-heparin because of its weak anticoagulant activity (Comper, 1981).

1.4.2 Primary structure of heparin, HS and DeS

The structure of the disaccharide units of these three GAG groups is given in Figure 8; the polysaccharides are linear poly-

Fig 8 Structure of GAGs

(From Lindahl and Höök, 1978)



mers of alternating A and B monosaccharide units. Whereas there is almost total consistency of the structure of the chondroitins (not shown) and of the DeS structure (90% consists of disaccharide units of a non-sulphated iduronic acid and 4-sulphated acetyl galactosamine), there is wide heterogeneity within the heparin and HS classes. This variation can be in the nature, number and distribution of the various substituents (e.g. acetyl and sulphate groups) along the carbohydrate chain. Other possible differences are the configuration and position of the intermonosaccharide glycosidic linkages (Jeanloz, 1974).

Heparin is distinguished from other GAGs both by its more pronounced biological activity (anticoagulant and antilipaemic) and by its high degree of sulphation. More than 70% of most commercial heparins consist of repeating tri-sulphated disaccharide units of O-sulphated iduronic acid and O-and N-sulphated glycosamine joined by 1 to 4 glycosidic linkages (Casu, 1979), as shown in Figure 9.

The latter stages of the biosynthesis of heparin involve the enzymic modification of a non-sulphated chain made up of disaccharide units of D-glucuronic acid and N-acetyl-D-glucosamine (Jacobsson et al., 1984). These steps are summarized in Table 4 for a representative section of chain. Each of these steps is dependant on the extent of the previous reaction because of the substrate specificity of the enzyme. For example, glucuronic acid (G) can only be epimerized to iduronic acid (I) if it is located between two N-sulphated glucosamine residues (Jacobsson et al., 1984). The biosynthesis of HS is by the same pathway, but none of these enzymic conversions goes as far, resulting in more N-acetyl groups and glucosamine residues and so less overall sulphation.

Fig 9 The major disaccharide of heparin

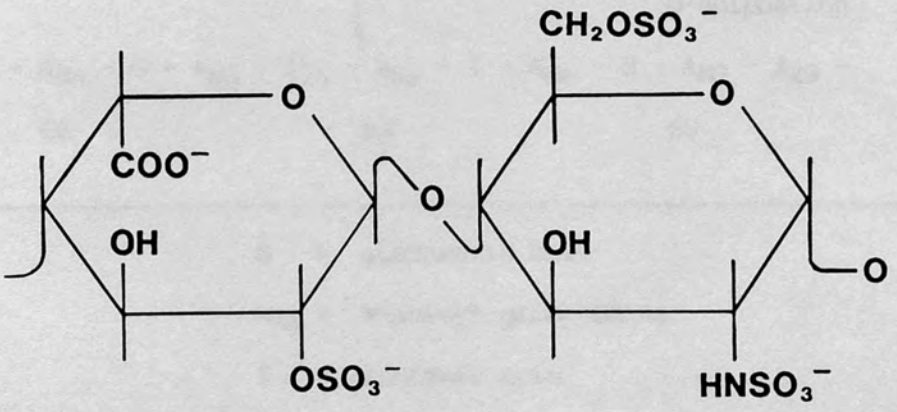
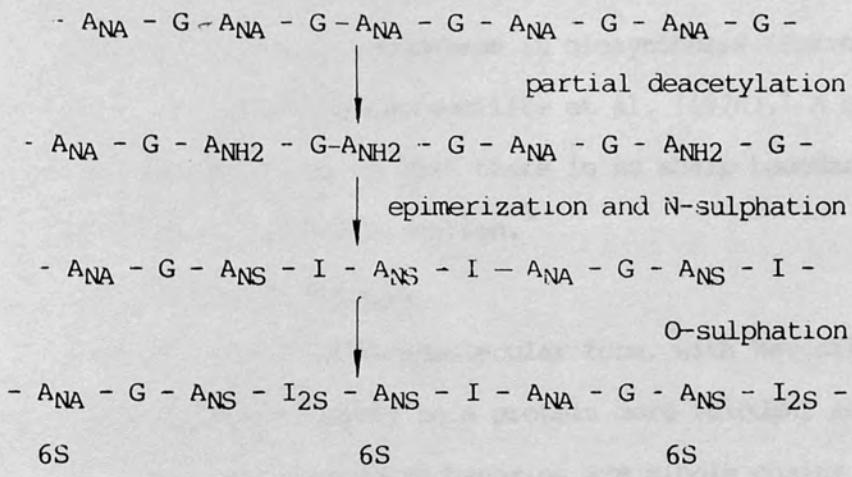


TABLE 4: Steps of enzymic modification of N-acetyl glucuronic acid-glucosamine polymer to form heparin



G = glucuronic acid

A_{NA} = N-acetyl glucosamine

I = iduronic acid

The existence of discrete molecular species has been postulated on the basis of electrophoretic studies with the use of ampholytes, but this has been challenged, and it seems more likely that the heterogeneity is due to randomness in biosynthesis (Barrowcliffe et al., 1978). As stated by Barrowcliffe et al. (1978)," A consequence of the heterogeneity is that there is no sharp boundary between purification and fractionation."

1.4.3 Protein moiety of heparin

Native GAGs exist in a macromolecular form, with several carbohydrate chains bound covalently to a protein core (Lindahl and Höök, 1978). However, commercial heparins are single chains and contain only very small amounts of amino acids (Johnson et al., 1983).

1.4.4 Molecular shape of heparin

X-ray crystallography and NMR data have suggested that heparin exists in a helical form (Casu, 1979); however, there is very little evidence that this can be related to how it exists in solution. Since there is rotational freedom between monosaccharide units of the GAG it is flexible and so could assume any of a range of conformations.

1.4.5 Anticoagulant activity of heparin

The anticoagulant action of heparin is usually regarded as based mainly on its binding to At III, the serine protease inhibitor for which it acts as a co-factor in the inactivation of several clotting factors in the 'coagulation cascade'. The presence of a specific pentasaccharide At III-binding sequence is important, but other parameters are also involved. This will be further discussed in Section 6 of this Chapter.

1.4.6 Mechanism of lipase release by GAGs

The release of LPL and HTGL by heparin from their position bound to the capillary endothelium is thought to be through the formation of a heparin-enzyme complex (Olivecrona et al., 1977). The dissociation from cell surface HS may be either a result of a conformational change induced by the binding of heparin, or by actual competition for the same GAG-binding site on the enzyme (Olivecrona et al., 1977).

1.4.7 Structural requirements for lipase release

The structural requirements for the lipase-releasing activity of GAGs do not appear to be as specific as those for At III binding. Binding seems to be largely dependent on charge density, resulting in an ability to form electrostatic interactions with lipases. In vivo work has been done mainly in rats and humans (Jansen and Hülsmann, 1974; Ganesan and Bass, 1976; Krauss et al., 1974; Casu et al., 1983) and the affinity of different GAGs for the lipases has also been studied in vitro using affinity chromatography (Bengtsson et al., 1980).

Casu et al. (1983) studied a large range of fractionated heparins and HS from different sources on total lipase release when injected into rats. Their results showed the importance of the degree of sulphation, and they concluded that a GAG must have > 50% trisulphated disaccharide units to have significant releasing ability. However, since activity does not rise linearly with the sulphation level, other factors must be involved, for example, the length of the sulphated sequences along the chain. The in vitro work by Bengtsson and co-workers (1980) using Sepharose columns substituted with a range of GAGs measured the affinity for lipase in

terms of the salt concentration needed to displace the bound enzyme. The order of affinity for both LPL and HTGL - heparin > HS ≃ DeS > chondroitin - was confirmed (Bengtsson et al., 1980) by in vivo studies in the rat, where only iduronic acid-containing GAGs released lipase (none with chondroitin sulphate). The HS they used, however, was of low sulphation and gave lipase levels less than 10% of those achieved with heparin, whereas Casu et al. (1983) have shown that more highly sulphated HS may be a good releaser. It should also be noted that artificially sulphated chondroitin (SSHA, semi-synthetic heparin analogue) (Thomas et al., 1980) and a heavily sulphated synthetic polypentosan (Fischer et al., 1982) have also been shown to release lipase in in vivo studies, so that iduronic acid groups are not essential. Heparin loses most of its affinity for lipase (as measured using substituted gels) on partial N-desulphation, but this can be largely restored by N-substitution with either acetyl or sulphate groups (Bengtsson et al., 1980). This could mean that the reduction in binding ability on desulphation is due in part to the presence of free amino groups and that the nature of the substituted group is not all-important. However, reacetylation of completely N-desulphated heparin does not restore affinity for the enzyme (measured by the same method).

Various heparin fragments were compared for their ability to release LPL from immobilized, unmodified heparin (Bengtsson et al., 1980). Their affinity for LPL was shown to decrease with molecular weight, with no releasing ability present below the hexasaccharide. The effect of heparin molecular weight has also been studied by fluorescence polarization spectroscopy (Clarke et al., 1983). The results suggest that the length of the heparin chain determines the

type of complex formed with the enzyme. The strong binding achieved with heparins of > 10,000 MW could be due to their ability to interact with sites on both LPL subunits, whereas lower MW molecules are not sufficiently long to bind both, so have lower affinity. These results are supported by the molecular dimensions and results of crystallographic studies. They also find that the LPL dissociation constant with HS is much greater than with heparin. No data are available on the effect of MW on the interaction of heparin with HTGL, but LMW heparins have been reported to be low releasers of the enzyme in vivo (de Swart et al., 1980).

1.4.8 Plasma lipase levels after polyanion injection

The levels of lipase activity and the proportion of the two enzymes after injection of heparin and other polysaccharides may depend on the time after injection, the dose and type of polysaccharide used (Jansen and Hülsmann, 1974; Ganesan and Bass, 1976). It has also been shown in the human that lipase activity depends on sex, age and nutritional state and is also altered in certain disease conditions (Krauss et al., 1974: Huttenen et al., 1976). All these factors, in addition to differences in measurements achieved using different assay conditions, make comparison of results by different workers difficult (especially as full details are not always given).

As an example of this, significant differences have been reported on the effect of sex and age on human LPL levels. Huttenen et al. (1976) report that LPL in PHP is higher in females than males at all age groups, and that it decreases with age in both sexes. Krauss et al. (1974), however, report that LPL levels are the same in adult men and women, but lower in adolescent girls

than boys. This would mean that LPL increases with age in females, but stays constant in males. There is more agreement between these two sets of workers on HTGL levels: HTGL is higher in males than females, ranging from 49-95% of total lipase activity (Krauss et al., 1974) and does not change with age in either sex.

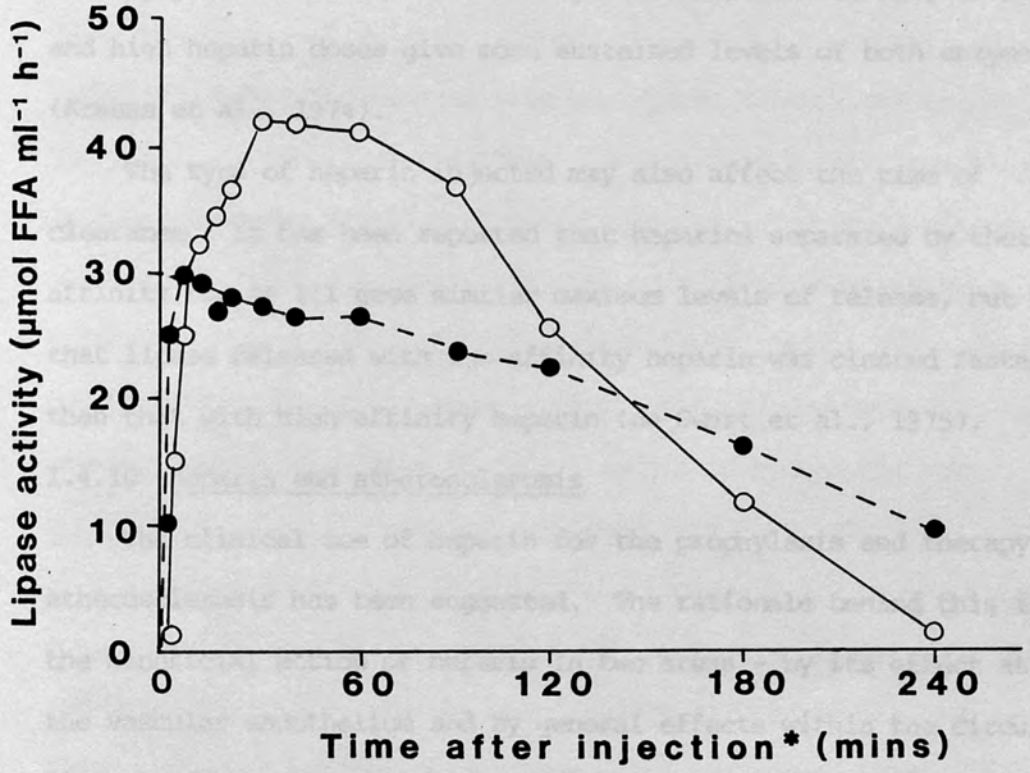
Low heparin doses are reported (Jansen and Hülsmann, 1974) to release mainly LPL into the circulation, but as dose is increased so does the contribution of HTGL to total lipolytic activity. The peak of HTGL release has been found to be reached at lower heparin doses than that required for LPL (Huttenen et al., 1975).

1.4.9 Time course of lipase in plasma after heparin injection

The time course of release and clearance of LPL and HTGL after heparin injection has been studied in man. HTGL is released first of the two enzymes, to reach peak activity at 2-5 mins. after injection, and is reported to remain at this level for at least 15 mins. (Huttenen et al., 1975) after which it is slowly removed (half-life in the plasma of approximately 4 hours) (Krauss et al., 1974; de Swart et al., 1980). LPL is released more slowly than HTGL, and is at a maximum 15-30 mins. after injection, after which it declines quickly to be completely removed by 2-4 hours (Huttenen et al., 1975; de Swart et al., 1980). Figure 10 shows a representative time course after 100 u/kg heparin (Huttenen et al., 1975). The removal of HTGL especially has been reported to be slower at higher heparin doses (Krauss et al., 1975). Use of radiolabelled LPL (Wallinder et al., 1979) has shown that this enzyme is taken up from the blood and degraded by the liver. The liver receptor responsible has not yet been identified, but it is not among the known galactose- or mannose-binding protein receptors which remove other

Fig 10 Time course of lipase in plasma after release by heparin

(From Huttenen et al., 1975)



* 100 i.u./kg in humans i.v.

glycoproteins (Wallinder et al., 1979). Other workers (de Swart et al., 1980) followed the disappearance from plasma of both ^{35}S-heparin (used for injection) and the HTGL released by it, and the similar curves achieved suggested that they both exist in the blood, and are taken up, as a complex. The lipase-bound heparin may influence removal of the complex (de Swart et al., 1980). Clearance of purified LPL injected into rats was greatly slowed down by prior administration of heparin (Wallinder et al., 1979) and high heparin doses give more sustained levels of both enzymes (Krauss et al., 1974).

The type of heparin injected may also affect the time of clearance. It has been reported that heparins separated by their affinity for At III gave similar maximum levels of release, but that lipase released with low-affinity heparin was cleared faster than that with high-affinity heparin (de Swart et al., 1975).

1.4.10 Heparin and atherosclerosis

The clinical use of heparin for the prophylaxis and therapy of atherosclerosis has been suggested. The rationale behind this is the beneficial action of heparin in two areas - by its effect at the vascular endothelium and by general effects within the circulating blood (Engelberg, 1980). At the endothelium, heparin may reduce platelet adhesion and the formation of thrombi, and also slow down the uptake of serum lipoproteins, possibly by maintaining the negativity of the cell surface. The effects in the bloodstream are considered to be mediated mainly through the action of released lipolytic enzymes.

It has been repeatedly noted (see Mahley and Innerarity, 1983) that there is a positive correlation between blood LDL levels and

the incidence of coronary heart disease. On the other hand, the reverse is true of HDL levels (Twu et al., 1984) and above normal levels in individuals appear to be associated with longevity (Engelberg, 1984). For this reason HDL has been termed a 'protective factor' against atherosclerosis. The difference between the lipoproteins is due to differences in their roles in cholesterol transport. Whereas cholesterol-rich LDL delivers cholesterol to tissues, which may lead to overloading of cells with the LDL (apo B, E) receptor, HDL acts in 'reverse cholesterol transport' by accepting excess cholesterol from peripheral tissues and transporting it to the liver for removal (Mahley and Innerarity, 1983). It has been known for some time that heparin injection leads to an increase in blood HDL levels and that this is accompanied by an increase in cholesterol excretion (Engelberg, 1984). These changes can be attributed to the release of LPL and HTGL by heparin, leading to an increase in TG and phospholipid hydrolysis and cholesterol esterification and transfer (see Fig. 3).

A second beneficial effect of the lipases would be via their action against the fatty lesions which develop on vessel cell walls and which interfere with the blood supply of these cells, since there are several lines of evidence which suggest that hypoxia accelerates the atherosclerotic process (Engelberg, 1984).

Only a small number of clinical trials of heparin against atherosclerosis have as yet been conducted (see Engelberg, 1980), possibly because of the risk of haemorrhage and osteoporosis in heparin therapy. The results of the majority of these trials however, did suggest that use of heparin can cut down the number of deaths in patients with established coronary heart disease

(Engelberg, 1980). It has also been shown that some heparans, as well as heparin, slow down the development of atherosclerosis in cholesterol-rich animals (Grossman et al., 1971). However, as in the case of the human studies, there have also been negative results (Engelberg, 1980), so that more study into this is needed.

1.5 LIPASE ASSAY METHODS

1.5.1 Development of Lipase Assays

From the time of the early work by Korn on 'clearing factor' (Korn, 1955), many different means of measurement of lipase activity in PHP and tissue extracts have been used. These can be divided into different groups depending on the nature of the substrate and the method employed to measure product release. Physiological substrates such as CM or VLDL preparations, have been used, as well as artificial triglyceride emulsions, which can be radiolabelled. Enzymic activity is measured by the release of glycerol or FFA, either by chemical means or by scintillation counting when radiolabelled TGs have been used.

The assay substrates used by Korn and co-workers (1955; 1957) were CM suspensions or coconut oil emulsions, with quantitation of activity by converting the glycerol released into formaldehyde which was then measured spectrophotometrically. Other early work by Angervall and Hood (1957) followed the decrease in the optical density at 570 nm on addition of enzyme to lipaemic plasma or homogenized cow's milk cream/saline mixtures. There are problems in using natural substrates, among them the instability of the lipoprotein suspensions, and the difficulty in standardization of both these and the lipaemic plasma used for the turbidity measurements. CM or VLDL suspensions are now used mainly for specificity tests,

or for studies of kinetics or mechanism (Eisenberg et al., 1981). Despite the problems of using these substrates, Korn (1955) did show some important properties of post-heparin lipase activity, although it was not realized at this time that there are two enzymes of different specificities and properties present in PHP. These findings included the inhibition of activity by incubating the enzyme mixture with salt or protamine, the need for fatty acid acceptors (such as albumin) and also for serum components when artificial substrates are used.

Due to the drawbacks of using natural substrates, later workers have mainly used commercial emulsions, such as Intralipid (made from soya bean triglycerides emulsified with phosphatidylcholine (PC)) and 'Ediol' (coconut oil in a detergent mixture), or purified triglycerides prepared for use daily in the laboratory. Intralipid, because of its structural similarity to the surface layer of native lipoproteins for which the LPL binding site is adapted (Bengtsson and Olivecrona, 1981b), has been shown to compare favourably with CMs or VLDL as a substrate when testing LPL both in adipose tissue extracts (Lithell and Boberg, 1977) and PHP (Riley and Robinson, 1974). HTGL, however, does not bind well to either, and Intralipid has been used by some workers to separate LPL and HTGL on the basis of their different affinities (Ehnholm et al., 1975).

The predominantly non-polar nature of lipids means that emulsifiers/stabilizers have to be employed in making up TG substrates (except when short chain TGs such as tributyrin are used (Rapp and Olivecrona, 1978)). A range of these has been used in different laboratories (Riley and Robinson, 1974). Phosphatidylcholine (PC)

and its derivatives have been a common choice, but other agents used include Triton X-100, gum arabic or acacia, Tween 60 and bulk egg yolk lipids. These emulsions are prepared by sonication immediately before use. The Nilsson-Ehle and Schotz assay (1976) (see Ch. 2), however, uses a substrate emulsified with PC and stabilized with glycerol. This allows its use for several weeks after a single sonication which is an obvious advantage.

For measurement of enzymic activity, glycerol or more commonly, FFA are extracted from the incubation mixture and quantitized. The early methods for FFA extraction based on those of Dole (1956) or Kelley (see Iverius and Östlund-Lindqvist, 1976) are time-consuming and require large enzyme volumes for accuracy. Assay improvements were made by the adoption of methods such as that of Belfrage and Vaughn (Nilsson-Ehle and Schotz, 1976), which is a one-stage, liquid-liquid partition method using organic solvents. Once extracted, the FFA can be titrated (Havel et al., 1973). Measurements were made quicker and easier by the use of radiolabelled substrates; the products once extracted can be measured directly by scintillation counting (Nilsson-Ehle and Schotz, 1976).

There have also been reports of determination of FFA by fluorescence with the use of a coumarin derivative (Curzon and Kantamanteni, 1977) and by enzymic means (Woollett et al., 1984). In this latter method, aliquots of the incubated enzyme-substrate mixture are added to a series of purified enzymes and cofactors, and the eventual oxidation of NADH to NAD^+ is measured spectrophotometrically. It should be noted, however, that these FFA measurements are carried out, without their prior extraction, on reaction

mixtures containing albumin which as it binds FFA may interfere with the quantitation.

Since the work of La Rosa et al. (1972) and Krauss et al. (1973) led to the understanding that there are at least two lipase enzymes in PHP, assays have been developed which purport to measure them selectively. These may use conditions that i) inhibit LPL or HTGL, ii) involve the precipitation of one of the enzymes by specific antibodies or iii) separate the enzymes by the difference in their affinity for heparin.

1.5.2 Methods of Selective Measurement

i) Inhibition of LPL or HTGL activity

a) Use of salt to inhibit LPL It is well documented (Nilsson-Ehle et al., 1980) that the two PHP lipases are affected differently by high salt concentrations. LPL is increasingly inhibited by NaCl, up to > 90% at 1.0 M, whereas HTGL is resistant to, and perhaps stimulated by, this high ionic strength (Krauss et al., 1973; Augustin and Greten, 1979a). The basis of the effect on HTGL is not known, although it has been suggested that high salt disrupts a high MW complex of HTGL into more active subunits (Ehnholm et al., 1974). The mechanism of LPL inhibition by high salt has been well investigated. Fielding and Fielding (1976) found it to be fully reversible and to be mediated to the same extent by different cations (tested with the same anion), but to significantly different extents by a range of salts which varied in their anionic component (with reactivity following the Hofmeister series). Since they also found a relationship between activation by apo CII and the extent of inhibition by salt, they concluded that salt acts through a reaction with a positively charged site on the apo CII.

Other workers (Bengtsson and Olivecrona, 1983) suggest that the salt acts either by reducing the degree of binding of the enzyme to the substrate, or through a direct effect on the enzyme.

The differential action of salt on the enzymes has been used to estimate the proportions of the two in PHP. Total lipase activity can be assayed at physiological salt concentrations in the presence of serum (to provide apo CII for full LPL activity), and HTGL activity under high salt conditions in the absence of serum, where there should be very little LPL activity (Huttenen et al., 1975; Östlund-Lindqvist and Boberg, 1977). The degree of salt inhibition has been reported to vary between substrates (La Rosa et al., 1972: Bengtsson and Olivecrona, 1983). Notable is the limited salt inhibition achieved when Intralipid is used, and this is thought to be due to the strong binding of LPL to this substrate (Bengtsson and Olivecrona, 1983). Other workers (Fielding and Fielding, 1976) find no difference between Intralipid and other substrates. Conflicting reports like this seem to be commonly encountered in this field.

b) Use of protamine sulphate to inhibit LPL Protamines are simple LMW proteins which are rich in arginine and strongly basic (Goodman and ~~Gilson~~ Gillman, 1970). They inhibit heparin through binding to acid groups, and have been found (like other polycations) also to inhibit PHP lipases (Korn, 1955). LPL is more sensitive to protamine inactivation than HTGL, and under carefully specified conditions, this can be exploited to achieve maximum inhibition of LPL (90%) with minimum inhibition of HTGL (10%) (Krauss et al., 1973). It is not possible to inhibit all the LPL without some effect on HTGL. Protamine is thought to act by decreasing enzyme stability

(although there have been reports that purified LPL is not affected), but it may also act by decreasing binding to substrates (Augustin and Greten, 1979b).

c) Use of sodium dodecyl sulphate to inhibit HTGL A chemical method for the removal of HTGL activity in PHP has been described (Baginsky, 1981). Baginsky and Brown (1979) report that the two lipases differ markedly in their response to SDS over a limited concentration range, and this can be used for selective measurement. They preincubated the enzyme sample with 35-50 mM SDS in buffer before testing with a gum arabic-stabilized triolein substrate, and under these conditions they report that 80-90% of the HTGL is inhibited whereas the LPL is unaffected.

ii) Precipitation of lipase by specific antibodies

LPL and HTGL possess different antigenic determinants, so that specific antibodies to each can be raised (Huttenen et al., 1975; Augustin et al., 1978). Removal of one then allows measurement of the remaining lipase under its optimal conditions. These antibodies are not available commercially.

iii) Separation by heparin-Sepharose chromatography

The assay method of Boberg et al. (1977) makes use of the different affinity of the two PHP lipases for heparin. Each sample is loaded on to a small column of heparin-Sepharose, washed, then HTGL and LPL are eluted stepwise with increasing salt concentrations. Fractions at each salt concentration are assayed under the conditions optimal for the two enzymes. This method was devised to overcome possible interference by lipoproteins present in PHP. However, it is a time-consuming method and there are extensive losses during chromatography.

1.5.3 Miscellaneous Points on Assay Conditions

i) Product inhibition and the use of albumin

Both LPL and HTGL are subject to product inhibition by FFA (Bengtsson and Olivecrona, 1980a). In vivo FFA move by lateral diffusion into surrounding tissues where they are utilized, but in vitro a FFA-acceptor has to be present. Normally serum albumin is used in assays (Eisenberg et al., 1981), although calcium ions are as effective (Korn, 1955). Several factors appear to contribute to the inhibitory effect of FFA (Olivecrona and Bengtsson, 1980). FFA are more surface-active than TGs, so may displace TGs from the lipid-water interface and compete for the active site. The enzyme would then form di- and triglycerides from FFA and partial glycerides. It is also thought that the lipase forms complexes with FFA which prevent binding of the enzyme to emulsion surfaces. In the case of LPL, it has been shown (Bengtsson and Olivecrona, 1980a) that there is no activation by apo CII in the absence of albumin, so it is also possible that FFA hinder the enzyme-activator interaction. The relieving of product inhibition by albumin is due to the latter binding FFA stronger than do the lipases (Bengtsson and Olivecrona, 1980a), via ç 7 high affinity binding sites.

ii) Effect of heparin

There have been many reports on the in vitro effect of heparin on lipase activity (Bengtsson and Olivecrona, 1981a). Early work (Korn, 1955) showed that heparin stimulated the activity of crude enzyme preparations, and that the presence of bacterial heparinase led to a decrease in enzyme activity. Heparin was thus thought to be a lipase co-factor. However, tests of highly purified enzyme

have shown that stimulation occurs only under sub-optimal conditions, and it has been suggested that heparin acts to stabilize the enzyme and keep it in solution (Bengtsson and Olivecrona, 1981a).

1.6 THE ANTICOAGULANT ACTION OF HEPARIN

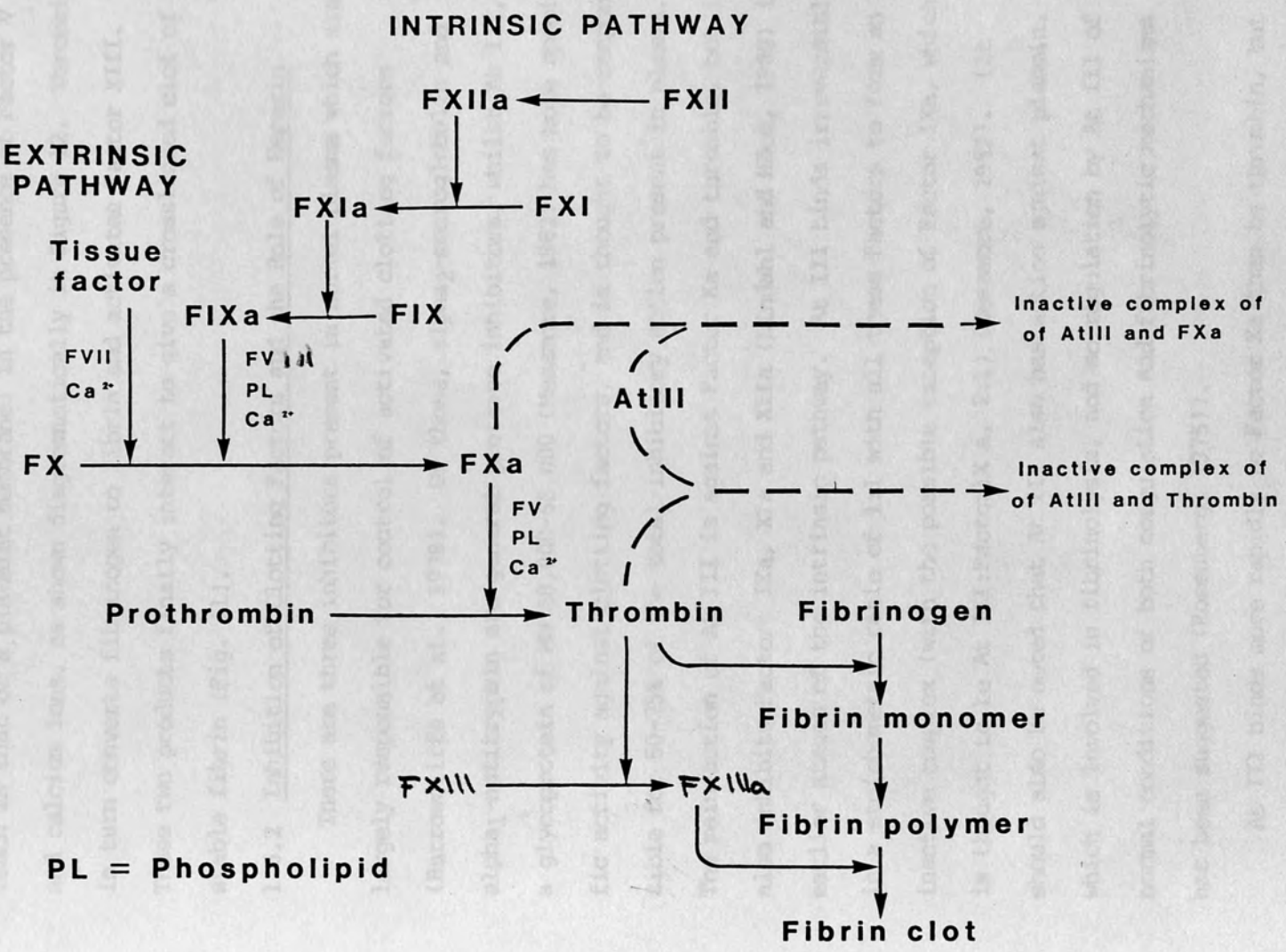
The action of heparin as an anticoagulant is now widely exploited clinically both in the control of thromboembolism and in maintaining blood fluidity outside the body (such as with dialysis machines) (Barrowcliffe et al., 1978). Use of heparin heightens the risk of haemorrhage, so different heparin types are continually being assessed for effectiveness and reduced risk.

As mentioned in Section 4 of this Chapter, the anticoagulant activity of heparin and HS appears to be dependent on the presence of a number of physicochemical parameters, which affects their interaction with At III (the major plasma serine protease inhibitor), and in some cases with the serine protease itself, for example thrombin.

1.6.1 Blood Coagulation

The physiological formation of a thrombus is a result of a series or cascade of linked reactions involving the conversion of enzyme zymogens into active serine proteases (for reviews see Esnouf, 1977; Jackson and Nemerson, 1980). The initial signal is the contact of blood either with a foreign surface (to start the intrinsic pathway) or with damaged tissues (the extrinsic pathway). These two pathways converge at the activation of Factor X (see Fig. 11) into a common pathway, and at each step there is an amplification of the signal. The conversion of Factor X and prothrombin to active serine proteases are both key steps in the coagulation sequence. The activation of prothrombin (Factor II) by Factor Xa

Fig 11 The blood coagulation cascade and the inhibitory action of AtIII



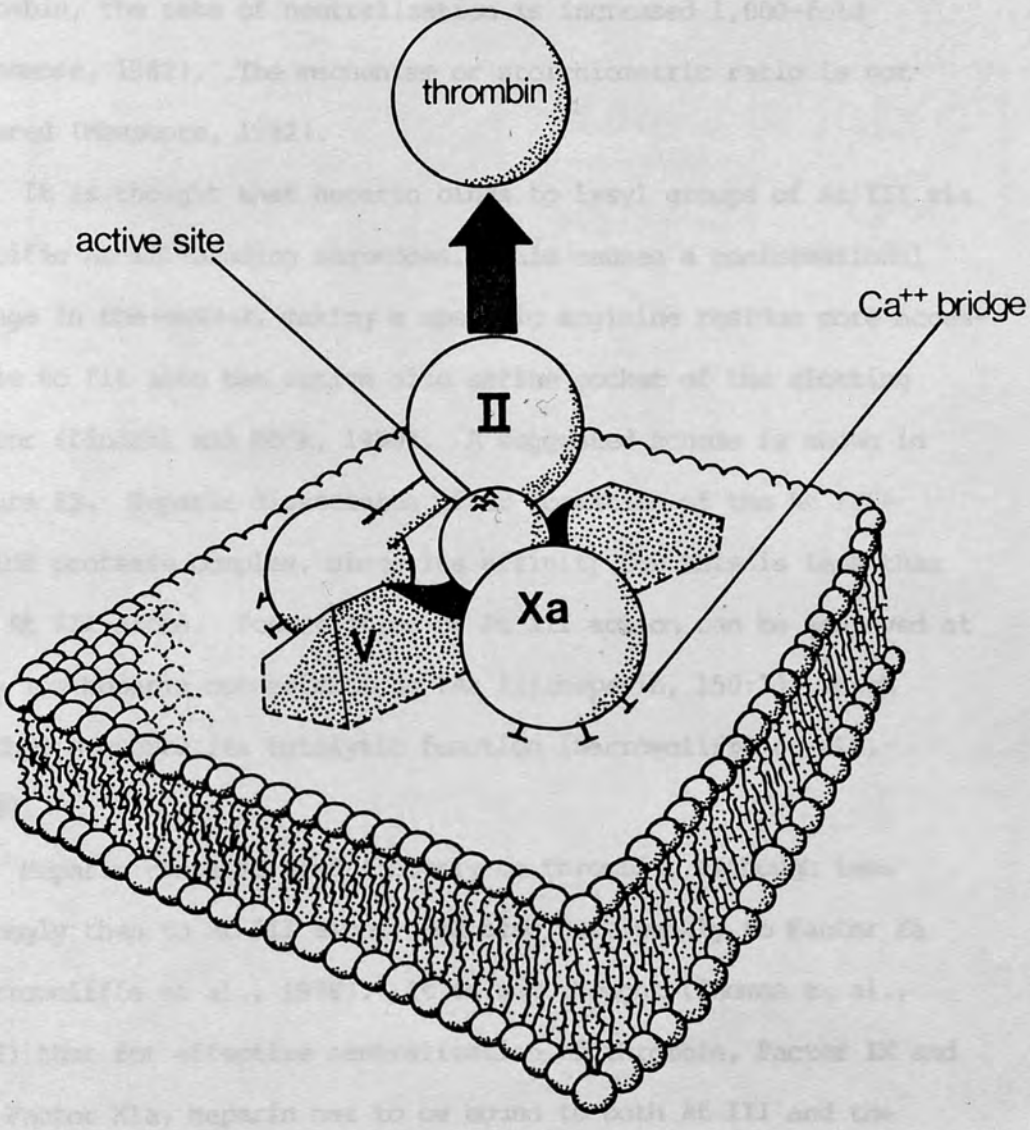
to form thrombin (Factor IIa) is the best characterized stage (Jackson and Nemerson, 1980); it takes place on a phospholipid surface (such as that of a platelet membrane) in the presence of Factor V and calcium ions, as shown diagrammatically in Figure 12. Thrombin in turn converts fibrinogen to fibrin and activates Factor XIII. These two products finally interact to give a crosslinked clot of stable fibrin (Fig. 11).

1.6.2 Inhibition of Clotting Factors and the Role of Heparin

There are three inhibitors present in normal plasma which are largely responsible for control of activated clotting factors (Barrowcliffe et al., 1978). Of these, $alpha_2$-macroglobulin and $alpha_1$-antitrypsin are general protease inhibitors, whilst At III, a glycoprotein of MW 58,000-65,000 (Messmore, 1982) has more specific activity against clotting factors, and is thought to be responsible for 50-75% of the total inhibitory action present in plasma. The main action of At III is against Factor Xa and thrombin, but it also inhibits Factors IXa, XIa and XIIa (Lindahl and Höök, 1980) in earlier stages of the intrinsic pathway. At III binds irreversibly in a stoichometric ratio of 1:1 with all these factors to form an inactive complex (with the possible exception of Factor IXa, which is thought to be At III:Factor IXa, 2:1) (Messmore, 1982). (It should also be noted that At III also has action against plasmin, which is involved in fibrinolysis, and so regulation by At III of normal conditions of both coagulation and fibrinolytic mechanisms has been suggested (Rosenberg, 1975)).

At III binds more rapidly to Factor Xa than to thrombin, but in mixtures of the two binds preferentially to thrombin (Barrowcliffe et al., 1978). In normal coagulation there is 100

Fig 12 Activation of prothrombin



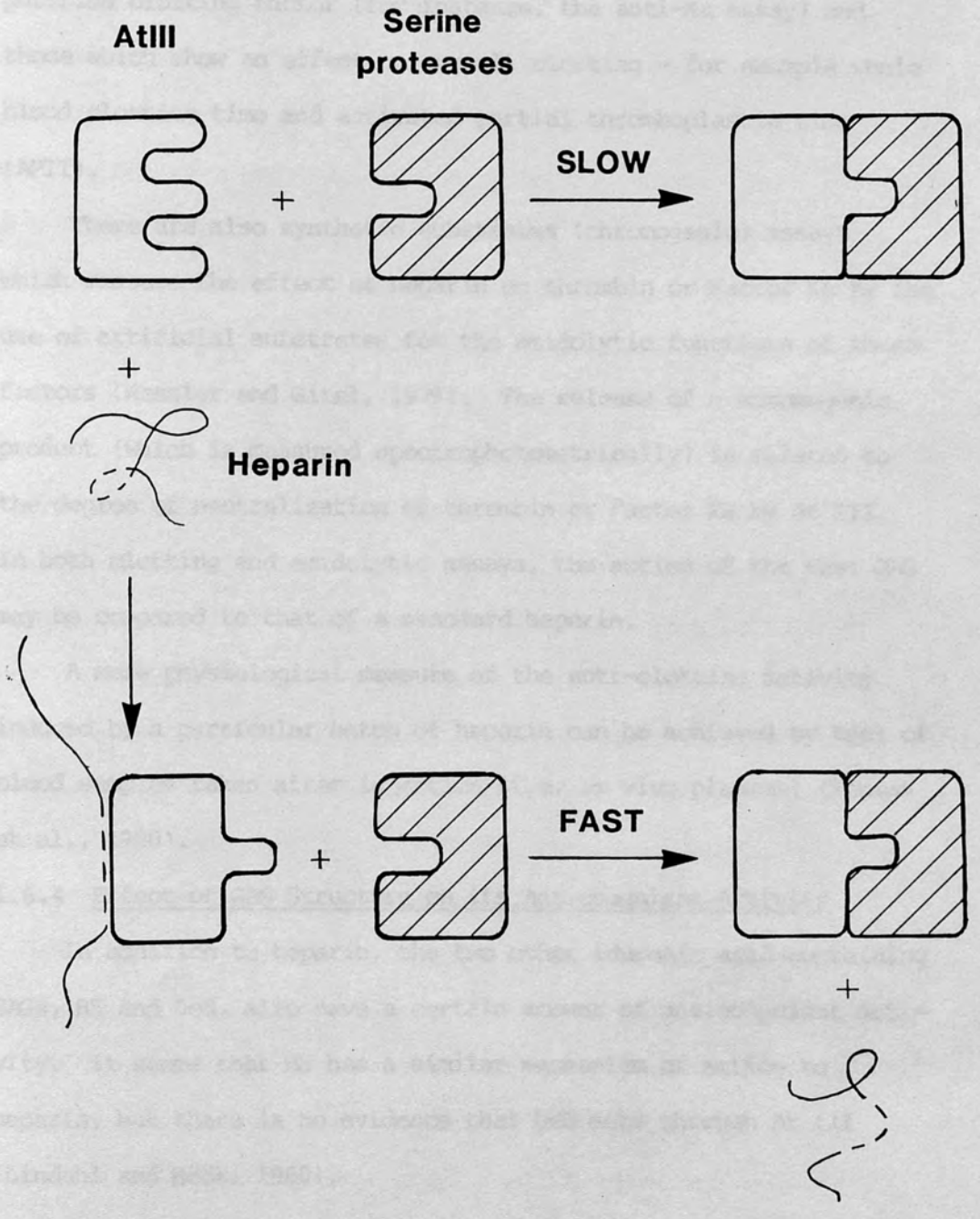
times more thrombin present than Factor Xa, since the latter is 'one step up' in the enzyme cascade, but because of the At III affinity difference both are neutralized within 15 minutes of clotting. There appears to be only little activity against the other serine proteases (Wessler and Gitel, 1979). However, heparin acts as a potent catalyst for all these reactions; in the case of thrombin, the rate of neutralization is increased 1,000-fold (Messmore, 1982). The mechanism or stoichiometric ratio is not altered (Messmore, 1982).

It is thought that heparin binds to lysyl groups of At III via specific At III-binding sequences. This causes a conformational change in the inhibitor, making a specific arginine residue more accessible to fit into the active site serine pocket of the clotting factor (Lindahl and Höök, 1980). A suggested scheme is shown in Figure 13. Heparin dissociates after formation of the At III-serine protease complex, since its affinity for this is less than for At III alone. Potentiation of At III action can be achieved at very low heparin concentrations (At III:heparin, 150:1), which further supports its catalytic function (Barrowcliffe et al., 1978).

Heparin can also bind directly to thrombin, although less strongly than to At III alone, and also very weakly to Factor Xa (Barrowcliffe et al., 1978). It is now thought (Thomas et al., 1982) that for effective neutralization of thrombin, Factor IX and and Factor XIa, heparin has to be bound to both At III and the clotting factor, whilst binding of At III alone is sufficient for inhibition of Factor Xa.

Fig 13 Inactivation of serine proteases by AtIII

(From Lindahl and Höök, 1978)



1.6.3 Heparin Assays

There are many clotting assays that may be used to assess the anticoagulant effect of a GAG (Barrowcliffe et al., 1978; Wessler and Gitel, 1979). These assays may be divided into two major groups: those which test the effect of heparin on one particular purified clotting factor (for instance, the anti-Xa assay) and those which show an effect on overall clotting - for example whole blood clotting time and activated partial thromboplastin time (APTT).

There are also synthetic substrates (chromogenic) assays which measure the effect of heparin on thrombin or Factor Xa by the use of artificial substrates for the amidolytic functions of these factors (Wessler and Gitel, 1979). The release of a chromogenic product (which is measured spectrophotometrically) is related to the degree of neutralization of thrombin or Factor Xa by At III. In both clotting and amidolytic assays, the action of the test GAG may be compared to that of a standard heparin.

A more physiological measure of the anti-clotting activity induced by a particular batch of heparin can be achieved by test of blood samples taken after injection (i.e. ex vivo plasmas) (Thomas et al., 1980).

1.6.4 Effect of GAG Structure on its Anticoagulant Activity

In addition to heparin, the two other iduronic acid-containing GAGs, HS and DeS, also have a certain amount of anticoagulant activity. It seems that HS has a similar mechanism of action to heparin, but there is no evidence that DeS acts through At III (Lindahl and Höök, 1980).

