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A STUDY OF LIPOPROTEIN LIPASE AND LIPASES OF BOVINE MILK AND LACTATING MAMMARY GLAND

A thesis presented in partial fulfilment
of the requirements for the degree of
Master of Science in Biochemistry

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Chapter 1

INTRODUCTION

1:1 The Role and Occurrence of Lipoprotein Lipase

Lipoprotein lipase is the enzyme involved in the hydrolysis of blood lipoprotein TG to yield FFAs and glycerol; the FFAs are taken up by the tissue organ to be esterified into new TG or metabolized and the glycerol released into the blood stream to be metabolized by the liver. The enzyme requires the presence of apolipoproteins surrounding the TG core for activation of the enzyme and these remain in the blood stream after hydrolysis of the TG to give a lipoprotein complex of higher density.

Lipoprotein lipase has been detected in chicken adipose tissue and rat adipose tissue by Korn and Quigley (1955) (1957), Angervall (1960), mammary tissue of guinea pigs by McBride and Korn (1963), rabbits by Fiddler and Falconer (1968), goats by West *et al* (1967) and bovine mammary gland by Askew, Emery and Thomas (1970), post heparin plasma of rats by Fielding (1969) and bovine milk by Korn (1962). It is released into the circulating blood after injection of heparin as shown by Heald *et al* (1965).

In cow TG of chylomicra and LDL (d less than 1.019) circulating in the blood are taken up by the mammary gland as shown by Emery *et al* (1965), Glassock *et al* (1966) and Welch *et al* (1968). The main source of long chain FAs removed from the blood by the mammary gland are TG fatty acids of circulating LDL and chylomicra as reported by Emery *et al* (1965), Glassock *et al* (1966) Welch *et al* (1968) and Huber *et al* (1969).

1:2 Lipoprotein lipase and fatty acid uptake

Barry *et al* (1963) using arteriovenous studies across the mammary gland of the lactating goat found that there was a large arteriovenous difference in the TGFAs of chylomicra and LDL of

density 1.005 - 1.019 with no significant arteriovenous differences in FFAs of plasma or in FAs of LDL of density 1.019 - 1.063 or HDL indicating most of the milk fat TG are derived from VLDL or chylomicrons of serum. The FAs taken up from VLDL and chylomicrons are mainly palmitic and stearic acids (90%) with moderate amounts of oleic and linoleic acids. They conclude that TG of chylomicra and VLDL are the main source of FAs of milk fat TG and possibly glycerol indicating the importance of LDL in the mammary gland. Annison et al (1967) found that milk FAs of chain length C4 - C14 arise largely from blood acetate and palmitate is derived partly from acetate and partly from plasma TG, the latter being a major source of oleate and stearate. Glassock et al (1966) estimated by tritium labelled TG of olive oil that 35 - 48% of the milk fat by weight was derived from B lipoprotein TG. Lascelles et al (1964) obtained similar high incorporation of chylomicron TG into milk fat.

West et al (1972) using chylomicron TG emulsions labelled with H^3 and C^{14} TG and passing these through the mammary arterial vein of lactating goat showed that both glycerol and