The effect of MW of heparin on its activity was studied by

separation on gel filtration followed by APTT and anti-Xa assays (Andersson et al., 1979). The different values achieved in these two assays can be expressed in terms of the anti-Xa/APTT ratio, which for unfractionated heparin is generally around 1 (Thomas et al., 1982). By definition the activity of the standard heparin gives the same value by anti-Xa and APTT assays, so any heparins which are similar will have a ratio close to unity. It was found that for heparins of MWs below 15,000 daltons, the relative value by APTT decreases compared to that by anti-Xa assay, giving a ratio of > 1 (Andersson et al., 1979). Low MW (LMW) heparins with high anti-Xa/APTT ratios have been investigated for possible clinical use (Thomas et al., 1982). It was thought that such heparin fractions, whilst having the desired therapeutic effect, would be less likely to cause haemorrhage since they have less effect on overall clotting. (Comparison of the use in animals of LMW and HMW heparin has also shown that only in the latter is there evidence of osteoporosis (thinning of bones) (Murray, 1984)). However, this has been shown to be an over-simplification which is not supported by in vivo work (Thomas et al., 1982). Thomas and co-workers (1982) found that whilst heparin fragments of both 10 and 16-18 monosaccharide units long have potent anti-Xa activity in vitro, only the latter has some antithrombotic effect. One factor in this may be the need for the GAG to bind thrombin as well as At III for neutralization.

It is now known that the binding of At III by heparin is accomplished most effectively via a specific pentasaccharide sequence in the heparin molecule (Lindahl et al., 1983). This sequence is present in approximately 30% of heparin molecules of an unfrac-

tionated samples, as determined by their affinity for At III-Sepharose (Lindahl and Höök, 1980). This is the only specific action of heparin that is known at present, in that it depends on a defined molecular sequence for maximum effectiveness. However, recent work (Merton et al., 1984) has indicated that heparin which has little At III-binding ability (low affinity (LA) heparin) acts synergistically with the heparin containing the specific At III-binding sequence (high affinity (HA) heparin). A mixture of HA and LA heparin was found to be more effective in preventing stasis thrombosis than HA heparin alone, although the mechanism for this is not yet known.

Work by Thomas et al. (1980) on heparin analogues further emphasized that in vitro assays alone cannot be relied upon in assessment for clinical use. They compared the in vitro and in vivo effects of SSHA (Thomas et al., 1980) and SP54 (Fischer et al., 1982) and found that although they were virtually inactive by in vitro assays, post-injection plasmas had enhanced anti-Xa activity. These results are compared in Table 5 to that using an unfractionated heparin. This suggests that some factor has been released into the blood by the heparin analogues which has, or induces anti-Xa activity. One of the suggestions given (Thomas et al., 1980) was that endogenous GAGs (possibly HS) with anti-Xa activity had been released. However, the activity could not, unlike heparin in ex vivo plasmas, be neutralized by platelet factor 4 (a heparin inhibitor occurring naturally in plasma) or by protamine sulphate. Yin and co-workers (1979) have noted the presence of factors in pre-injection plasma which protect Factor Xa from neutralization, and it has been suggested that these factors

TABLE 5: Comparison of the anti-Xa clotting activity of heparin, SSHA and SP54 by in vitro assay and that measured in plasma 2 hours following subcutaneous injection

	Anti-Xa clotting activity	
	In vitro iu/mg	Ex vivo iu/ml
Heparin*	180	0.05
SSHA†	< 5	0.06
SP54*	6	0.02

* Fischer et al., 1982

† Thomas et al., 1980

are lipoproteins (Thomas et al., 1980). In addition to this, it has been reported (Barrowcliffe et al., 1982) that certain lipoprotein classes (particularly LDL and HDL) have anti-Xa activity. Since triglyceride lipases are released into the circulation after injection of SP54 and SSHA, it is possible that these enzymes are responsible for the additional anti-Xa activity, by an effect on plasma lipoproteins.

1.7 WORK COVERED AND AIMS

The primary aim of this project was to compare various assay methods for ease and reliability in measurement of PHP lipase activity, including, if possible, selective measurement of LPL and HTGL. Literature surveys show the danger of directly relating results from different assays. For example, as described in Sections 5.2 and 2.7 of this chapter, descriptions of the effect of salt and of various serum components on the enzymes' activity have varied widely depending on the assay conditions used by the separate groups of workers.

Three lipase assays have been compared for reliability and reproducibility. Possible improvements of one assay in particular were studied. This assay purports to allow selective measurement of the two lipase enzymes. The effect of dilution of PHP by either buffer or pre-injection plasmas were also studied - results from preliminary experiments suggested that LPL and HTGL were differently affected by these two diluents.

A series of sulphated polysaccharides was selected to cover a range of MWs, sulphate content and sulphate/carboxyl ratios. This included several heparins and HS, a DeS, a non-GAG polysaccharide (SP54) and an over-sulphated chondroitin (SSHA). These

were used to investigate further possible correlations between polysaccharide structure and the ability to release lipase on injection, and also to see if there were differences in the proportions of LPL and HTGL released. Most of these in vivo studies were carried out in the rat; a range of doses of each polysaccharide (with blood sampled a set time after injection), and time courses of lipase release and clearance (at one dose per polysaccharide) were investigated. A limited amount of work was carried out using human subjects.

Various methods of inducing lipase release in vitro were also investigated using preparations of rat adipose tissue. This was tried as a potential alternative, or support, to the in vivo work, which is subject to animal variation. This would also have the advantage of reducing the number of experimental animals required.

LPL and HTGL were purified by affinity chromatography from bovine milk and both rat and human PHPs. These enzyme preparations were used in clotting tests to investigate whether their presence contributes to the anti-Xa clotting activity present in post-injection plasmas. These tests were carried out both in the absence and presence of heparin.

Heparin is widely used clinically as a drug for the control and treatment of thromboembolism, although other polysaccharides and heparin fractions are continuing being assessed for anticoagulant action with reduced risk of haemorrhage. It was hoped that this work would contribute to the overall knowledge of the various effects of injection of heparin and heparin 'analogues' in vivo.

CHAPTER 2

MEASUREMENT OF LIPASES IN POST-HEPARIN PLASMA

2.1 INTRODUCTION

In order to study in detail the effectiveness of various polyanions in releasing lipase into the circulation, it is desirable to have a reliable, sensitive and convenient assay of the lipase in post-injection plasmas. The assay used by Crinos SpA (see Appendix I) uses Intralipid as substrate, from which FFA are extracted by Dole's method after incubation with the enzyme, and titrated for quantitation (Casu et al., 1983). It has the disadvantage of requiring relatively large volumes of plasma for accuracy (0.5-1.0 ml) and being fairly lengthy to perform, thus limiting the number of samples which can be assayed at one time. This method does not differentiate between the two lipolytic enzymes released into plasma.

An improved procedure was desirable, and so possible alternative assays were investigated. Most of the work was done on the assay of Nilsson-Ehle and Schotz (1976) (the 'Modified NES Assay'), using a glycerol-stabilized radiolabelled TG substrate, with adaptations to allow selective measurements of HTGL and LPL. In addition, two other assays involving radiolabelled substrates were tried, one using a TG emulsion stabilized with gum arabic (the 'Gum Arabic Assay') and the other using the commercial TG emulsion, Intralipid (the 'Intralipid Assay').

2.2 Assay No. 1: the Modified Nilsson-Ehle and Schotz-Assay

The Nilsson-Ehle and Schotz assay was selected for initial investigation since, according to the authors, it offered improvement in the 'sensitivity, precision and practicality' of lipase measurements (Nilsson-Ehle and Schotz, 1976). The advantages of the assay include the use of a stable emulsion which can be used

for at least six weeks, the radiolabelled tracer which allows sensitive determinations on small enzyme volumes and the quick and easy extraction and quantitation of FFA's. An adaptation was made, due to Dr. J. Stocks (Lipid Research Laboratory, St. Bartholomew's Hospital, London), which was described as permitting the distinction of the two PHP lipases by using of different substrates.

2.2.1 Materials

Glycerol tri [9,10-^{3}H] oleate (triolein) (5 mCi/ml) The Radiochemical Centre Ltd., Amersham, Bucks., U.K.

Unlabelled triolein (made from technical grade oleic acid). BDH Chemicals Ltd., Poole, Dorset, U.K.

Glycerol (Analar grade). BDH Chemicals Ltd.

Phosphatidyl choline (10 mg/ml in chloroform and 10% methanol) Type V-E from egg yolk. Sigma, Poole, Dorset, U.K.

Bovine serum albumin, fraction V. BDH Chemicals Ltd..

Tris (Tris (hydroxymethyl) aminomethane). BDH Chemicals Ltd.

Sodium chloride (Analar grade). BDH Chemicals Ltd.

Human serum prepared from a six-donor pool, heated for 60 mins. at 56°C to remove any endogenous lipase activity.

Chloroform	Analar	used in ratios 141)	Belfrage
Methanol	Analar	125)	reagent
n-Heptane	GPR	100)	

Potassium carbonate BDH Chemicals Ltd.

Cocktail T 'Scintran' BDH Chemicals Ltd.

Assay tubes: Sterilin screw-cap, glass 10 ml.

2.2.2 Methods

i) Preparation of stock substrate

Labelled triolein was diluted to 20 µCi/ml with hexane

(Analar) and stored at 4°C. Counts were checked each time before use by comparison with a ^{3}H Packard standard and by adjusting if necessary for radioactive decay. 1 ml of this diluted triolein was mixed in a sonication vial (glass, round-bottomed) with 0.7 ml phosphatidylcholine (10 mg/ml) and the solvents blown off under nitrogen. 120 mg of unlabelled triolein and 2.52 g glycerol were then added and the mixture sonicated for 5 mins. in 30 second bursts. This was done using a 3/8 inch titanium probe (with the tip placed 2 mm below the surface) set at amplitude 18 microns of a MSE 150 watt Ultrasonic Disintegrator. The vial was placed in an ice/water mixture during sonication to prevent overheating.

ii) Preparation of working substrate

Made on day of use

a) For measurement of both enzymes

0.625 g stock substrate

2.0 ml of 0 .2 M Tris/HCl buffer, pH 8.5 containing 30 mg albumin/ml (= 'low salt buffer')

0.5 ml human serum

b) For measurement of HTGL only

0.625 g stock substrate

2.0 ml of 0.2 M Tris/HCl buffer, pH 8.5, 30 mg albumin/ml, 1 M NaCl (= 'high salt buffer')

0.5 ml 1 M NaCl (in distilled water)

These mixtures were well vortexed.

Final substrate concentration

The working substrate concentrations and that in the assay based on a 30 µl enzyme volume are given in Table 1.

TABLE 1: Final substrate concentrations of the NES assay

	Working substrate		In assay (30 µl sample volume)	
Unlabelled triolein	10.7 mmoles/ml (9.5 mg/ml)		9.3 mmoles/ml (8.3 mg/ml)	
Labelled triolein	3.4 nmoles/ml (1.7 µCi/ml)		3.0 nmoles/ml (1.5 µCi/ml)	
Glycerol	0.21 g/ml		0.19 g/ml	
Phosphatidyl-choline	0.58 mg/ml		0.5 mg/ml	
Tris	0.13 M		0.11 M	
Albumin	20 mg/ml		17.4 mg/ml	
	Low salt	High salt	Low salt	High salt
Serum	0.17 µl/ml	-	0.15 µl/ml	-
Sodium chloride	-	0.83 M	-	0.77 M

iii) Assay procedure

200 µl of working substrate were dispensed with Finn automatic pipettes into glass tubes, and pre-incubated for 10 mins. in a water-bath at 37°C. All samples were tested in duplicate. The assay was started by the addition of 30 µl of enzyme, or buffer (for blank) with mixing at staggered intervals. The reaction was stopped after 10 mins. by the addition of 3.25 ml of Belfrage reagent, and 1.0 ml of 1.0 M potassium carbonate (pH 10.5) using 'Zippette' automatic dispensers (Jencons). The contents of the tubes were well-mixed on a vortex mixer then centrifuged at 3,000 g for 15 mins. 1 ml of the upper methanol/water phase was aliquoted into scintillation vials, and 10 ml of scintillation fluid added. The radioactivity was counted on a Packard Tri-Carb Liquid Scintillation Counter 3255 for 10 mins. Aliquots (50 µl) of each of the working substrates were also counted for radioactivity to enable calculation of specific activities. To copy the quenching characteristics of the counted samples, these were added with 0.95 ml of blank upper layer (no enzyme, no substrate) of Belfrage reagent (3.25 ml) and potassium carbonate (1.0 ml).

Calculation of enzymic activity

The following have to be taken into account:

1. Blank values
2. Enzyme volume (x µl)
3. Adjustment for % extraction of FFA (76%)
4. Total volume of upper layer (2.45 ml)
5. Incubation time (minutes)
6. Conversion to nanomoles
7. Specific activity of working substrate (y = cpm in 50 µl)

8. Molar ratio of triglyceride to FFA

$$mU/ml = \text{net cpm} \times \frac{1000\mu l}{x\ \mu l} \times \frac{100}{76} \times 2.45 \times \frac{1}{n\ \text{mins}} \times 1000 \times \frac{1.617\mu mol\ FFA\ x3}{y}$$

= nanomoles oleic acid released $minute^{-1}$ ml^{-1}

2.2.3 Results and Comments

Test of assay parameters and possible improvements

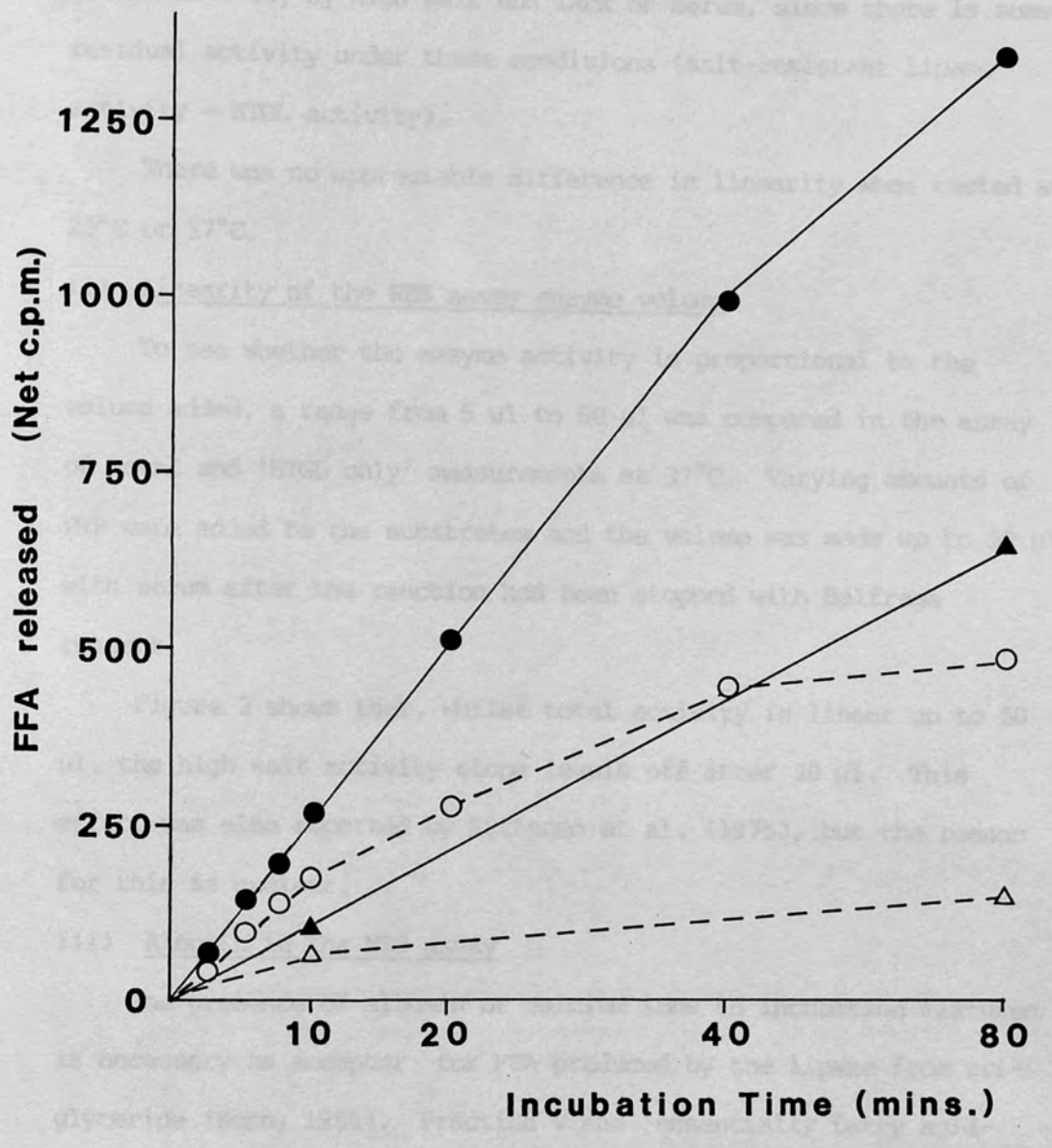
i) Linearity of the NES assay with incubation time

Nilsson-Ehle and Schotz report the measured enzyme activity to be linear with time for up to 120 mins. However, their tests were done on enzyme extracts of acetone powders from rat adipose tissue, and the authors suggest that shorter incubation times are used when PHPs of higher activity are assayed.

Tests of linearity were carried out on human PHP using both low and high salt substrates. A rabbit anti-human HTGL serum which binds and inactivates the HTGL was also used (kindly donated by Dr. Bengtsson, University of Umeå, Sweden). PHP and anti-serum were mixed in a ratio of 4:1 and left for two hours at 4°C before assaying.

Linearity of activity was measured under four sets of conditions: with or without anti-HTGL serum, and with using low or high salt substrates. The results in Figure 1 show that whilst 'total' and 'LPL only' activities are linear up to 40 mins., the 'HTGL' is linear only up to 10-15 mins. Possible reasons for the non-linearity were tested by the addition after 45 mins. of further enzyme, albumin (to bind FFA which may be causing product inhibition) or substrate. None of these additions led to the resumption of a linear reaction rate. Subsequent assays were run for 10 mins.

Fig 1 Time course of the NES assay



● Total lipase
▲ LPL only (using anti-HTGL serum) } *

○ 'HTGL' (total salt-resistant lipase, SRL)
△ Total SRL - HTGL (using anti-HTGL serum) } **

* Measured in low salt substrate

** Measured in high salt substrate

These results also show that there is not complete inhibition of LPL activity by high salt and lack of serum, since there is some residual activity under these conditions (salt-resistant lipase activity - HTGL activity).

There was no appreciable difference in linearity when tested at 25°C or 37°C.

ii) Linearity of the NEFS assay enzyme volume

To see whether the enzyme activity is proportional to the volume added, a range from 5 µl to 50 µl was compared in the assay of total and 'HTGL only' measurements at 37°C. Varying amounts of PHP were added to the substrates and the volume was made up to 50 µl with serum after the reaction had been stopped with Belfrage reagent.

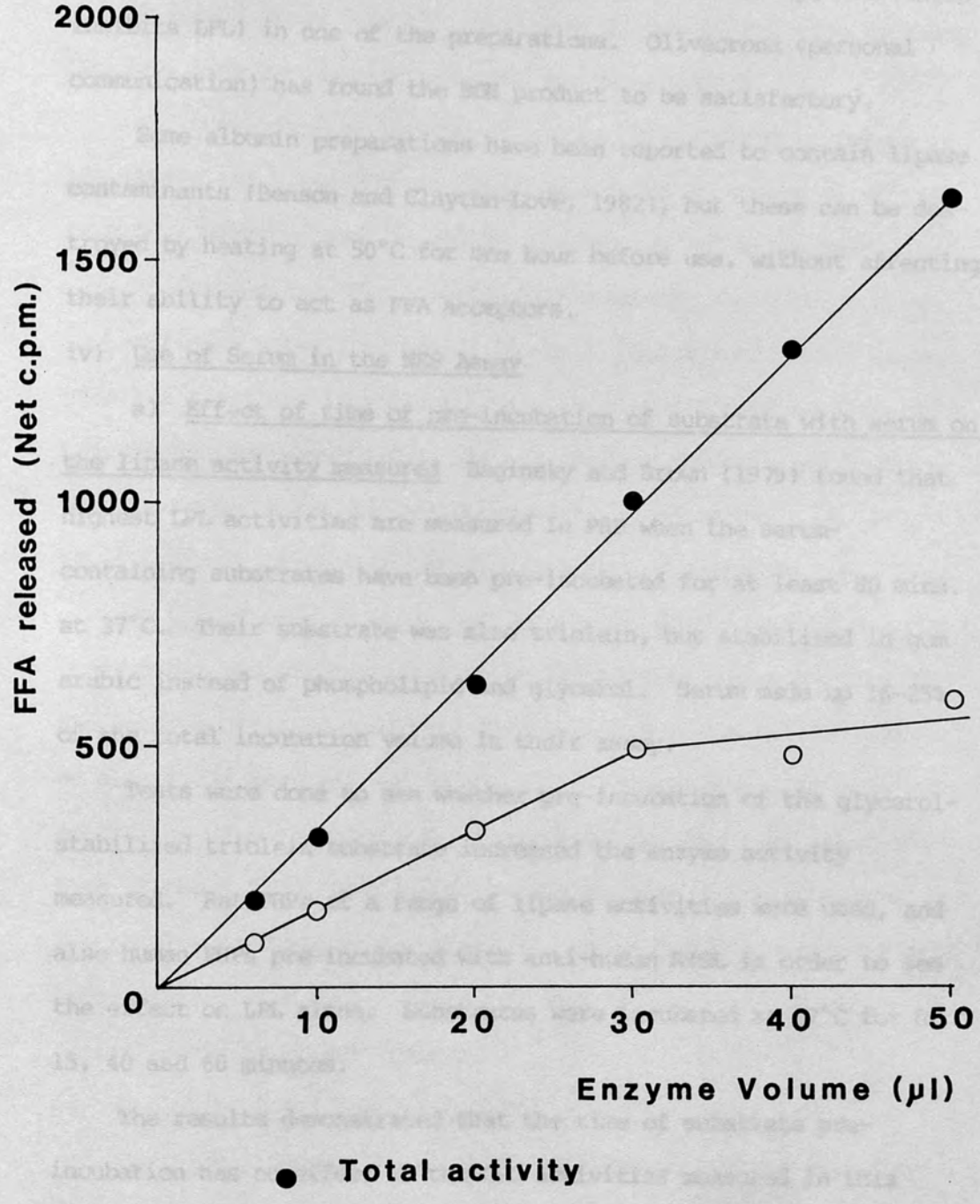
Figure 2 shows that, whilst total activity is linear up to 50 µl, the high salt activity slope levels off after 30 µl. This effect was also reported by Huttenen et al. (1975), but the reason for this is unclear.

iii) Albumin in the NEFS assay

The presence of albumin or calcium ions in incubation mixtures is necessary as acceptor for FFA produced by the lipase from triglyceride (Korn, 1955). Fraction V and 'essentially fatty acid-free' grades of albumin (both from BDH Chemicals Ltd.) were compared for use in the assay, but no differences were found, either in the blank volume of the substrates or in the enzyme activities achieved. (Results not shown).

Eisenberg et al. (1981) have reported differences in lipase activities measured when albumins from different suppliers (of the same grade) are used in lipase assays. They explain the discrepancy

Fig 2 Effect of enzyme volume in the NES assay



as being due to the presence of contaminants such as apo A-I (which inhibits LPL) in one of the preparations. Olivecrona (personal communication) has found the BDH product to be satisfactory.

Some albumin preparations have been reported to contain lipase contaminants (Benson and Clayton-Love, 1982), but these can be destroyed by heating at 50°C for one hour before use, without affecting their ability to act as FFA acceptors.

iv) Use of Serum in the NES Assay

a) Effect of time of pre-incubation of substrate with serum on the lipase activity measured Baginsky and Brown (1979) found that highest LPL activities are measured in PHP when the serum-containing substrates have been pre-incubated for at least 80 mins. at 37°C. Their substrate was also triolein, but stabilized in gum arabic instead of phospholipid and glycerol. Serum made up 16-25% of the total incubation volume in their assay.

Tests were done to see whether pre-incubation of the glycerol-stabilized triolein substrate increased the enzyme activity measured. Rat PHPs of a range of lipase activities were used, and also human PHPs pre-incubated with anti-human HTGL in order to see the effect on LPL alone. Substrates were incubated at 37°C for 0, 15, 40 and 60 minutes.

The results demonstrated that the time of substrate pre-incubation has no effect on the LPL activities measured in this assay (results not shown).

b) Test of optimal amount of serum to be used in the substrate Baginsky (1981) reports that the level of serum needed in the low salt substrate for maximal stimulation of LPL varies with the serum batch. To test this two PHPs were assayed in substrates

with three serum batches at four different concentrations.

Figure 3 shows that the pattern of lipase stimulation by serum can vary with serum batch and with the PHP assayed.

c) Comparison of the use of rat and human serum in assays of LPL in rat and human PHP Rat and human serum as source of apoprotein C-II were compared in assays of both rat and human PHPs.

In testing rat PHPs, substrates containing human serum gave higher lipase activities at all serum concentrations. With human PHPs the trend was less clear. One PHP tested showed no difference between rat and human serum, whereas another showed a 20% increase when using rat serum (at all concentrations of serum).

v) The Effect of Salt on NES Lipase Assay Measurements

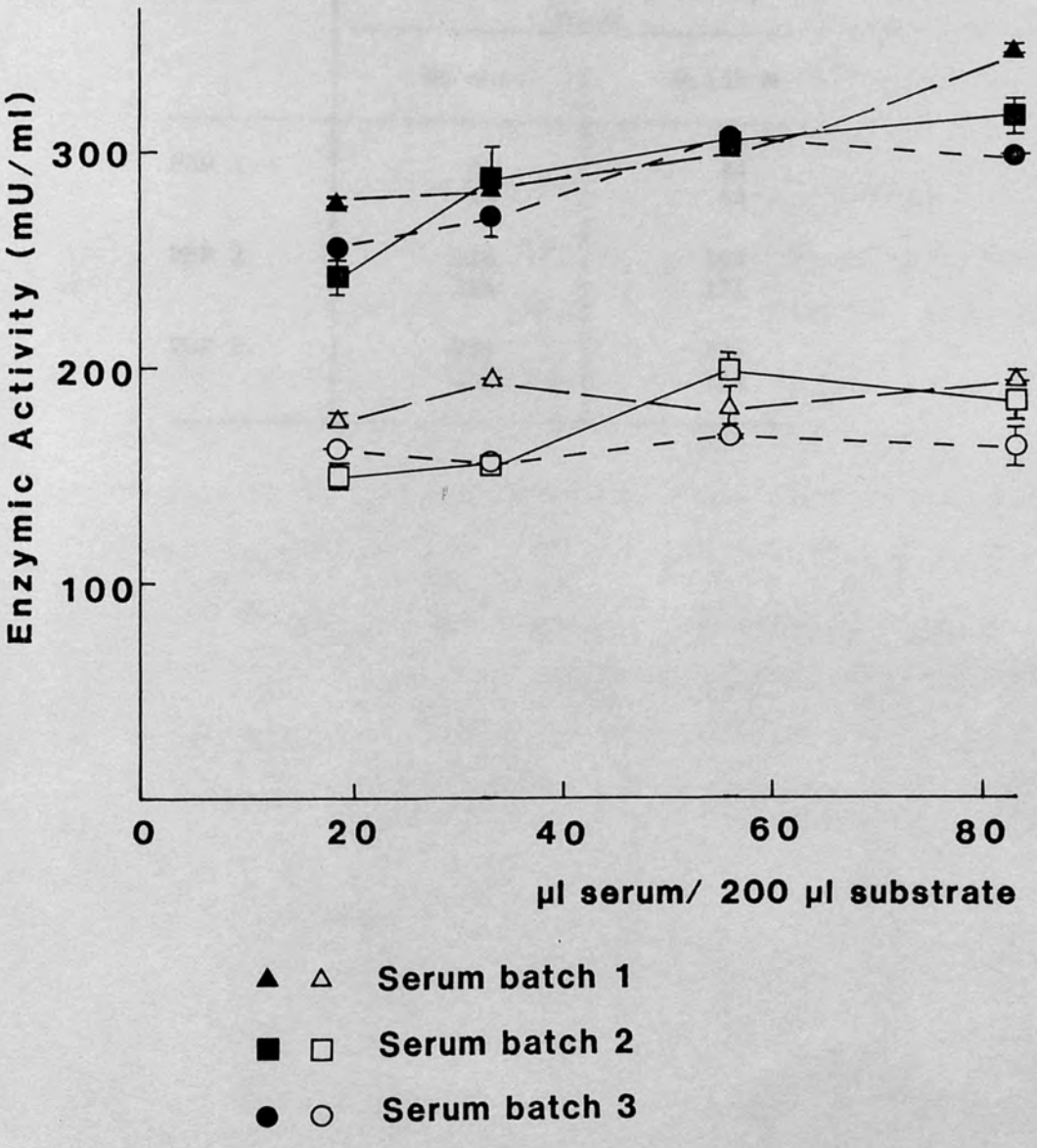
a) Use of physiological salt concentrations Several methods suggest the use of physiological (0.145 M) salt concentrations in the serum-containing substrate for maximal activity of LPL (Krauss et al., 1973; Baginsky and Brown, 1977). Measurements at this concentration were compared with those using no added salt other than that of the 0.2 M Tris-HCl buffer used to make up the substrate. The results in Table 2 show that in this emulsion, increase of salt concentration with NaCl to 0.145 M leads to a slight inhibition of 'total' activity of PHP samples.

b) Use of high salt concentrations

Purified lipases LPL and HTGL purified by heparin-Sepharose chromatography (see Chapter 3) were assayed in 'low salt' (0.1 M Tris-HCl buffer) and 'high salt' (1.0 M NaCl in 0.2 M Tris-HCl buffer) substrates, both in the presence and absence of serum.

The results in Table 3 clearly show the inhibitory effect of high salt on serum-stimulated LPL, although there is little effect

Fig 3 Effect of serum batch and volume on the enzymic activity measured in the NES assay



(T =37°C)

TABLE 2: Effect of physiological salt concentration on the enzymic activity of PHP

	Total enzymic activity mU/ml	
	No salt	0.145 M
PHP 1	51	44
	49	44
PHP 2	186	164
	186	171
PHP 3	234	225
	231	214

TABLE 3: Effect of salt concentration on the enzymic activity of LPL and HTGL in the presence and absence of serum using the NES assay

	Enzymic activity (mU/ml)			
	Low salt		High salt	
	No serum	With serum	No serum	With serum
LPL	42.3	126.1	46.3	39.8
HTGL	83.3	67.7	133.6	27.9

in the absence of serum. HTGL is inhibited by serum, especially at 1.0 M NaCl. HTGL has the highest activity at high salt in the absence of serum, and LPL at low salt in the presence of serum.

PHP A range of salt concentrations (0 - 1.2 M NaCl) was used to make up the substrates, and to assay whole PHP lipase activity in the absence and presence of serum. The results (see Table 4) show that at salt concentrations below 1.0 M there is little effect of added serum on the enzymic activity measured of PHP. At higher concentrations where the LPL would be inhibited the activity is generally higher in the absence of added serum.

Comparison of the results of Tables 3 and 4 emphasizes that the effect of conditions observed with purified enzymes does not necessarily apply also for PHP samples.

vi) Use of Sodium Dodecyl Sulphate in Selective Measurement of Lipase Enzymes

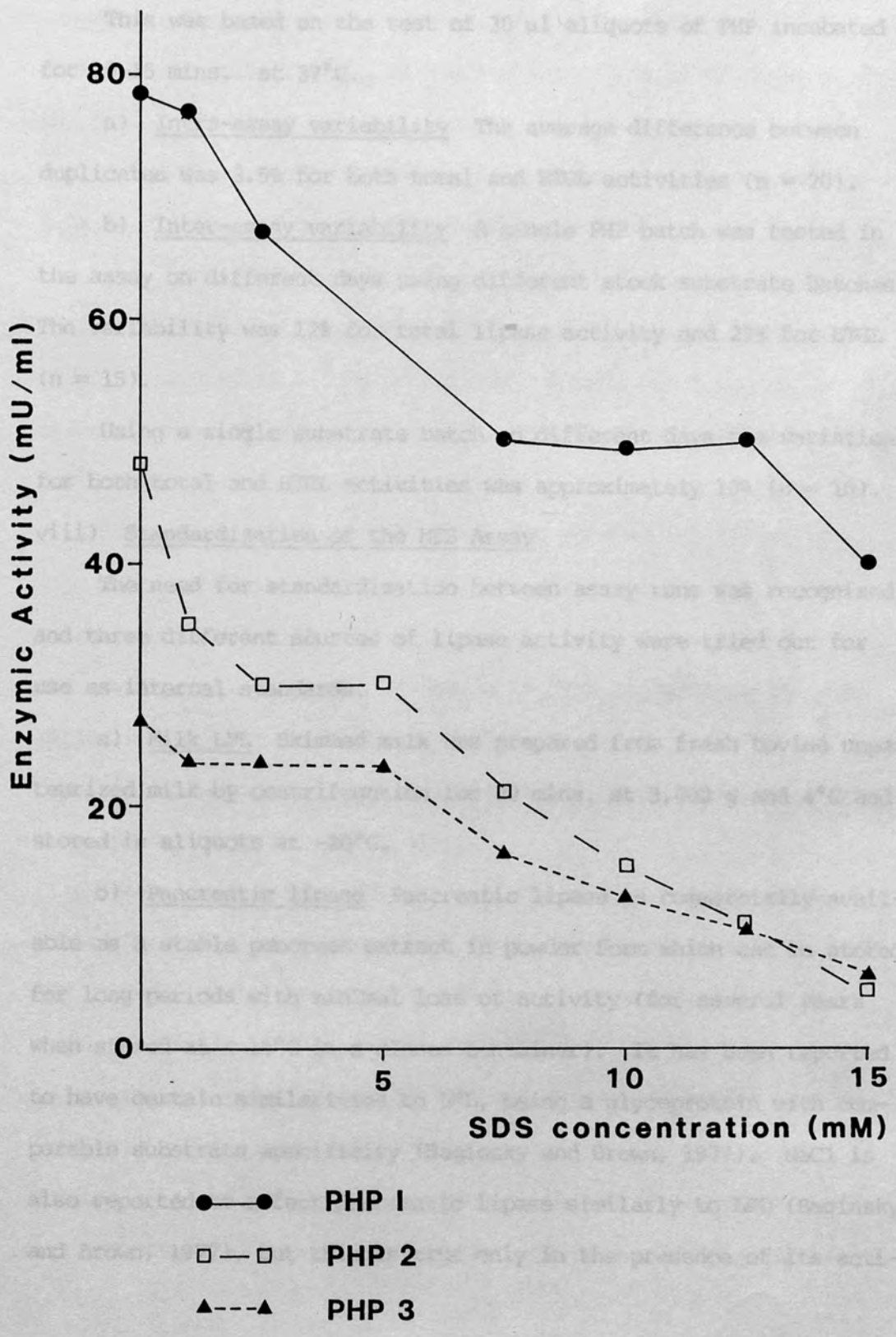
The method reported by Baginsky (1981) aims to selectively inhibit HTGL activity in PHP with SDS, to allow direct measurement of LPL alone. A one-step adaptation (i.e. without pre-incubation of the enzyme sample with SDS) was tried. This involved the use of buffers containing a range of SDS (BDH Chemicals Ltd.) concentrations to make up a series of substrates.

The results in Figure 4 show the action of SDS on three PHPs in low salt substrates containing serum. The results seem to suggest that the higher the activity of the PHP the more SDS is needed to inhibit the HTGL, and that going much above this level leads to inhibition of the LPL also. This means that the approximate level of activity would have to be known before selecting the SDS concentration to use.

TABLE 4: Effect of salt concentration on lipase activities measured by the NES assay in the presence and absence of serum

	NaCl concentration (M)					
	0	0.2	0.5	0.75	1.0	1.2
PHP 1 + serum	240	215	181	73	66	66
- serum	226	209	173	73	85	94
PHP 2 + serum	31.1	25.7	21.7	13.5	13.0	15.7
- serum	27.5	30.9	17.0	11.4	15.2	15.6
PHP 3 + serum	181				39	
- serum	177				50	

Fig 4 Effect of SDS on lipase measurement of PHP by the NES assay



Better results were not achieved by pre-incubation of the PHP sample with SDS (results not shown).

vii) Test of Variability of the NES Assay

This was based on the test of 30 µl aliquots of PHP incubated for 10-15 mins. at 37°C.

a) Intra-assay variability The average difference between duplicates was 3.5% for both total and HTGL activities (n = 20).

b) Inter-assay variability A single PHP batch was tested in the assay on different days using different stock substrate batches. The variability was 12% for total lipase activity and 23% for HTGL (n = 15).

Using a single substrate batch on different days the variation for both total and HTGL activities was approximately 10% (n = 10).

viii) Standardization of the NES Assay

The need for standardization between assay runs was recognised and three different sources of lipase activity were tried out for use as internal standards.

a) Milk LPL Skimmed milk was prepared from fresh bovine unpasteurized milk by centrifugation for 20 mins. at 3,000 g and 4°C and stored in aliquots at -20°C.

b) Pancreatic lipase Pancreatic lipase is commercially available as a stable pancreas extract in powder form which can be stored for long periods with minimal loss of activity (for several years when stored at < 15°C in a closed container). It has been reported to have certain similarities to LPL, being a glycoprotein with comparable substrate specificity (Baginsky and Brown, 1977). NaCl is also reported to affect pancreatic lipase similarly to LPL (Baginsky and Brown, 1977), but this is true only in the presence of its acti-

vator 'co-lipase', a low MW polypeptide thought to be analagous to the apo CII of LPL (Bengtsson and Olivecrona, 1982b).

Solutions of Pancreatin (Pancrex V Powder, Paines & Byrne Ltd., Greenford, England) were prepared in 0.2 M Tris/HCl buffer (pH 8.2), of a concentration range 0.01 - 0.5 mg/ml. Initial dispersion of the powder was aided by sonication in an ultrasonic bath for 2-3 mins. Solutions could be made up with reasonable reproducibility (± 4.6%, n = 4 assayed on the same day).

c) Human PHP A human volunteer was injected i.v. with 1,000 u heparin (Pularin, Evans Medical), blood taken after 10 mins. and mixed 9:1 with 3.8% trisodium citrate. Plasma was prepared by centrifugation at 38,000 g for 10 mins. at 4°C, snap-frozen in aliquots and thawed immediately before assaying.

Skimmed milk lipase could generally be used to correct total lipase values between assays with good agreement (± 15%). However, since milk contains no HTGL it could not be used for adjustment of the levels of this enzyme. There is no firm evidence on the similarity under assay conditions of LPL preparations from PHP and milk and this leads to doubts on the reliability of using the milk enzyme as a standard for the PHP activity.

There is an obvious advantage of using aliquots from a single pooled batch (as in milk and PHP) where the enzyme is stored fresh and thawed just before use. The enzymes have been reported to be stable for at least six months when stored at -20°C (Krauss et al., 1973; Huttenen et al., 1975). Although pancreatic lipase is very stable in powder form, the manufacturers recommend that fresh solutions be made up every day. The stability of frozen aliquots was not investigated. A linear relationship was found to exist

between the concentration and lipase activity of Pancreatin within the range 0.025 - 0.25 mg/ml, which would cover the range of lipase activities found in PHP samples (this could not be done with skimmed milk or PHP where dilution led to significant non-linearity as described in Ch. 5). However, despite this advantage of Pancreatin, it was not found to be as reliable as a standard normal pooled PHP, presumably because the enzymes were not affected in the same way by variations in conditions.

An average value for the control PHP used was obtained by repeat assays in low and high salt substrates. It was found suitable for correcting lipase levels of the experimental PHP samples to within 10%. Subsequent to this, a PHP was used as an internal standard in all assays.

ix) Effect of Purity of Substrate on the Enzymic Activity Measured

a) Test of substrate purity

The purities of the radiolabelled and unlabelled triolein used to make up the substrate for the assay were checked using GC (gas chromatography) and reverse phase HPLC (high pressure liquid chromatography). This was done primarily as a check of the radioactive triolein, which by this time had been stored for two years, and may have contained significant amounts of impurities not present in the unlabelled triolein (which is used greatly in excess for the substrate) with possible effects on the enzyme activity. GC is a standard method of separating fatty acid mixtures. It is sensitive and allows the separation of a small amount of material with good accuracy. This was done with the help of Dr. C. Jones and Mrs. A. Wilson (Chemistry Section, NIBSC). HPLC is a rapid separation method and has the advantage that, as in conventional chromato-

graphy, fractions can be easily collected.

The system used was suggested by Dr. P. Corran (Chemistry Section, NIBSC) and run with his help. The methods of GC and HPLC used are given in Appendix II.

The unlabelled triolein had two main peaks by HPLC (see Fig. 5) and these were shown by GC to be the oleic (80%) and the palmitic (12%) glycerol esters (see Table 5). More than 95% of the radioactivity of the labelled triolein was associated with a peak that coincided with the major peak of the 'cold' triolein by HPLC (see Fig. 5). This suggests that there had not been a substantial amount of deterioration in the labelled substrate in terms of the position of the radiolabel (Table 5).

b) Comparison of the use of '99% pure' and technical grade triolein in the NES assay

Substrate batches were prepared using '99% pure' triolein (Sigma) and the values measured in the assay compared with those using technical grade substrate, testing the same PHP under the same conditions.

The levels achieved with the pure triolein were about twice as high as those using technical grade substrate for total lipase, and three times higher for HTGL (Table 6). In the original report on this assay (Nilsson-Ehle and Schotz, 1976), technical grade was used, but some other workers since have used purer substrates.

Further work could be done to compare the composition of various TG preparations and the activity measured with their use.

Fig 5 HPLC of technical grade triolein

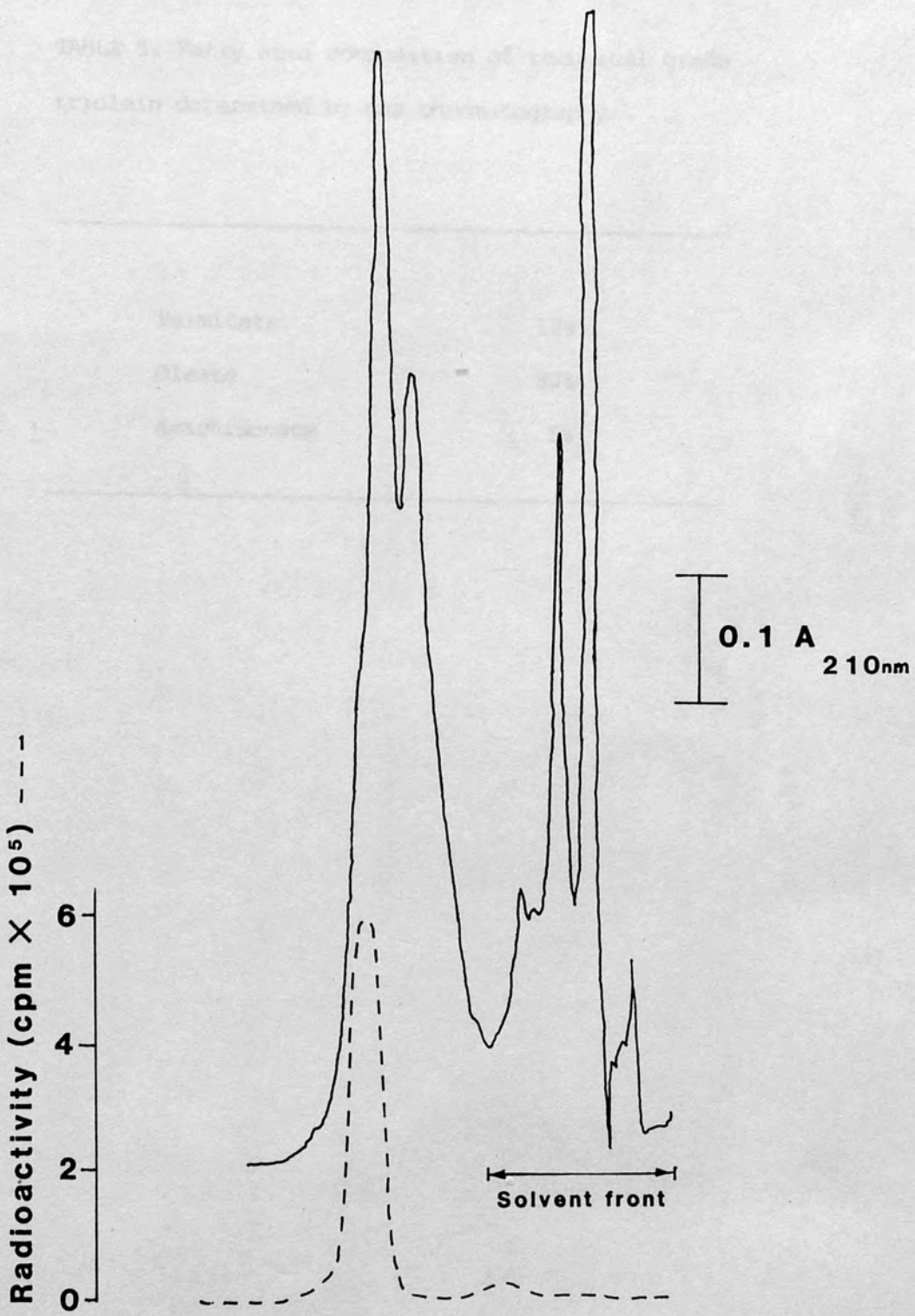


TABLE 5: Fatty acid composition of technical grade triolein determined by gas chromatography

Palmitate	12%
Oleate	80%
Arachidonate	5%

TABLE 6: Effect of triolein purity on enzymic activity measured in the NES assay

Triolein used in substrate	Enzymic activity (mU/ml)		
	Total lipase	HTGL only	% HTGL of total
Technical substrate			
Batch I	237	124	55%
Batch II	223	121	54%
99% pure	521	462	89%

2.3 ASSAY NO. 2: The Gum-Arabic Assay

Gum arabic-stabilized TG emulsions have been used in lipase assays by many groups of workers (Huttenen et al., 1975; Baginsky and Brown, 1977; Havel et al., 1973; Hernall et al., 1975). Like the NES assay, the incorporation of radioactive triolein allows many samples to be tested in one run making it a convenient assay, and differential use of salt and serum in the substrates may allow selective measurements of LPL and HTGL in PHP samples (Havel et al., 1975). However, some non-linearity with respect to time and volume has been found (Huttenen et al., 1975; Goldberg et al., 1983) and it is reported to be a poor substrate for purified LPL (Havel et al., 1973), although it is a good one for HTGL and it is less sensitive to other plasma proteins (especially lipoproteins) (Olivecrona, personal communication). Preliminary tests were done using this assay to investigate its usefulness in testing PHP as a possible replacement for the NES assay. The particular assay method used was suggested by Dr. Olivecrona (Umeå, Sweden).

2.3.1 Materials

Glycerol tri [9, 10-^{3}H] oleate)
Bovine serum albumin, fraction V)
Tris) As for NES assay
Human serum)
Sodium chloride)

Gum arabic, BDH Chemicals Ltd.

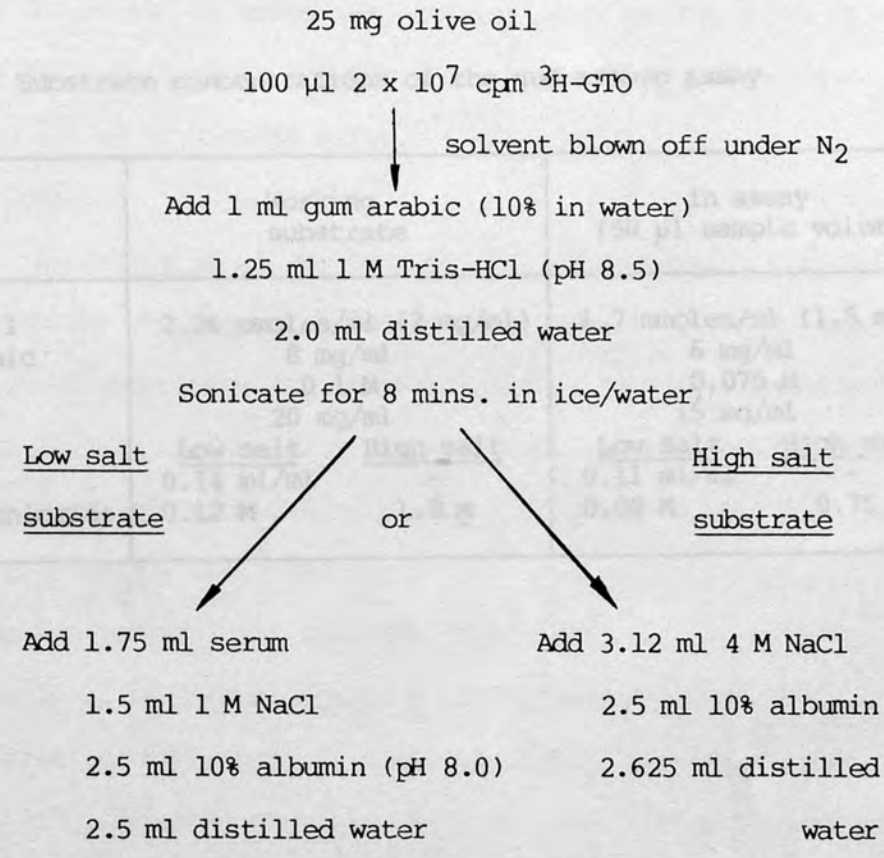
Olive oil, local supplier

2.3.2 Methods

i) Preparation of substrate

The emulsion has to be prepared daily with sonication using

fresh gum arabic solutions. A batch of albumin solution is made up and frozen in aliquots.



Substrate concentrations The working substrate concentrations and assay concentrations based on a 50 µl enzyme volume are given in Table 7.

ii) Assay procedure

150 µl of the appropriate substrate were dispensed per tube, and preincubated at 25°C or 37°C for 15 mins. 15 µl enzyme were added at zero time and the reaction stopped, the FFA extracted and the radioactivity counted as in the NES assay.

Calculation of enzymic activity The olive oil used as source of triglyceride in the substrate contains mixed triglycerides, the fatty acids of which are mainly oleic acid (approx. 85%) (Merkx Index, 1976), but for the purposes of calculation (relating labelled

TABLE 7: Substrate concentrations of the gum arabic assay

	Working substrate		In assay (50 µl sample volume)	
Olive oil	2.26 mmoles/ml (2 mg/ml)		1.7 mmoles/ml (1.5 mg/ml)	
Gum arabic	8 mg/ml		6 mg/ml	
Tris	0.1 M		0.075 M	
Albumin	20 mg/ml		15 mg/ml	
	Low salt	High salt	Low salt	High salt
Serum	0.14 ml/ml	-	0.11 ml/ml	-
Sodium chloride	0.12 M	1.0 M	0.09 M	0.75 M

to unlabelled TG) 100% oleic acid was assumed. On this basis, 50 µl of working substrate contains 0.33 µmoles of oleic acid. Further factors which must be taken into account, such as the % FFA extraction by Belfrage reagent and the total upper layer volume, are adjusted for as in the NES assay.

2.3.3 Results

i) Linearity of enzyme activity measurements with time

Human PHP samples were assayed in 'low salt' (0.15 M) and high salt (1.0 M) substrates for incubation times ranging from 10 - 80 mins. at 25°C and 37°C. Anti-serum to HTGL was also used in tests to allow measurement of LPL only after incubation with PHP for 2 hours (anti-serum: PHP, 1:8).

The non-linearity of the HTGL (high salt) measurements is immediately obvious from Figures 6 and 7, especially at 37°C. The FFA released by HTGL after 10 mins. incubation are lower at 37°C than at 25°C, and this suggests that the inability of the enzyme to work on the substrate (whatever the cause) develops progressively. It would not appear to be due solely to inactivation of the enzyme, since further addition of fresh enzyme after 40 mins. incubation did not result in further hydrolysis. This would suggest that the active enzyme is in some way prevented from interacting with the substrate.

Total and LPL activities (measured in low salt substrates) were generally linear with time at least up to 40 mins. at 25°C and to 20 mins. at 37°C (Figs. 6 and 7). No LPL is measurable in the high salt substrate as shown by the use of anti-HTGL serum.

The difference in suitability of the two substrates for LPL and HTGL is shown most clearly at 25°C (Fig. 7) where HTGL activity is

Fig 6 Time course of the gum arabic assay at 37°C

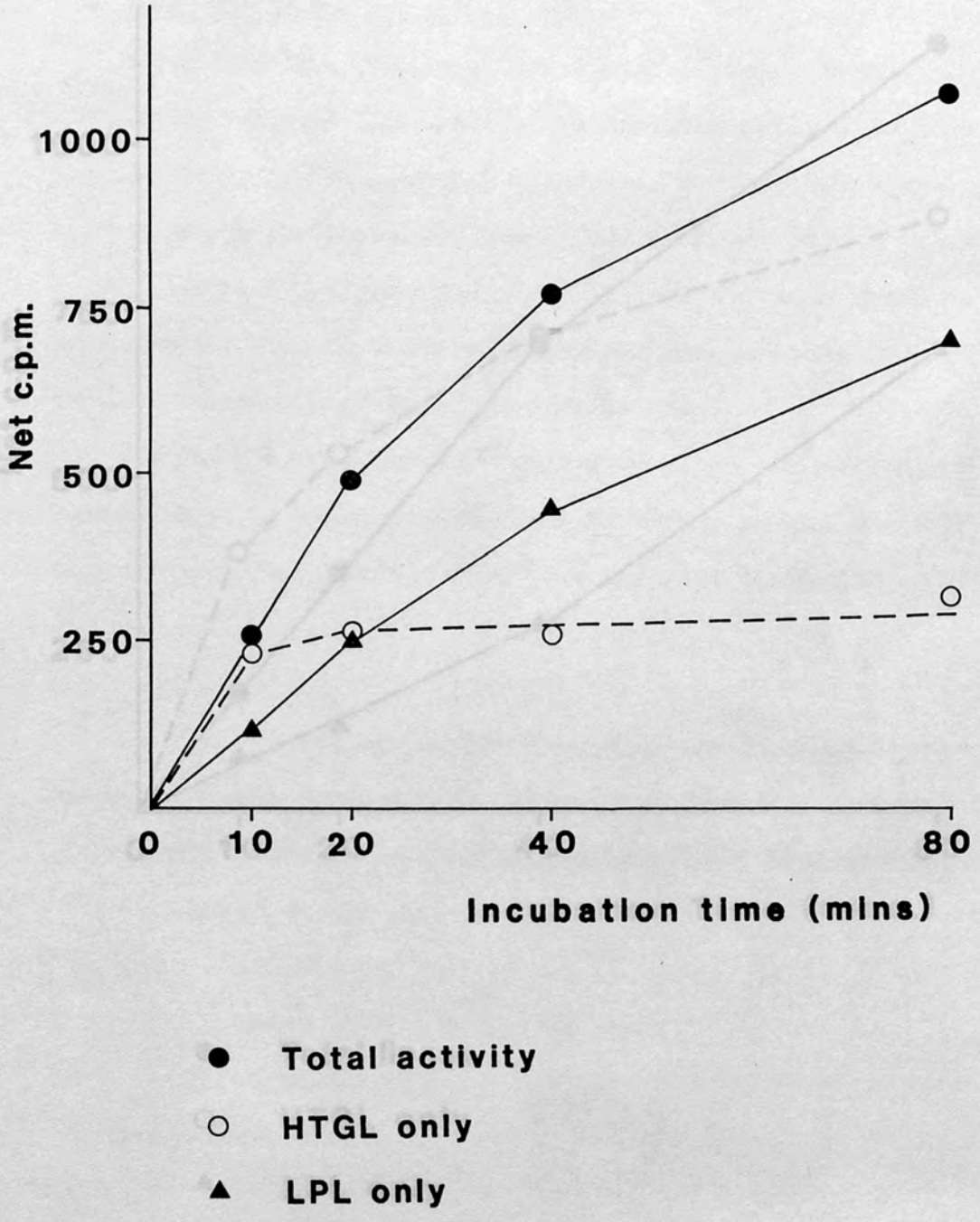
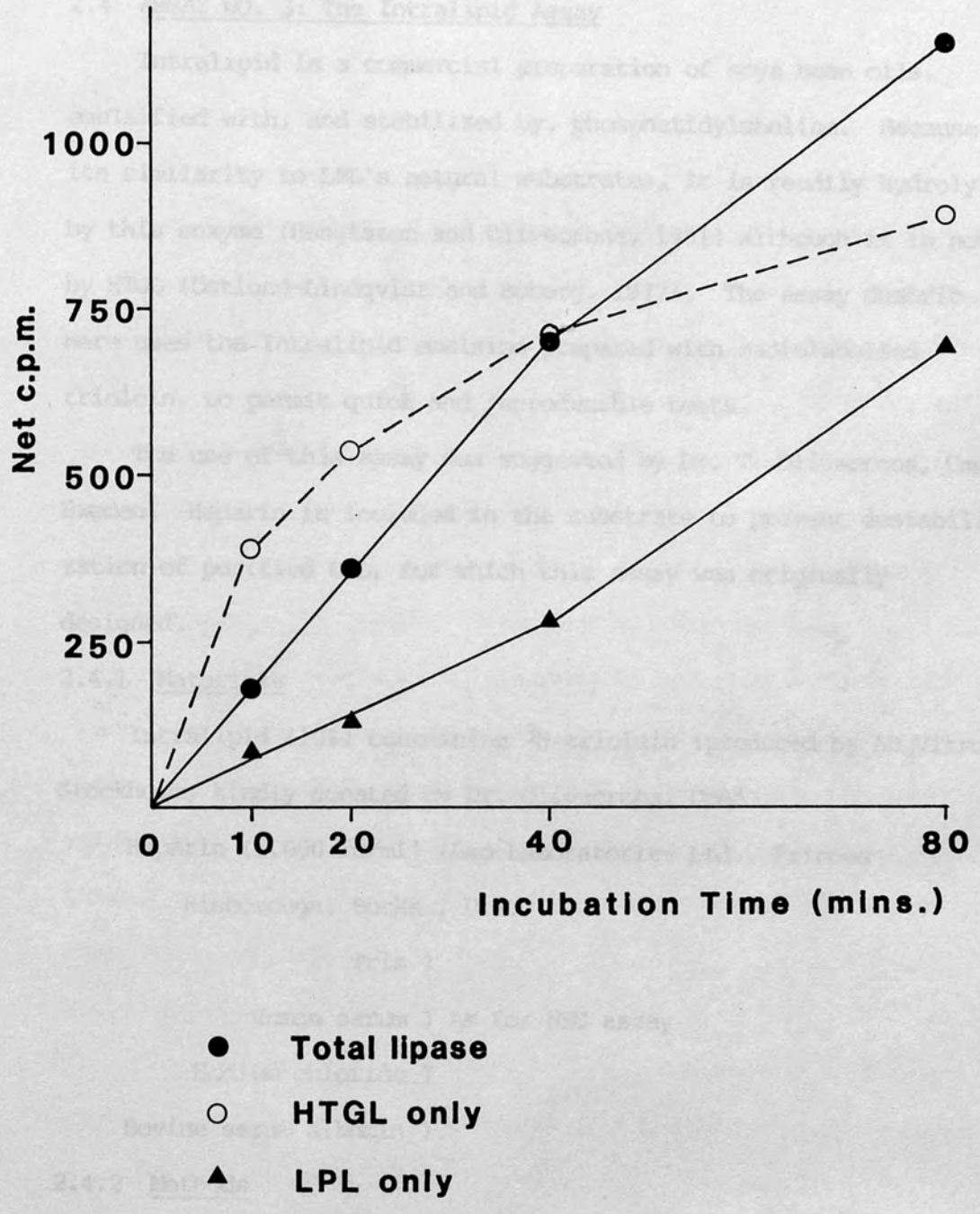


Fig 7 Time Course of the gum arabic assay at 25°C



greater than total, up to incubation times of 40 mins.

The overall level of PHP activities measured by this assay was of the same order as those calculated from use of the NES assay.

2.4 ASSAY NO. 3: The Intralipid Assay

Intralipid is a commercial preparation of soya bean oils, emulsified with, and stabilized by, phosphatidylcholine. Because of its similarity to LPL's natural substrates, it is readily hydrolyzed by this enzyme (Bengtsson and Olivecrona, 1981) although it is not by HTGL (Östlund-Lindqvist and Boberg, 1977). The assay described here uses the Intralipid emulsion prepared with radiolabelled triolein, to permit quick and reproducible tests.

The use of this assay was suggested by Dr. T. Olivecrona, Umeå, Sweden. Heparin is included in the substrate to prevent destabilization of purified LPL, for which this assay was originally designed.

2.4.1 Materials

Intralipid (10%) containing ^{3}H-triolein (produced by AB Vitrum, Stockholm, kindly donated by Dr. Olivecrona, Umeå).

Heparin (5,000 iu/ml) (Leo Laboratories Ltd., Princes Risborough, Bucks., U.K.)

Tris)
Human serum) As for NES assay
Sodium chloride)
Bovine serum albumin)

2.4.2 Methods

i) Preparation of substrate

The substrate is prepared by thorough vortex mixing of the following:

160 µl ^{3}H-Intralipid

100 µl heparin (5,000 iu/ml)

100 µl 1 M Tris/HCl buffer pH 8.5

400 µl 1 M NaCl

40 µl distilled water

1000 µl serum

ii) Assay procedure

90 µl of the above substrate per tube are pre-incubated at 25°C or 37°C for 10 mins. and 60 µl of 20% albumin (w/v in 0.5 M Tris-HCl buffer, pH 8.5) added immediately prior to the addition of 50 µl enzyme at zero time. The reaction is stopped, the FFA extracted and the radioactivity counted as described for the NES assay.

Substrate concentrations The final concentrations in the assay substrate using an enzyme volume of 50 µl are given in Table 8.

Calculation of results Soya bean oil used in the Intralipid substrate contains a mixture of saturated and unsaturated fatty acids, the major ones of which are given as linolenic acid (51%), oleic acid (29%) and palmitic acid (10%) (Merkx Index, 1976). A weighted average triglyceride MW was calculated on the basis of these figures, and used in the conversion of radioactive counts to mU/ml as in the NES assay.

2.4.3 Results

Linearity of the assay The assay was linear for incubation times at least up to 60 mins. and with a volume range up to 50 µl (results not shown).

Calculated values of PHP activities using this assay were 3 - 4 times higher than those from the NES or gum arabic assays.

TABLE 8: Final substrate concentrations of the Intralipid assay

Intralipid	
Soya bean oils	4 mg/ml
Fractionated egg phospholipids	0.48 mg/ml
Glycerol	0.88 mg/ml
Tris	0.17 M
Albumin	60 mg/ml
Serum	0.25 ml/ml
Sodium chloride	0.1 M
Heparin	125 u/ml

2.5 DISCUSSION

The range and variety of the lipase assays used by different workers suggest the difficulties in finding a satisfactory method. The work described in this Chapter has mainly been concerned with ways of improving the reliability and reproducibility of a modified NES assay. An adaptation to allow selective measurement of LPL and HTGL was incorporated, using substrate preparations containing either serum or a high concentration of salt. Another assay investigated used a gum arabic-stabilized TG emulsion and the same method of distinguishing between the two enzymes. With a third assay using the commercial substrate, Intralipid, no such differentation was employed, since inclusion of salt produces only little inhibition of LPL activity with this substrate (Bengtsson and Olivecrona, 1983), and it was reported to be hydrolysed much more readily by LPL than by HTGL (Olivecrona, personal communication).

Measurements of 'total' or 'LPL only' activities did not deviate from linearity with respect to both time and sample volume in all three assays (up to 40 mins. and 50 µl of PHP (300 mU/ml)). However, results for 'HTGL activity' (in high salt and the absence of serum) in both the NES and gum arabic assays were significantly non-linear with respect to incubation time and sample volume, especially the gum arabic assay. Various means of overcoming the reduction in enzymic activity were not successful, so the assay incubation time and enzyme volume were reduced to within the linear range in the NES assay, and tests on other parameters of this assay were continued. However, linearity of response was so poor in the gum arabic assay that its use was discontinued. The Intralipid assay gave measured activities 3 - 4 times higher than those from the

other assays tried, as was found by Riley and Robinson (1974) but, as stated, it cannot be reliably used for assessment of the proportion of LPL and HTGL in PHP samples.

The variability between NES assay runs was reduced by use of a pooled PHP as an internal standard. Assays of this nature are sensitive to slight variations in procedure, especially in the preparation of the substrate emulsion. The degree of sonication used has to be constant since in altering the emulsion particle size, binding of the enzymes (and apo CII) and so the measured activity are also altered. LPL measurement is reported to be especially sensitive to sonication changes (Huttenen et al., 1975).

The scatter of 'total lipase' values obtained using varying amounts of different serum batches in the low salt substrate, gives an indication of the complicated activation/inhibition effects operating on the enzymes in this measurement. This is obviously a source of inter-assay variability, especially of higher activity PHPs (see Section 2.2.3, iv), b) of this Chapter). Even greater differences were found however, by Baginsky (1981) using a gum arabic-stabilized substrate. As a consequence, fresh serum batches were compared with the current batch to check for major variations.

Pre-incubation (other than for temperature equilibration) was found to be unnecessary for the serum-containing substrate of the NES assay. In other assay systems (Korn and Quigley, 1957; Baginsky and Brown, 1979) it was necessary to pre-incubate the substrate with the serum prior to addition of the enzyme, to allow incorporation of apo CII into the emulsion. Apo CII is reported to be taken up readily by phospholipid-containing emulsion since these resemble VLDL and CMs in structure (Bengtsson and Olivecrona, 1981b).

Different workers have used substrates of different grades of purity - ranging from chromatographically pure TGs (for example Baginsky, 1981) to relatively crude mixtures such as olive oil and soya bean oils. Results given in 2.2.3, ix) of this Chapter comparing the use of substrates prepared with '99% pure' and technical grade triolein show the great effect this can have on the activity measured. There have been reports that the chain length and degree of saturation of the fatty acid acyl chains affect the hydrolysis of TGs by both LPL (Demel et al., 1984) and HTGL (Hülsmann et al., 1980). Since oleic acid and its main contaminant in technical grade triolein, palmitic acid, differ only by two carbons in chain length the effect may perhaps be explained in terms of the degree of saturation. Packing of TG emulsions are 'looser' with unsaturated fatty acid chains than with saturated chains, so hydrolysis by lipase of the former is quicker since the lipid is more accessible (Demel et al., 1984). This could account for the lower values obtained with the technical grade triolein, although the presence of inhibitory contaminants cannot be discounted.

The differential use of serum and salt in the two substrates to distinguish LPL from HTGL is not totally reliable. This was shown by assaying both purified enzymes and PHP before and after anti-HTGL treatment; in both systems the figures were not additive (i.e. 'LPL only' and 'HTGL only' $\neq$ total). This was also found by Hernall et al. (1975) using a gum arabic-stabilized substrate, where the activity of purified HTGL against the serum-stimulated substrate was only 40% of that in the high salt substrate.

Other chemical methods of inhibiting one or other of the lipases also have proved not to be totally selective. Pre-

incubation with SDS has been used (Baginsky and Brown, 1971) at concentrations which inhibit HTGL but allow full LPL activity to be measured. However, neither this nor a subsequent report (Baginsky, 1981) appears to investigate its use with a wide range of PHP lipase activities. The results reported here in Section 3.2.3, vi) using the NES assay indicate that a precise SDS concentration is needed to inhibit the HTGL without affecting LPL. If this is so, the concentration required will not only be different for high and low activity PHPs, but may also differ with the proportion of the two enzymes present. Preliminary testing of SDS levels necessary for accuracy would considerably lengthen the assay time making it an unsatisfactory method. However, use of SDS may be more effective with a gum arabic-stabilized emulsion. It should be noted, however, that SDS will affect many proteins other than HTGL with unknown consequences in so complex a system.

A surer means of selective inhibition is to use antisera specific to each enzyme: following inhibition of one enzyme, the other may be assayed under its optimal conditions. An attempt was made to raise antiserum in the rabbit against rat HTGL, but this was not successful (see Appendix III).

In assays of an enzyme's activity, similarity to the natural substrate and environment does not necessarily allow reliable quantitation of the enzyme. The nature of the substrate emulsifier/stabilizer may greatly affect the measured activities of LPL and HTGL. Bengtsson and Olivecrona (1981b) found considerable variation in activity between different types of substrate emulsion used, and gave the explanation that 'the quality of the lipid-water interface determines to what extent the enzyme becomes properly orientated for

effective catalysis'. In the case of LPL this will also depend on how well apo CII is bound, which as mentioned earlier, is improved by the use of a phospholipid emulsifier.

Phospholipid may also affect the 'presentation' of TGs at the emulsion surface (Bengtsson and Olivecrona, 1980c). Also relevant is the fact that, although inclusion of phospholipids in (preparation of) emulsions provide a good model for lipoproteins, they may also interfere with measured activity since both the lipases have some phospholipase activity. This will affect especially the HTGL measurements, since this enzyme has higher phospholipase activity than LPL. This may provide an explanation for the report (Jansen, personal communication) that the substrate of the NES assay is not a 'good one' for HTGL. Such interference may be eliminated by substitution of phosphatidylcholine with its lyso-derivative which is not hydrolysed by LPL or HTGL (Bengtsson and Olivecrona, 1980c).

Other detergents used for the preparation of the substrate may also affect the two lipases in different ways (Baginsky and Brown, 1977). For example, SDS can protect and even activate LPL (by preventing its aggregation and reducing denaturation) whilst inhibiting HTGL . It has also been reported (Bengtsson and Olivecrona, 1980c) that although some of the problems of using phospholipid are circumvented by use of gum arabic or Triton X-100, there may be non-specific binding of protein to the substrate which reduce its availability for LPL. In various ways then, optimal activities of the two enzymes would be achieved by using different emulsifiers/ stabilizers in preparation of their test substrates.

In conclusion, in determining what sort of substrate preparation to use for a lipase assay it is necessary to decide first what

is the purpose of the measurement. In some studies, for example that described in Chapter 6 comparing release of LPL and HTGL by various polysaccharides, perhaps measurements in terms of mg protein by a radioimmuno assay (Olivecrona and Bengtsson, 1983) would also be appropriate. Measurement of activity is of course also important, but it must be emphasised that LPL and HTGL are different enzymes, with different (albeit overlapping) substrate specificities. Instead of using the same substrate for both, a more reliable measure of the in vivo effect of the enzyme released into the bloodstream might be achieved by using TG-radiolabelled emulsions for LPL and phospholipid-labelled substrates for HTGL.

The NES assay has been modified to allow reliable measurement against a glycerol/phospholipid-stabilized TG substrate, with a degree of differentiation between LPL and HTGL. The '% HTGL' calculated from the results of low and high salt substrates will be lower than that of many other assays, because of the presence of phospholipid competing with TG for hydrolysis. However, so long as this and other limitations of the assay are appreciated, it can be usefully employed to make valid comparison of LPL and HTGL in a series of post-injection plasmas.

CHAPTER 3

PURIFICATION OF LIPASES FROM POST-HEPARIN PLASMA AND BOVINE MILK

3.1 INTRODUCTION

Heparin-Sepharose chromatography has been widely employed for purification of triglyceride lipases from milk and PHP since its use was first reported by Olivecrona and co-workers in 1971. The method permits a many-fold purification from the high protein content present in these starting materials. However, heparin, being a highly charged ionic species, also binds several other proteins including At III and some lipoprotein classes. Removal of contaminatory At III especially has caused problems since like LPL and HTGL it is a glycoprotein with an approximate MW of 60,000. (However, there has been a single report (Becht et al., 1980) of the use of gel filtration for separation from At III. The method relies on LPL being aggregated after heparin-Sepharose, so that it is eluted in the void volume on gel filtration).

Purification of LPL and HTGL in this project was undertaken primarily to allow the study of the effect of these enzymes on plasma anti-Xa activity. Since At III is responsible for most of the anti-Xa activity present in normal plasma, various methods of avoiding contamination with At III have been investigated.

Unpasteurized bovine milk contains no At III and is a convenient source of LPL, so was used as starting material. Both HTGL and LPL can be purified from PHP using their difference in affinity for heparin. Despite the extra problems in using PHP, LPL was purified from PHP as well as from milk since complete identity of action of the enzyme from the two sources cannot be assumed (although there have been reports (Wallinder et al., 1982) of similar immunotitration curves, and molecular and kinetic properties).

Alternative ligands to normal heparin were tried because of

their lower affinity for At III in order to reduce the degree of adsorption of At III to the gel.

3.2 MATERIAL AND METHODS

3.2.1 Enzyme source

i) Bovine milk Fresh unpasteurized bovine milk was obtained from a local farm and used on the same day as collection.

ii) Human PHP Healthy human volunteers were injected i.v. with 5,000 u heparin and plasma obtained after 10 mins. by plasmapheresis. The PHP was stored at -40°C until used.

iii) Rat PHP PHP was obtained from male Sprague-Dawley rats by cardiac puncture 10 mins. after i.v. injection of 250 u heparin/kg. The blood was mixed 9:1 with 3.8% trisodium citrate, spun at 48,000 g for 10 mins., and the plasma stored at -20°C or used fresh.

3.2.2 Affinity gels

i) Heparin-Sepharose Sepharose 4B (Pharmacia (GB) Ltd., Hounslow, Middx., U.K.) was activated with cyanogen bromide and then coupled with heparin as described in Appendix IVa. In addition, Sepharose CL6B was purchased from Pharmacia. Although the cross-linked Sepharose has lower capacity, it is more resistant to packing down under the pressure used in pumping.

ii) Modified heparin-Sepharose Heparin with low affinity for At III was prepared from commercial heparin by N-desulphation and reacetylation as described in Appendix IVc. The At III-binding ability was determined by APTT clotting assay (Casu et al., 1983) and gave a value of 16 u/mg compared to 150 u/mg for the starting (unmodified) heparin. The modified heparin was coupled to Sepharose 4B using the one-step method of Andersson et al. (1975) as described in Appendix IVb. In this method heparin, cyanogen bromide and

Sepharose are all mixed together which should allow a more complete binding of heparin to the gel.

The gel was compared to normal heparin-Sepharose for its ability to bind At III from plasma. Normal human plasma was mixed with the gels, and the supernatant tested immunologically for At III (Barrowcliffe et al., 1982). The modified heparin-Sepharose could bind just 0.9 u At III/ml* gel compared with 8.2 u/ml normal heparin-Sepharose gel, so there is a 90% reduction of At III bound using the modified heparin gel.

iii) Dextran sulphate-Sepharose Dextran sulphate (Pharmacia (GB) Ltd.) was coupled to Sepharose 4B (Pharmacia) by the method described in Appendix IVa.

3.2.3 Protein determinations

Estimation of protein concentrations were made by measuring absorption at 280 nm.

3.2.4 Concentrating methods

i) Ultrafiltration An Amicon 50 ml cell was used with a Diaflo PM10 membrane (approximate MW cut-off of 10,000 daltons) and a pressure of 15-20 psi supplied by nitrogen. Since proteins of smaller size than that of the membrane pore will pass through, there is also a degree of purification by this method.

ii) Precipitation by ammonium sulphate This technique of 'salting out' of proteins is based on the tendency of hydrophobic sections of proteins to aggregate at high salt concentrations.

The protein solutions to be concentrated were dialysed overnight at 4°C against 3.6 M ammonium sulphate - 0.01 M sodium phos-

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* Normal plasma contains 1.0 u At III/ml by definition.

phate (pH 6.5) (Östlund-Lindqvist and Boberg, 1977). After centrifugation at 40,000 g for 15 mins., the protein pellet was made up in approximately two volumes of 0.01 sodium phosphate - 0.145 M NaCl (pH 7.5), the insoluble protein separated by further centrifugation, and salt removed by dialysis against 0.01 M sodium phosphate (pH 7.5) - 50% glycerol (v/v).

iii) Use of polyethylene glycol High MW complexes of polyethylene glycol can be used to remove water and low MW salts by solid dialysis.

The solution to be concentrated was put into dialysis tubing and packed round with 'Carbowax' (20 M Flaked (MW 20 x 10^6) from Union Carbide Corporation, New York, USA).

3.2.5 Polyacrylamide gel electrophoresis

Final lipase preparations were tested for purity using a method for gel electrophoresis in SDS modified from that of Weber and Osborn (Olivecrona, personal communication). Gels were made up as slabs or rods by the method described in Appendix VI containing SDS concentrations of 10% for the main gel and 4% for the stacking gel.

Samples were prepared for electrophoresis by incubation for 1 hour at 60°C in 0.01 M phosphate buffer containing 4 M urea and 1% SDS. A preparation of low molecular weight calibration proteins (Pharmacia) was similarly treated.

Approximately 1 µg of protein was loaded of each sample in sucrose containing bromophenol blue as tracker dye. The gels were run in a Biorad Protean Dual Vertical Slab Gel electrophoresis Cell (Biorad Laboratories, Richmond CA, USA) at room temperature for 2 hours at 100 v then for 18 hours at 60 v. The gels were stained in 0.2 % Coomassie brilliant blue in a mixture of water:ethanol:acetic

acid (4:5:1) for 2 hours at room temperature, then de-stained in the same solvent.

3.2.6 Lipase assay

Lipase activity of the starting material and fractions at all stages of the procedure were assayed by the modified NES assay as described in Chapter 2. Unless stated, eluted column fractions for total lipase measurements were diluted before assaying to give salt concentrations of not more than 0.2 M.

3.3 RESULTS

3.3.1 Purification of milk LPL with heparin-Sepharose

Five litres of fresh unpasteurized milk were centrifuged at 3,000 g for 20 mins. to separate the cream which was discarded. Solid NaCl was added to the skimmed milk to give a final concentration of 0.5 M, and this was equilibrated for 90 mins. with 150 ml heparin-Sepharose (prepared as described in Appendix IIIa). The gel was then washed with 3 volumes of 0.5 M NaCl, 5 mM sodium barbitone buffer (pH 7.4) and packed into a column. The column was further washed until no protein could be detected by absorbance at 280 nm, then the LPL eluted with 1.5 M NaCl-sodium barbitone buffer (pH 7.4) at a flow rate of 0.8 ml/min. Lipase activity was eluted in a single peak, the fractions of which were pooled and dialysed extensively against the washing buffer and then stored at -40°C.

The purification scheme is summarized in Table 1. Under the conditions given, only 40% of active LPL from the starting material bound to the heparin-Sepharose. Protein concentrations could not be reliably determined at 280 nm because of the interfering effects of the barbitone buffer.

TABLE 1: Purification of LPL from bovine milk by heparin-Sepharose chromatography

Fraction	Volume (ml)	Total enzymic activity (mU)	Protein concen. (mg/ml)	Yield
Skimmed milk	4,500	765,000	30	100%
Supernatant + column wash	5,500	440,000		
Peak elution fraction	80	60,000*	0.05**	8%
Dialysed peak fractions	100	70,000		10%

* The lipase activity was measured without prior dilution of the eluting buffer, thus a considerable proportion of LPL would have been inhibited.

** Estimated

3.3.2 Chromatography on dextran sulphate-Sepharose

The suitability of using dextran sulphate-Sepharose gel for purifying LPL and HTGL from human PHP was tested. Dextran sulphate has low affinity for At III and is used in the purification of Factor Xa from plasma.

Human PHP was loaded on to a column of dextran sulphate-Sepharose, washed with 0.05 M NaCl in 0.05 M Tris/HCl buffer (pH 8.2) and either eluted stepwise with increasing salt concentrations of 0.8, 1.0 and 1.5 M or by a linear salt gradient of 0.5-1.5 M (in the same buffer). Immunological measurement of At III showed that there was no difference in the amounts of it bound by unsubstituted Sepharose and dextran sulphate-Sepharose. Over 85% of total plasma At III loaded was detectable in the supernatant and the 0.5 M NaCl wash.

However, although there was close to 100% binding of both lipases by the gel, there were difficulties in eluting them. Very little of either enzyme was eluted up to 1.5 M NaCl; a small amount of HTGL was eluted at all concentrations above 0.7 M and continued after 1.0 M which was where the LPL started to elute (results not shown). This meant that LPL and HTGL could not be resolved under the conditions used, and was therefore not a useful system.

3.3.3 Use of aluminium hydroxide

Aluminium hydroxide ($Al(OH)_3$) absorption is a standard method of removing At III and other clotting factors from plasma. Its use was investigated as a step prior to heparin-Sepharose chromatography to remove At III. 0.1 ml of 20% (w/v) of $Al(OH)_3$ was mixed with 1 ml human PHP for 30 mins. at 4°C, and the lipase activity tested before and after the treatment. As well as removing At III however, total

lipase activity of the PHP was reduced by > 50%, so this technique was not looked at further.

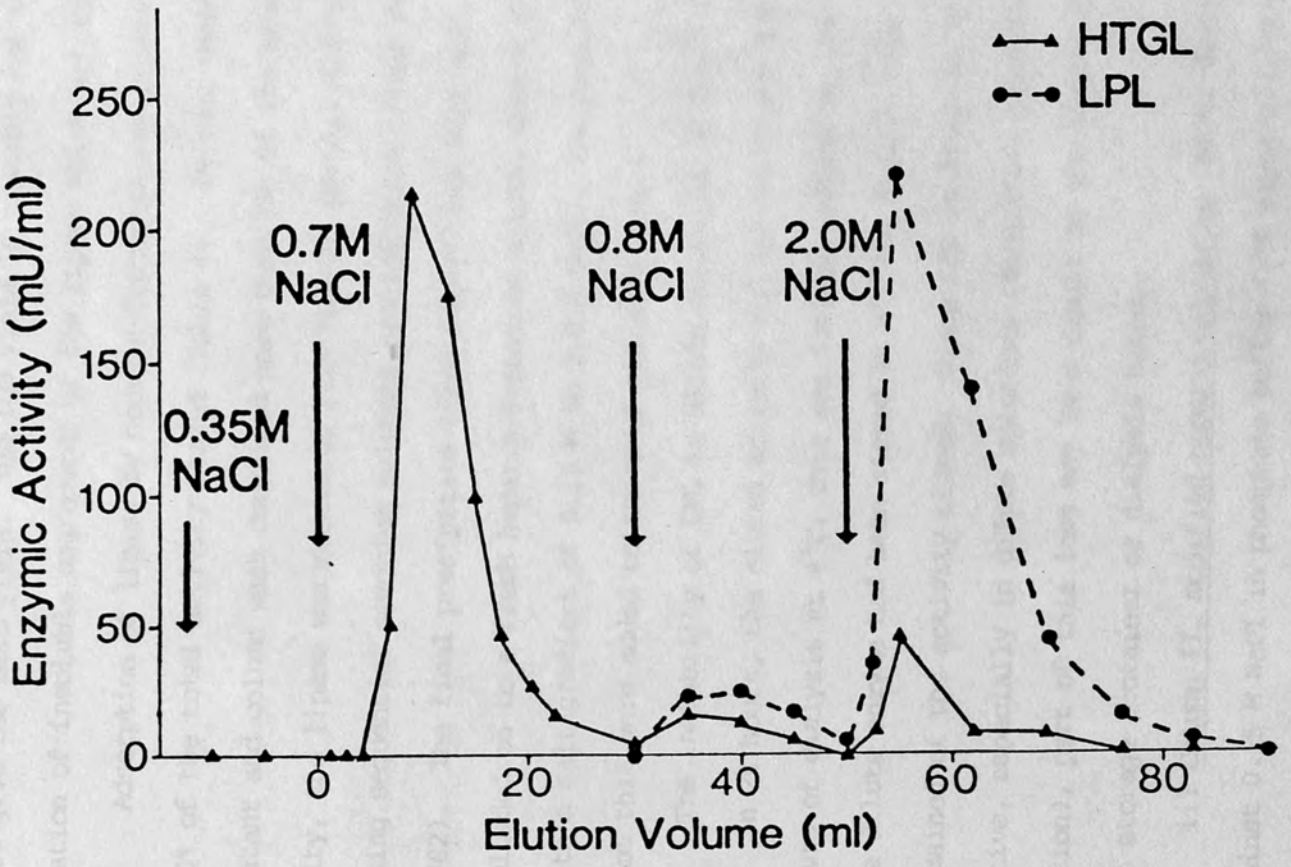
3.3.4 Use of heparin-Sepharose chromatography for purification of LPL and HTGL from PHP

LPL and HTGL were purified from PHP either by chromatography on a single column of normal heparin-Sepharose (Column I) or by using in addition a second column (Column II) of Sepharose substituted with heparin modified to have low affinity for At III (as used by Östlund-Lindqvist and Boberg, 1977).

i) Column I, heparin-Sepharose Solid sodium chloride was added to human or rat PHP to give a final concentration of 0.35 M, then the plasma mixed with heparin-Sepharose in a ratio of 10:1 (v/v) and left to stir for 2 hours. This and all subsequent steps were carried out at 4°C. In some runs EDTA was added with the sodium chloride to a concentration of 5 mM to prevent any formation of fibrin strands. The column was poured and washed with six volumes of 0.35 M NaCl - 0.1 M phosphate buffer (pH 7.4) containing 20% glycerol (v/v) for stabilization of the lipases. Stepwise elution at a flow rate of 0.5 ml/min. was carried out with increasing salt concentrations in phosphate buffer and 20% glycerol. The bulk of the loaded HTGL (generally > 60%) eluted with 0.7 M NaCl; a mixture of LPL and HTGL with 0.8 M NaCl, and finally LPL (50-80% of loaded) eluted with 2.0 M NaCl (see Fig. 1). There appeared to be contaminating HTGL present in the LPL fractions, but this may be due to a lack of absolute differentiation in the lipase assay used (see Ch. 2).

The peak activity fractions of HTGL and LPL were separately pooled, and if column II was not used, concentrated by one of the methods described in section 2.5 of this Chapter. Their effective-

Fig 1 Affinity chromatography of PHP on heparin-Sepharose



ness is compared in Table 2. Precipitation by dialysis against concentrated ammonium sulphate solution was found to be the most useful technique in producing a concentrated sample, although it did not give the best yield. The low yield is possibly due to the formation of insoluble aggregates of the lipase which are inactive.

Adsorption of lipase by heparin-Sepharose was generally around 80% of the total activity loaded (Table 4). In runs where the supernatant and column wash contained more than 25% of the starting activity, the lipase was recovered from this by partial fractionation using sequential ammonium sulphate precipitation (Frost et al., 1982). The final precipitate (from 30-60%) was then well dialysed, reloaded on to a fresh heparin-Sepharose column, washed then eluted with a salt gradient of 0.15 M to 2.0 M NaCl. The peak fractions from this were added to those of the main run.

The instability of LPL in dilute solutions is shown in Table 3. In 24 hours, the eluted activity had fallen by 80% and after 24 hours of dialysis at 4°C, this was further reduced by 50%. After the dilute enzyme had been stored at 4°C for 5 days, only 5% remained of the activity eluted. Since LPL is known to be surface-active, especially in dilute solutions (Bengtsson, personal communication), part of this loss may be a result of the enzyme sticking to the storage container or dialysis tubing.

ii) Column II, modified heparin-Sepharose After dialysis against 0.15 M NaCl in phosphate buffer (20% glycerol), the LPL and HTGL samples from column I were separately applied to 2 ml columns of modified heparin-Sepharose. The gels were washed with 0.35 M NaCl and HTGL eluted with 1.0 M NaCl (Fig. 2a) and LPL with 2.0 M NaCl (Fig. 2b).

TABLE 2: Comparison of methods for concentrating lipase fractions

Technique	Enzymic activity of concentrated sample (mU/ml)	% recovery
Ammonium sulphate precipitation	430	37
Ultrafiltration	70	58
Carbowax	23	72

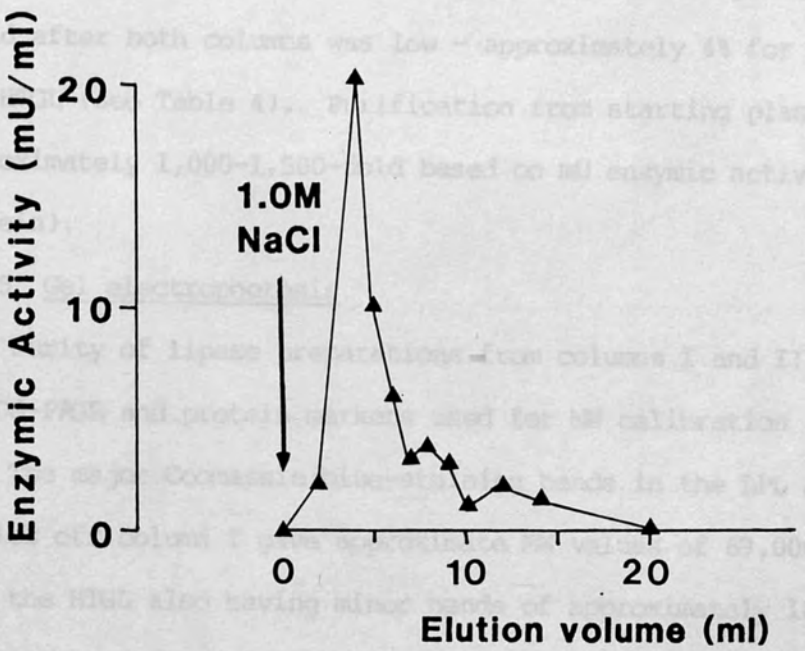
The starting volume and enzyme activity were the same for each method used

TABLE 3: Stability at 4°C of dilute LPL solutions

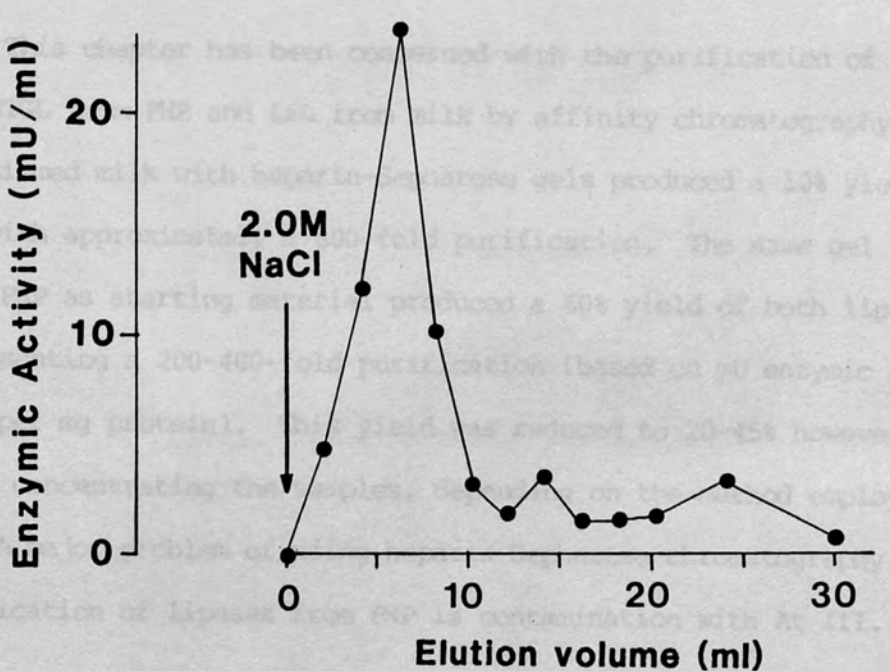
	Storage time after elution off heparin-Sepharose column	Total enzymic activity (mU)
Calculated total activity in eluted fractions	30 mins.	6,500
Pool of above fractions	24 hours	1,200
Dialysed pool	48 hours	680
	5 days	290

Fig 2 Affinity chromatography on modified heparin-Sepharose

a. Elution of HTGL



b. Elution of LPL



The results of protein estimations of loaded and eluted fractions indicate that > 80% of protein was removed from the lipase sample by use of this column. This was accompanied, however, by a loss of approximately 50% of overall lipase activity, and the final yield after both columns was low - approximately 4% for LPL and 3% for HTGL (see Table 4). Purification from starting plasma was approximately 1,000-1,500-fold based on mU enzymic activity per mg protein).

3.3.5 Gel electrophoresis

Purity of lipase preparations from columns I and II was studied by SDS-PAGE and protein markers used for MW calibration (see Fig. 3). The major Coomassie blue-staining bands in the LPL and HTGL samples off column I gave approximate MW values of 69,000-70,000, with the HTGL also having minor bands of approximately 185,000 and two or three other high MW proteins close to the origin. The protein concentration of the samples eluted from column II was too low to be detected by the staining method used.

3.4 COMMENTS

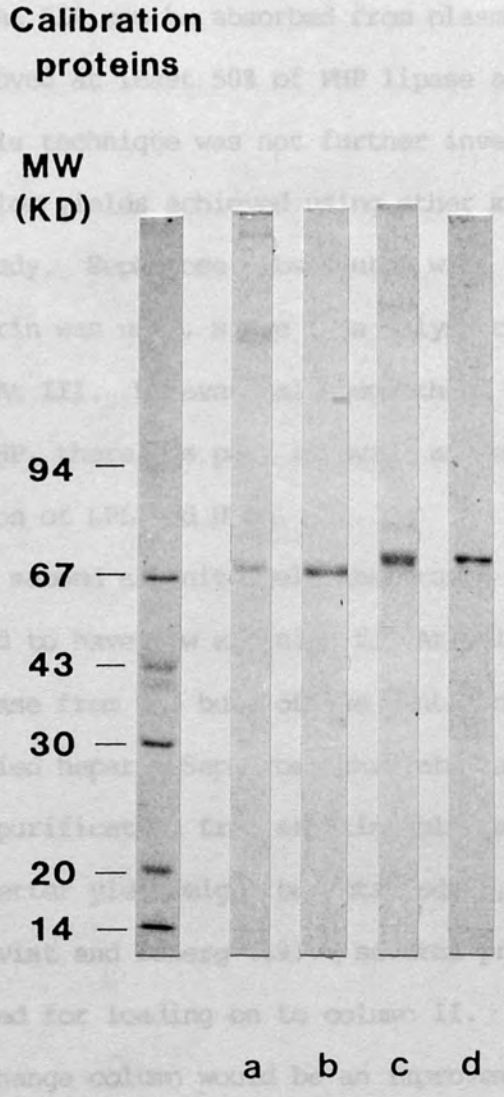
This chapter has been concerned with the purification of LPL and HTGL from PHP and LPL from milk by affinity chromatography. Use of skimmed milk with heparin-Sepharose gels produced a 10% yield of LPL with approximately a 600-fold purification. The same gel used with PHP as starting material produced a 60% yield of both lipases representing a 200-400-fold purification (based on mU enzymic activity per mg protein). This yield was reduced to 20-45% however after concentrating the samples, depending on the method employed.

A major problem of using heparin-Sepharose chromatography for purification of lipases from PHP is contamination with At III. This

TABLE 4: Heparin-Sepharose chromatography of PHP

Fraction	Volume (mL)	Total enzymic activity (mU)		Specific activity (mU/mg)		% yield	
		LPL	HTGL	LPL	HTGL	LPL	HTGL
Starting plasma	33	3,400	2,500	1.7	1.3	100	100
Column I:							
supernatant	20	70	40				
wash	25	10	5				
0.7 M elution	15	0	110		350		65
2.0 M elution	25	76	9	250		60	
Column II:							
LPL	50	100		1,200		4	
HTGL	30		90		1,500		3

Fig 3 SDS-PAGE of lipases purified from PHP



a Rat HTGL
b Rat LPL
} from Column I

c Major heparin-binding protein (AtIII)

d Rat albumin

is especially so for LPL since > 90% of immunoreactive At III has been shown by McKay and Laurell (1980) to be eluted at NaCl concentrations above 0.8 M. Various ways of avoiding this contamination were tried. At III can be absorbed from plasma using $Al(OH)_3$, but this also removed at least 50% of PHP lipase activity. Because of this loss, this technique was not further investigated, but in the light of the low yields achieved using other methods, this would be worth more study. Sepharose substituted with dextran sulphate in place of heparin was used, since this polysaccharide has only low affinity for At III. However, although there was good binding of lipase from PHP, there was poor recovery and elution did not allow good separation of LPL and HTGL.

Use of a second affinity gel substituted with a heparin chemically modified to have low affinity for At III was successful in purifying lipase from the bulk of the contaminating protein obtained after unmodified heparin-Sepharose chromatography. This allowed > 1,000-fold purification from starting plasma but with a low yield of 3-4%. A better yield might be obtained if, as was done by Östlund-Lindqvist and Boberg (1977), several preparations from column I were combined for loading on to column II. Also, desalting with a small ion-exchange column would be an improvement on dialysis because of the surface active properties of the enzymes.

Both lipases, and especially LPL are extremely unstable in dilute solutions (Iverius and Östlund-Lindqvist, 1976) and this was only partly overcome by inclusion of 20% glycerol in all buffers. Heparin has been used by some workers as a stabilizing agent, and is also useful in deaggregating lipase after ammonium sulphate precipitation (Olivecrona, personal communication), but this could

not be used since the lipase was to be used in clotting assays. Use of BSA as a 'carrier' for LPL has also been suggested (Olivecrona, as above). The purified enzyme was stored in 50% glycerol and 0.01 M phosphate buffer (pH 7.4) both at +4°C and -20°C; there was no loss in activity over a two-month period at -20°C or for at least 10 days at +4°C.

Protein in lipase samples run by SDS-PAGE could only be detected in those from column I, (normal heparin-Sepharose), where the MW of the major band from both LPL and HTGL preparations was 69,000-70,000. The detected protein may be At III however, since its contamination of these samples is both possible in HTGL and likely in LPL.

CHAPTER 4

RELEASE OF LIPASES IN VIVO

4.1 INTRODUCTION

Most of the studies on the lipase-releasing activity of GAGs have been carried out using heparin and there have only been limited reports of the use of other sulphated polysaccharides (Bengtsson et al., 1980; Casu et al., 1983). The work described in this Chapter investigates the effect of a number of different GAGs and of partially synthetic heparin analogues on lipase release in rats under controlled conditions. Dose and time related studies of lipase release were carried out.

4.2 MATERIALS AND METHODS

4.2.1 Polysaccharides used for injection

Physicochemical parameters are given in Table 1 determined as in Casu et al. (1983).

Heparin I Prepared from porcine mucosa. A significant amount of free NH_2 groups are detectable by NMR, so it is partially N-desulphated (probably a result of it being subjected to acid pH during preparation). Supplied by Bioiberica, Barcelona, Spain (Batch F4).

Heparin II Prepared from porcine duodenum. Supplied by Crinos SpA, Milan, Italy. (PD in Casu et al., 1983).

Heparin III A mucosal heparin (sodium salt). Supplied by Leo Laboratories, Princes Risborough, Bucks., U.K. (Batch H 64578).

Depolymerized heparin (LMW heparin) A mucosal heparin treated by partial nitrous acid degradation. Supplied by Riker Laboratories Ltd., Loughborough, Leics., U.K. (Batch P2658).

Heparan sulphates HSI and HSII Prepared by E.A. Johnson as described in Casu et al. (1983) (where they are termed HSI and HSIIA, respectively) from a pig mucosal fraction supplied by

TABLE 1: Physicochemical parameters of the polysaccharides injected

	Mean MW	Sulphate (meq/g)	*Ratio Sulphate / carboxylate
Heparin I	12,500	3.66	2.11
Heparin II	15,000	3.43	2.15
LMW heparin	5,500	3.44	2.0
HSI	20,000	1.94	1.0
HSII	7,500	3.03	1.67
SP54	3,000	5.48	no carboxyl groups
SSHA	8,000	4.22	2.75
DeS	n.d.	2.03	1.05

* residues per disaccharide

n.d. values not determined

Laboratori Derivati Organici, Milan, Italy.

Sodium pentosan polysulphate (SP54) A semi-synthetic polymer prepared from vegetable raw materials. It is composed of β-D-xylopyranose pentose base units, linked (1→4). Supplied by Benechemie, Munich, West Germany (see Fig. 1).

Semi-synthetic heparin analogue (SSHA) An artificially sulphated bovine chondroitin. The SO_4 groups are thought to attach mainly to the 6-carbon of the galactosamine residues. Supplied by Luitpold-Werk, Munich, West Germany (Eleparon M705A).

Dermatan sulphate Prepared by E.A. Johnson from porcine mucosal GAGs supplied by Laboratori Derivati Organici, Milan, Italy.

Other materials

Trisodium citrate BDH Chemicals Ltd., Poole, Dorset, U.K.

Diethyl ether (containing pyrogallol as antioxidant). BDH Chemicals.

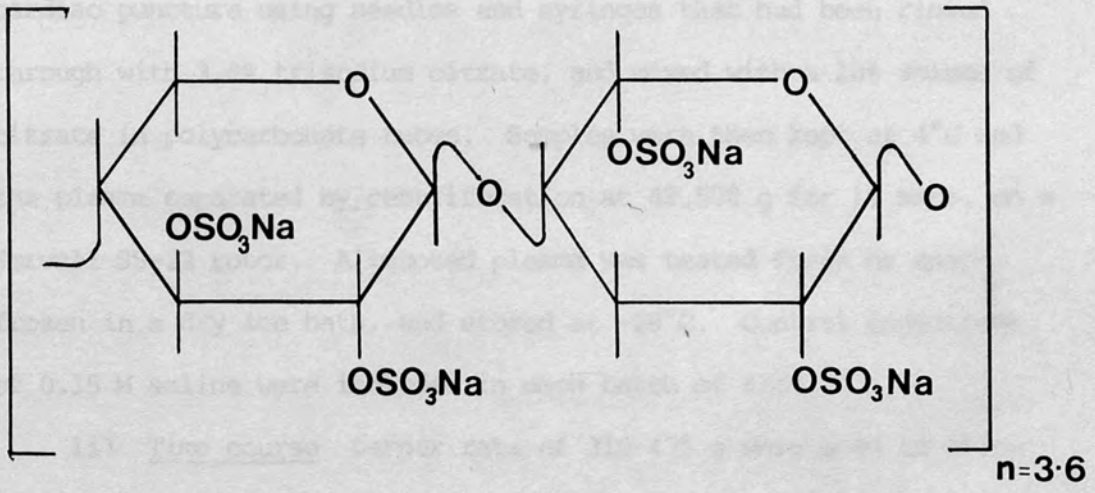
Hypnorm (Janssen) Crown Chemical Co. Ltd., Lamberhurst, Kent, U.K.

Animals Male Sprague-Dawley rats were used throughout supplied by Olac 1976 Ltd., Bicester, Oxon., U.K. The rats were fed on R and M diet No. 1 (BP Nutrition) and water ad lib.

4.2.2 Preparation of the solutions for injection

The polysaccharides were made up from the solid in 0.15 M NaCl (with the exception of Heparin III, which is supplied as a solution). 12% by weight was allowed for water in the sample (average loss on drying of stored samples). A range of doses was made up for each polysaccharide so that the same volume per kg could be injected into the rat irrespective of the dose.

Fig 1 Structure of SP54



4.2.3 Lipase release in the rat

i) Single time samples Rats of 200 ± 10 g were used without prior fasting. To avoid possible changes in lipase activity due to the diurnal rhythm of the rat, injections were always made between 2-5 pm (see Footnote 1). The rats were anaesthetized with diethyl ether and the polysaccharide injected into the blood stream via a tail vein (0.1 ml $second^{-1}$). Blood was sampled 10 mins. later by cardiac puncture using needles and syringes that had been rinsed through with 3.8% trisodium citrate, and mixed with a 10% volume of citrate in polycarbonate tubes. Samples were then kept at 4°C and the plasma separated by centrifugation at 48,500 g for 15 mins. on a Sorvall SS-22 rotor. Aliquoted plasma was tested fresh or snap-frozen in a dry ice bath, and stored at -20°C. Control injections of 0.15 M saline were included in each batch of rats.

ii) Time course Larger rats of 310-475 g were used to allow several blood samples to be taken. The animals were anaesthetized with an intra-muscular injection of Hypnorm (Janssen) (0.1 ml/100 g). The neck area was exposed and either jugular vein used for administration of the polysaccharide and for blood sampling. In both cases the needle was passed through the overlying muscle to prevent bleeding from the vein. A pre-injection blood sample of 0.2-0.5 ml was taken and further samples taken at specified times after injection.

Footnote 1: Recent data presented by Jansen (1984) indicate that in the rat the amounts of heparin-releasable enzyme may vary noticeably in the afternoon and that the morning may thus be a better time to obtain PHP samples.

Before the experiment, known blood volumes had been carefully weighed to calculate a conversion factor for blood weight to volume. This enabled quick estimation of blood sample volumes by weighing, and so the volume of citrate solution to be added.

4.2.4 Lipase assays

Lipase activity was assayed on fresh plasma by the NES assay using 10 min. incubation times and samples volumes of 30 µl. 50 µl of plasma samples that had been frozen were also tested in the Intralipid assay using 20 min. incubation times.

4.3 RESULTS

4.3.1 Control injections

Normal saline (0.1 ml/kg) was injected into rats and blood taken after 10 mins. as for test samples. A low level of lipase was measured, with no differences found between different rat batches. The total lipase activity detectable in control plasmas was 1.3 mU/ml (n = 20) and that of HTGL only 1.1 mU/ml (n = 10).

4.3.2 Effect of anaesthetic on lipase release

Ether stress has been reported (Jansen, 1984) to decrease HTGL activity releasable by heparin six hours after the use of ether as anaesthetic. In order to determine whether ether stress affects the release of lipase within the time scale used in these experiments, male rats were injected with identical doses of heparin with and without the use of ether. Blood was taken after 10 mins. in the normal way using ether anaesthesia. No significant differences were found in the lipase activities of plasmas from the two sets of rats (results not shown).

4.3.3 Effect of freezing on lipase activity

The effect on PHP lipase activity of freezing and thawing was

tested. PHP aliquots were either kept at 4°C, or quickly frozen and re-thawed, then the two samples assayed. No differences were found in the lipase activity measured (results not shown).

4.3.4 Lipase activities 10 minutes post-injection

The results quoted for total, HTGL and LPL activities are those calculated from use in the NES assay of low salt substrate, high salt substrate and the difference of these two respectively, as described in Chapter 2. The limitations of these distinctions should be borne in mind.

As shown in Figures 2-4, the polysaccharides used for injections can be divided into four major groups based on their dose-response curves. These four groups are:-

1. The heparins
2. The heparin analogues, SP54 and SSHA
3. LMW heparin and HSII
4. DeS and HSI

The highest release at all doses was achieved by the three heparins, with a peak total lipase of approximately 300 mU/ml at 2.0 mg/kg (Fig. 2). The analogues gave low release up to 0.5 mg/kg, then the total activity rose to a peak at 2.0 - 4.0 mg/kg with 180 mU/ml. This is approximately 60% of that of the heparin peak. The LMW heparin produced a dose response curve very similar to that for HSII. Like the analogues they gave poor release at low doses (only approx. 30 mU/ml at 0.5 mg/kg compared with approx. 150 mU/ml for the heparins at this dose), but a steady increase approaching a plateau at 8.0 mg/kg gave a value 70% of that of the heparin peak, at around 200 mU/ml.

DeS and HSI both gave low release. At doses up to 1.0 mg/kg,

Fig 2 Total lipase released in rats after injection of sulphated polysaccharides

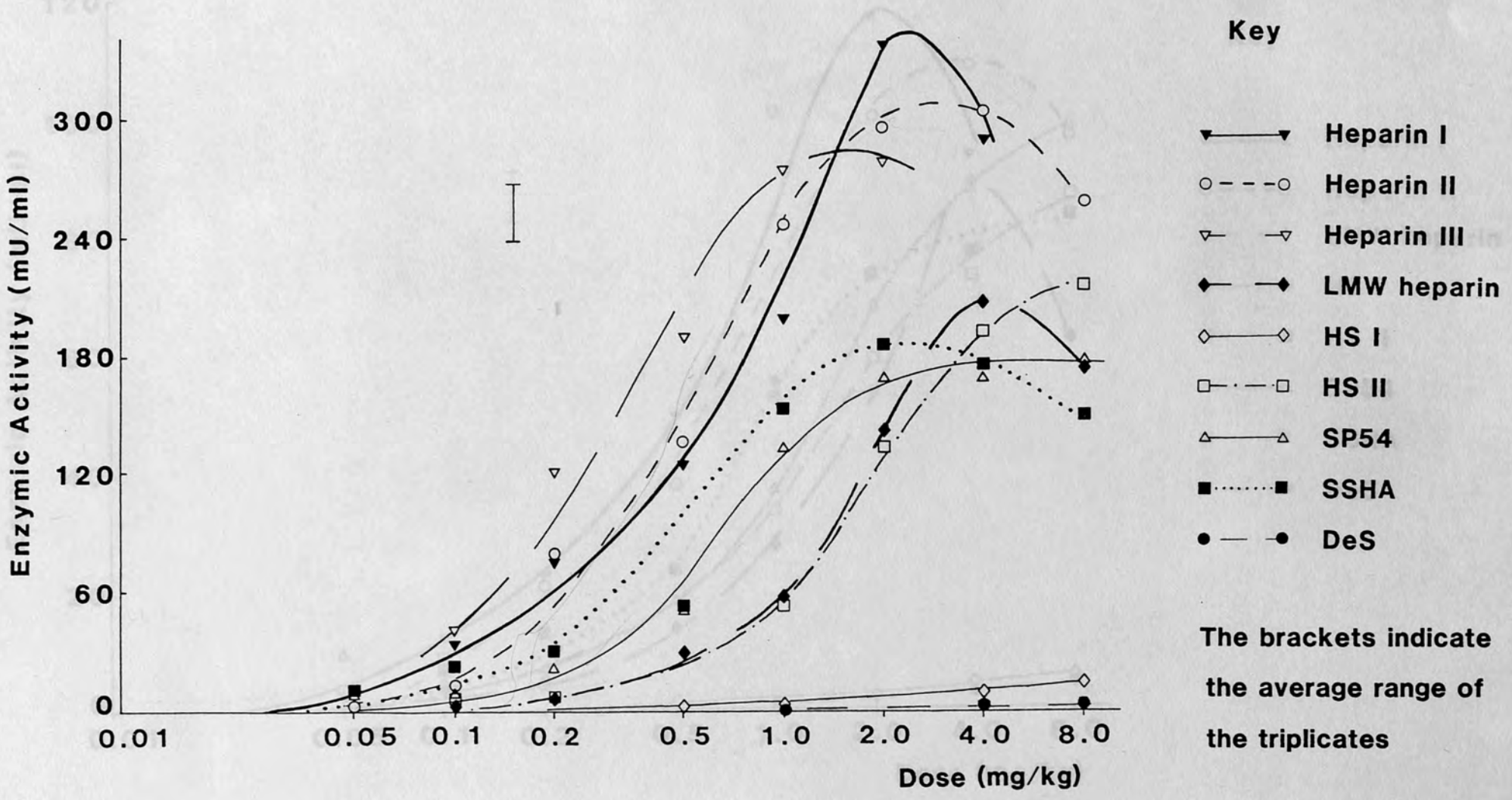
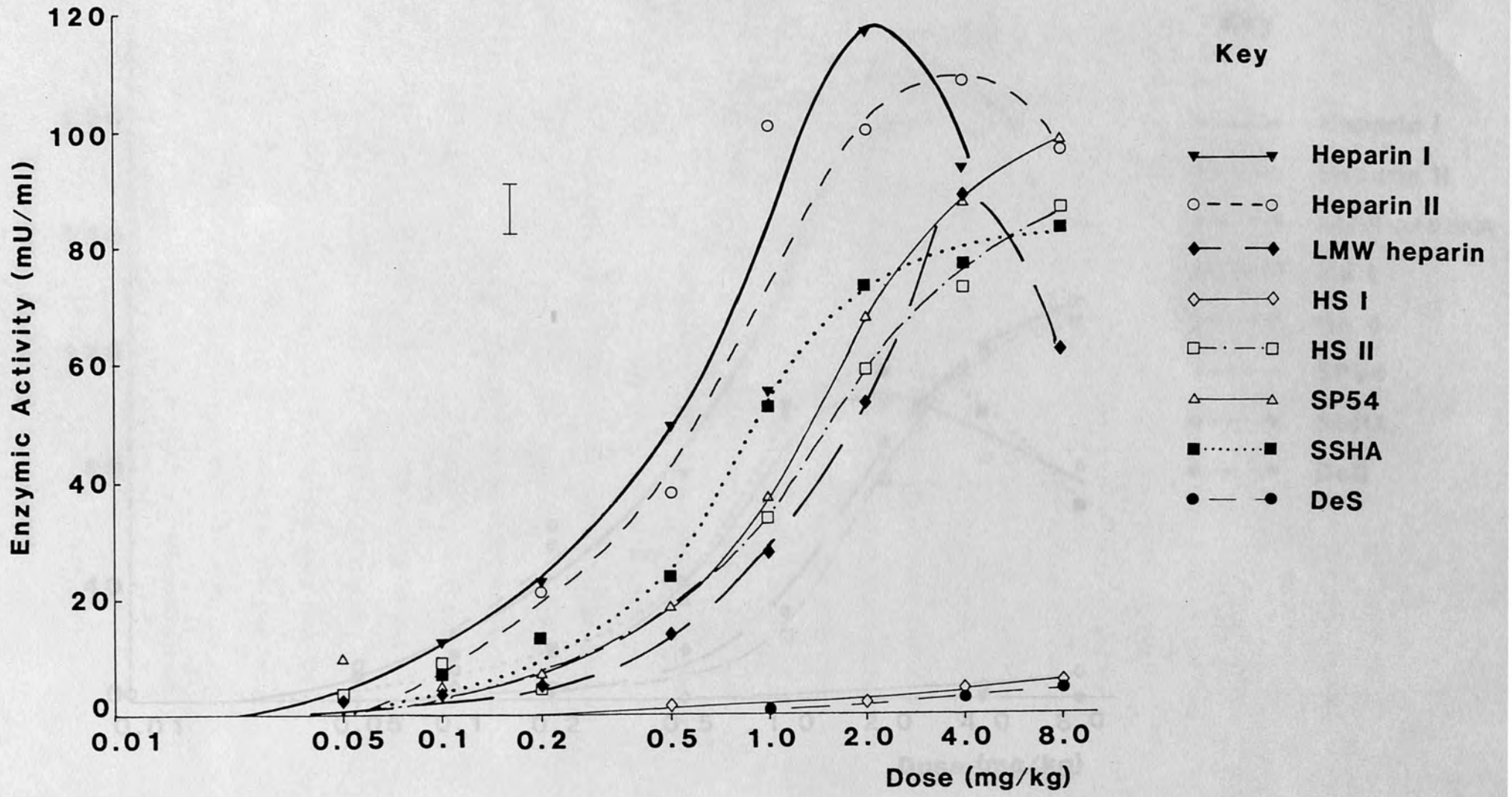
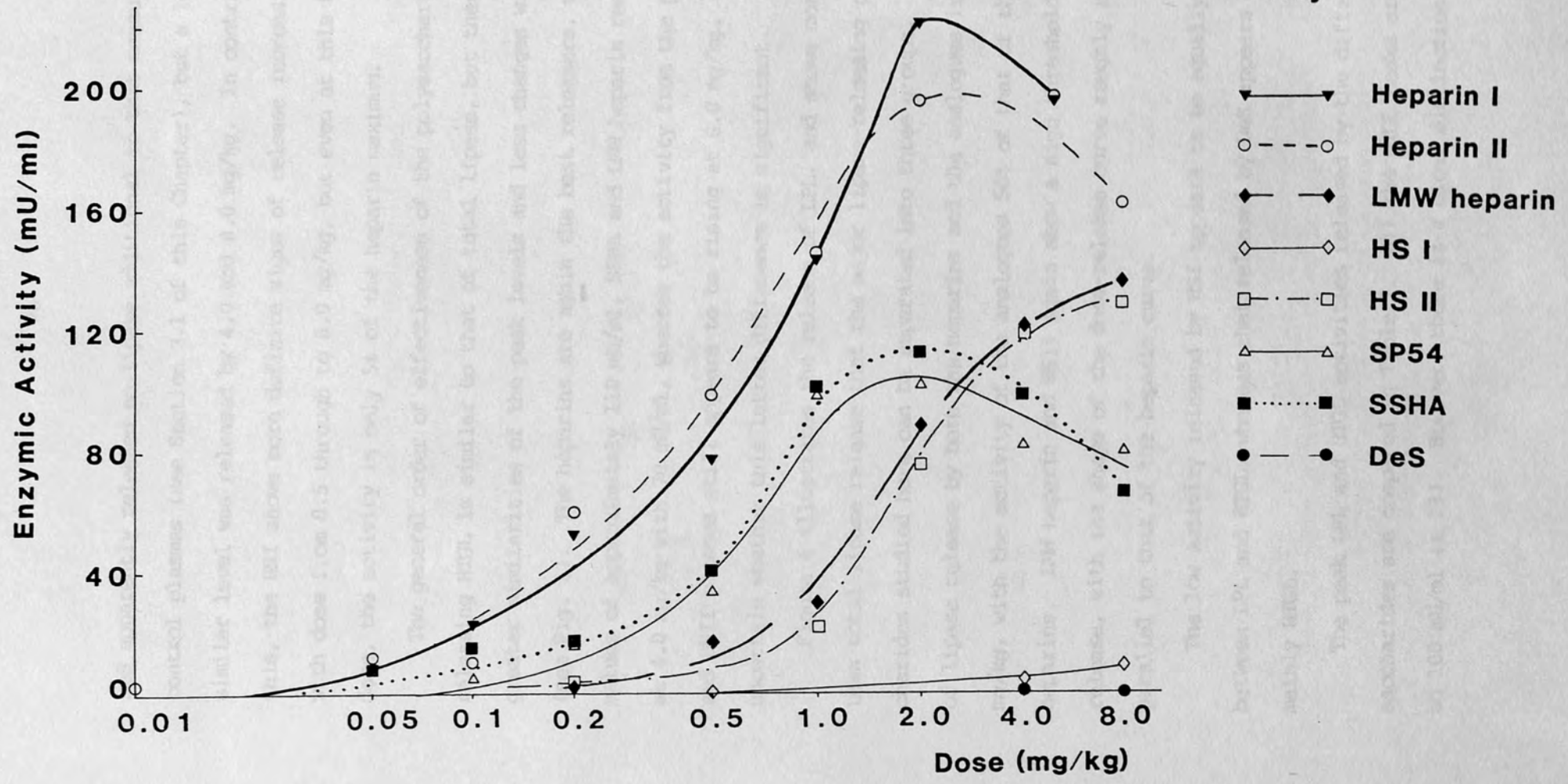


Fig 3 HTGL released in rats after injection of sulphated polysaccharides



95b

Fig 4 LPL released in rats after injection of sulphated polysaccharides



DeS apparently released no lipase additional to that measurable in control plasmas (see Section 3.1 of this Chapter), but a low and similar level was released by 4.0 and 8.0 mg/kg. In contrast to this, the HSI shows more definite signs of release increasing with dose from 0.5 through to 8.0 mg/kg, but even at this highest dose, the activity is only 5% of the heparin maximum.

The general order of effectiveness of the polysaccharides in releasing HTGL is similar to that of total lipase, but there are greater similarities of the peak levels and less changes with dose (see Fig. 3). The heparins are again the best releasers, with peak values of approximately 110 mU/ml, SSHA and LMW heparin reach a peak at 4.0 mg/kg with 70 mU/ml, whereas the activity from the post-SP54 and HSII plasmas still appears to be rising at 8.0 mg/kg. It is uncertain whether this latter difference is significant.

Figure 4 illustrates the release of LPL, and shows more clearly than total lipase release that the major lipase-releasing polysaccharides studied here can be separated into three groups. The peak of lipase release by both the heparins and the analogues is at 2.0 mg/kg, with the activity of the analogues 50% of that of the heparins. LMW heparin and HSII again show a high threshold of release, with the shape of the dose-release curve roughly running parallel to that of the heparin curve.

The low activity released by HSI appears to be equally divided between LPL and HTGL, whereas that released by DeS appears to be mainly HTGL.

The peak LPL and HTGL activities released by the different polysaccharides are compared in Table 2. All the HTGL peaks are close to 100 mU/ml (± 13). However, there is a clear distinction in

TABLE 2: Peak lipase activities released by sulphated polysaccharides by i.v. injection

	Enzymic activity mU/ml	
	HTGL	LPL
Heparin I	116	220
Heparin II	108	196
LMW heparin	90	> 136*
HSII	> 86*	> 129*
SP54	> 98*	101
SSHA	83	112

For dose at peak, see Figures 3 and 4

* Indicates lipase activity at the highest dose given (8.0 mg/kg). In all these cases, the lipase level was approaching a plateau.

release of LPL between the high level from that of the two heparins (at 200 mU/ml) and that of the other four polysaccharides. Of these latter, the LMW heparin and HSII are the more potent LPL releasers, with a peak value of around 130 mU/ml compared with the analogues which released an average of 105 mU/ml.

In all three lipase activities determined (that is, total, 'HTGL only' and 'LPL only') an increase of dose above that required to give peak release resulted in lower measured activity (see Figs. 2-4).

Plasma lipase activities were also measured using the Intralipid assay. The dose-response curves thus obtained (Fig. 5) were of a similar shape to those from use of the high salt substrate of the NES assay (Fig. 3), although with higher calculated activities, as is usual with Intralipid. Both substrates are stabilized with phosphatidylcholine, with all that this means in the relative measurement of the two lipases (see Ch. 2) but, since Intralipid has been reported to be a much better substrate for LPL than for HTGL, it is surprising that the activity curves do not fit in closer with calculated NES LPL measurements.

The tables of values relating to Figures 2-4 are given in Appendix VI. The lipase levels released at a single dose (0.5 mg/kg) are compared with the anticoagulant activity of the polysaccharides (as determined by the in vitro APTT and anti-Xa assays) in Table 3. These figures show, as discussed in Chapter 1, that different sets of physicochemical parameters are important in determining the two activities of the polysaccharides.

4.3.5 Time course of lipase

Polysaccharides were chosen out of the group studied previ-

Fig 5 Total lipase released by sulphated polysaccharides as tested by the Intralipid assay

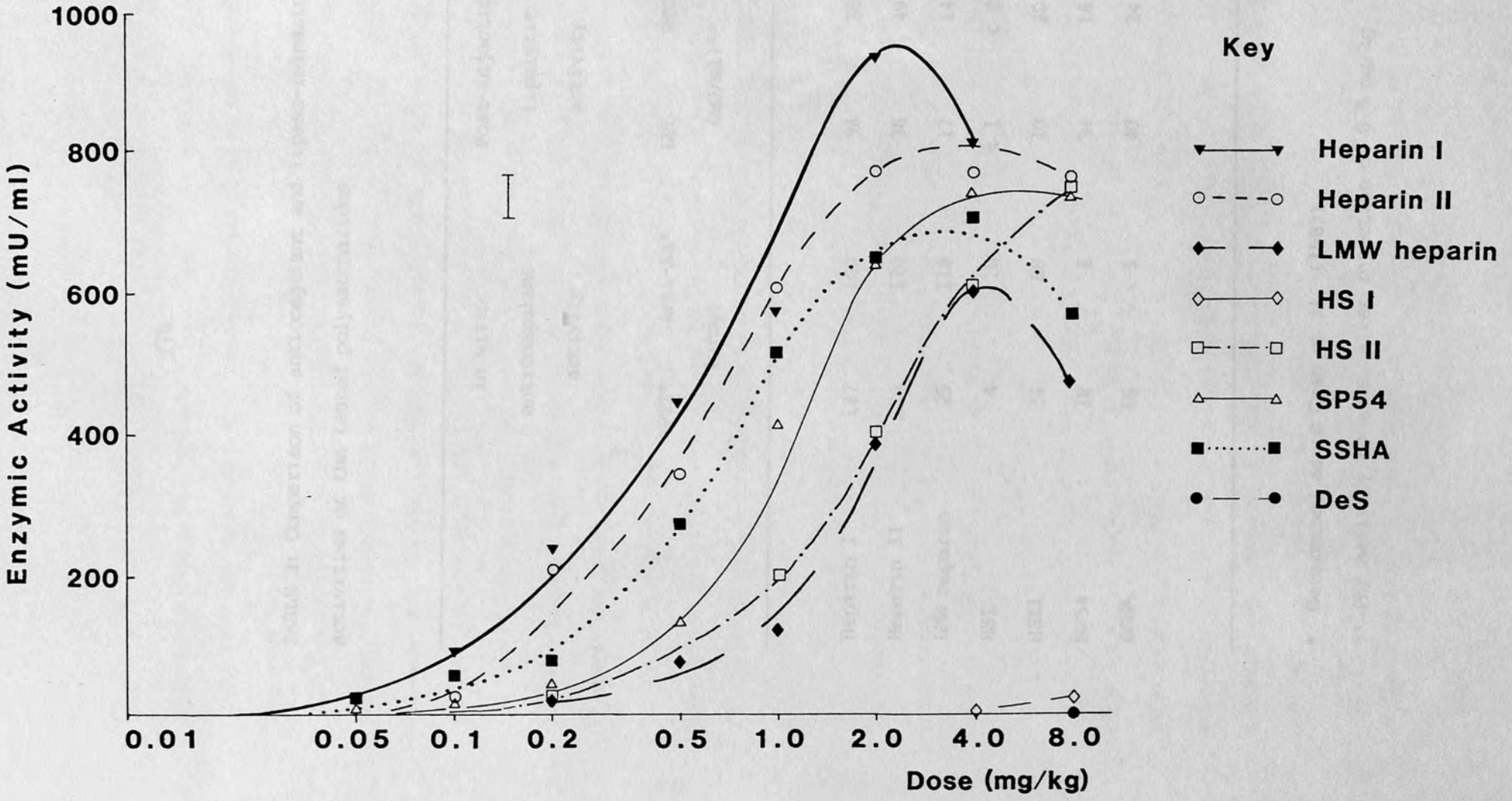


TABLE 3: Comparison of anticoagulant and lipase-releasing activities of the tested polysaccharides

	In vitro anticoagulant activity		Post-injection lipolytic activity	
	APTT*	Anti-Xa*	LPL	HTGL
	(u/mg)		(mU/ml)**	
Heparin I	187	160	98	38
Heparin II	175	170	76	49
LMW heparin	25	118	17	14
HSI	4	10	< 1	< 2
HSII	34	88	10	20
SP54	18	6	34	18
SSHA	16	< 5	40	24

* Determined as in Casu et al. (1983)

** PHP activity 10 mins. after injection of 0.5 mg/kg

ously: a 'normal' heparin (Heparin 1), LMW heparin, HSII and the two analogues. Since HSI and DeS released such low levels of lipase, these were not studied. Doses were selected to give approximately the same level of release.

Generally, both enzymes were released quickly and cleared in an exponential manner after all the polysaccharides tested. HTGL release (Fig. 7) was complete within 5.0-7.5 mins. after all polysaccharide injections. LPL release (Fig. 8) tended to be more gradual, most notably after SSHA and SP54 injections, where the levels of lipase reached a peak only after 15 mins. Once the peak was reached, the activity of both enzymes was cleared from the blood stream in a similar way independent of the polysaccharide used. However, by comparing peak values to the activity after 90 mins. (Table 4) it can be seen that LPL is cleared quicker than HTGL.

4.4 DISCUSSION

4.4.1 Lipase-releasing activity of different polysaccharides

The release of HTGL and LPL by different polysaccharides has been compared. It appears that the release of HTGL is less specific than that of LPL, and much of the following discussion will thus apply more to LPL than to HTGL.

Comparison of the physicochemical parameters (Table 1) and the peak lipase results (Table 3) shows that there is strong correlation between sulphate content and lipase release in that all good releasers have > 3.00 meq sulphate/g. This is not, however, a quantitative correlation like that described for a group of heparins and heparans by Casu et al. (1983).

MW is also important in determining lipase-releasing ability, as shown by the LMW heparin which gave reduced release of both

Fig 6 Time course of total lipase after injection of sulphated polysaccharides

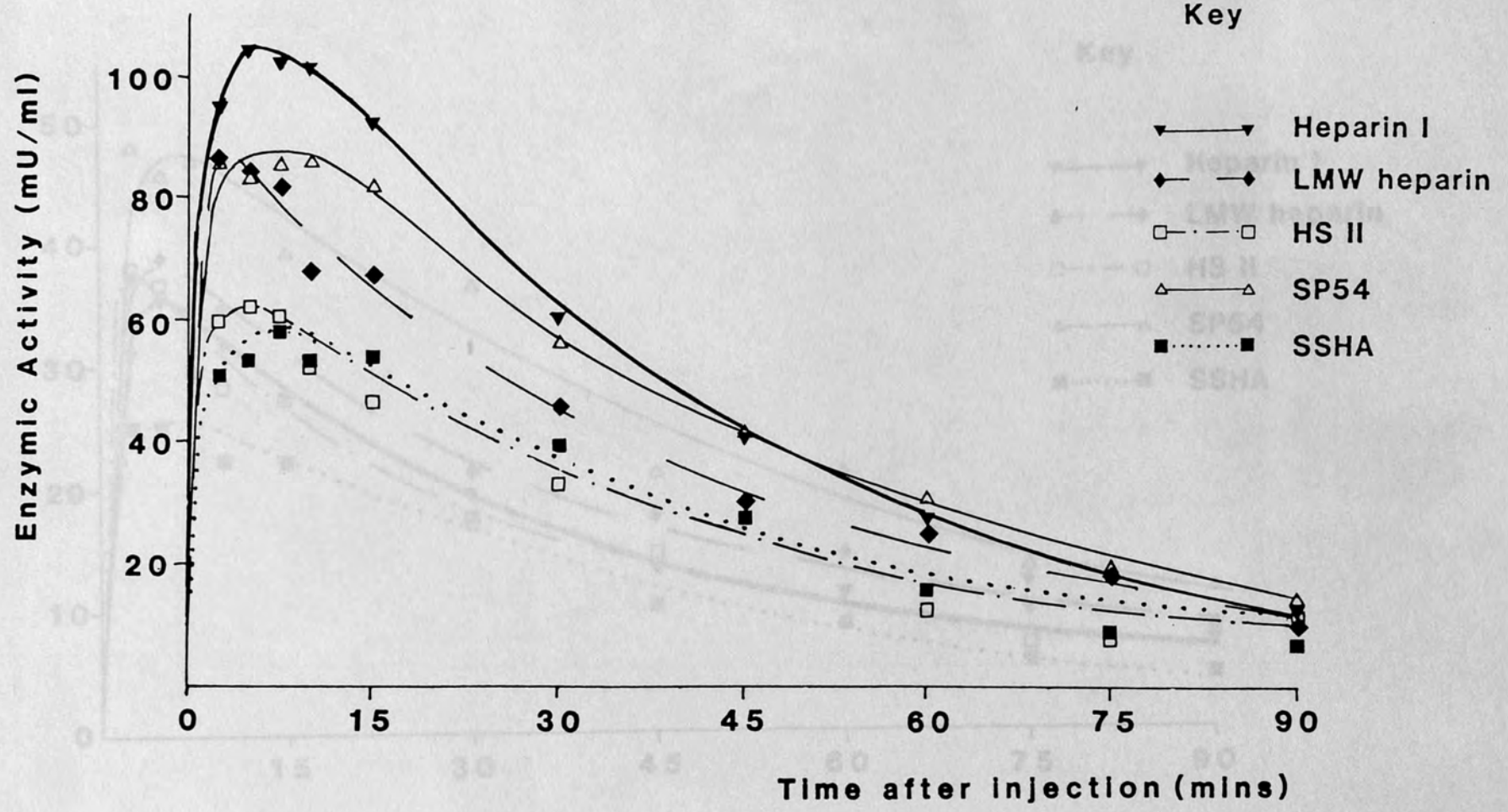
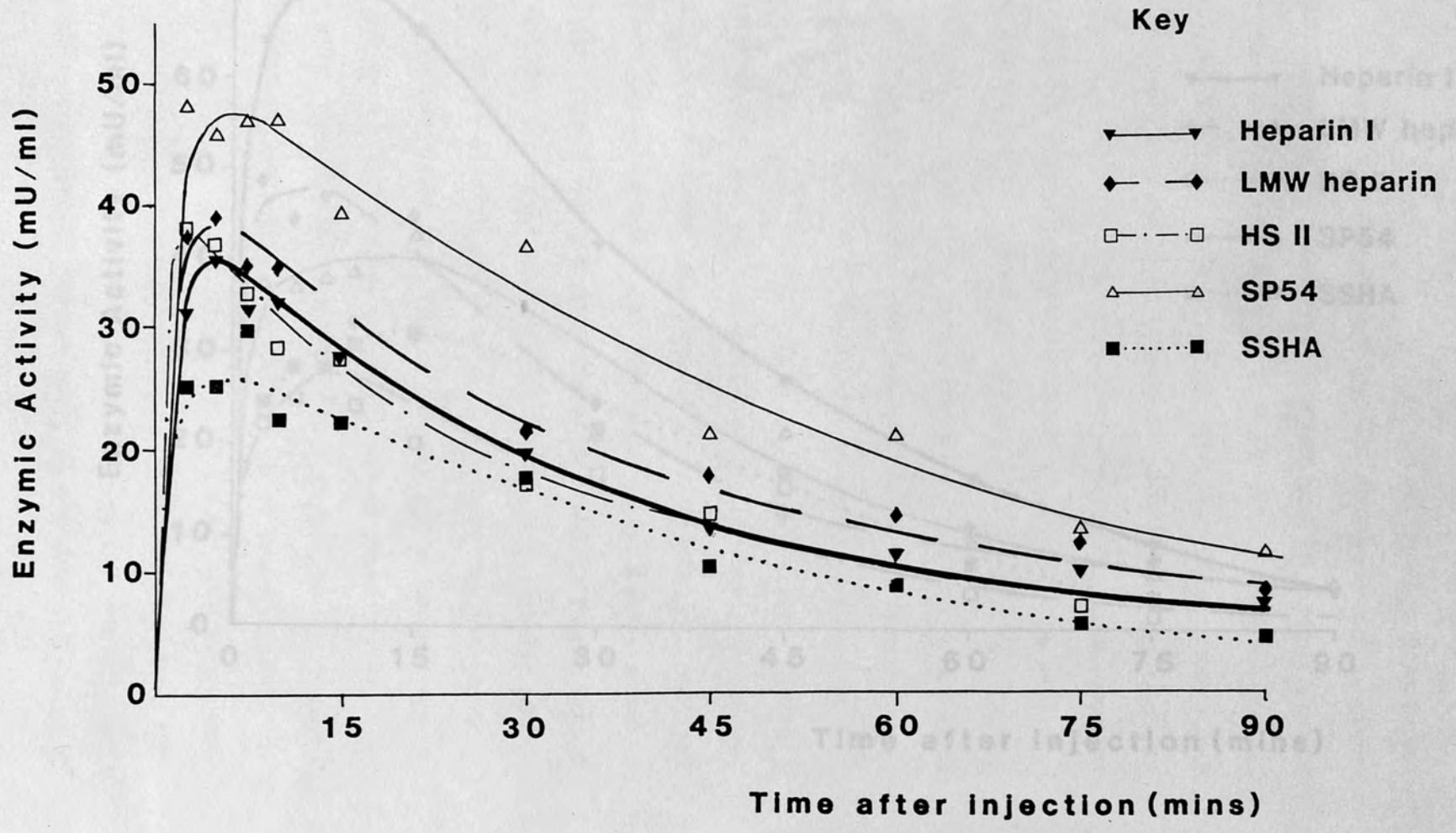


Fig 7 Time course of HTGL after injection of sulphated polysaccharides



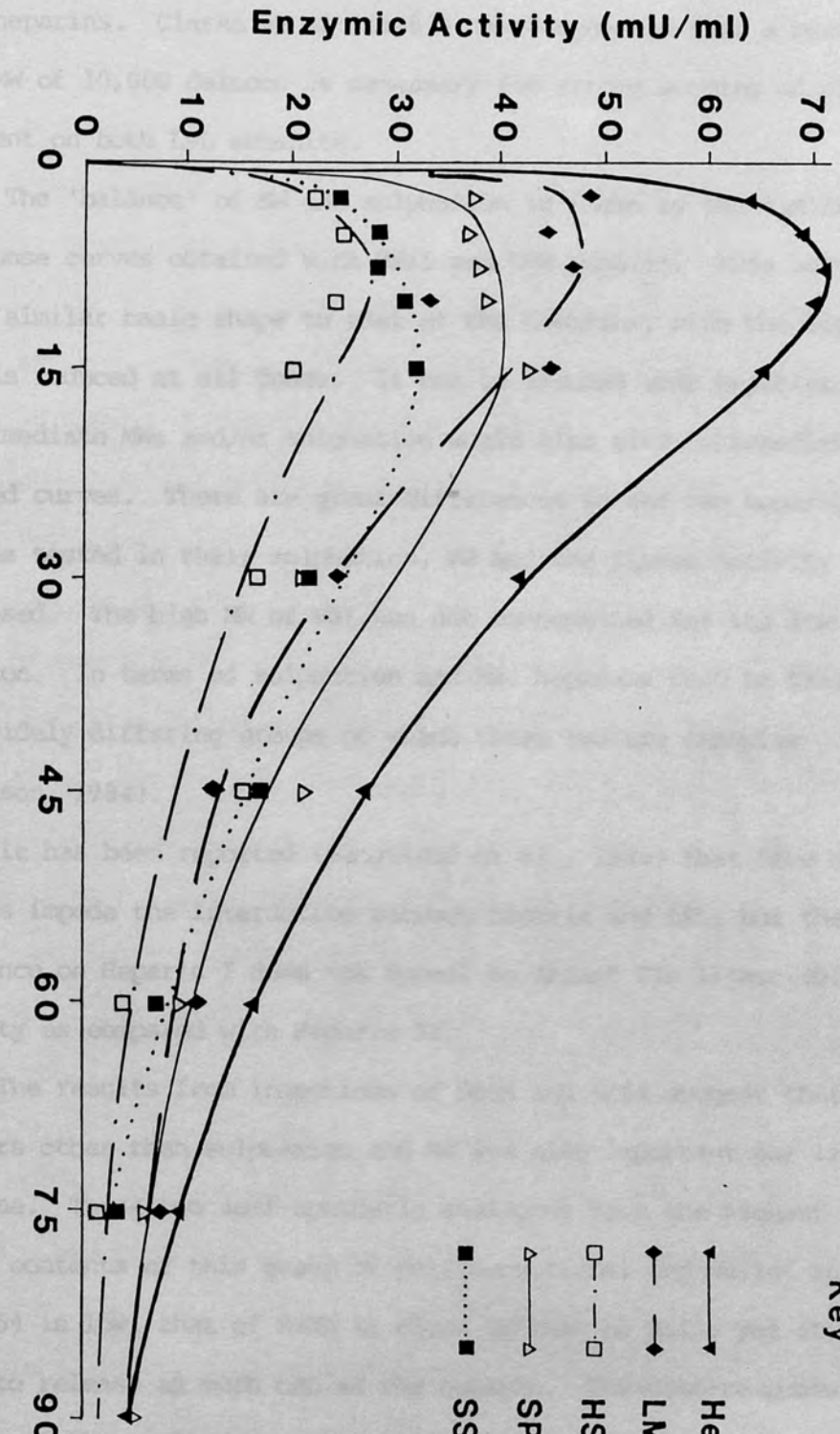


Fig 8 Time course of LPL after injection of sulphated polysaccharides

lipases, although its sulphate content is similar to that of the heparins. Clarke et al. (1983) have suggested that a minimum GAG MW of 10,000 daltons is necessary for strong binding of sites present on both LPL subunits.

The 'balance' of MW and sulphation is shown by the similar dose-response curves obtained with HSII and LMW heparin. This curve is of a similar basic shape to that of the heparins, with the lipase levels reduced at all doses. It can be assumed that heparins with intermediate MWs and/or sulphation would also give intermediate shaped curves. There are great differences in the two heparan sulphates tested in their sulphation, MW and the lipase activity released. The high MW of HSI has not compensated for its low sulphation. In terms of sulphation and MW, heparans tend to fall into two widely differing groups of which these two are examples (Johnson, 1984).

It has been reported (Bengtsson et al., 1980) that free amino groups impede the interaction between heparin and LPL, but their presence on Heparin I does not appear to affect its lipase-releasing ability as compared with Heparin II.

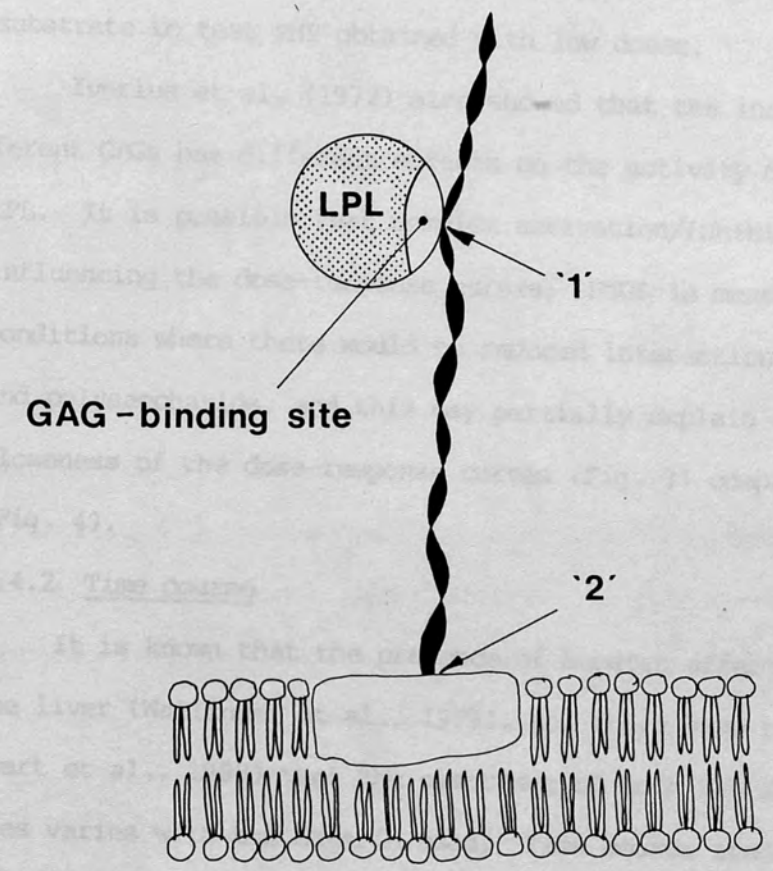
The results from injections of SSHA and SP54 suggest that factors other than sulphation and MW are also important for lipase release. These two semi-synthetic analogues have the highest sulphate contents of this group of polysaccharides, and whilst the MW of SP54 is low, that of SSHA is close to that of HSII, yet it is not able to release as much LPL as the heparan. The dose-response curves of the analogues are also distinctly different from those of the natural GAGs tested.

As mentioned in Chapter 1 (Section 4) it has been suggested

that the release of lipase by sulphated polysaccharides from its site on the endothelium is mediated in one of two ways: by competition with membrane-bound HS for binding of the LPL, or by induction of a conformational change of the LPL (Olivecrona et al., 1977). The former view is now generally accepted (see '1' in Fig. 9). Results from cultured endothelial cells have indicated that whilst some of the HS proteoglycans are firmly embedded in the membrane core protein, others can be displaced from the surface by the addition of heparin (see Williams et al., 1983). There is an excess of HS chains over LPL molecules bound on porcine endothelium (HS:LPL, 4:1) (Williams et al., 1983), and it is possible that all the LPL is attached via the covalently-bound HS. If, however, LPL is bound by both HS populations, there is the possibility of two 'sites' of release. In addition to competition by the injected polysaccharide with the HS for the LPL GAG-binding site (as already described), it could also compete at the membrane protein (see '2' on Fig. 9). There is no published evidence that the structure of the covalently bound HS from the electrostatically bound one, but if it is, then this would mean that there are effectively three sites of release, which could have different affinities for the injected polysaccharides.

The decrease in enzyme activity in the dose-response curves at doses above the plateau has also been noted by other workers (R. Pescador, personal communication). It is unlikely that the 'excess' polysaccharide is affecting the clearance rate of the lipase (Wallinder et al., 1979), and probably affects instead the assay measurements of activity. Work with purified milk LPL has shown that heparin does not affect the enzyme's catalytic activity under

Fig 9 Possible sites of release of lipase by sulphated polysaccharides from the endothelium



otherwise optimal conditions (Olivecrona et al., 1977). However, the effect in crude systems such as that using skimmed milk (Iverius et al., 1972) can be substantial, with activation at low and inhibition at high heparin concentrations. The highest dose injected in the present study (8.0 mg/kg) would give a final assay concentration of the order of 30 µg/ml,* a level which was shown to inhibit skimmed milk LPL (Iverius et al., 1972). This could be checked by the addition of heparin and the other polysaccharides to the assay substrate to test PHP obtained with low doses.

Iverius et al. (1972) also showed that the inclusion of different GAGs has different effects on the activity of skimmed milk LPL. It is possible that complex activation/inhibition effects are influencing the dose-response curves. HTGL is measured in high salt conditions where there would be reduced interaction between lipase and polysaccharide, and this may partially explain the relative closeness of the dose-response curves (Fig. 3) compared with LPL (Fig. 4).

4.4.2 Time course

It is known that the presence of heparin affects LPL uptake by the liver (Wallinder et al., 1979), and there have been reports (De Swart et al., 1980) that the clearance of both LPL and HTGL activities varies with the heparin used. Time course studies on selected polysaccharides were carried out to investigate whether there were significant differences in the group studied.

In agreement with published results (Huttenen et al., 1975) LPL

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* Based on: rat blood volume of 64 ml/kg, packed red blood cell volume of 45%, use of 30 µl enzyme and 200 µl substrate in the assay.

is found to be released more slowly and cleared more quickly than HTGL. The analogues differ from the heparins and HS by releasing LPL more slowly, but the subsequent clearance is the same. Similar HTGL time courses in all respects were achieved with different polysaccharides. De Swart et al. (1980) compared the time course of the enzymes with that of the heparins used and on the basis of their results suggested that the removal of HTGL especially is determined by the polysaccharide used for injection. The current work provides no support for this (at least for the polysaccharides studied).

A fuller investigation of lipase time courses could be carried out, using a wider series of polysaccharides tested over a more extensive range of doses. The rates of clearance of LPL from the circulation appears to be largely independent of its levels present over a very wide range (Wallinder et al., 1979). These workers found that purified LPL injected at a level 100 times that normally present in PHP, was cleared from the circulation by the liver at the same rate as much smaller doses. Heparin injected prior to the LPL slowed down clearance substantially. No such studies (with purified enzyme) have been carried out with HTGL however. Huttenen et al. (1975) found that clearance of HTGL was slower after high heparin doses, suggesting that uptake by the liver is affected by the GAG.

CHAPTER 5

EFFECT OF DILUTION OF POST-HEPARIN PLASMA ON THE ENZYMIC ACTIVITY MEASURED IN THE NILSSON-EHLE AND SCHOTZ LIPASE ASSAY

5.1 INTRODUCTION

Because of the problems encountered in achieving intra-assay reproducibility of the modified NES lipase assay (see Ch. 2). the possibility of using a series of PHP dilutions to provide a 'standard curve' was investigated. Preliminary studies of diluting PHP with either saline/buffer or pre-injection (normal) plasma, gave the unforeseen result of significant deviation from the expected value. Total and HTGL activities were differently affected by the two diluents. At this stage a thorough re-appraisal of the literature was undertaken.

Previous work on the effect of a range of serum concentrations on LPL and HTGL activities has been done mainly on either the enzymes purified by heparin-Sepharose or as whole PHP with only total lipase measurements (with no attempt at differentiation). As discussed in Chapter 1 (section 2.5.ii), it is well accepted that LPL requires apo CII from serum for full activity under sub-optimal conditions. However, it has also been reported that assaying LPL in the presence of serum concentrations greater than that needed for maximal stimulation (generally 15-25% of total substrate volume) may lead to inhibition of both the activity in PHP (Huttenen et al., 1975) and of the purified enzyme (Hernall et al., 1975; Feliste et al., 1982). The extent of this may vary between serum batches (Baginsky, 1981). The apo CI and CIII components of CMs and VLDL appear to be responsible for this inhibition (Chung and Scanu, 1977; Owen and McIntyre, 1982). There have also been reports (Baginsky and Brown, 1977) that incubation at 25°C of purified LPL diluted with buffer may lead to aggregation with consequent loss of activity.

The wide range of reported results on the effect of serum components on the activity of purified HTGL has also been covered in Chapter 1. This disparity is itself worthy of note. There is disagreement both as to whether serum stimulates or inhibits HTGL activity at low concentrations (see Ch. 1) (all agree that it inhibits at high concentrations) and also to which serum components are responsible. Apo A has been implicated as the activator of HTGL at low concentrations (Jahn et al., 1983) and as the inhibitor by other workers (Haberbosch et al., 1984) in addition to apo CI and III (Jahn et al., 1984; Bengtsson and Olivecrona, 1980b). Huttenen et al., (1975), testing the effect of serum on the activity of HTGL in whole PHP, reported inhibition with serum levels > 10% of the total substrate volume.

It was considered that further study of the effect of dilution of PHP lipases on their assay measurements was warranted, to clarify the position under the specific conditions used.

5.2 MATERIALS

5.2.1 Source of plasma

i) PHP Rat PHPs (prepared from blood as described in Ch. 4) of mid range lipase activity (i.e. total activity 70-120 mU/ml) were generally selected for use. A record was kept of the heparin type and dose used for injection. These included Leo heparin (heparin III in Ch. 6), Crinos F5, Bioiberica heparin (heparin I in Ch. 6) and LMW heparin.

ii) Diluting plasma Normal blood samples were taken from rats with or without a prior injection of saline. Some tests for comparison were done using serum.

Buffer

The buffer used for dilution was 0.2 M tris/HCl (pH 8.3) containing 20 mg albumin/ml, as was used for preparation of the assay substrate.

5.3 RESULTS

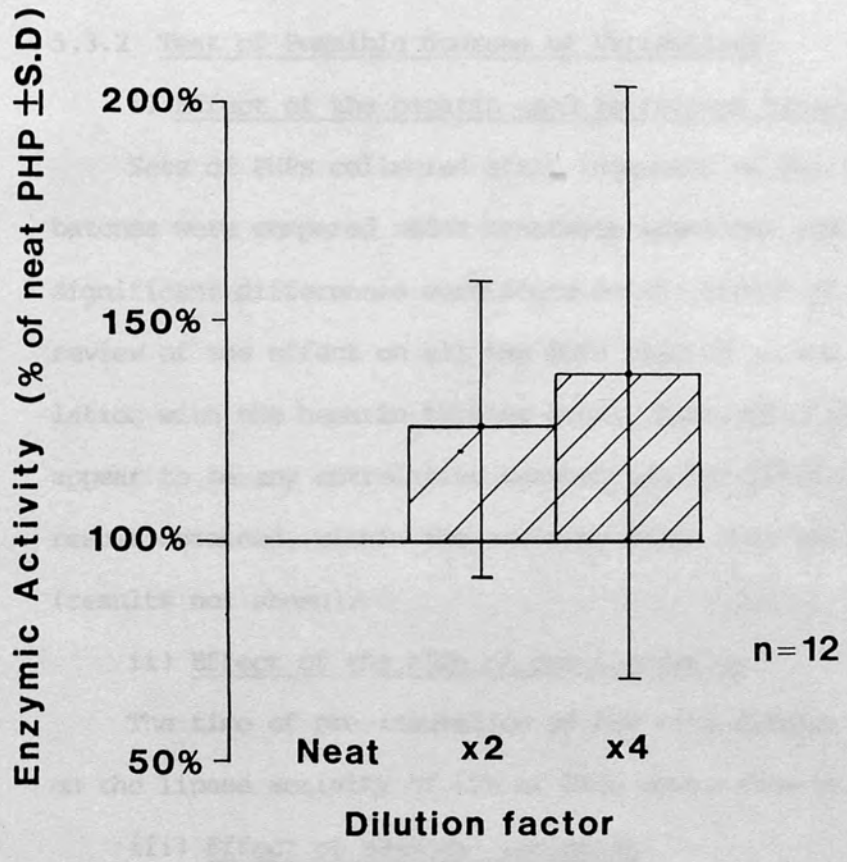
5.3.1 General Findings

PHPs were mixed with either plasma or buffer to give a final PHP volume of 50% or 25% (1 in 2 and 1 in 4 dilution respectively). Aliquots of each mixture were assayed by the NES lipase assay within 15 mins. of mixing, alongside undiluted PHP. As mentioned in the Introduction, the first results appeared to follow a pattern. The effect of the two diluents on LPL and HTGL activity can be summarized as:-

HTGL	decreases	when diluted with plasma
activity	increases	when diluted with buffer
LPL	increases	when diluted with plasma
activity	decreases	when diluted with buffer

However, as more tests were done, it became apparent that many results did not fit this pattern and there were great differences in the direction and extent of the effect on measured activity. As an example of this variation, Figure 1 shows the effect of buffer on HTGL activity using the results from 15 experiments using different PHPs. The activity values from the assays were adjusted for dilution and compared to the neat plasma which was given a value of 100%. Figure 1 illustrates the great range of responses obtained (total and LPL only results are not shown). It should be noted, however, that in any given test the direction of the effect on activity was consistent, so that with increasing dilution the

Fig 1 Effect of dilution of PHP with buffer on the HTGL activity measured in the NES assay



increase (or decrease) in activity was more marked. In some tests there was no significant change. Duplicates were within the normal variation of the assay (< 4%). There was no difference between the effect of plasma and serum prepared from the same blood sample, or between saline and buffer, when used to dilute the same PHP. Various possible reasons for the variability of the results were investigated.

5.3.2 Test of Possible Sources of Variability

i) Effect of the heparin used to release lipase

Sets of PHPs collected after injection of different heparin batches were compared under otherwise identical conditions. No significant differences were found on the effect of dilution. A review of the effect on all the PHPs studied showed up no correlation with the heparin type or dose. Similarly, there did not appear to be any correlation between the PHP lipase levels and the result obtained, within the activity range that was studied (results not shown).

ii) Effect of the time of pre-incubation

The time of pre-incubation of PHP with diluent had no effect on the lipase activity of LPL or HTGL measurable in this assay.

iii) Effect of seasonal variation

The effect of dilution of PHPs collected from rats in June and November after an identical heparin dose was compared, using the same control plasma and buffer batch. No difference was found in the response (results not shown).

5.3.3 Effect of dilution of purified lipase

HTGL purified by single heparin-Sepharose chromatography (as described in Ch. 3, section 3.4 (i)) was diluted with plasma and

buffer and tested as described for PHP. The results in Figure 2 show that whilst there was little change after dilution with buffer, there was increasingly reduced activity when diluted with plasma. Although the results of just a single experiment are reported here, confirmation of this effect came from assays run during the purification procedures, where activities were always lower in the presence of plasma than with saline.

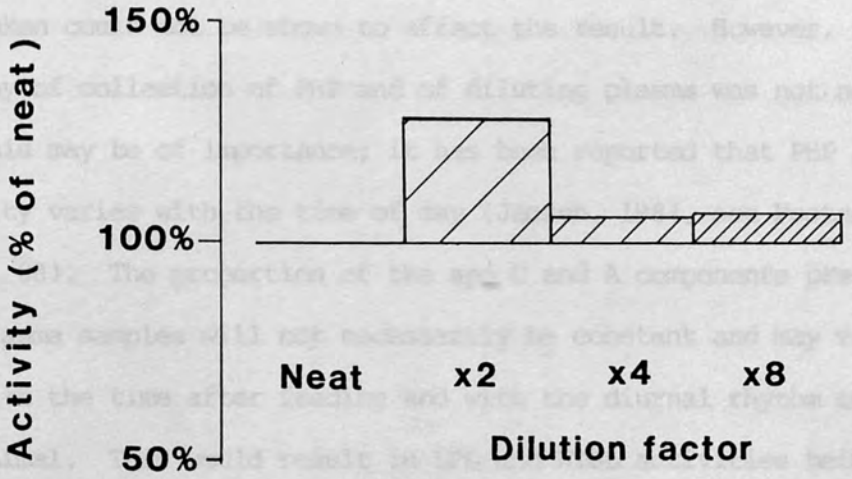
5.4 DISCUSSION

Whilst the effects of serum on the activity of purified triglycerides have been well-studied, it was thought necessary to continue investigation of the discrepancies found on dilution of full PHP prior to assay. The results in this chapter have shown that pre-incubation of PHP with plasma and buffer can affect the measured activity in this assay but the direction of this effect appears to be unpredictable. Different results are obtained dependent on the PHP and/or the plasma diluent used, both as to the extent and direction of the change, and to whether LPL and HTGL are similarly or differently affected.

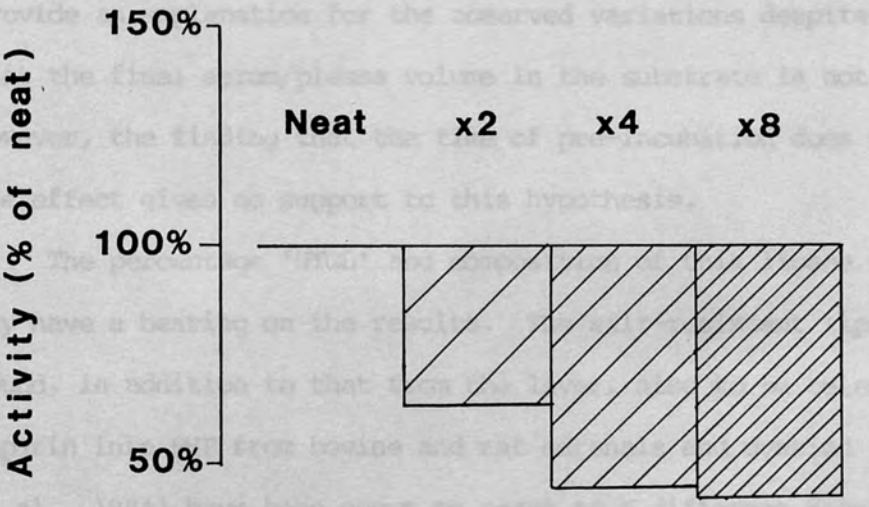
Since the effect of diluents on purified HTGL is in line with results of other workers, it is difficult to attribute these irregularities to some peculiarity of the assay used (modified NES assay). No simple explanation can be offered for them - no correlation could be made with the heparin type and dose used, to the level of enzymic activity of the PHP or to the time of pre-incubation. It seems likely that the differences are due to variations in the plasma composition, especially apoproteins, although it should be noted that some workers (Kubo et al., 1983) have found that, in its effect on HTGL, the best equivalent to serum is a mixture of ultracentri-

Fig 2 Effect of dilution of purified HTGL on the activity measured in the NES assay

(a) Dilution with buffer



(b) Dilution with plasma



fugally-separated HDL and d = 1.21 fractions (the latter containing most of the serum proteins). This indicates that the lipases may also be affected by proteins other than the apoproteins.

As would be expected from a laboratory animal such as the rat, kept under controlled conditions, the time of the year the PHP was taken could not be shown to affect the result. However, the time of day of collection of PHP and of diluting plasma was not noted, and this may be of importance; it has been reported that PHP lipase activity varies with the time of day (Jansen, 1984, see Footnote 1, p. 88). The proportion of the apo C and A components present in the plasma samples will not necessarily be constant and may vary both with the time after feeding and with the diurnal rhythm of the animal. This would result in LPL and HTGL activities being differently affected - the complications of the inhibitors and activators of the enzymes have been suggested by the results of Feliste et al. (1982), using irradiated rabbit lipoprotein fractions.

As well as the effect of the apoproteins, inhibition may be caused by competition of lipoprotein with the assay substrate. This should show up particularly with short assay times, and could provide an explanation for the observed variations despite the fact that the final serum/plasma volume in the substrate is not altered. However, the finding that the time of pre-incubation does not alter the effect gives no support to this hypothesis.

The percentage 'HTGL' and composition of this lipase fraction may have a bearing on the results. The salt-resistant lipases found, in addition to that from the liver, also to be released by heparin into PHP from bovine and rat adrenals and ovaries (Cordle et al., 1984) have been shown to react to a different degree to

serum inhibition, depending on tissue origin.

This work has left many questions unanswered as to the reason for the effect of pre-dilution on PHP lipases. However, it may serve to caution against over-simplified interpretation of lipase assay results. Measured enzyme activity may not always be directly proportional to the amount of enzyme present because of variations in other interacting components from one sample to another.

CHAPTER 6

RELEASE OF LIPASE FROM ISOLATED TISSUES

6.1 INTRODUCTION

In order to reduce the number of experimental animals used to test the effectiveness of various sulphated polysaccharides in releasing lipases, the suitability of using adipose tissue preparations was investigated. This would provide a back-up to the results of post-injection plasmas on LPL release in vivo, and if enough tissue could be prepared in one batch the different GAGs and doses could be tested together without the risk of animal variability.

Isolated mammalian tissues have been widely used, in addition to PHP and milk, to provide a source of triglyceride lipases. The enzymes have either been released from perfused tissues, most commonly of the liver, with heparin-containing buffer (Augustin and Greten, 1979a) or extracted (with or without the use of heparin) after treatment of the tissue. Adipose tissue has been used in one of three forms for the extraction of LPL: as tissue pieces, as aqueous homogenates, or as acetone-ether powders (Chilliard et al., 1977). These methods have generally been used as the first step in the purification of the enzyme, but there have also been reports on the use of isolated tissue for clinical studies of LPL activities such as that of Lithell and Boberg (1977) on adipose tissue, or Reitman et al. (1982) on adipose tissue and muscle.

6.2 METHOD 1 - Incubation of tissue pieces

The stimulatory effect of heparin in releasing lipase in vitro from adipose tissue has previously been studied both as whole paired fat bodies (Salaman and Robinson, 1966) and tissue slices (La Rosa et al., 1973) from the rat, and as tissue pieces from man and goat (Lithell and Boberg, 1977; Chilliard et al., 1977). This method is thought (Chilliard et al., 1977) selectively to release, during

short-term incubations (30-60 mins.), the extra-adipocyte fraction of the LPL which is localized at the surface of the capillary lumen (see Footnote 1). In extended incubations of 3-5 hours however, surface LPL is replaced by newly- synthesized enzyme from inside the cell, as judged by the inhibitory effect of puromycin (a protein synthesis inhibitor). Furthermore, adequate oxygenation of the tissue was required for continued release of the enzyme (Wing et al., 1966).

In this study, adipose tissue pieces were used to compare in vitro release of LPL by heparin with other sulphated polysaccharides. Short incubation times of one hour were used, so that only extra-adipocyte LPL should be released. Oxygen was not supplied. The incubation medium used was that suggested by J. Stocks (personal communication). In preliminary studies on human and rat adipose tissue (unpublished, St. Bartholomew's Hospital, London), Stocks obtained both a graded response with dose, and differences in the level when using different polysaccharides to release LPL.

6.2.1 Preparation of tissue

Male Sprague-Dawley rats weighing 300-500 g were killed by a blow on the head and the fat bodies immediately removed and placed in ice-cold physiological saline (0.145M NaCl). The fat was blotted dry then cut into small pieces of 5-10 mg and about 100 (± 0.1) mg weighed accurately into tubes. The samples were kept on ice until

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Footnote 1: 'Hormone-sensitive lipase' (responsible for intracellular TG breakdown during fat mobilization) is also present in adipose tissue could also be released under these conditions (e.g. through cell damage). However, this enzyme would not be active at the pH used for the LPL assays (Chilliard et al., 1977).

used (within 2 hours of being removed from the rat).

6.2.2 Incubation medium

Materials

Earles balanced salt solution (BSS) x 10 (without phenol red, without $NaHCO_3$) (from Media room, NIBSC)

4.2% (w/v) $NaHCO_3$ BDH Chemicals Ltd.

Bovine serum albumin (Fraction V) BDH Chemicals Ltd.

Heparin 1,000 u/ml, 'Pularin', Duncan Flockart, U.K.

SSHA (see Ch. 4).

Heparan sulphate (HSII - see Ch. 4).

Method of Preparation

5 ml Earles BSS (x 10) were mixed with 2.5 ml $NaHCO_3$, and water added to 30 ml. After addition of 1.5 g albumin, the volume was made up to 5 ml with water. This medium was used in control incubations, with 1 or 2 u/ml heparin added for the tests. In further tests, SSHA and HSII were added to give similar concentrations to these (i.e. 6.5 and 13.0 µg/ml respectively) based on an approximated heparin potency of 150 u/mg.

6.2.3 Incubation of tissue

At zero time 1 ml of medium was added to the tissue (n = 3 or 4) and shaken well to ensure adequate suspension of tissue pieces in the medium. The suspension was then incubated in a shaking water bath for 1 hour at 37°C.

100 µl of each incubation mixture were tested in duplicate in the NES assay (low salt substrate only, since only LPL will be present). The results were calculated to give mU released g^{-1} fat in 1 hour.

6.2.4 Results

Different tissues gave very different levels of activity and also varied in their response to the two concentrations of heparin used. Results from two fats are shown in Figure 1.

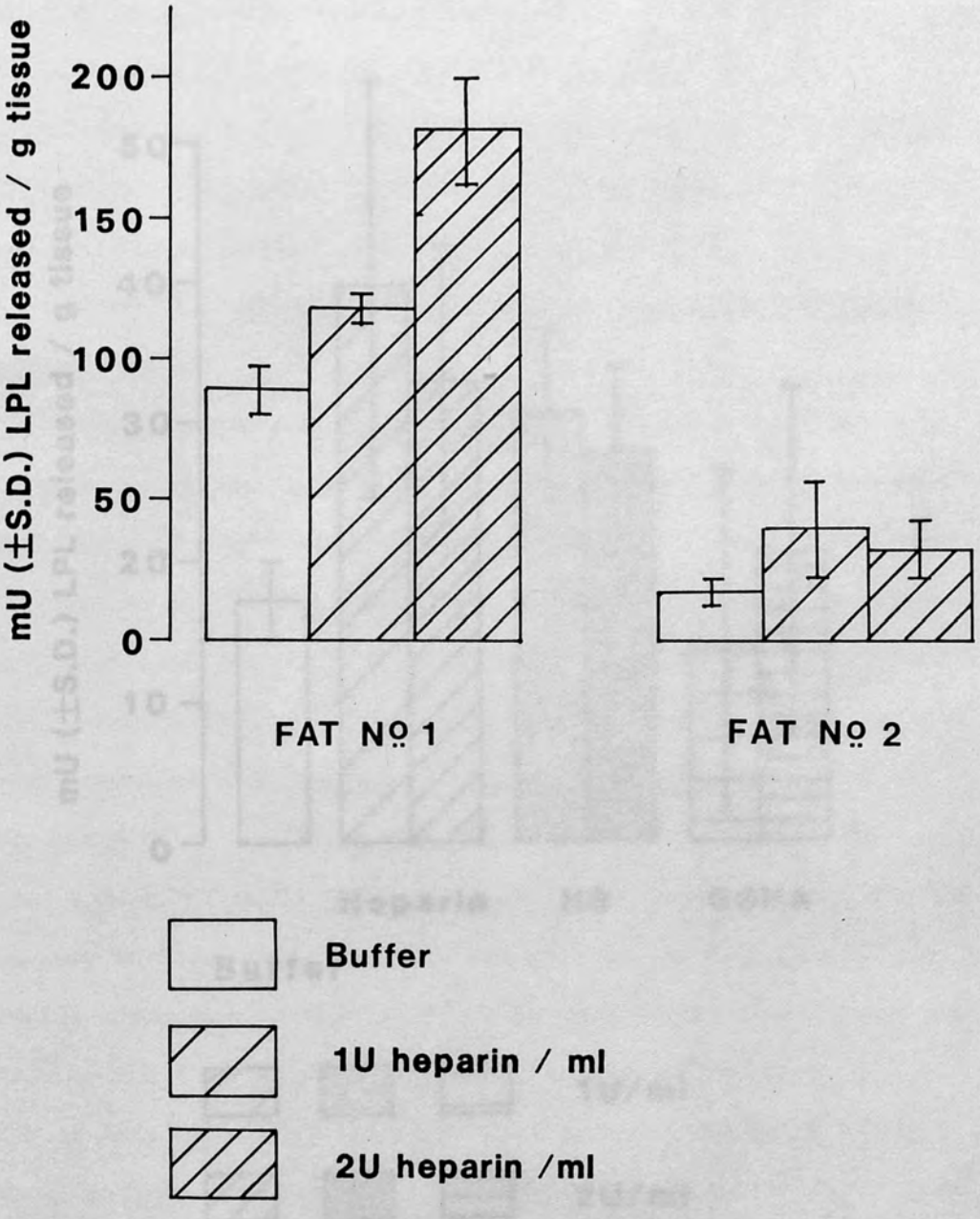
With Fat No. 1 there was good release of LPL even in the absence of heparin (approx. 90 mU LPL/ml g^{-1} fat) and a graded increase with heparin concentration of 30% and 100% for 1 u/ml and 2 u/ml respectively. Fat No. 2 gave much lower release overall (blank of approx. 20 mU/ml g^{-1} fat) with an approximate doubling of activity with both 1 u/ml and 2 u/ml heparin. Figure 2 shows the effect of the use of different sulphated polysaccharides. It was found that HS was not as effective as heparin in releasing lipase under these conditions (approx. 70% increase for both concentrations compared with 100% for heparin) but better than SSHA, which gave values not significantly different from those of the blank. It must be noted however, that the enzyme levels per tube for these incubations were very low.

6.2.5 Comments

The relatively high values of LPL activity obtained in the blank incubation (no heparin present) indicate that under these conditions a large proportion of the enzyme is released non-specifically from the tissue surface. This has also been reported by other workers using a similar method (Lithell and Boberg, 1977).

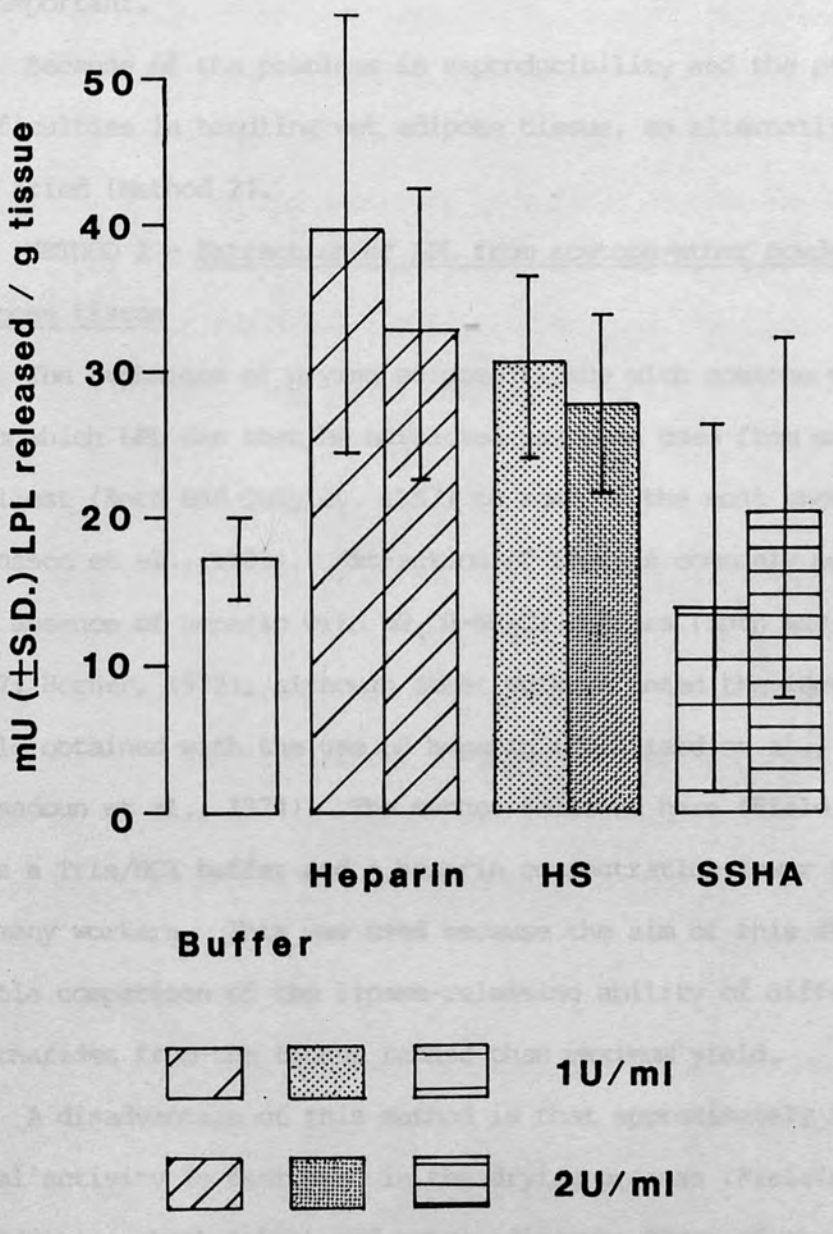
The great difference in the general levels obtained from incubation of tissues from different animals emphasizes that direct comparisons can only be made from the tissue of one animal. The poor repoducibility between duplicate samples is particularly evident with use of tissues that give low levels of release (e.g. Fat No. 2,

Fig 1 LPL released from adipose tissue pieces on incubation with heparin



Incubation time = 1 hour

Fig 2 LPL released from adipose tissue on incubation with a range of sulphated polysaccharides



Incubation time = 1 hour

Fig. 1). Sources of variability would include how finely the tissue is cut and the amount of moisture left on the tissue by blotting before weighing. The surface area presented to the medium is crucial in determing lipase release, so the size of the tissue piece is important.

Because of the problems in reproducibility and the practical difficulties in handling wet adipose tissue, an alternative method was tried (Method 2).

6.3 METHOD 2 - Extraction of LPL from acetone-ether powders of adipose tissue

The technique of drying adipose tissue with acetone and ether from which LPL can then be extracted has been used from some of the earliest (Korn and Quigley, 1957) to some of the most recent work (Hansson et al., 1983). Extraction of LPL was commonly performed in the absence of heparin with NH_4OH-NH_4Cl buffers (Korn and Quigley, 1957; Horner, 1972), although other workers noted the increased yield obtained with the use of heparin (Chilliard et al., 1977; Bensadoun et al., 1974). The method followed here (Fielding, 1968) uses a Tris/HCl buffer and a heparin concentration lower than that of many workers. This was used because the aim of this study is to enable comparison of the lipase-releasing ability of different polysaccharides from the tissue rather than maximum yield.

A disadvantage of this method is that approximately 60% of the total activity is destroyed in the drying process (Fielding, 1968). In addition, both intra- and extra-adipocyte forms of the LPL enzyme are thought to be extractable after this treatment (Chilliard et al., 1977) and this may mean that there is a large degree of non-specific release, i.e. that occurring in the absence of heparin.

However, there are indications (Horner, 1972) that specific release by heparin can still occur.

6.3.1 Preparation of tissue

The method used was based on that of Fielding (1968). Adipose tissue taken out of freshly-killed rats was at once placed into ice-cold 0.145 M NaCl (0.5 ml saline/g tissue). The mixture was homogenized in an overhead Silverson blender for 10 secs. The homogenate was added to approximately 40 volumes of acetone:ether (2:1), and stirred for 1 hour at 4°C. It was then either spun at 7,000 g for 15 mins., or rapidly filtered on a Buchner. The precipitate was dried at 4°C under vacuum over phosphorus pentoxide to constant weight.

6.3.2 Incubation of tissue

Approximately 5 or 10 mg portions of the dried tissue were accurately weighed out and incubated with 0.5 or 1.0 ml respectively or 0.05 M Tris/HCl buffer (pH 8.1) containing heparin (1 u/ml) or other polysaccharides (equivalent weight 6.5 µg/ml). These were shaken at 4°C for set lengths of time, then briefly centrifuged and 100 µl aliquots of the medium immediately tested in the NES lipase assay.

6.3.3 Results

Different tissue preparations gave one of two results. In approximately 50% of preparations the activity released was low (< 10 mU/ml per 10 mg dried tissue) and with these the inclusion of heparin in the incubation buffer had little or no effect on the activity (see Table 1a). Increasing incubation times to more than 15 mins. commonly led to a fall off in both sets of lipase activity (i.e. in the presence and absence of heparin). In the other tissue

TABLE 1: Release of LPL from acetone-ether dried adipose tissues on incubation in the presence and absence of heparin

Incubation time (mins.)	Enzymic activity mU/ml (10 mg)$^{-1}$ tissue			
	(a)*		(b)*	
	Buffer only	Heparin (1 u/ml) in buffer	Buffer only	Heparin (1 u/ml) in buffer
1	10.0	12.1	20.4	23.2
	10.3	12.2	19.2	23.9
5	8.4	9.7	25.5	30.7
	9.3	11.8	24.1	30.5
15	10.4	9.8	20.6	31.7
	9.9	7.6	23.6	34.9
30	9.0	8.0		
	7.9	8.5		
60	7.2	8.0	22.1	27.3
	7.4	7.8	20.5	27.3

* a) and b) refer to different tissue preparations

preparations however, the activity was approximately twice that of the first group, and in all such cases the presence of heparin in the incubation buffer gave a modest but definite increase in the enzymic activity measured (see Table 1b). This increase was of the order of 25% after 5 mins., rising to 50% after 15 mins., then declining after 30-60 mins.

Polysaccharides other than heparin that are known to release the lipase to different extents in vivo were also incubated with the defatted tissue. The enzymic activities obtained after use of DeS and SP54 were low, and not significantly different from the blank value (results not shown).

6.3.4 Comments

Extraction of LPL from acetone-ether dried adipose tissue has been extensively used as an early step in the preparation of purified enzyme (Korn and Quigley, 1957; Havel et al., 1973; Bensadoun et al., 1974). It does not appear from the results reported here however, to be a method that can be used to quantify the lipase-releasing ability of different polysaccharides. In only half the tissue preparations did the presence of heparin result in additional LPL activity after incubation. This inconsistency of tissue response (also found in Method 1), may be a result of real differences in tissue state from different rats, or to slight variation in the method of preparation, which has an effect both on overall enzyme activity and on how much can be released.

In those tissue preparations where heparin did increase activity on incubation, it is possible that heparin acted as a stabilizer rather than an active releaser. A recent paper by Parkin et al. (1982) describes the fine sensitivity of LPL extracts from

adipose tissue over a small range (0.05-1.0 u/ml) of heparin concentrations. The results (not shown) of a single experiment using Earles BSS and albumin medium (as used in Method 1) in place of Tris/HCl buffer support the importance of the stabilizing effect of heparin. In this test, the addition of heparin to the medium made no difference to the enzymic activity measured after 20 mins.' incubation, suggesting that the LPL was already well stabilized by the medium. However, other results (Table 1a) show that the released enzyme incubated for an hour at 4°C does not appear to be more stable in the presence than in the absence of heparin.

This method was not further considered for future use, both because of the variability of the results and because of the uncertainty that heparin does actually act, under these conditions, to release the enzyme from its endothelial-bound site. Due to the inflammability of acetone and ether, the large volumes used make this an unsuitable method for routine use.

6.4 METHOD 4 - Adipose tissue homogenates

Preliminary studies were performed using adipose tissue homogenates, in an attempt to circumvent some of the unreliability and unsuitability of the other two methods. In making a homogeneous mixture, it was hoped to eliminate some of the errors due to weighing problems of Method 1, whilst being a more physiological model than the acetone-dried tissue of Method 2. The preparation of large homogeneous batches, using adipose tissue from different rats, would allow many polysaccharide samples to be tested together.

6.4.1 Preparation of tissue

Adipose tissue was homogenized in 0.145 M NaCl (2 g tissue/ml saline) in a Silverson blender. 150 µl (approx. 150 mg) aliquots of

the homogenate were dispensed with an automatic pipette into weighed tubes. These were incubated for 1 hour at 37°C in a shaking water bath, in 1 ml 0.05 M Tris/HCl (pH 8.1) with or without heparin (1 or 2 u/ml).

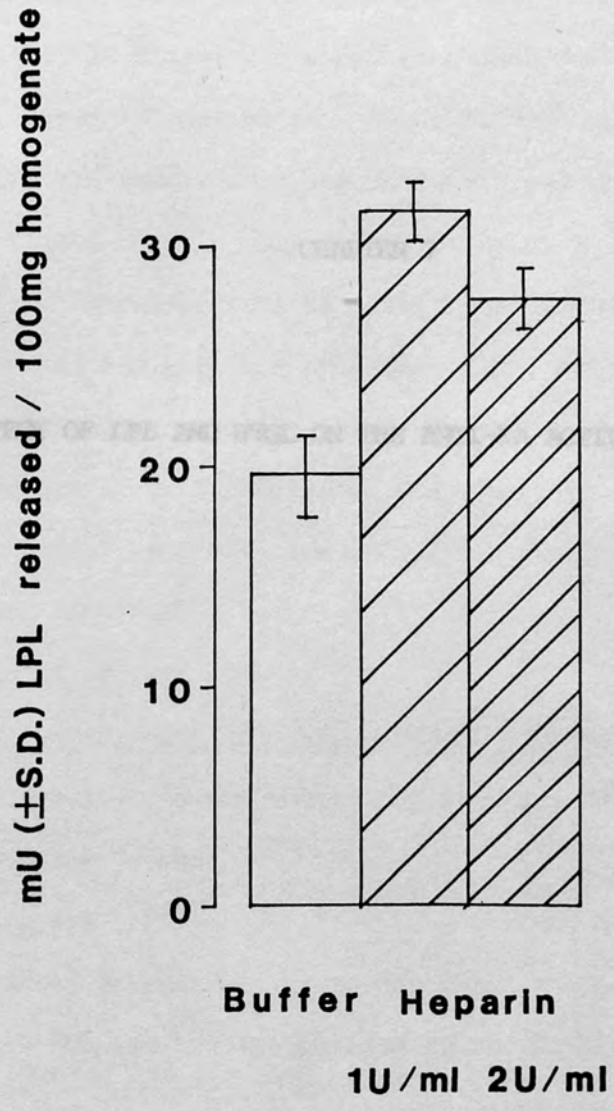
6.4.2 Results and Comments

Figure 3 shows the results of the incubation in the presence and absence of heparin. Although these are definite increases in the presence of heparin, the lipase levels are very low.

6.5 CONCLUSIONS

It was desirable to have a usable in vitro model for LPL release, in order to reduce both the number of animals used in in vivo work and the risk of animal variability. Three methods of preparing adipose tissue prior to inducing release with heparin were tried, but none was found to be satisfactory. The best results were obtained with incubation of tissue pieces, but even with this method the lipase levels released were generally low with relatively high blank values. There were also great differences between tissue batches, and difficulties in handling the tissue made it a cumbersome method. Use of other polysaccharides showed up only general differences in their ability to release lipase.

Fig 3 LPL released from adipose tissue homogenates on incubation with heparin



Incubation time = 1 hour

CHAPTER 7

THE EFFECT OF LPL AND HTGL ON THE ANTI-XA ACTIVITY OF PLASMA

7.1 INTRODUCTION

Several pieces of recent research have pointed to the possibility that the triglyceride lipases released by heparin and other polysaccharides have a direct or indirect effect on plasma coagulability. The heparin analogues, SSHA and SP54, have virtually no anti-Xa activity in vitro, but there is enhanced activity present in ex vivo plasmas (Thomas et al., 1980; Fischer et al., 1982). This suggests that some factor has been released into the blood. The work described in this chapter was designed to test the hypothesis that the increased anti-Xa activity of post-SSHA and -SP54 plasmas is associated with the presence of LPL and/or HTGL, since both these analogues are potent releasers of the lipases. This was tested by the use of purified lipase enzymes (both from PHP and milk) in the anti-Xa clotting and amidolytic assays.

7.2 MATERIALS AND METHODS

7.2.1 Source of LPL and HTGL

i) Post-heparin LPL and HTGL LPL and HTGL were purified by single heparin-Sepharose chromatography from rat PHP as described in Chapter 3 (Section 3.4.1).

ii) Milk LPL LPL was purified from skimmed bovine milk by heparin-Sepharose chromatography as described in Chapter 3. A sample of milk LPL was kindly provided by Dr. T. Olivecrona, Umeå, Sweden.

7.2.2 Plasmas for Incubation

i) Human lipid-rich plasma Blood was collected from normal volunteers 1½ hours after a fatty meal, and mixed 9:1 with 8.3% trisodium citrate. Plasma was prepared by centrifugation at 48,000 g for 10 mins. and stored at -40°C until used.

ii) Normal human plasma Plasma from six donors was provided by the North London Blood Transfusion Centre, Edgware, Middx. (anticoagulant CPD and adenine) and the chylomicra were removed by centrifugation at 90,000 g for 90 mins.

iii) Lipoprotein-free plasma This was prepared (by Ms. E. Gray, Division of Blood Products, NIBSC) from normal human plasma by density gradient ultracentrifugation at 200,000 g for 3 hours in a Sorvall TV-850 vertical rotor. It was dialysed overnight against 0.05 M Tris, 0.15 M NaCl (pH 7.4), and stored at -40°C until used.

7.2.3 Anti-Xa Clotting Assay

i) Principle of Assay This is a two-stage assay designed to test the effect of plasma (plus any additions) against one particular clotting factor. Test plasma is allowed to act against a precise amount of purified Factor Xa for a set length of time. The amount of Factor Xa remaining is then determined by addition to Factor Xa-deficient plasma. In this assay, the higher the anti-Xa activity in the test plasma, the longer the clotting time.

ii) Reagents

Calcium chloride

50 mM (supplied as 1 M solution, BDH Chemicals, Poole, Dorset, U.K.)

CAG buffer, pH 7.4

150 volumes glyoxaline buffer (0.05 M glyoxaline, 0.1 M NaCl, pH 7.4)

30 volumes 3.8% trisodium citrate (BDH Chemicals)

1 volume 20% (w/v) bovine albumin, Fraction V (Sigma, Poole, Dorset, U.K.)

Factor X-deficient plasma Diagnostic Reagents Ltd., Thame, Oxon.

Factor Xa (bovine) Diagnostic Reagents Ltd.

Phospholipid (bovine brain) NIBSC reagent 79/508

BHA water

2 mM butylated hydroxyanisole (BDH Chemicals) in ethanol, diluted 1 in 1000 in distilled water.

BHA saline

2 mM butylated hydroxyanisole in ethanol, diluted 1 in 10 with distilled water, then 1 in 100 in 0.145 M NaCl.

The Factor Xa is made up in 0.5 ml distilled water, then diluted 1:100 or 1:1000 in CAG buffer. The freeze-dried phospholipid is reconstituted with 1 ml BHA water then diluted 1 in 400 with BHA saline, and mixed 1:1 with Factor X-deficient plasma (reconstituted as directed by the manufacturers, with distilled water) to prepare the 'substrate mix'.

iii) Method

1. All reagents are preincubated at 37°C.
2. Prepare 100 µl calcium chloride in clotting tubes.
3. Mix 300 µl Factor Xa with 100 µl of the plasma + lipase mixture. Start a clock at the same time.
4. After 1 min. 45 secs. subsample 100 µl from the above and add to the calcium chloride.
5. At exactly 2 mins. add 200 µl 'substrate mix' and measure clotting time from this final addition.

The tests were performed using semi-automated coagulometers (Burkard Scientific, Uxbridge, Middx., U.K.)

7.2.4 Amidolytic Assay

i) Principle of assay Factor Xa catalyzes the hydrolysis of p-nitroaniline from the substrate Bz-Ile-Glu-Gly-Arg-pNA (S-2222).

This release is measured spectrophotometrically at 405 nm, and the rate of release can be related to the concentration of Factor Xa present.

ii) Reagents

Bovine Factor Xa Diagnostic Reagents Ltd., Thame, Oxon, U.K.

Chromogenic substrate, S-2222 Kabi Diagnostica AB, Stockholm, Sweden

CAG buffer (pH 7.4) (see anti-Xa clotting assay)

EDTA/tris buffer (pH 8.4) 0.05 M Tris, 7.5 mM EDTA, 0.175 M NaCl

The Factor Xa is made up as directed by the manufacturer with 0.5 ml distilled water, and then diluted 1:4 with CAG buffer. The S-2222 is made up to 4 mM with distilled water, diluted to 0.4 mM with distilled water then mixed 1:1 with EDTA/Tris buffer.

Incubation mixtures of enzyme and lipid-rich plasma were made up as described for the clotting assays (in Section 7.3.1).

iii) Method

1. All reagents are preincubated at 37°C.
2. At zero time add 0.1 ml plasma + lipase mixture 0.3 ml Factor Xa. Incubate at 37°C for 2 mins.
3. Add 1 ml of S-2222/buffer mixture, mix thoroughly and immediately measure absorbance at 405 nm.

7.3 RESULTS

7.3.1 Effect of lipases on plasma anti-Xa clotting activity in the absence of heparin

Human lipid-rich plasma was mixed with LPL or HTGL to give lipase activities similar to those achieved in man after i.v. injection of 1,000-2,000 U heparin (i.e. 30-50 mU/ml as tested by the modified NES assay, Ch. 2), and incubated at 37°C for the time indi-

cated under each section. Incubation mixtures were made up, in the order given, as follows:

8 volumes human lipid-rich plasma

+

1 volume LPL or HTGL (approx. 350 mU/ml)

+

1 volume 0.145 M saline

Controls where the active enzyme was replaced with an equal volume of either heat-denatured enzyme (treated at 56°C for 15 mins.) or 0.15 M NaCl in 0.01 M phosphate buffer - 50% glycerol (pH 7.4), were included in all assays.

The incubation mixtures were tested by anti-Xa clotting assays as described in Section 7.2.3, using Factor Xa diluted 1:1000 to increase sensitivity. Clotting times were determined by duplicate or triplicate runs.

i) Effect of LPL No change in anti-Xa activity of human plasma was detected after incubation with LPL preparations from milk at 37°C for 45 mins. (see Table 1). The same result was obtained with several preparations of the enzyme purified in one step by heparin-Sepharose chromatography, and that provided by Dr. Olivecrona.

The LPL enzyme from PHP was also incubated with four different plasmas (3 lipid-rich and 1 normal) and the anti-Xa activity measured. There was little or no change in clotting time; an average lengthening of 2.3 secs., with a maximum difference of 3.5 secs. for one of the plasmas (batch IV) (see Table 2).

The time course of the action of LPL purified from PHP was followed by subsampling at various times up to 1 hour. There was a

TABLE 1: Effect of LPL from bovine milk on plasma anti-Xa clotting activity in the absence of heparin

Source of lipase	Mean clotting time (secs.) Buffer	Lipase
NIBSC[1]	31.6	30.6
Umeå[2]	50.0	50.3

1. Prepared in one step by heparin-Sepharose chromatography as described in Chapter 3, Section 3.4.1.

2. Provided by Dr. Olivecrona, Umeå.

TABLE 2: Effect of LPL purified from PHP on plasma anti-Xa clotting activity in the absence of heparin

Plasma batch	LPL prep.	Mean clotting time (secs.) ± s.d. Buffer	Lipase
I Lipid-rich	a	56.7 ± 1.3	56.9 ± 0.3
II " "	a	45.2 ± 1.4	47.2 ± 1.3
III Normal	a	44.3 ± 1.3	47.6 ± 2.5
IV Lipid-rich	b	52.1 ± 0.6	55.6 ± 1.4

slight lengthening in clotting time of 2-3 secs. after all incubation times, including the zero time (see Fig. 1).

ii) Effect of HTGL Incubation of HTGL with lipid-rich plasma for 45 mins. at 37°C led to a significant lengthening of clotting time (i.e. anti-Xa activity increased) by an average of 12.6 secs. A smaller increase was obtained using HTGL with normal plasma (batch III), suggesting the role of lipid in alteration of anti-Xa activity on incubation with lipase (see Table 3).

The time course of HTGL action on lipid-rich plasma was also studied, and showed a steady increase in anti-Xa activity to a maximum after 45 mins.' incubation, when it reached a plateau (see Fig. 2). The buffer control showed little change during this time.

Unless stated, incubation times of 45 mins. were used for all subsequent tests.

iii) Heparin equivalence of HTGL action The heparin equivalence of HTGL action was estimated by substituting HTGL with a range of heparin concentrations in the clotting assay, and comparing the clotting times using the same plasma. The increase in clotting times induced by HTGL (mean, 15.4 secs.) was equivalent to using 0.05 iu/ml heparin (mean 13.1 secs.).

iv) Specificity of HTGL action It is possible that the lengthening in clotting times found with HTGL is due to a non-specific action of the enzyme preparation, or its incubation products, independent of the anti-Xa activity. To test this, the 2 mins. normally allowed for action of plasma against Factor Xa in the first stage of the assay were omitted. Instead, the plasma and HTGL were added to the Factor Xa and the plasma was immediately subsampled for the second stage of the assay. In Control 1 (see Fig.

Fig 1 Time course of LPL action on plasma anti-Xa activity in the absence of heparin

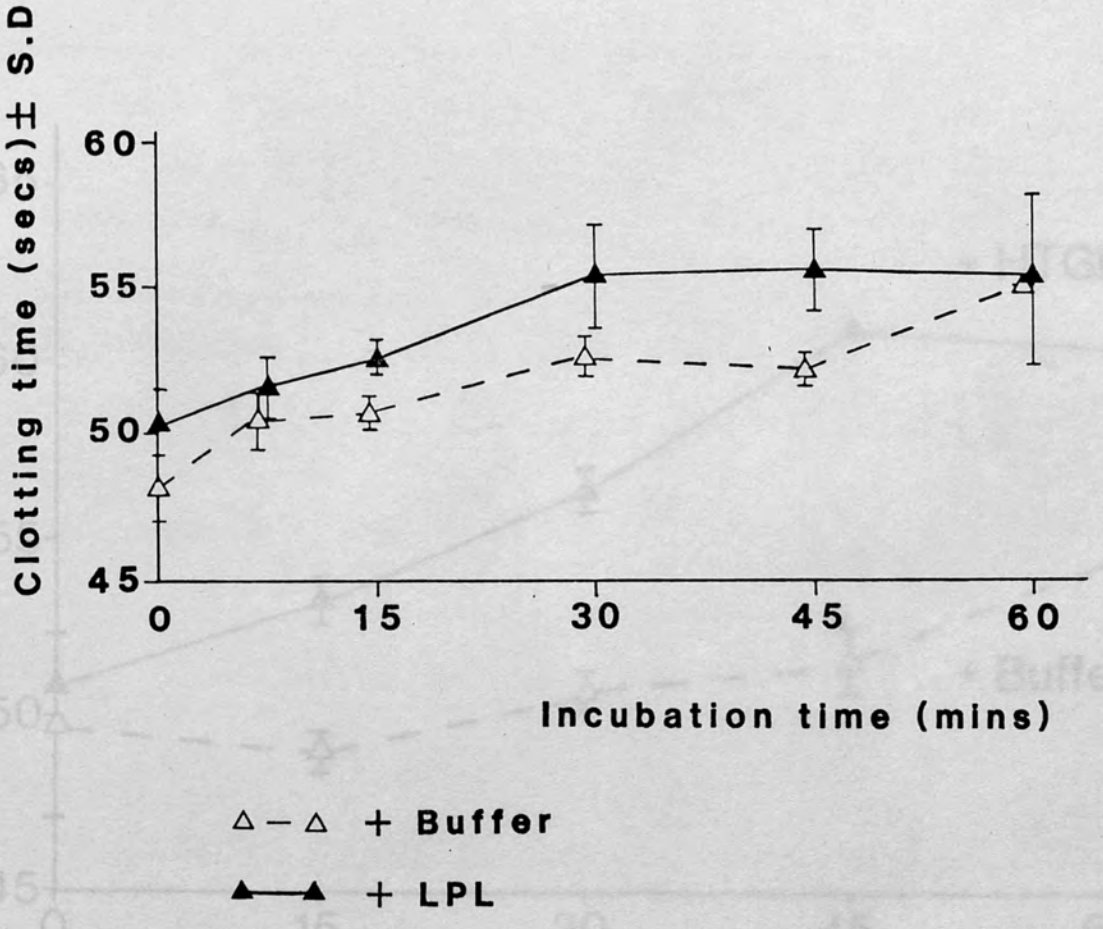


Fig 2 Time course of HTGL action on plasma anti -Xa activity in the absence of heparin

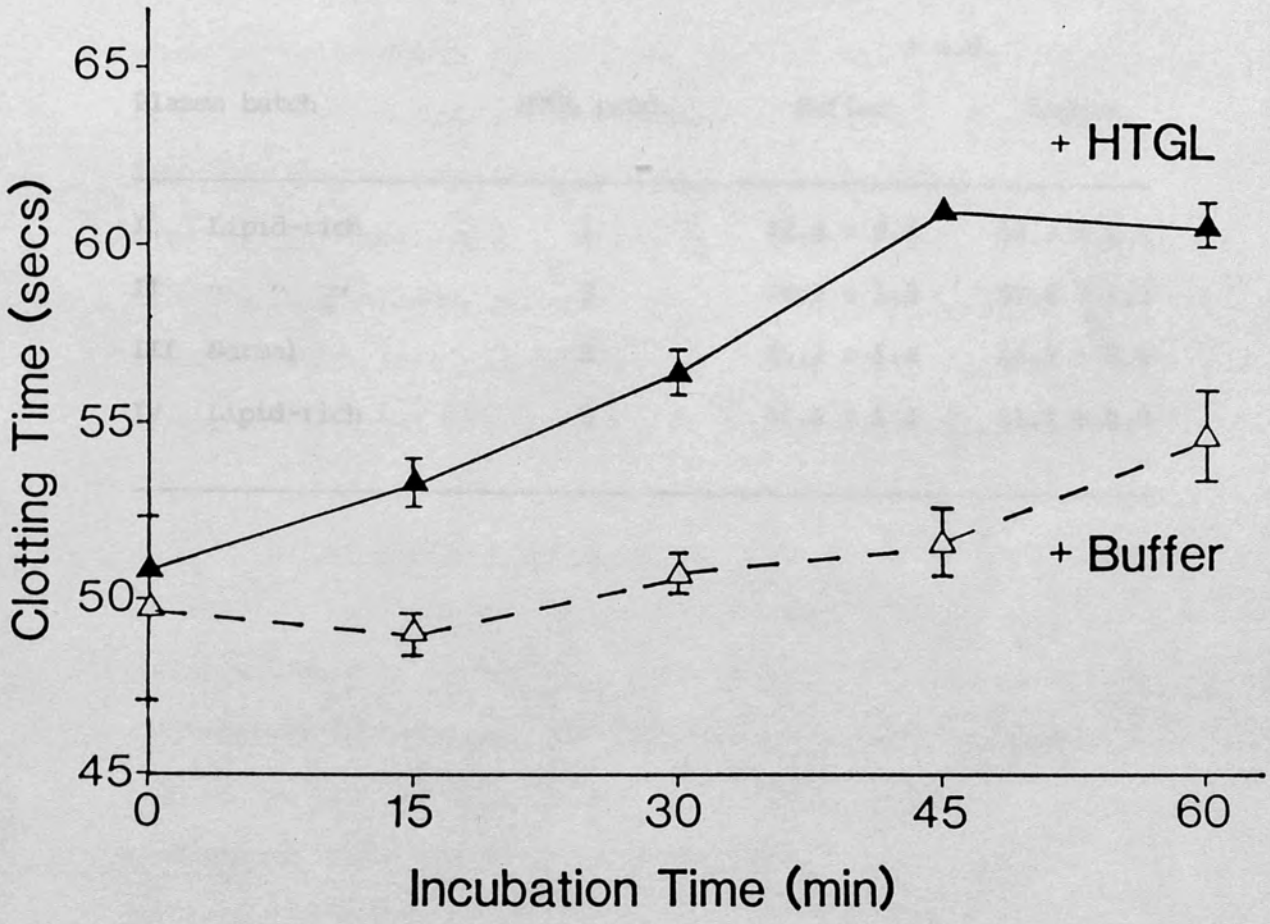


TABLE 3: Effect of HTGL on plasma anti-Xa clotting activity in the absence of heparin

Plasma batch		HTGL prep.	Mean clotting time (secs.) ± s.d. Buffer	Lipase
I	Lipid-rich	1	42.8 ± 0.6	58.2 ± 1.1
II	" " "	2	44.3 ± 1.3	57.6 ± 1.3
III	Normal	2	45.2 ± 1.4	48.2 ± 0.8
IV	Lipid-rich	3	51.6 ± 1.4	61.1 ± 0.0

3), to investigate the effect of the enzyme preparation itself, plasma and HTGL were mixed and added without prior incubation to Factor Xa. To test the effect of the incubation products, plasma and HTGL were incubated together at 37°C for 45 mins. before subsampling into Factor Xa (Control 2). These differences in procedure are compared in Figure 3 to the normal test.

As shown in Table 4, no significant differences in clotting times were obtained by the addition of either the HTGL preparation or the incubation products, as compared with buffer. This result indicates that the effect of HTGL to increase clotting times (as shown in the 'normal test', Table 4) is through action against Factor Xa. The longer plasma clotting times obtained with buffer in the normal test compared with the controls, is due to the action of plasma At III which acts on Factor Xa during the 2 minute' incubation.

v) Use of lipoprotein-free plasma The results from the time course of HTGL action and its use with plasmas of different lipid contents (Table 3) suggest that the effect is through enzymic activity against lipoproteins. This was tested by using lipoprotein free plasma (lipoproteins removed by ultracentrifugation). Using this plasma, there was no change in anti-Xa clotting activity on incubation with HTGL or LPL for 45 mins. at 37°C (Table 5). This result shows the importance of lipoproteins in the effect HTGL has in lengthening plasma clotting times in the absence of heparin.

7.3.2 The effect of lipases on anti-Xa activity measured by the amidolytic assay

Thomas et al. (1980) have reported that although post-SSHA plasmas have good anti-Xa activity by clotting assays, little acti-

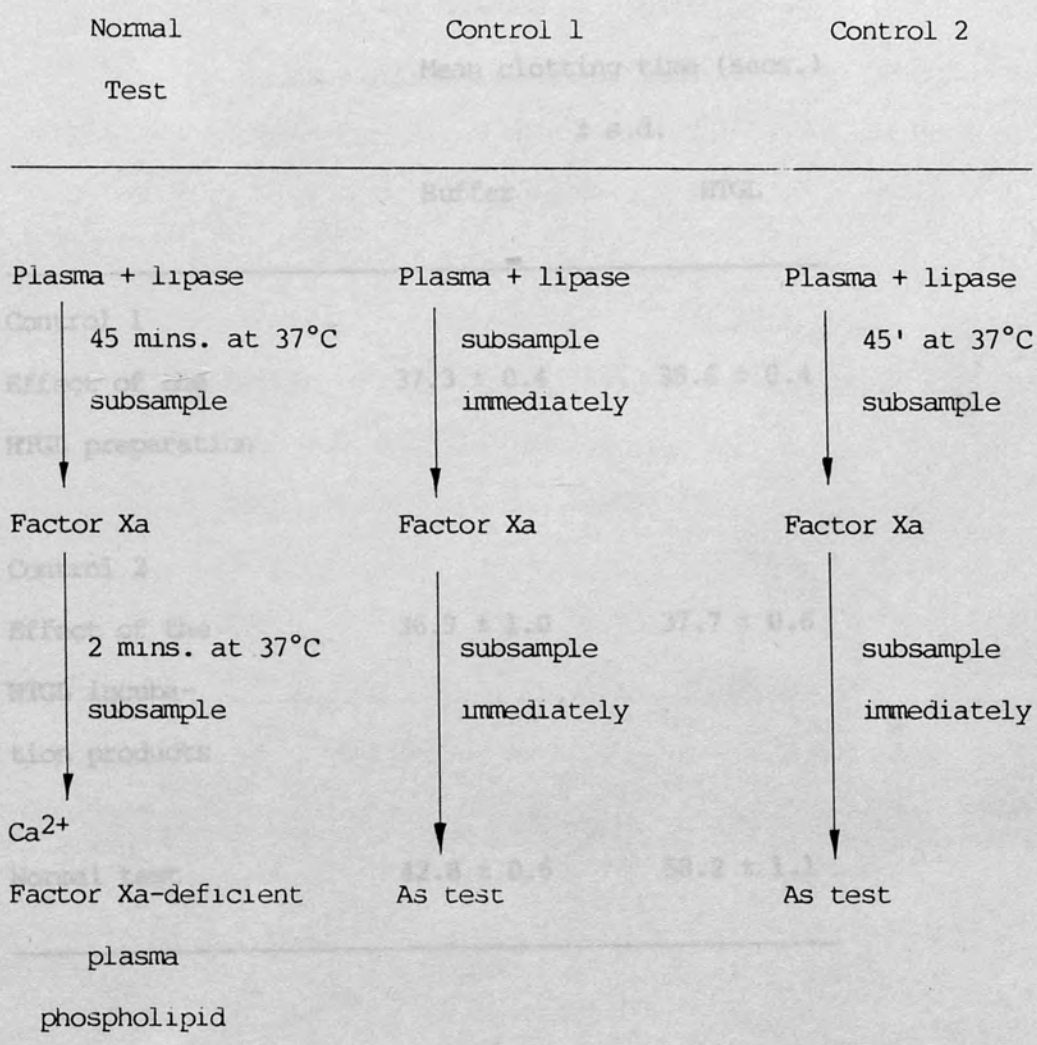


FIGURE 3

Summary of test and control procedures

TABLE 4: Test of the specificity of the action of HTGL or its incubation products on anti-Xa activity

	Mean clotting time (secs.) ± s.d.	
	Buffer	HTGL
Control 1 Effect of the HTGL preparation	37.3 ± 0.4	35.6 ± 0.4
Control 2 Effect of the HTGL incubation products	36.9 ± 1.0	37.7 ± 0.6
Normal test	42.8 ± 0.6	58.2 ± 1.1

TABLE 5: Effect of LPL and HTGL on anti-Xa clotting activity of lipoprotein-free plasma in the absence of heparin

	Mean clotting time (secs.) ± s.d.
Buffer	51.4 ± 0.4
HTGL	51.4 ± 0.4
LPL	50.8 ± 1.1

vity is detected using the amidolytic assay. The results reported in Section 7.3.1 indicate that HTGL increases plasmna anti-Xa activity. Preliminary tests were carried out to see whether this effect of HTGL can also be detected by the amidolytic assay or whether, like post-SSHA plasmas, it can only be shown by the clotting assay.

As in the clotting tests, HTGL was incubated with lipid-rich plasma for various times at 37°C, and aliquoted into Factor Xa and left for 2 mins. The amount of amidolytically-active Factor Xa remaining in the incubation was compared with that of the buffer control by the rate of formation of the coloured product from the synthetic substrate, S-2222. A decrease in the ΔOD would indicate a reduction in Factor Xa and so greater anti-Xa activity in the incubation mixture.

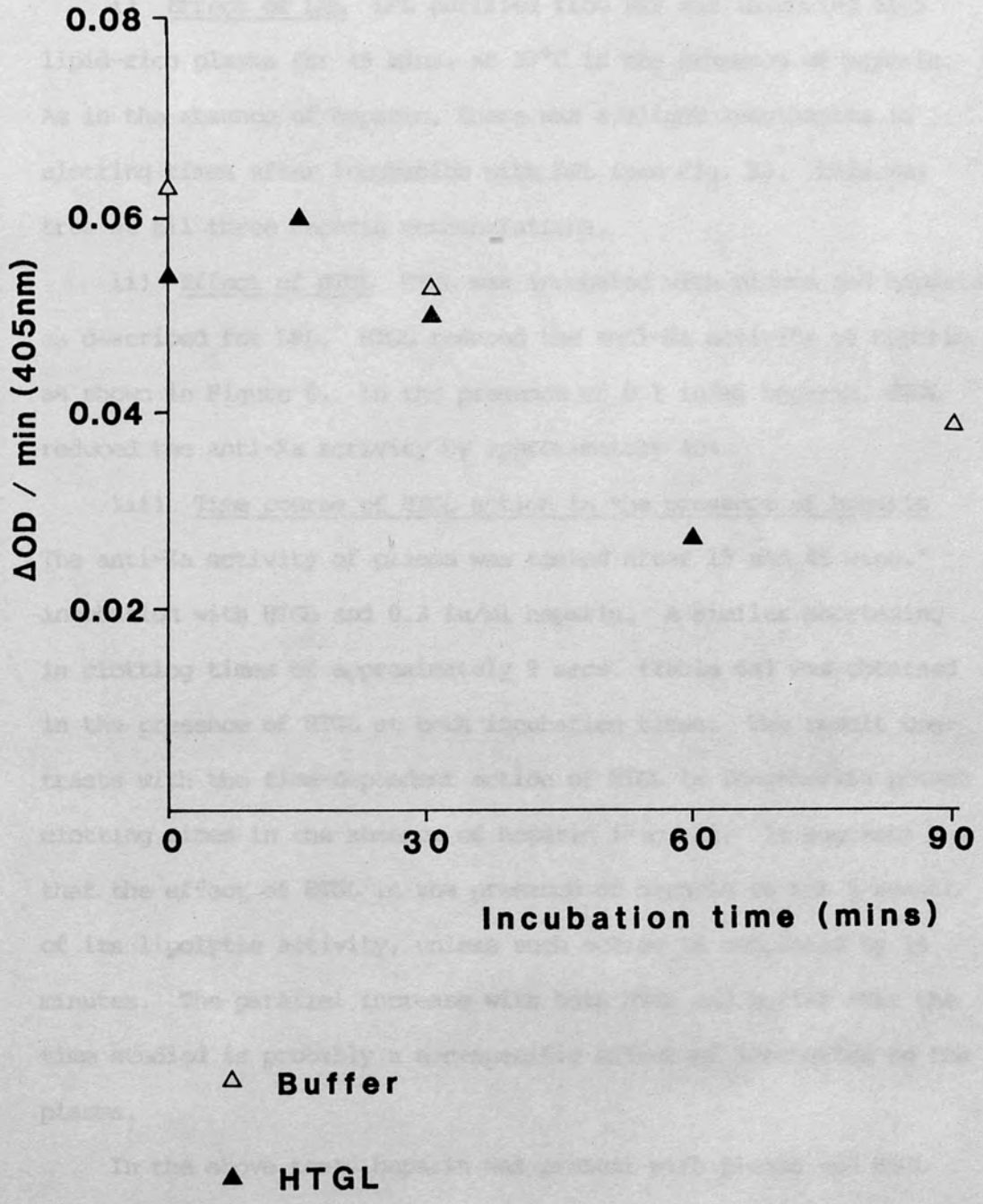
The results given in Figure 4 show that there was little change of plasma anti-Xa activity with either HTGL or buffer up to incubation times of 30 mins. There was an apparent increase in anti-Xa activity with HTGL after 60 mins.' incubation.

7.3.3 <u>The effect of lipases on plasma anti-Xa clotting activity in the presence of heparin</u>

To simulate ex vivo PHP where heparin and lipase are both present, the in vitro effect of lipase was tested in the presence of heparin. As reported in Section 3.1 of this chapter, both heparin and HTGL increase the anti-Xa activity of plasma, and so a further enhancement of activity would be expected when both are present.

To test the effect of added heparin with both lipases, incubation mixtures were made up as described for tests in the absence

Fig 4 Effect of HTGL on plasma anti-Xa activity as tested by the amidolytic assay



of heparin, except that 50 µl heparin (3rd International Standard, 65/69 NIBSC) were added in place of saline to give a final incubation concentration of 0.05, 0.1 or 0.2 iu/ml. These mixtures were tested in the anti-Xa clotting assay using purified Factor Xa diluted 1:100.

i) Effect of LPL LPL purified from PHP was incubated with lipid-rich plasma for 45 mins. at 37°C in the presence of heparin. As in the absence of heparin, there was a slight lengthening in clotting times after incubation with LPL (see Fig. 5). This was true at all three heparin concentrations.

ii) Effect of HTGL HTGL was incubated with plasma and heparin as described for LPL. HTGL reduced the anti-Xa activity of heparin as shown in Figure 6. In the presence of 0.1 iu/ml heparin, HTGL reduced the anti-Xa activity by approximately 40%.

iii) Time course of HTGL action in the presence of heparin The anti-Xa activity of plasma was tested after 15 and 45 mins.' incubation with HTGL and 0.2 iu/ml heparin. A similar shortening in clotting times of approximately 9 secs. (Table 6a) was obtained in the presence of HTGL at both incubation times. The result contrasts with the time-dependent action of HTGL in lengthening plasma clotting times in the absence of heparin (Fig. 2). It suggests that the effect of HTGL in the presence of heparin is not a result of its lipolytic activity, unless such action is completed by 15 minutes. The parallel increase with both HTGL and buffer over the time studied is probably a non-specific effect of incubation on the plasma.

In the above test, heparin was present with plasma and HTGL for the full 45 mins. of incubation. In one incubation mixture

Fig 5 Effect of LPL on plasma anti-Xa clotting activity in the presence of heparin

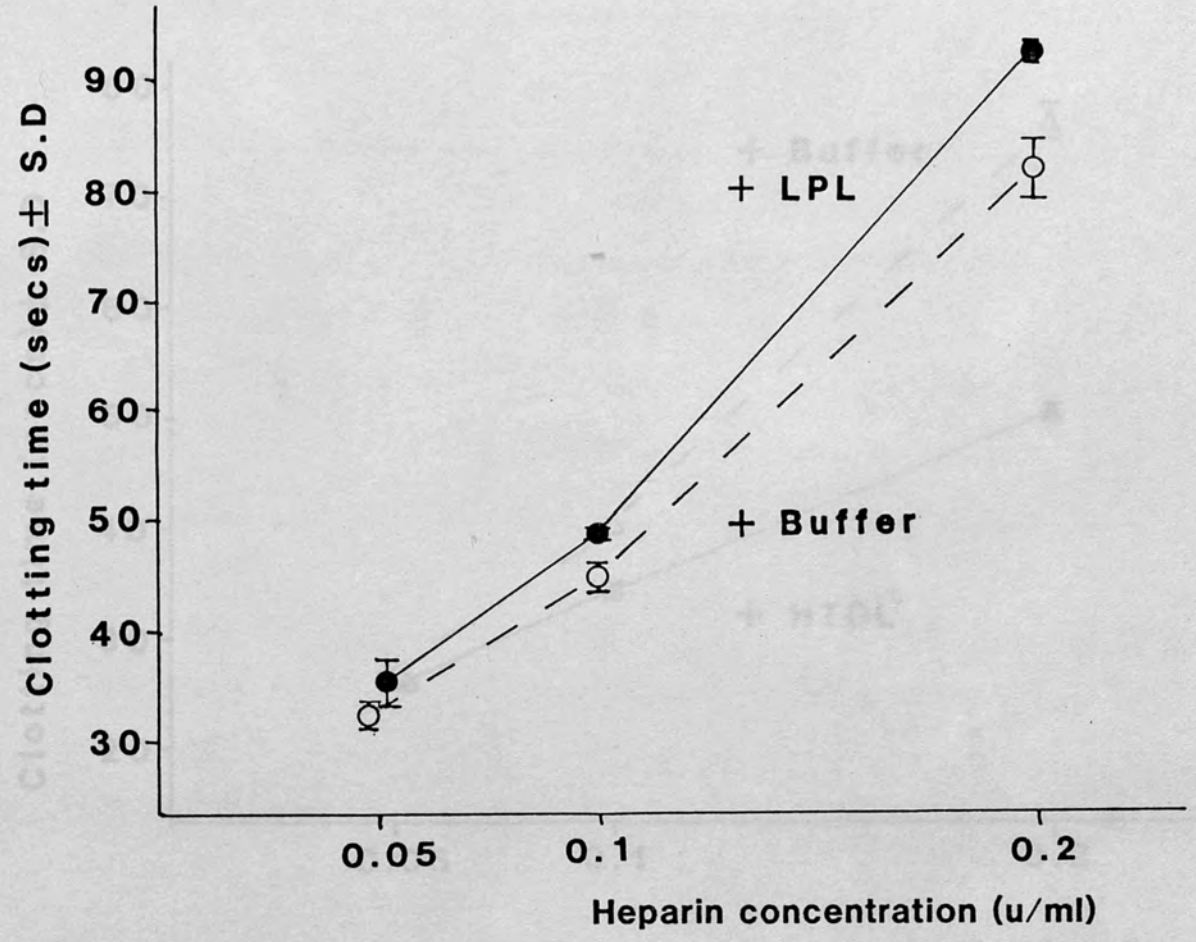


Fig 6 Effect of HTGL on plasma anti-Xa clotting activity in the presence of heparin

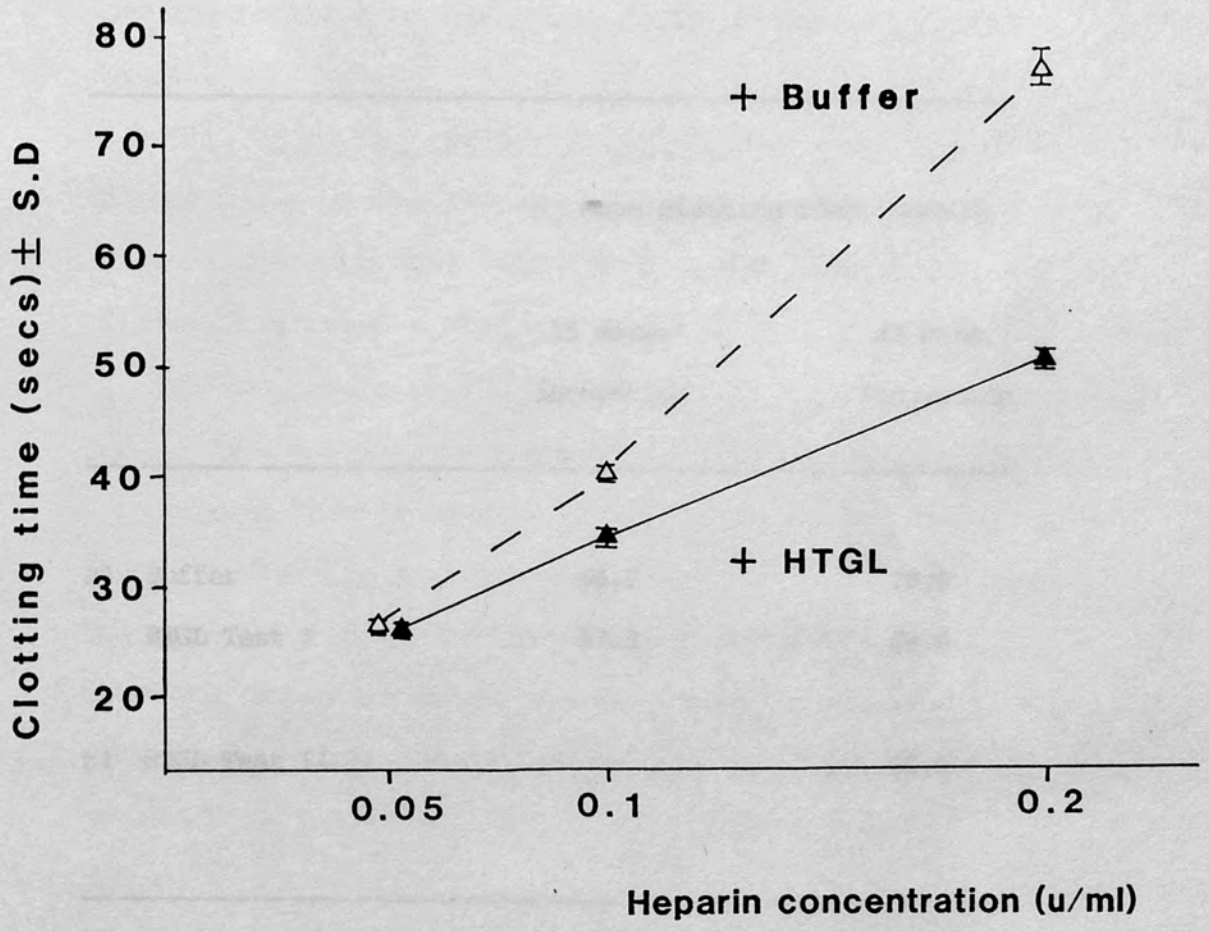


TABLE 6: Time course of HTGL action on plasma clotting time in the presence of heparin (0.2 iu/ml) (Test I) and the effect of preincubation in the absence of heparin (Test II)

	Mean clotting time (secs.)	
	15 mins.' incubation	45 mins.' incubation
a) Buffer	66.5	78.6
HTGL Test I	57.3	69.6
b) HTGL Test II		68.6

however (Test II, Table 6b) heparin was added just before the mixture was tested in the assay. Comparison of Tests I and II in Table 7 shows that there is no difference in the clotting times obtained. This means that although HTGL would presumably have had the normal effect of increasing plasma anti-Xa activity during incubation in the absence of heparin, the effect is negligible compared with the reduction it immediately causes in the anti-Xa activity of heparin.

iv) Effect of a mixture of LPL and HTGL The effect of HTGL alone and that of both HTGL and LPL on plasma clotting times in the presence of heparin (0.2 iu/ml) was compared. There was not a significant difference in clotting times (Table 7).

v) Test of lipase and heparin with the use of lipoprotein-free plasma HTGL and LPL were incubated with lipoprotein-free plasma, with heparin added to give a final concentration of 0.2 iu/ml. As shown in Table 8, there was a significant 14 sec. shortening of plasma clotting time after incubation with HTGL but little change with LPL. This is a similar result to that obtained with lipid-rich plasma, which suggests that the effect of HTGL in the presence of heparin is a non-specific one, not involving its lipolytic action.

7.4 DISCUSSION

This study was designed to test the hypothesis that the anti-Xa activity present in post-SP54 and SSHA plasmas (Thomas et al., 1980; Fischer et al., 1982) is due to the presence of triglyceride lipases. The polysaccharides have little detectable anti-Xa activity in vivo, and it has been suggested that the activity ex vivo is due to the presence of some factor(s) which has been

TABLE 7: Comparison of the effect of HTGL and a HTGL/LPL mixture on plasma anti-Xa clotting activity in the presence of heparin (0.2 iu/ml)

	Mean clotting time (secs.)
HTGL	69.4
HTGL + LPL	68.1
Buffer	78.6

TABLE 8: Effect of HTGL and LPL on anti-Xa clotting activity of lipoprotein-free plasma in the presence of heparin (0.2 iu/ml)

	Mean clotting time (secs.) ± s.d.
Buffer	73.6 ± 2.3
HTGL	59.1 ± 0.0
LPL	75.1 ± 1.8

released into the circulation.

LPL from rat PHP or bovine milk did not significantly alter the anti-Xa activity of plasma. A slight increase in anti-Xa activity was detectable immediately on addition of the LPL from PHP. This is probably due to the presence of At III which co-elutes with LPL when purified by normal heparin-Sepharose chromatography as described in Chapter 3 (Östlund-Lindqvist, 1979). This explanation is supported by the finding that LPL from bovine milk - which should have no contaminating At III - gave no change in plasma clotting times.

In contrast, significant lengthening in plasma clotting times were induced by HTGL, especially in lipid-rich plasmas. The peak lengthening was similar to that achieved by the in vitro addition of 0.05 iu/ml heparin to the same plasma. This level of anti-Xa activity is also equivalent to that found in ex vivo plasma samples after subcutaneous injection of 5,000 units of heparin. Since this dosage of heparin has been used to prevent post-operative deep vein thrombosis (Kakkar et al., 1978) the anti-Xa activity mediated through lipase release after SSHA and SP54 may be of some clinical significance.

Virtually no change was found on incubation of HTGL with lipoprotein-free plasma. Further tests showed that the effect in lipid-rich plasma was not due to the non-specific action of the enzyme preparation nor its incubation products on the clotting process itself. There should be little At III contamination of the HTGL sample (from work on human plasma, McKay and Laurell, 1980). All these results indicate that it is the HTGL action against lipoproteins which is causing the increase in anti-Xa activity of the plasma.

Work done by Barrowcliffe et al. (1982) shows that LDL and HDL possess anti-Xa clotting activity and that the activities of these lipoproteins are increased after they have been batch absorbed with $Al(OH)_3$. It is feasible that HTGL acts on these lipoproteins in the same way as $Al(OH)_3$, particularly since HDL and LDL appear to be the major natural substrates of HTGL (Nilsson-Ehle et al., 1980). In support of a common underlying mechanism, preliminary results showed that the increased anti-Xa activity induced by HTGL was not detectable by amidolytic assay up to 30 mins.' incubation, as was found both for $Al(OH)_3$-treated lipoproteins (Barrowcliffe et al., 1982) and for post-injection plasmas of SSHA and SP54 (Thomas et al., 1980; Fischer et al., 1982).

The anti-Xa activity demonstrable in plasma after heparin injection is generally assumed to be due totally to the presence of heparin, and the lipase released is not considered. The results shown in the first part of this Chapter suggest that, since HTGL does affect anti-Xa activity, the actual measured activity may be a result of the combined action of heparin and of the lipase. For this reason, the effect of both lipases on plasma anti-Xa activity in the presence of heparin was also studied. Plasma clotting times after incubation with LPL were lengthened by a few seconds, as was the case in the absence of heparin, and this can be similarly ascribed to At III contamination of the LPL preparation. The results using HTGL, however, are more puzzling. Although both HTGL and heparin alone have, or induce anti-Xa activity, plasma clotting times were reduced when they were incubated together. In terms of heparin units this shortening due to the HTGL represented, when using 0.2 iu/ml heparin, a decrease of 40% in anti-Xa activity (i.e.

0.2 to 0.125 iu/ml). However, at 0.05 iu/ml, the lowest heparin concentration tested, there was little change in clotting time. This effect of HTGL on heparin anti-Xa activity appeared to be immediate and non-specific (i.e. not related to lipolytic activity) since the same shortening of clotting times by HTGL was also found using lipoprotein-free plasma (see Table 8).

The lengthening of clotting times suggests that HTGL binds heparin and prevents it from full interaction with plasma At III to catalyse anti-Xa action. However, from work with heparin-Sepharose gels (Bengtsson et al., 1980; McKay and Laurell, 1980) both LPL and At III have higher affinity for heparin than does HTGL, since higher salt concentrations are needed to elute them from the gel. Thus the action of HTGL and not of LPL in reducing heparin action is surprising.

This apparent inconsistency could be due to a difference in the mode of binding of heparin by the two enzymes. Investigations have been carried out on the structure and stability of the LPL-heparin complex (Clarke et al., 1983) but no such studies have as yet been reported for HTGL. By the model proposed by Clarke et al., heparins of MW > 10,000 attach at GAG-binding sites on both of the two LPL subunits, which means that the heparin used in these studies - an unfractionated heparin of mean MW 15,000 - would be well bound by the LPL.

The lower affinity of HTGL for heparin is due to the active enzyme being monomeric and so only having one GAG-binding site compared with two on the dimeric LPL (Bengtsson et al., 1980). It has been suggested that in binding LPL, heparin wraps itself around the enzyme to reach the two binding sites (Clarke et al., 1983). With

HTGL however, with only one binding site, the major part of the heparin molecule is likely to be only loosely associated with the protein, if at all, which may allow simultaneous binding of At III to the unbound section of the heparin chain. This means that although At III may then firmly bound to the heparin via specific At III-binding sequences (not required for binding of lipase: Bengtsson et al., 1980), its action against Factor Xa is prevented by steric hindrance due to HTGL. Thus, despite the fact that HTGL has lower affinity than LPL, for heparin, it will have a greater effect on heparin co-factor activity since it is not in direct competition with At III for its binding. This theory could be further tested out by use of heparins of different MWs, and also of other sulphated polysaccharides known to bind lipase.

An alternative explanation of the findings in the presence of heparin is that the effect of the HTGL is due to the co-purification of lipoproteins. It has been reported (Bleyl et al., 1975) that lipoproteins, present in normal serum or as added isolated fractions, bind heparin and thus compete with At III and reduce its anticoagulant action. The 'anti-heparin' properties of LDL in particular have been suggested (MacGregor et al., 1980). However, although lipoproteins do bind heparin-Sepharose as used for lipase purification, more than 90% would be eluted at the NaCl concentrations used to wash the gel prior to elution of HTGL (Iverius, 1971).

In summary, this work has shown that HTGL can induce anti-Xa activity in lipid-rich plasma, which leads to significant lengthening of clotting times in the absence of heparin. It can however also decrease the anti-Xa clotting activity of heparin. LPL has little effect in the presence or absence of heparin.

CHAPTER 8

FINAL DISCUSSION

The prime concern of this thesis has been to compare the activity of heparin with other strongly anionic sulphated polysaccharides in releasing triglyceride lipases from mammalian tissues. A major part of the work was the necessary assessment of various methods to measure the relative contribution of HTGL and LPL to total activity released, since these enzymes play different roles in lipoprotein metabolism.

The lipase-releasing effect of parenteral heparin has generally been studied as a topic separate from its effects on coagulation; in most coagulation work (including that on possible heparin substitutes), the anti-lipaemic effect has been completely ignored. In an attempt to balance this, some of the work described in this thesis has explored possible relationships between the anti-lipaemic and anticoagulant activity of heparin and related compounds.

8.1 Assay Methods

A major problem encountered at the inception of this work concerned the reliability of some of the methods employed to assay lipase activities. These methods were compared and none was found to be entirely satisfactory in regard to all of the three necessary qualities of reproducibility, selectivity and precision. However, one was selected for use which, under defined conditions, gave reliable results. This assay (the modified NES assay) permits some differentiation of HTGL and LPL activities in PHP samples. Total lipase activity is measured in low salt concentrations in the presence of serum, and HTGL is selectively assayed in high salt concentrations and in the absence of serum. LPL activity is then obtained by difference.

Alternative differentiation procedures for LPL and HTGL have been suggested in the literature such as that of using SDS, which has been observed to inhibit HTGL in PHP selectively with only minimal effect on LPL (Baginsky, 1981). This adaptation was investigated with the NES assay, but was unpractical as the concentration of SDS required appeared to vary substantially between PHP samples (see Ch. 2, Section 2.3.vi).

The use of specific anti-sera against LPL, or HTGL is a useful alternative method if these sera are readily available. There may be problems in raising these antibodies, however; the anti-serum raised from rabbits in the present work after challenging with enzymes purified from rat PHP were not specific enough for use in the assay (see Appendix III).

Assay substrates which use gum arabic as stabilizers are commonly employed by workers in the lipase field. This system (called the 'gum arabic assay' in this thesis) was tried in conjunction with selective use of serum and salt (as used in the NES assay), but it was repeatedly found that product release was not linear as a function of time, especially in assays of HTGL (Ch. 2, Section 3.3). Satisfactory linearity has been reported for the gum arabic assay with use of specific antibodies against both enzymes (Huttenen et al., 1975) and also for measurement of LPL in the presence of SDS (Goldberg et al., 1983). However, no data regarding linearity when measuring PHP HTGL by the salt/serum differentiation have been reported, despite the widespread use of this assay method (Benson and Clayton-Love, 1982). Since I obtained poor linearity of HTGL measurements using this assay in two different laboratories, it is suggested that this is not a suitable

method, although it may be satisfactory for LPL. These results emphasize the importance of checking all parameters when setting up an assay, especially when testing the activity of lipases of origin different from that used in the published method. For example, assays designed to test purified lipases will not necessarily be the best ones to measure the activity present in crude tissue extracts or in unfractionated PHPs.

Substrate preparations may differ in chemical composition, i.e. in the source and purity of their components, which are not always precisely defined. This means that different types of substrate preparations are not interchangeable with different assay methods without risk of altering the measurement of one or both of the enzymes. As example of this is the difference between the commercial TG emulsion Intralipid and TG emulsions prepared in the laboratory - use of the former resulted in the release of 3-5 times more FFA than that found with the latter substrate (also noted by Riley and Robinson, 1975). Whilst the composition of Intralipid is known (Table 8, Ch. 2), the method of preparation has not been disclosed by the manufacturers. Use of this substrate however, does not allow inhibition of LPL with high salt concentrations. The two lipases have different specificities for TG and phospholipid and the 'mode of presentation' of the hydrolysable substrate in the preparation (including homogeneity and droplet size) may greatly influence the measured hydrolysis rate. Also, in the case of phospholipid-emulsified radiolabelled TG preparations,the true value of total action of the enzyme would not be measured. Especially in the case of HTGL - which has better phospholipase activity than LPL - this would consist of measured TG hydrolysis (by release of

^{3}H-FFA) but also of unmeasured phospholipid hydrolysis.

8.2 Effect of Serum Factors on Lipase Measurements

Serum factors, whether present in the PHP sample to be assayed, or added to the substrate mixture, also affect LPL and HTGL differently. The modulatory apolipoproteins are probably responsible, but the lipoproteins themselves may also compete with the artificial substrate. The proportion of inhibitory and stimulatory apoproteins may vary from sample to sample, and assessment of this is not easy because of the complexity of the interactions. A clearer view of the situation may be provided by testing the activity of purified enzymes on addition of various serum factors - purified apoproteins or whole lipoprotein fractions - to different substrates.

The unpredictable effects of serum factors provide support for the use of the assay method (see Ch. 1, Section 5.2.iii) which removes most of them by passing the PHPs to be assayed through heparin-Sepharose affinity columns. Lipoproteins are eluted in the void volume or at low salt concentrations.

8.3 The Lipase-Releasing Ability of Different Polysaccharides

A group of heparins and related polysaccharides previously characterized chemically and by clotting assays was investigated for lipase release and provided data on the structural requirements for this activity. Some preliminary work in vitro on release using preparations of isolated animal tissues was undertaken but since variability and non-specific release was high, no useful results were obtained. However, perfused tissue systems, such as that of the liver (Groot et al., 1983) may be worth further investigation.

The bulk of the work has been done in vivo using the rat.

Most previous reports have either been of binding studies in vitro using affinity gels (Bengtsson et al., 1980) or in vivo of very limited scope (e.g. only 2 or 3 selected doses). In the present work fuller comparison of the different polysaccharides has been made with the dose response curves ranging from the threshold of release up to peak activity. Some general comments can be made. There were no significant differences between LPL and HTGL either as regards the dose of polysaccharide at which they were first detected, or between the doses of different polysaccharides at the threshold of release. The major lipase-releasing compounds tested fell into three main groups which had differently shaped dose-response curves. This makes 'ranking' of the compounds (i.e. efficacy of lipase release) difficult, but based on the peak levels reached and the dose needed for this, the most potent were the heparins, followed by SSHA and SP54 then LMW heparin and HSII. The levels achieved by DeS and HSI were extremely low at all doses.

Comparison of the total, HTGL and LPL activity curves allows the tentative conclusion that the differences between the lipase release by different polysaccharides are primarily the result of variations in LPL rather than HTGL. In specific terms, it has been shown here that whilst heparin is more effective in releasing LPL than the other polysaccharides tested, it is only marginally better at releasing HTGL. This probably indicates a difference in the binding of these enzymes in situ. The finding that only the heparins are able to compete successfully at the endothelial surface for the release of the extra 40-50% of the LPL suggests that at least this proportion of the population may be bound in situ to GAGs with a charge density approaching that of heparin.

Time courses of lipase release by the various polysaccharides agree with published work in that LPL is released more slowly and cleared more quickly than HTGL. This was particularly marked with the heparin analogues SSHA and SP54. The overall difference in this respect between the two enzymes however, was not found to be as great as that reported by other workers (Huttenen et al., 1980; Krauss et al., 1974). It would be interesting to try a range of doses in time course studies, and also to test the plasmas using different assays.

No firm conclusions can be drawn on the structural requirements for lipase release, other than (as was previously known) that both MW and the degree of sulphation affect the efficacy. There also appears to be some effect associated with the heparin-heparan family of GAGs, other than that of sulphation or MW.

8.4 The Effect of Lipases on Coagulation

It has been suggested (Thomas et al., 1980) that lipase release may be indirectly responsible for all the anti-Xa activity present in plasma after injection of heparin analogues, and part of that after heparin. Use of purified lipases has produced interesting but not easily explained results. HTGL, but not LPL, was found to have significant effects on plasma anti-Xa activity, to lengthen clotting times in the absence of heparin, and to shorten them in its presence. The HTGL action in the absence of heparin was especially marked in lipid-rich plasma, but absent in lipoprotein-free plasma, which is consistent with its proposed action via lipoproteins. It is suggested that this effect, which progresses with time of incubation of HTGL with plasma, is due to the production of LDL and certain HDL sub-fractions. If this is so, it is

somewhat surprising that the co-addition of LPL to the incubation mixture did not further augment the increase in clotting times. By itself, LPL affected clotting times only marginally and although this was tentatively explained as an effect of the presence of contaminating At III in the LPL sample, further work on more highly purified enzymes is required. Tests on the effect of purified lipoprotein fractions on plasma anti-Xa activity in the presence and absence of HTGL may provide more evidence.

The above test conditions do not of course truly represent the position in post-injection plasmas, since the polysaccharides which release the lipases are absent. As mentioned, when these tests were performed in the presence of heparin, the effect of HTGL was to shorten clotting times, i.e. to reduce the normally heightened action of At III or other protease inhibitors. Suggested explanations for this finding are that HTGL binds loosely to heparin thereby preventing full potentiation of At III activity, or alternatively that certain lipoprotein fractions (produced by HTGL action) themselves neutralize heparin action. The latter is less likely since the observed effect was not dependent on the time of lipase action. Such a mechanism might be checked by testing lipoprotein fractions from PHP and normal plasmas, possibly with the use of antisera to selected fractions.

The wide therapeutic use of heparin as an anticoagulant or antithrombotic agent, with its drawbacks of risk of haemorrhage and osteoporosis, has led to many investigations on the use of alternative or modified preparations. In addition to their anticoagulant activity, there is a possible use of heparin and related compounds against atherosclerosis. This has been only scantily investigated,

but is presumably related to their ability to release lipases into the circulation. As stated by Morton et al. (1984), "the occurrence in mammalian tissues of a native polysaccharide that affects LPL but lacks effect on blood coagulation may have important physiological and therapeutic implications." In this study heparins, heparans and DeS were employed, together with SSHA (also of animal origin but chemically modified) and SP54 (a chemically sulphated plant polysaccharide). The previously reported finding (Thomas et al., 1980; Fischer et al., 1982), that the anti-Xa activities in vitro and ex vivo of these latter two compounds differ, is important - whatever its origins - since it emphasizes the inadequacy of in vitro tests alone. Leaving aside the effects of lipase, assessment of activity even of closely related GAGs such as heparins and HS is not easy because of the non-parallelism of their dose response curves in clotting tests.

With drugs such as heparin, which display a wide spectrum of physiological effects, some of which are poorly understood, use of a number of assay procedures directed at different aspects of their behaviour may allow better selection of the preparation and of the minimum dose required.

APPENDIX I

LIPASE ASSAY USED BY CRINOS SpA (No. 3120/054)

REAGENTS

Substrate

Bovine albumin (fatty acid-free, Sigma))
10% (w/v) in 0.05 M Tris/HCl (pH 8.15)) Mixed 1:1 for
Intralipid (20%, KabiVitrum)) substrate
Diluted x 9 with Tris buffer (as above))

Extraction mixture

40 vol. Isopropanol
10 vol. n-heptane
1 vol. 1 N H_2SO_4

n-heptane

CO_2-free H_2O

0.005% H_2SO_4

Indicator

0.0025% bromothymol blue in n-heptane

Titrant

Prepared and stored (at 4°C) as 10 N NaOH in CO_2-free distilled water; diluted for use by 1 in 10 then 1 in 400 with absolute alcohol.

Fatty acid standard

1 µmole/ml stearic acid

METHOD

1. 2 ml substrate are preincubated with shaking at 37°C for 5 mins. At zero time 1 ml enzyme/PHP is added to the substrate. For the 'test blank' 1 ml of buffer is added in place of enzyme.
2. 0.5 ml aliquots are sampled at zero time and after 30 mins. incubation, and mixed with 5 ml of the extraction mixture.

This is left for 30 mins. at 4°C.

3. Add 3 ml n-heptane, mix and leave further 30 mins. at 4°C.
4. Add 2.5 ml CO_2-free H_2O, mix and leave 30 mins. at 4°C.
5. Remove the heptane (upper) phase and mix with 5.5 ml of 0.005% H_2SO_4. Centrifuge for 15 mins. at 3,000 g.
6. Transfer 2.5 ml of the heptane phase into titration tubes, add 1 ml of indicator and pass a stream of nitrogen through for 10-15 secs.
7. Titrate this against 1/400 N NaOH, with a constant stream of nitrogen to mix and to expel CO_2.

Standard curves and blanks

1. Add 5 ml of the extraction mixture to 0.1, 0.1, 0.4, 0.6, 0.8 and 1.0 ml of the stearic acid solution. Leave for 30 mins. at 4°C.
2. Add 2.9-2.0 ml of n-heptane to give final volume of 8 mls. For the 'standard blank' add 3 mls. of n-heptane to 5 ml of extraction mixture. Mix and leave at 4°C for 30 mins.
3. Add 3 ml of CO_2-free H_2O. Mix well and leave at 4°C for 30 mins.
4. Continue as in Method from 5) onwards.

CALCULATION OF ENZYMIC ACTIVITY

$$\frac{\mu\text{moles FFA}}{\text{ml plasma 30 mins.}} = \frac{6 \times (\text{test} - \text{test blank}) \times \text{standard } (\mu\text{moles})}{\text{standard} - \text{standard blank}}$$

APPENDIX II

METHODS USED TO TEST THE PURITY OF TRIOLEIN

a) Gas chromatography This was done on a Perkin-Elmer 115, using a SGE BP1 bonded phase WCOT column (25 m x 0.1 mm i.d.) with helium as carrier, and a flame ionization detector. Samples were prepared as shown in Figure 1. Methyl oleate (BDH) was also run as a standard. Injection of solutions was made in nonane.

b) HPLC The column used was a ODS Hypersil (Shandon Southern) of dimensions 150 mm x 2.2 mm (i.d.) with a mobile phase of ethanol (Analar, 96%) and a flow rate of 0.2 ml/min. Unlabelled triolein (BDH, normal grade) was dissolved in 96% ethanol to a concentration of 6 mg/ml, and 5 µl (30 µg of this injected. Elution was monitored at 210 nm. Radioactive triolein is supplied in toluene, and this solent was blown off under nitrogen, and the triolein made up in ethanol (0.45 mg/ml, 0.25 mCi/ml). 0.25 ml fractions were collected off the column and the radioactivity counted with 10 ml of scintillation fluid.

The triolein was prepared for G.C. as shown in Figure 1.

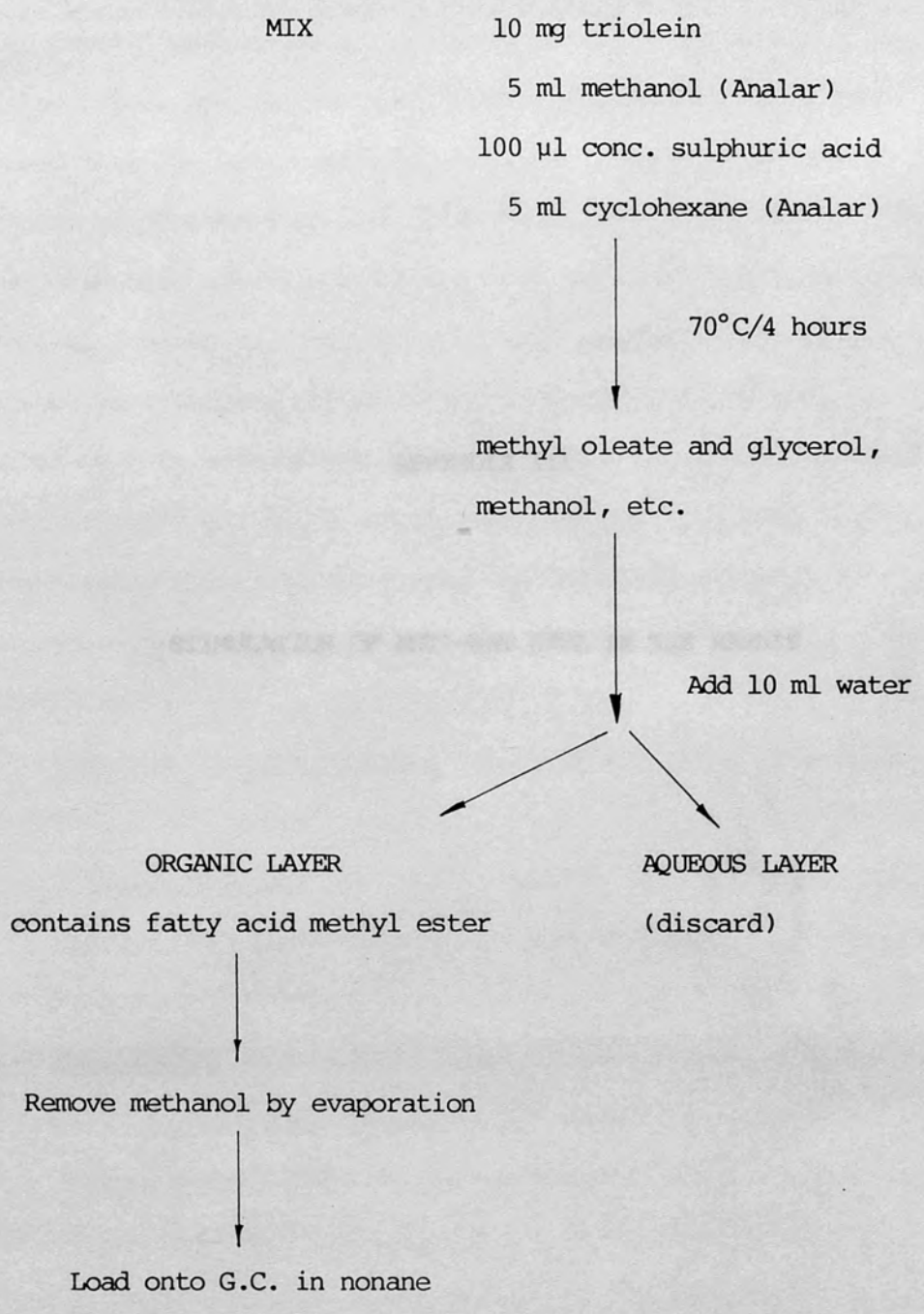

MIX
10 mg triolein
5 ml methanol (Analar)
100 µl conc. sulphuric acid
5 ml cyclohexane (Analar)
70°C/4 hours
methyl oleate and glycerol,
methanol, etc.
Add 10 ml water
ORGANIC LAYER
contains fatty acid methyl ester
AQUEOUS LAYER
(discard)
Remove methanol by evaporation
Load onto G.C. in nonane

FIGURE 1

APPENDIX III

STIMULATION OF ANTI-RAT HTGL IN THE RABBIT

INTRODUCTION

Several methods have been described which selectively inhibit either LPL or HTGL in PHP samples before assaying, which then allows their selective measurement under their optimal assay conditions (Huttenen et al., 1974; Goldberg et al., 1983). Since there had been only partial success in selective measurements using chemical inhibition (NaCl and SDS) with the NES assay, it was decided to raise antibodies to the HTGL enzyme (from the rat, the animal used in most of the lipase-releasing experiments). This involved purification of the enzyme from PHP, injecting it in a concentrated form into the rabbit and testing the rabbit sera at intervals for an inhibitory response.

MATERIALS

Complete Freund's Adjuvant Gibco Laboratories, New York, USA.

Ammonium Sulphate BDH Chemicals Ltd., Poole, Dorset, U.K.

Agarose Gel (A37) Reactifs IBF, Pharmindustrie, Clichy, France.

Rat Albumin (Fraction V) Sigma, Poole, Dorset, U.K.

Swine Anti-Rabbit Immunoglobulin (SWAR/75), Nordic Immunological Laboratories, Maidenhead, Berks., U.K.

METHODS AND RESULTS

Stimulation of anti-rat hepatic lipase in the rabbit

HTGL was purified from rat PHP by heparin-Sepharose chromatography as described in Chapter 3. The enzyme preparation was mixed 1:1 (v/v) with complete Freund's adjuvant and injected into a New Zealand White rabbit intradermally at multiple sites, three times, at roughly two-week intervals. To avoid loss of

enzymic activity by concentrating methods, two further batches of HTGL eluted off the affinity column were dialysed and re-bound to fresh heparin-Sepharose, which was then injected into the rabbit mixed 1:1 with 0.15 M NaCl in phosphate buffer (pH 7.4). The gel should allow slow release of the enzyme for stimulation of the antibody response.

The rabbit was bled from the ear, and the blood left to clot in glass at 37°C for 2-3 hours then overnight at 4°C before spinning at 3,000 g for 15 minutes to obtain serum. Any endogenous lipase activity was inactivated by heating at 56°C for 30 mins. Blood samples from an ear vein of the rabbit were taken at three-week intervals and tested for the presence of antibody both by Ouchterlony plates and by its effectiveness to inhibit HTGL in the lipase assay. After detection of anti-HTGL was detected by both techniques the immunoglobulin fraction (Ig) fraction was partially purified from the serum. This was done to enable smaller volumes to be used of a more concentrated sample, and to avoid complications that may arise from adding further serum into the assay system.

Preparation of partially-purified immunoglobulin

Ice-cold saturated ammonium sulphate (pH 6.5) was added dropwise to the rabbit serum with stirring (on ice) to give a final concentration of 40% of saturated. This was left to stir for 30 mins. then spun at 12,000 g for 10 mins. at 4°C. The pellet was washed with further 40% saturated ammonium sulphate and re-spun. The Ig pellet was extensively dialysed against phosphate buffered saline at 4°C, then spun at 3,000 g to remove any insoluble protein.

Test of antisera by Ouchterlony immunoprecipitation

The rabbit antiserum and Ig preparation were tested by the Ouchterlony double immunodiffusion technique, using a 1% agarose gel in 0.1 M phosphate buffer pH 7.4. 25 µl of the serum or Ig preparation were added to the centre well and 10 µl of antigen to the surrounding wells. The plates were left overnight at 37°C.

Figure 1 shows the protein precipitates from various rat plasma fractions by the Ig preparation. The protein present in samples 1-5 is probably antithrombin III, which is likely to be the major contaminant of the HTGL sample used for antibody stimulation (since heparin-Sepharose gel was used for purification). No protein is precipitated from the albumin sample. By comparing samples 1, 2 and 3, it would appear that a protein not present in pre-heparin plasma is precipitated from PHP and the HTGL. It is possible that this is the hepatic lipase. No immunoprecipitate is formed with the LPL.

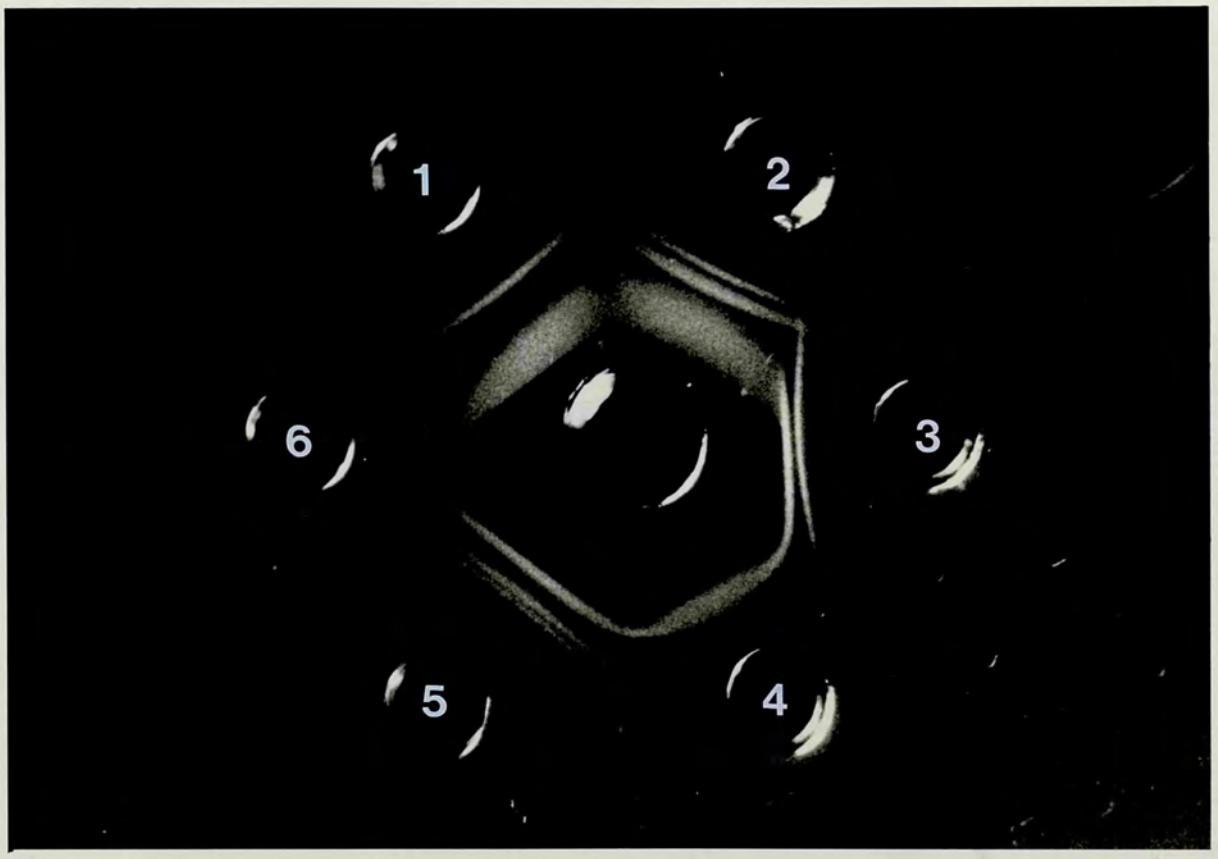
Plates were also run using rabbit anti-human HTGL serum, supplied by Dr. Olivecrona in Umeå. The bonds of precipitated protein are not so clear here, since serum was used instead of Ig preparation (see Fig. 2).

Anti-HTGL activity of antiserum and Ig fraction

Rat PHP was mixed in varying proportions with neat serum or Ig preparation, and left 2-3 hours at 4°C before testing in the lipase assay. Controls of normal rabbit serum (NRS) and buffer were run alongside.

To aid immunoprecipitation of the HTGL-Ig complex, swine anti-rabbit immunoglobulin (SWAR) was used. Lysophilized SWAR was made up in distilled water, and 10 µl of different concentrations added

Fig 1 Ouchterlony immunoprecipitation using the Ig preparation against rat HTGL



1 Normal rat plasma

2 Rat PHP

3 Purified rat HTGL

4 Purified rat LPL

5 Heparin-binding fraction from normal rat PHP

6 Rat albumin

Centre well = Ig prep. against rat HTGL

to 250 µl of either neat PHP or PHP/Ig (1:1) mixture. This was left for a further 2-3 hours or overnight at 4°C, spun in an Eppendorf 5412 bench centrifuge for 2 mins. at 7,500 rpm then assayed. The results are shown in Table 1.

Since this additional treatment appeared to aid removal of lipase activity without a similar effect on the control, 5 µg/ml of SWAR were used in subsequent incubations.

Increasing amounts of Ig preparation were incubated with rat PHP to determine the amount necessary for inhibition of HTGL. The effect on lipase activity under high salt conditions (1.0 M NaCl) is shown in Figure 3, with the decrease in activity appearing to be nearing completion. Although theoretically there should be no lipase measurable with anti-HTGL under high salt conditions, similar results were obtained in this assay system using anti-HTGL supplied by Dr. Olivecrona in Umeå (results not shown).

However, the total lipase values appeared to be continuing to decrease even at a 1:1 ratio of Ig preparation:rat PHP. This was confirmed using a second Ig preparation from the antiserum which, when used in increasing proportions effected almost total inhibition of lipase activity measured in low and high salt substrates (see Figs. 4 and 5). These were run against controls of Ig preparation from normal rabbit serum. So, the antiserum stimulated in rabbit is not usable to selectively inhibit HTGL since both enzymes are affected, directly or indirectly. Although LPL was definitely excluded from the HTGL preparation by differential salt elution from the affinity column, it is possible that LPL cross-reacts with an antibody produced against HTGL. This was found to be the case using antiserum against HTGL prepared in chickens (Augustin et al.,

TABLE 1

Effects of SWAR on enzyme activity measured in lipase assay

	(SWAR) in incubation mixture	Enzymic activity (adjusted) for dilution	
		Total	SRL
Neat PHP	-	214.7	73.5
	5 µg/l	224.4	74.4
Serum:PHP (1:1)	-	72.4	18.3
	50 µg/ml	59.8	16.6
	10 µg/ml	61.7	15.8
	5 µg/ml	59.4	16.6

Fig 3 Effect of Ig prep. on PHP HTGL activity

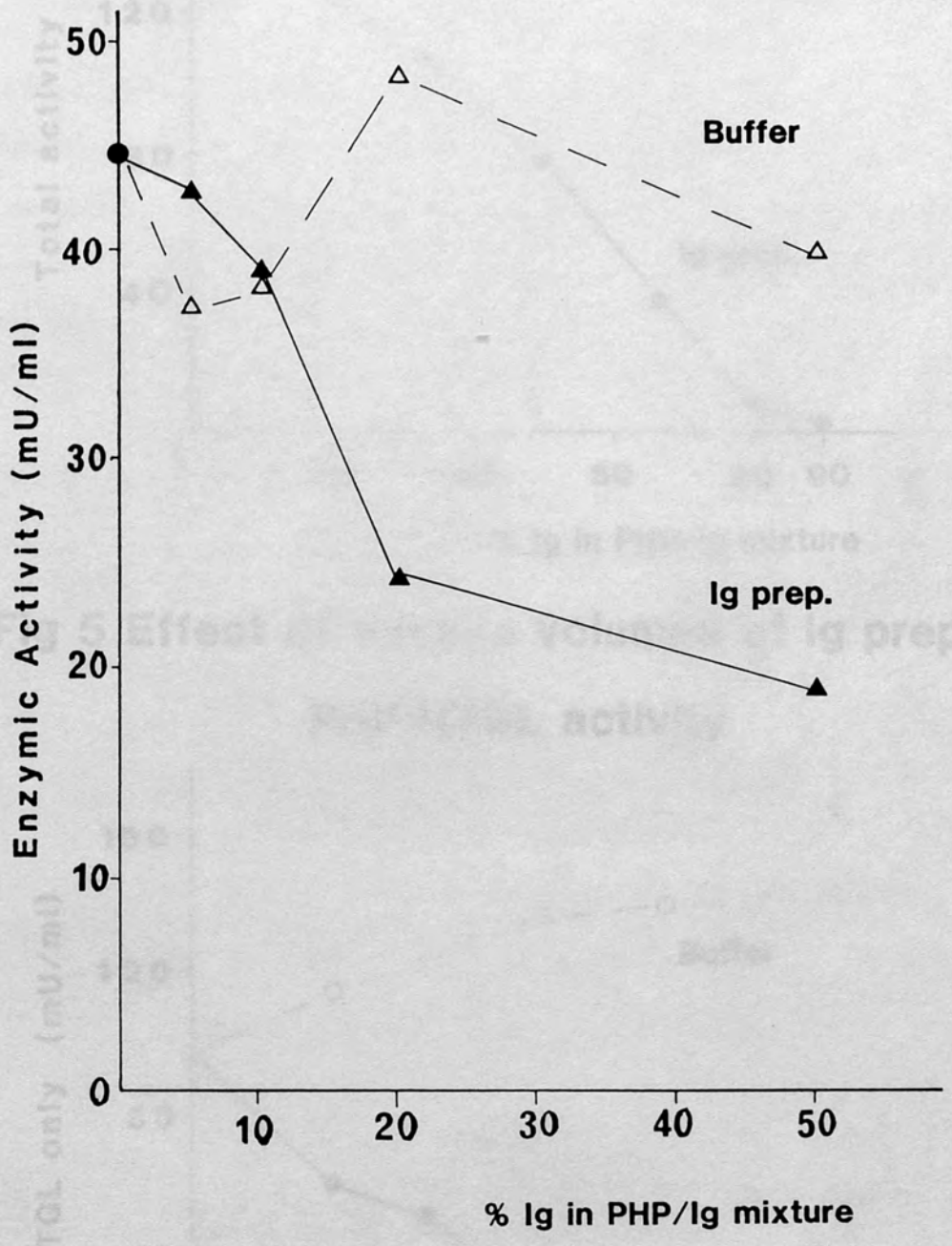


Fig 4 Effect of Ig prep. on total lipase

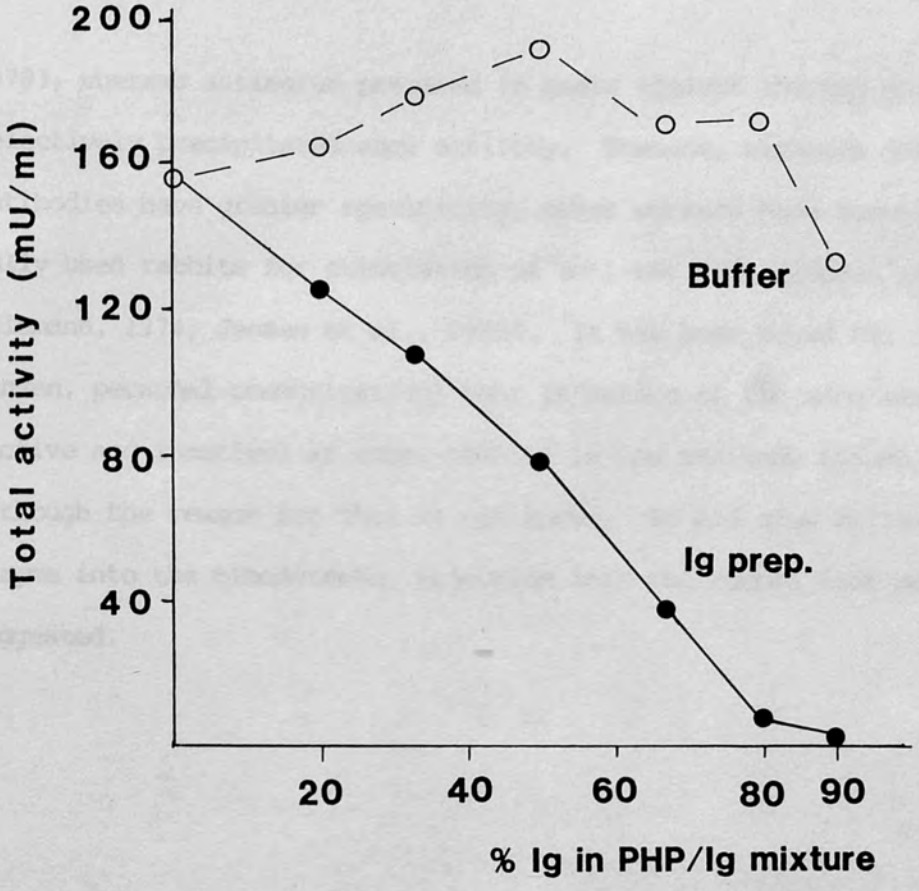
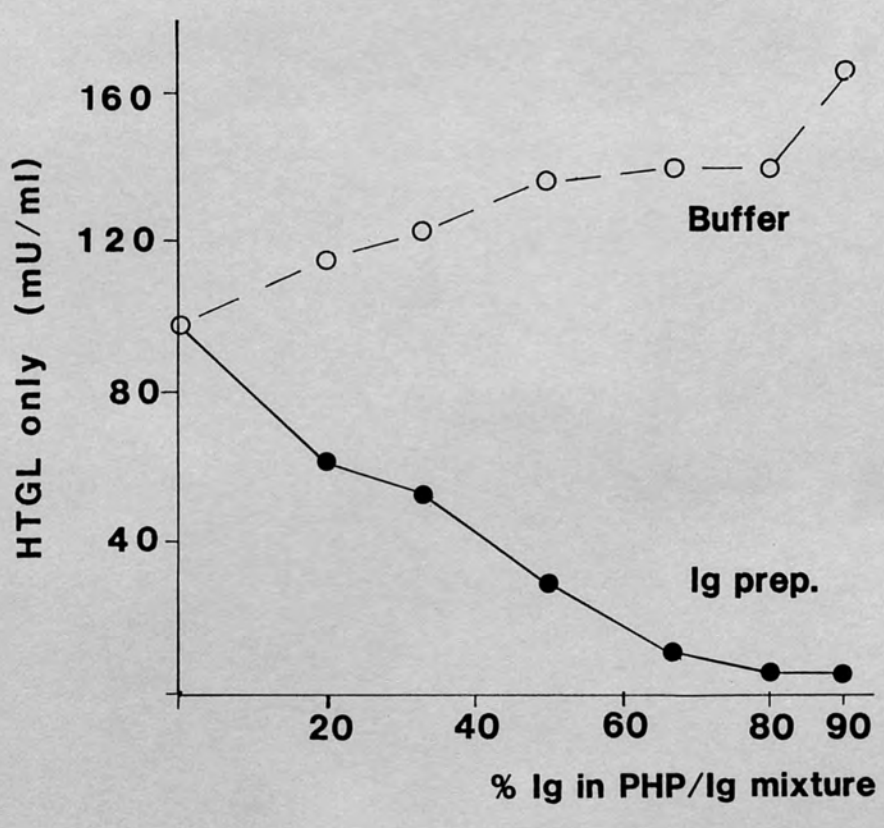


Fig 5 Effect of excess volumes of Ig prep. on PHP HTGL activity



1978), whereas antiserum prepared in goats against the two enzymes selectively precipitated each activity. However, although goat antibodies have greater specificity, other workers have successfully used rabbits for stimulation of anti-rat HTGL (Jansen and Hülsmann, 1974; Jensen et al., 1982). It has been found (H. Jansen, personal communication) that injection of two much enzyme (active and inactive) at once, results in low antibody titres, although the reason for this is not known. To aid slow release of enzyme into the bloodstream, injection into the rabbit foot pads is suggested.

APPENDIX IV

PREPARATION OF HEPARIN-SEPHAROSE GELS

a) Method 1

MATERIALS

Sepharose 4B (Pharmacia U.K. Ltd.)

Ethanolamine 1 M pH 8.0

Cyanogel bromide

Heparin

Sodium hydroxide 10 N

Buffers

Coupling buffer A: 0.2 M sodium bicarbonate, pH 11.0

Coupling buffer B: 0.2 M sodium bicarbonate, pH 9.0

Washing buffer A: 0.1 M sodium acetate, 1.5 M sodium chloride, pH 4.0

Washing buffer B: 0.2 M sodium bicarbonate, 1.5 M sodium chloride, pH 9.0

METHOD

1. Suspend 100 ml of Sepharose 4B in an equal volume of coupling buffer A and add 5 g of solid CNBr. Maintain the pH at 11.0 for 20 mins.

2. Wash with ice-cold distilled water and add to 1.2 g heparin (dissolved in a small volume of coupling buffer B). Leave to stir overnight at room temperature.

3. Filter gel and wash with coupling buffer B then washing buffer B.

4. Stir for 30 mins. with 200 ml 1 M ethanolamine, then wash with further fresh ethanolamine.

5. Wash exhaustively with alternate volumes of washing buffers A and B.

6. Wash and leave in running buffer.

b) Method 2

MATERIALS

Sepharose

Ethanolamine 0.75 M (pH 8.0)

Cyanogen bromide

Heparin

Sodium hydroxide

Buffers

0.5 M sodium bicarbonate, 1.5 M sodium chloride, pH 9.0

0.1 M sodium acetate, 1.5 M sodium chloride, pH 4.0

METHOD

1. Dissolve 1 g heparin in 100 ml distilled water and add 10 g cyanogen bromide.

2. Add 150 ml Sepharose and stir in an ice bath for 15 mins., maintaining the pH at 11.0. Leave to stir overnight at room temperature.

3. Wash gel with bicarbonate buffer, then stir at 37°C with two changes of ethanolamine.

4. Wash extensively, alternating bicarbonate and acetate buffers.

APPENDIX V

PREPARTION OF SDS-POLYACRYLAMIDE GELS FOR ELECTROPHORESIS OF LIPASES

Based on the method of Weber, K. and Osborn, M.

(J.Biol.Chem 244, 4406-4412 (1969)

Under gel (10%)

6.72 ml 45% (w/v) acrylamide

1.01 ml 1.6% (w/v) N, N'-methylene bisacrylamide (bis)

7.5 ml 1.5 M Tris, pH 8.8

0.8 ml 4% SDS

12.6 ml distilled water

The above mixture is de-gassed and the following added:-

1.6 ml 1% ammonium persulphate (freshly prepared)

40 µl TEMED (N,N,N',N'-tetramethylethylene-diamine)

Over gel (4%)

0.89 ml 45% acrylamide

0.13 ml 1.6% bis

0.84 ml 1.5 M Tris, pH 6.8

0.27 ml 4% SDS

7.34 ml distilled water

De-gas and add:-

0.53 ml 1% ammonium persulphate

13.3 µl TEMED

Electrode buffer (pH 8.3)

0.025 M Tris

0.192 M glycine

0.1% SDS

APPENDIX VI

LIPASE ACTIVITIES RELEASED IN THE RAT IN VIVO AFTER INJECTION OF A RANGE OF SULPHATED POLYSACCHARIDES

i) Total lipase values

ENZYMIC ACTIVITY (mU/ml)* measured by the NES assay

Polysaccharide injected: dose (mg/kg)	Heparin I	Heparin II	LMW heparin	HSII	HSI	SP54	SSHA	DeS
0.05	-	16	1	5	-	6	11	-
0.1	35	13	4	9	-	10	23	-
0.2	76	80	7	9	2	23	31	-
0.5	126	136	31	-	3	52	64	-
1.0	197	245	58	80	3	133	154	-
2.0	336	295	141	133	-	168	185	-
4.0	288	304	188	191	9	168	174	4
8.0	-	257	198	214	15	176	149	4

* Mean value for three post-injection plasmas

ii) HTGL values

ENZYMIC ACTIVITY (mU/ml)* measured by the NES assay

Polysaccharide injected: dose (mg/kg)	Heparin I	Heparin II	LMW heparin	HSII	HSI	SP54	SSHA	DeS
0.05	-	4	2	4	-	10	3	-
0.1	13	3	4	9	-	5	7	-
0.2	23	21	5	4	-	7	13	-
0.5	49	28	14	-	2	18	24	1
1.0	55	100	28	34	-	36	22	1
2.0	116	99	53	59	2	67	73	-
4.0	93	108	68	72	5	87	77	3
8.0	-	96	62	86	5	98	83	5

* Mean value from three plasmas

iii) LPL values

ENZYMIC ACTIVITY (mU/ml)* measured by the NES assay

Polysaccharide injected: dose (mg/kg)	Heparin I	Heparin II	LMW heparin	HSII	HSI	SP54	SSHA	DeS
0.05	-	13	-	1	-	0	9	-
0.1	23	11	0.1	0	-	5	16	-
0.2	53	59	2	5	-	17	18	-
0.5	76	98	17	-	0.5	34	40	0.4
1.0	143	145	30	21	-	97	101	0.6
2.0	220	195	88	74	-	101	112	-
4.0	195	196	120	118	4	81	98	1
8.0	-	161	136	129	9	79	66	0

*Mean values from three plasmas

iv) Total values from use of the Intralipid assay*

ENZYMIC ACTIVITY (mU/ml)

	Heparin I	Heparin II	LMW heparin	HSII	HSI	SP54	SSHA	DeS
0.05	8	15	2	8	-	4	1	-
0.1	85	20	4	12	-	15	25	-
0.2	236	206	18	22	-	41	55	-
0.5	443	344	77	-	-	127	75	-
1.0	574	605	119	197	-	413	268	-
2.0	931	768	384	399	-	637	513	-
4.0	809	765	598	602	5	734	645	0
8.0	-	759	474	756	25	732	564	0

*Mean value from two plasmas

APPENDIX VII

RELEASE OF LIPASE IN MAN

Polysaccharides for injection

Heparin (Leo Laboratories, Princes Risborough, Bucks., U.K.), batch H 64578

SP54 (Benechemie, Munich, West Germany)

LMW heparin (Laboratoire Choay, Paris) (as used by Thomas, D.P. and Merton, R.E., Thrombosis Res. 28, 343-350 (1982)).

Procedure Six healthy volunteers were injected subcutaneously with SP54 (50 mg), heparin (5,000 units*) and LMW heparin (40 mg). At least one week was allowed between injection of the different polysaccharides. Blood was taken pre-injection and also 1, 2, 3 and 6 hours post-injection. Blood was mixed with 9 volumes of 3.8% trisodium citrate and plasma obtained by centrifugation at 48,500 g for 15 mins. Lipase activity was measured by the NES assay as described in Chapter 2.

The results from injection of SP54 and heparin have been published in Fischer et al., Thrombos.Haemos. 47, 109-113 (1982).

Results

Figure 1 shows the time course of plasma lipase activity after SP54, heparin and LMW heparin.

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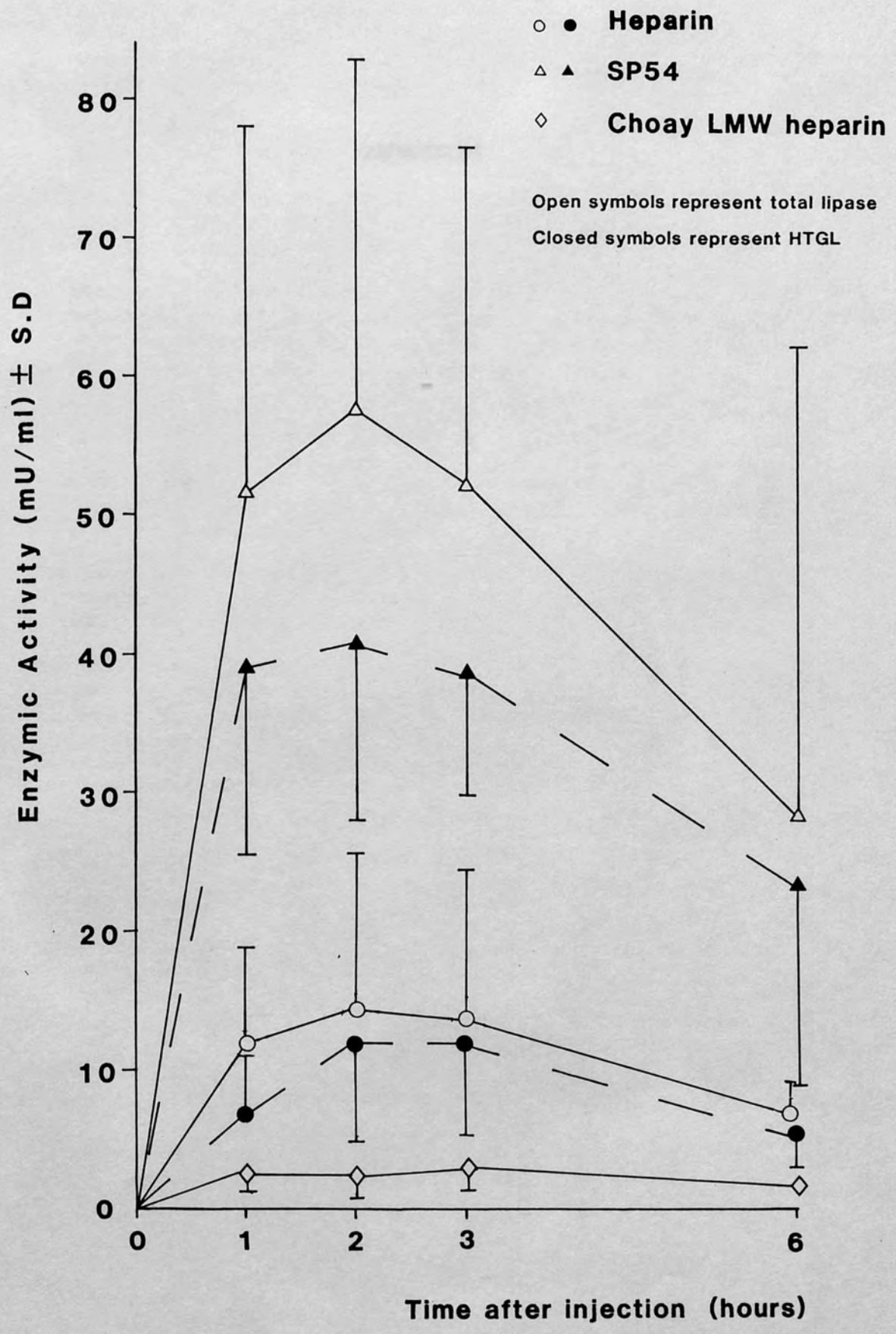
* Approximately 35 mg.

Fig 1 Release of lipases in man



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THE EFFECTS OF POST-HEPARIN PLASMA LIPASES ON ANTI-XA CLOTTING ACTIVITY

S.P. Williams* and T.W. Barrowcliffe†
*Chemistry Section and †Division of Blood Products,
National Institute for Biological Standards and Control, Holly Hill,
Hampstead, London NW3 6RB, U.K.

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ABSTRACT

The effects of hepatic triglyceride lipase (HTGL) and lipoprotein lipase (LPL) on the anti-Xa clotting activity of plasma were studied. LPL had no effect, but HTGL enhanced anti-Xa activity. This enhancement was shown to be due to a time-dependent action of HTGL on lipoproteins. These results could explain the increases in anti-Xa clotting activity previously observed after injection of heparin analogues, SSHA and SP54, which are potent releasers of lipase enzymes.

INTRODUCTION

It is well known that injection of heparin leads to the release of lipolytic activity into the blood (1). This property of lipase release is shared by other naturally occurring glycosaminoglycans (GAGs) (2) and by analogues of heparin, such as the semi-synthetic heparin analogue, SSHA (3), and pentosan polysulphate, SP54 (4). There are two distinct enzymes released: lipoprotein lipase (LPL), from extrahepatic tissues, and hepatic triglyceride lipase (HTGL) from the liver (1). LPL requires apoprotein C-II for maximal activity and is inhibited by high salt, whereas HTGL does not require an apoprotein co-factor and is stimulated by high salt. In human post-heparin plasma, HTGL is generally present in larger amounts than LPL (5).

An interesting feature of the heparin analogues SSHA and SP54 is that both drugs, and especially SSHA, give rise to anti-Xa clotting activity after injection, even though they have virtually no anti-Xa activity in vitro (3,4). Evi-

Keywords: Lipase; Heparin; Anticoagulant

dence was found that the anti-Xa activity associated with SSHA injection was due to release of some component(s) into the blood (3) and it seems possible that the anti-Xa activity is associated in some way with lipase release. In the present study, this hypothesis was tested by studying the influence of purified lipase enzymes on the anti-Xa clotting assay.

MATERIALS AND METHODS

Plasma Human post-meal blood (9 vols) was collected from normal volunteers, 1½ hours after a fatty meal into 3.8% trisodium citrate (1 vol.). Plasma was separated by centrifugation at 48,000 g for 10 mins. and stored at -40°C. Normal human plasma (anticoagulant CPD + adenine) was supplied by the North London Blood Transfusion Centre, Edgware, Middx. and centrifuged at 90,000 g for 90 mins. to remove chylomicra. Lipoprotein-free plasma was prepared as described by Chung et al. (6), by ultracentrifugation at 200,000 g for 3 hours in a Sorvall TV-850 vertical rotor, using a discontinuous NaCl/KBr gradient from 1.006 to 1.33 g/ml. The protein containing fractions at the bottom of the tube were pooled, dialysed overnight at 4°C, and stored at -40°C. The plasma was checked for absence of lipoproteins by phospholipid and triglyceride analyses. Post-heparin plasma (PHP) was taken from male Sprague-Dawley rats by cardiac puncture 10 minutes after i.v. injection of 250 iu/kg heparin (sodium salt, Leo Laboratories, Princes Risborough, Bucks., England). The blood was mixed 9:1 with citrate as above, and spun for 48,000 g for 10 minutes and stored at -20°C or used fresh.

Measurement of lipase activity Triglyceride lipase activity was measured by the method of Nilsson-Ehle and Schotz (7) using ^{3}H-triolein stabilized with glycerol. Different assay conditions were used to allow selective measurement of the two enzymes. Total activity was assayed in a low salt substrate containing serum (as in 7) and HTGL only in a substrate containing 1 M NaCl and with serum omitted. Short assay incubation times (10 minutes) and small enzyme volumes (10-30 µl) were used to avoid assay non-linearity.

1 mU of enzyme activity is defined as the release of 1 nanomole of oleic acid per minute at 37°C.

In some experiments, enzyme activity after incubation with plasma was monitored by plasma-free fatty acid measurements, using a commercial kit (Boehringer Ltd., Mannheim).

Purification of lipase enzymes by affinity chromatography LPL and HTGL were purified on heparin-Sepharose CL-6B (Pharmacia). Solid NaCl was added to the PHP to a concentration of 0.35 M and mixed with the gel for 2 hours before packing. The column was washed with 0.35 M NaCl in 0.01 M phosphate buffer containing 20% glycerol (pH 7.4) and the enzymes eluted step-wise with salt. In some experiments, fresh, skimmed bovine milk was used as starting material instead of PHP. Highly purified milk LPL, supplied by Dr. T. Olivecrona, Umeå, Sweden, was also tested.

Peak fractions of the two enzymes were separately pooled and concentrated by overnight dialysis against ammonium sulphate (8). Precipitates were made up and dialysed against 0.15 M NaCl in phosphate buffer containing 50% glycerol.

All steps were carried out at 4°C.

Anti-Xa clotting assay Lipase enzymes were incubated with human plasma at 37°C for up to 45 minutes, to give levels similar to those achieved in man after injection of heparin analogues (30-50 mU/ml). The mixture was tested by a modi-

fied Denson and Bonnar anti-Xa clotting assay (9,10), using bovine Factor Xa (Diagnostic Reagents Ltd., Thame, Oxon.) and an incubation time of 2 minutes.

RESULTS

Release of lipases by heparin and related drugs The peak levels of total lipase released by s.c. injection of heparin, the heparin analogues SSHA and SP54 and a low molecular weight heparin fraction in human volunteers are summarised in Table 1. Further details of the injection experiments are given in the published references cited in the Table. Both analogues gave considerably higher lipase levels than heparin or LMW heparin. The percentage of the total lipase activity which was due to the hepatic enzyme ranged from 55-82% (Table 1).

TABLE 1
Release of Lipase Enzymes by s.c. Heparin and Analogues in Humans

Drug	Published Reference	Lipase release	
		Total mU/ml	% HTGL
Heparin (n = 6)	4	14.5	82
SSHA (n = 4)	3	68.7	55
SP54 (n = 6)	4	57.7	70
LMW heparin (n = 6)	13	8.3	64

40-60 mg of each drug were injected subcutaneously into normal volunteers (numbers in brackets). Further details are given in the published references.

Purification of lipases from post-heparin plasma The fractionation of rat post-heparin plasma on heparin-Sepharose is shown in Figure 1. The HTGL eluted at 0.7 M NaCl, with no detectable LPL contamination. The 0.8 M fraction contained small amounts of both enzymes and was discarded. The 2.0 M NaCl fraction contained mostly LPL, with a small amount of HTGL activity (this may be due to non-specificity of the assays). The enzymes were purified about 200 times from the post-heparin plasma.

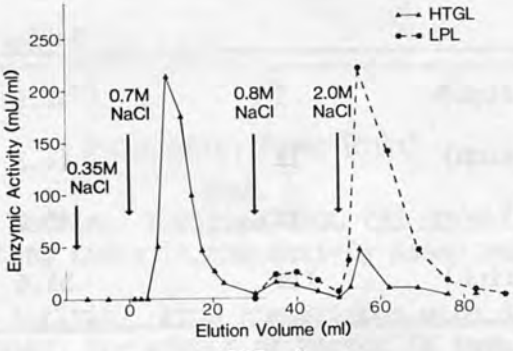

FIG. 1
Purification of HTGL and LPL from rat post-heparin plasma by chromatography on heparin Sepharose

Effects of LPL on anti-Xa activity of plasma Neither of the two enzyme preparations from milk gave any detectable change in anti-Xa activity when incubated with human plasma (Table 2). Negative results were obtained with several different batches of milk enzyme, at concentrations up to 250 mU/ml, with different batches of plasma, both from the Transfusion Centre and from normal individuals after a fatty meal, and also on incubation of milk LPL with whole blood. In the initial experiments, monitoring of plasma free fatty acids showed the expected increase, indicating that the enzyme preparations were active. Preparations of LPL from post-heparin plasma prolonged clotting times by a few seconds, the maximum being 3.5 seconds (Table 2). This prolongation was not time dependent, i.e. it was observed on immediate mixing of the lipase and plasma and did not change up to 45 minutes' incubation.

TABLE 2
Anti-Xa Activity of Post-Meal Plasma + LPL (45 mins. Incubation)

Enzyme prep.	Source	Concn. mU/ml	Clotting time (secs.) Buffer	Lipase
NIBSC 1	Milk	100	31.6	30.6
Umeå 1*	Milk	35	50.0	50.3
NIBSC 2	PHP	35	52.1	55.6

* Highly purified LPL, kindly donated by Dr. T. Olivecrona, University of Umeå, Sweden

Effects of HTGL on anti-Xa activity of plasma In contrast to the results with LPL, preparations of HTGL gave marked prolongations of clotting times in the anti-Xa assay. The results with three different enzyme preparations and four different batches of plasma are summarised in Table 3. With three batches of lipid-rich plasma, the average prolongation of clotting times was 12.6 seconds.

TABLE 3
Effect of HTGL (35 mU/ml) on Anti-Xa Activity of Plasma (45 mins. incubation)

Plasma batch	Enzyme prep.	Clotting time (secs.) Buffer	Lipase
1 (lipid rich)	I	42.8	58.2
2 (lipid rich)	II	44.3	57.6
3 (normal)	II	45.2	48.2
4 (lipid rich)	III	51.6	61.1

Plasma batch 3 was from the Transfusion Centre. Batches 1, 2 and 4 were from normal subjects, taken 1½ hours after a fatty meal.

However, with a fourth batch of plasma, collected from the Blood Transfusion Centre and centrifuged to remove chylomicra, the prolongation of clotting times was less marked, indicating that the effect of HTGL on anti-Xa activity depended on the lipoprotein content of the plasma. This was confirmed in a separate experiment; when the hepatic lipase was incubated with plasma from which all lipoproteins had been removed by ultracentrifugation, there was no increase in anti-Xa activity (Table 4).

TABLE 4
Involvement of Lipoproteins in Anti-Xa Activity of HTGL

Plasma	Clotting time (secs.)	
	Buffer	Lipase
Lipid-rich	51.6	61.1
Lipoprotein-free	51.4	51.4

Time-course of HTGL action HTGL was incubated with lipid-rich plasma for up to 60 minutes, and anti-Xa activity measured at intervals. As shown in Figure 2, there was no significant effect when the plasma was tested immediately after addition of lipase, but subsequently there was a progressive increase in anti-Xa activity up to a maximum of 45 minutes' incubation. The increase in clotting times observed in the buffer control after 60 minutes is due to instability of normal plasma anti-Xa activity.

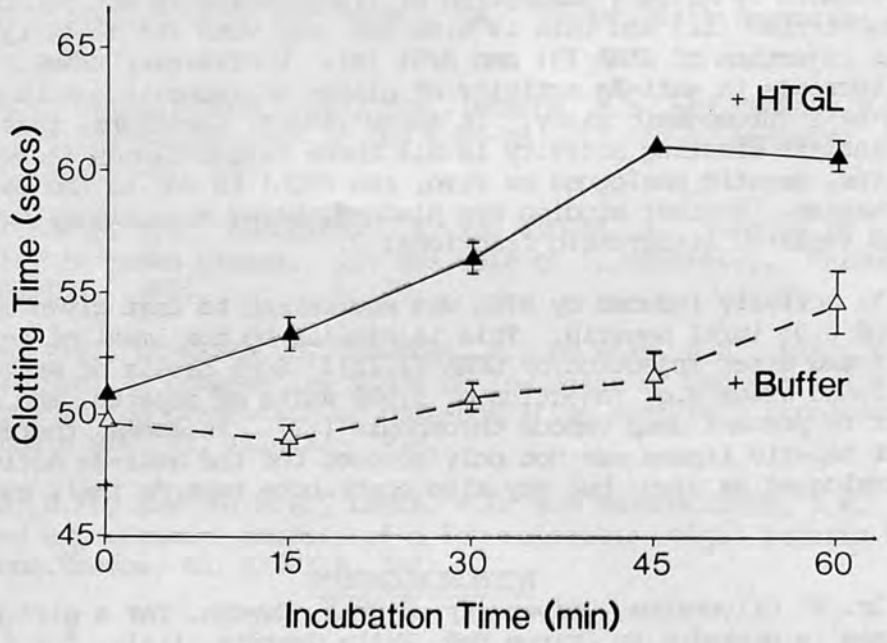

FIG. 2
Time course of HTGL action. Purified HTGL (35 mU/ml) was incubated with human plasma and clotting times in the anti-Xa assay measured at intervals.

Quantitation of anti-Xa activity From comparisons with dilution curves of the Factor Xa used in the assay, the amount of Factor Xa remaining after incubation with plasma + HTGL (35 mU/ml) was approximately 50% of that remaining after incubation with plasma + buffer. In one batch of lipid-rich plasma, the pro-

longation of clotting times by HTGL (35 mU/ml) was compared with that given by in vitro addition of various amounts of heparin. The anti-Xa activity of HTGL was found to be equivalent to that of 0.05 iu/ml heparin.

DISCUSSION

The results of this study show that, of the two lipases released by heparin and its analogues, LPL had no effect on anti-Xa activity, whereas HTGL gave a marked increase in anti-Xa clotting activity when incubated with plasma. The fact that there was a progressive increase in anti-Xa activity from 0-45 minutes' incubation (Fig. 2) and that lipase alone did not affect Xa clotting times suggests that the prolongation of clotting times is not artefactual. Also, it is unlikely that these results can be explained by contamination with antithrombin III (At III). Preliminary studies with human post-heparin plasma have shown that At III is mostly located in the LPL fraction (unpublished data) and this may be the explanation for the slight prolongation of clotting times with this fraction, which was not seen with the milk enzymes (Table 2) and was not time-dependent. The HTGL fraction contains only a small amount of At III, and did not prolong clotting times in plasma from which lipoproteins had been removed (Table 4).

It appears, therefore, that HTGL increases the anti-Xa clotting activity of plasma by a time-dependent interaction with lipoproteins. The exact mechanism is unknown, but may be related to previous studies, in which the anti-Xa clotting activity of lipoproteins, particularly LDL and HDL, was markedly increased by adsorption with $Al(OH)_3$ (11). The $Al(OH)_3$ appeared to be acting by revealing high-affinity phospholipid binding sites for Factor Xa in the lipoproteins and it is possible that HTGL could be acting in the same way. The anti-Xa activity induced by $Al(OH)_3$ adsorption of lipoproteins is not detected by chromogenic substrates (11) and this is also the case with the activity detected ex vivo after injection of SSHA (3) and SP54 (4). Preliminary results indicate that the increase in anti-Xa activity of plasma on incubation with HTGL is also not shown by a chromogenic assay. It seems likely, therefore, that the increase in anti-Xa clotting activity in all three circumstances (adsorption of lipoproteins, heparin analogues ex vivo, and HTGL) is due to the same underlying mechanism. Further studies are planned, using more highly purified enzyme and isolated lipoprotein fractions.

The anti-Xa activity induced by HTGL was equivalent to that given by in vitro addition of 0.05 iu/ml heparin. This is similar to the level of heparin-like activity found after injection of SSHA (3,12). Such levels of anti-Xa activity are also found after s.c. injection of 5,000 units of heparin, and appear to be sufficient to prevent deep venous thrombosis (12). It seems, therefore, that release of hepatic lipase can not only account for the anti-Xa activity of heparin analogues ex vivo, but may also contribute towards their antithrombotic action.

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The association of apolipoprotein-CII with lipoprotein lipase in the presence and absence of a triglyceride substrate

ANTHONY R. CLARKE, MOLLIE LUSCOMBE and J. JOHN HOLBROOK

Department of Biochemistry, University of Bristol Medical School, Bristol BS8 1TD, U.K.

It has been shown, both *in vitro* and *in vivo*, that apo-CII serves to enhance the activity of LPL against long-chain triglyceride and phospholipid substrates (for a review see Posner, 1982). This activation is physiologically relevant since apo-CII is a constituent of the surface film of very-low-density lipoproteins and chylomicra, and renders the core of these particles more susceptible to enzymic hydrolysis (Fitzharris *et al.*, 1981). Despite the numerous kinetic studies of LPL activation, no comprehensive description has emerged of the mechanism by which apo-CII achieves its effect.

The aim of the work reported here is to demonstrate and characterize the formation of an enzyme–apo-CII complex both in free solution and on the substrate surface, and to define the activation mechanism using a simple, globular triglyceride substrate.

The binding of LPL to a fluorescently labelled apo-CII in the absence of a substrate was followed by measuring the anisotropy of the extrinsic fluorescence. These experiments revealed that a weak complex is formed in which a single apo-CII molecule associates non-co-operatively with each subunit of the dimeric enzyme. The K_d for this interaction at 0.05M-NaCl is 0.2×10^{-6}M and it is weakened markedly by raising the salt concentration or by the binding of heparin to the enzyme (Clarke *et al.*, 1983).

In the presence of glycerol triolate (emulsified in gum-arabic) the binding of apo-CII to the enzyme was monitored by measuring the steady-state lipolytic rate as the concentration of the activator was increased. The results show that the higher the level of enzyme in the incubation, the more apo-CII is required to achieve maximal stimulation, thus demonstrating that activation proceeds through the formation of a protein–protein complex rather than through 'substrate-activation' (Fielding & Fielding, 1980). The data also show that, while the stoichiometry of the complex is preserved, the apo-CII–LPL interaction is much tighter than that in the absence of substrate (the apparent K_d being $15–20 \times 10^{-9}$M at a substrate concentration of 4.2mM). Additionally, it was observed that the complex formed under these conditions was not disrupted by heparin and was relatively insensitive to the concentration of salt.

The mechanism of activation was investigated further by an analysis of the first order pre-steady-state kinetics. The observed rate at which the system attained the steady-state on the addition of apo-CII was recorded as a function of the concentration of substrate, activator and enzyme. Plots of the observed rate constant versus concentration were hyperbolic with respect to substrate, linear with respect to apo-CII and the level of LPL had no influence (see Fig. 1). The simplest explanation of this kinetic pattern is that the enzyme and substrate combine in a rapid equilibrium, which is shifted by the preferential association of apo-CII with the form of the enzyme that is bound to the substrate. The mechanism can be represented as follows:

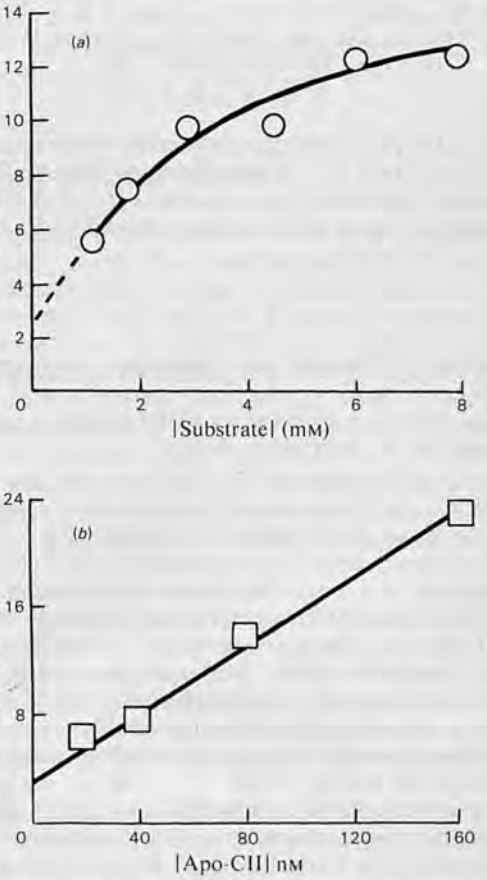

Fig. 1. *The effect of substrate and apo-CII concentrations on the pre-steady-state reaction*

(*a*) The observed rate constant ($k_{obs.}$) describing the approach of the reaction to the steady-state was determined as a function of the concentration of glycerol trioleate. The medium contained 0.05M-NaCl and 4nM-enzyme, and the activation was started by the addition of 40nM-apo-CII. (*b*) $k_{obs.}$ was also determined at varying levels of apo-CII. The medium contained 1.8M-substrate, 0.05M-NaCl and 4nM-enzyme.

$$E + S \underset{}{\overset{K_s}{\rightleftharpoons}} E\text{–}S \underset{k_{-2}}{\overset{k_2}{\rightleftharpoons}} C\text{–}E\text{–}S \quad (C)$$

Abbreviations used: apo-CII, apolipoprotein-CII; LPL, lipoprotein lipase.

where E = free enzyme, S = triglyceride substrate, E–S = lipid-bound enzyme, C = apo-CII and C–E–S = activated ternary complex.

The observed rate constant ($k_{obs.}$) for the approach of this reaction to the steady-state upon addition of apo-CII is described by the equation:

$$k_{obs.} = \frac{[S]k_2[C]}{K_s + [S]} + k_{-2}$$

From the kinetic plots of $k_{obs.}$ vs [S] and [C] the following constants were derived: $K_s = 3–4$ mM, $k_2 = 3.0–3.5 \times 10^5\,M^{-1}s^{-1}$, $k_{-2} = 2.5–3.5 \times 10^{-3}\,s^{-1}$.

The steady-state rate equation describing this reaction scheme is:

$$v = \frac{E_0V_fK_c + E_0V_f^{act.}[C]}{[C] + K_c + K_sK_c/[S]}$$

where V_f is the rate of product formation by the unactivated enzyme (E–S) and $V_f^{act.}$ by the activated enzyme (C–E–S), and K_c is the true dissociation constant (k_{-2}/k_2) describing the binding of apo-CII to substrate-bound LPL.

Further steady-state experiments in which substrate and apo-CII concentrations were varied gave results which were predicted accurately by the above equation and, additionally, revealed that $V_f^{act.}$ is approximately 1.4 times greater than V_f.

In conclusion, the study shows that apo-CII achieves activation in this system by associating preferentially with the form of the enzyme which is bound to the substrate ($K_d = 7–12$ nM) rather than to the free species ($K_d = 200$ nM), thus reducing the apparent K_s. Activated LPL forms products at a rate which is only slightly greater than that of the unactivated enzyme.

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Effects of lipase enzymes on procoagulant and anticoagulant activities of lipoproteins

ELAINE GRAY,* SUSAN P. WILLIAMS† and TREVOR W. BARROWCLIFFE*

**Division of Blood Products and †Chemistry Section, National Institute for Biological Standards and Control, Holly Hill, Hampstead, London NW3 6RB, U.K.*

Phospholipids are essential for several reactions in the blood coagulation pathway, providing an active surface which binds the clotting factors and effectively reduces their K_m (Jackson, 1981). Although plasma lipoproteins contain large amounts of phospholipid, this is normally inactive in the coagulation system, probably because it does not have the optimal structure for clotting factor binding (Vijayagopal & Ardlie, 1978).

Our previous studies indicated two areas in which lipoproteins can become more involved with the coagulation system. It was found that lipid peroxides, prepared by autoxidation of arachidonic acid, stimulated the generation of thrombin in platelet-free plasma (Barrowcliffe *et al.*, 1975), and subsequent studies showed that this procoagulant activity was due to interaction of the peroxides with triglyceride-rich lipoproteins (Barrowcliffe *et al.*, 1984). Studies on the inhibition of the enzyme Factor Xa showed that part of the plasma 'anti-Xa' activity is due to lipoproteins, especially LDL and HDL (Barrowcliffe *et al.*, 1982).

In the present study, we have examined the effects of various lipase enzymes on these interactions with a view to increasing our understanding of the mechanisms involved.

LPL and HTGL were separated and partially purified from human or rat PHP by chromatography on heparin–Sepharose. The lipase enzymes were assayed with a tritiated triolein substrate as described by Nilsson-Ehle & Schotz (1976); for HTGL, serum was omitted from the incubation mixture and the NaCl content was increased to 1 M. Purification was estimated at 200 times from PHP. LPL was also prepared by the same method from bovine milk. PLA_2, highly purified from *Vipera berus*, was a gift from Dr. M. C. Boffa (Paris). The remaining phospholipase enzymes used were purchased from Sigma Ltd.

The effects of lipase release on lipid peroxide-induced thrombin generation *in vivo* were studied by subcutaneous injection of a lipase-releasing drug, SP54, into six volunteers. The peak total lipase level averaged 57.7 munits/ml, of which 70% was due to HTGL. The total amount of thrombin generated on addition of lipid peroxides to the post-infusion plasmas was only 50% of that generated in the pre-infusion plasmas.

Thrombin generation induced by lipid peroxides was measured after incubation of fatty plasma with various lipases *in vitro*, as shown in Table 1. HTGL reduced the peak thrombin level to 30% of that in the buffer control, whereas LPL had little effect, suggesting that the reduction of thrombin generation in the experiments *in vivo* was largely due to release of HTGL. All except one of the phospholipase enzymes gave a similar reduction of thrombin generation to HTGL, indicating that the action of HTGL may be due to its phospholipase activity. The lack of activity of PLA_2 from *Naja naja* is in keeping with the fact

Abbreviations used: LDL, low-density lipoprotein; HDL, high-density lipoprotein; LPL, lipoprotein lipase; HTGL, hepatic triglyceride lipase; PHP, post-heparin plasma; PLA_2, phospholipase A_2.

Table 1. *Effects of lipase enzymes on lipid peroxide-induced generation of thrombin in chylomicra-rich plasma*

Enzymes were incubated with plasma at 37°C for 1 h at concentrations of 30–50 munits/ml (LPL and HTGL) or 1–4 μg/ml (PLA_2 and PLC). Peak thrombin generated was measured as a percentage of that in the buffer control.

Enzyme	Source	Peak thrombin (%)
PLA_2	*V. berus*	30
PLA_2	Pancreas	29
PLA_2	*Naja naja*	100
PLC	*Bacillus cereus*	7
LPL	Rat PHP	83
HTGL	Rat PHP	23
HTGL	Human PHP	29

that this enzyme, unlike PLA_2 from *V. berus*, is known to be a poor anticoagulant (Boffa *et al.*, 1980).

The anti-Xa activity of plasma was measured by a clotting assay after incubation with LPL or HTGL. LPL, purified from milk or PHP, had virtually no effect, but HTGL produced a marked increase in activity. The enhancement of activity increased on incubation of HTGL with plasma from 0 to 45 min, and was not apparent in plasma from which lipoproteins had been removed by ultracentrifugation. These results indicate that HTGL increases anti-Xa activity by a time-dependent action on lipoproteins.

Overall, it appears that release of lipase enzymes, especially HTGL, into plasma increases its anticoagulant potential, and this could be an important contribution to the antithrombic properties of heparin and other lipase-releasing drugs.

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Turnover of lipoprotein lipase in rat adipose tissue

SUSAN M. PARKIN, BRIAN K. SPEAKE and DONALD S. ROBINSON

Department of Biochemistry, University of Leeds, Leeds LS2 9JT, U.K.

There is a considerable amount of evidence that adipose tissue lipoprotein lipase has an exceptionally high rate of turnover (Wing *et al.*, 1967; Wing & Robinson, 1968; Patten, 1970). This evidence is, however, based largely on changes in the activity of the enzyme and the role of proteolytic degradation in this process has, as yet, received little attention.

Indirect evidence for the degradation of lipoprotein lipase is provided by the time-course of incorporation of [^{3}H]leucine into the enzyme during incubation of rat epididymal fat-bodies *in vitro* in the presence of insulin (2 m.i.u./ml). The incubation conditions and the method for the isolation of ^{3}H-labelled lipoprotein lipase are described by Speake *et al.* (1985). Whereas the incorporation of label into total fat-body protein is linear with time for at least 4 h, the radioactivity associated with lipoprotein lipase shows a linear increase only during the initial 1–2 h of incubation, and thereafter remains at an approximately constant level.

Additional experiments have indicated that the observed cessation of net incorporation of label into lipoprotein lipase which occurs between 1 and 2 h of incubation is due neither to secretion of labelled enzyme into the medium, nor to an inability to maintain the synthesis of the enzyme under these conditions. A possible explanation for these results is that lipoprotein lipase is degraded, presumably at a site distal to the site of its synthesis, and that the steady-state level of radiolabelled enzyme which prevails after the initial 1–2 h of incorporation represents a balance between the synthesis and the degradation of the enzyme.

In order to investigate this possibility, the degradation of lipoprotein lipase was measured by using pulse-chase incubations of epididymal fat-bodies *in vitro*. Groups of fat-bodies were incubated in the presence of [^{3}H]leucine for 1 h. At the end of this 'pulse' period, the fat-bodies were washed free of exogenous radioactivity and the incubation was continued for a further 3 h. Insulin (2 m-i.u./ml) was present throughout. Approx. 50% of the label incorporated into lipoprotein lipase during the 'pulse' period was lost during the subsequent 3 h decay period. Little or no degradation of total fat-body protein was observed under these conditions.

The extensive degradation of adipose tissue lipoprotein lipase thus described, provides a possible explanation for the rapid turnover of lipoprotein lipase activity which has been reported to occur both *in vivo* (Wing *et al.*, 1967) and *in vitro* (Wing & Robinson, 1968; Patten, 1970). An important consequence of this high turnover rate is that the level of the enzyme will change rapidly in response to variations in the rate of enzyme synthesis (Schimke, 1970). Thus the stimulation of lipoprotein lipase synthesis induced by insulin and glucocorticoids, as described by Speake *et al.* (1985), will result in a rapid increase in the amount of the enzyme to a new steady state level.

The degradation of lipoprotein lipase may itself be subject to regulation. In the presence of adrenaline (10 μM), the rate of degradation of the enzyme was increased by approx. 1.5-fold. This observation is consistent with the evidence that adrenaline reduces the activity of adipose tissue lipoprotein lipase by a post-translational mechanism (Ashby *et al.*, 1978).

The site and mechanism of the gradation of lipoprotein lipase have not yet been ascertained, although the report that a number of cell types have the ability to degrade ^{125}I-labelled lipoprotein lipase after endocytotic uptake (Friedman *et al.*, 1982) may be of significance.

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Regulation of the synthesis of lipoprotein lipase in rat adipose tissue

BRIAN K. SPEAKE, SUSAN M. PARKIN and DONALD S. ROBINSON

Department of Biochemistry, University of Leeds, Leeds LS2 9JT, U.K.

Studies involving the incubation of rat epididymal fat-bodies *in vitro* have shown that insulin and glucocorticoids can promote a protein-synthesis-dependent increase in the activity of lipoprotein lipase in the tissue, and it has been proposed that these hormones are responsible for the increase in the activity of the enzyme which occurs *in vivo* after the onset of feeding (Ashby & Robinson, 1980).

In order to investigate the mechanism of these hormonal effects, we have devised a novel method for measuring the synthesis of lipoprotein lipase in rat adipose tissue. Epididymal fat-bodies from 24h-starved rats were incubated for 1h at 37°C in Krebs–Henseleit bicarbonate buffer solution, supplemented with amino acids at the concentrations defined by Eagle (1955), glucose (10mM), casein (2% w/v) and L-[4,5-^{3}H]leucine (10μCi/ml). At the end of the incubation period, groups of four fat-bodies were removed from the incubation flasks, and homogenized in 10ml of 2% (w/v) casein, pH7.2. The homogenate was delipidated by extraction with acetone and diethyl ether as previously described (Ashby *et al.*, 1978). Each delipidated tissue residue was homogenized in 15ml of 5mM-sodium barbital, pH7.5, containing 20% (w/v) glycerol, 0.1% Triton X-100 and 50mM-NaCl, and the suspension was centrifuged at 60000*g* for 30min. Lipoprotein lipase was isolated from the resultant supernatant in a single step by affinity chromatography on heparin–Sepharose as described by Parkin *et al.* (1982). Sodium dodecyl sulphate/polyacrylamide-gel electrophoresis of this ^{3}H-labelled lipoprotein lipase preparation revealed a single sharp peak of radioactivity corresponding to a polypeptide of M_r 56000. This polypeptide has previously been identified as lipoprotein lipase (Parkin *et al.*, 1982). No other peaks of radioactivity were detected on the gel and losses of lipoprotein lipase during this procedure were minimal. Thus the ^{3}H-labelled enzyme can be rapidly and quantitatively isolated free from other radioactive adipose tissue proteins.

In the presence of insulin (2m-i.u./ml), the incorporation of [^{3}H]leucine into total adipose tissue protein and into lipoprotein lipase was increased by 1.3 ± 0.2 fold and 2.6 ± 0.6 fold respectively (mean $\pm$ S.D., $n = 7$), compared with controls. Thus the insulin-induced stimulation of lipoprotein lipase synthesis is partly due to a general increase in total protein synthesis in addition to a specific effect on the synthesis of the enzyme. Similar results have been reported by Vydelingum *et al.* (1983).

The regulation of lipoprotein lipase by glucocorticoids was investigated by preincubating fat-bodies in the presence or absence of dexamethasone (400nM) for 3h before the addition of [^{3}H]leucine, in order to allow for any lag in the glucocorticoid-induced effects. [^{3}H]Leucine (10μCi/ml) was then added and the incubation was continued for a further 1h. Insulin (2m-i.u./ml) was present in all the incubations. The rate of synthesis of lipoprotein lipase was increased by 1.99 ± 0.72 (mean $\pm$ S.D., $n = 5$) fold in the presence of dexamethasone plus insulin, compared with insulin alone. This effect represents a specific induction of lipoprotein lipase synthesis since total protein synthesis was not affected by dexamethasone.

These observed effects of insulin and glucocorticoids on the synthesis of lipoprotein lipase are consistent with the changes in enzyme activity which occur under these hormonal conditions. It thus seems likely that both hormones regulate the activity of lipoprotein lipase at the level of enzyme synthesis. Although the effect of insulin is partly explained by a non-specific increase in general protein synthesis, the effect of glucocorticoids apparently represents a specific induction of the enzyme.

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Role of apoproteins B and E in the receptor-mediated uptake of very-low-density lipoprotein remnants by the perfused rat liver

BHIM S. SURI, MICHAEL E. TARG and DONALD S. ROBINSON

Department of Biochemistry, University of Leeds, Leeds LS2 9JT, U.K.

Two distinct receptors are concerned in the binding and removal of remnants of plasma triglyceride-rich lipoproteins by the liver. The 'LDL receptor' is believed to be primarily concerned with the removal of VLDL remnant lipoproteins of hepatic origin containing apoprotein B_{100} whereas the 'chylomicron remnant receptor' appears to be specific for apoprotein B_{48}-containing remnant particles, which may originate either from the intestine or the liver (Brown & Goldstein, 1983). The apoprotein specificity of the two hepatic receptors remains, however, an area of much interest since all the remnant populations also contain additional apoproteins.

HDL_c, an apoprotein E-enriched lipoprotein, binds to the 'LDL receptor' with a 20-fold greater affinity than a predominantly apoprotein B_{100}-containing particle such as LDL (Innerarity *et al.*, 1980). Moreover, HDL_c will compete with ^{125}I-labelled chylomicron remnants for removal by the perfused liver (Sherrill *et al.*, 1980). These findings have led Mahley and his co-workers to suggest that apoprotein E is the major mediator of the hepatic removal of all the remnant classes (Mahley & Innerarity, 1983). However, the observed differences between the metabolism of apoprotein B_{100} and apoprotein B_{48} remnants cannot be reconciled on the assumption that the receptor-interaction of these particles is mediated through apoprotein E alone (Brown &

Abbreviations used: LDL, low-density lipoprotein; VLDL, very-low-density lipoprotein; HDL, high-density lipoprotein; CHD, 1,2-cyclohexanedione.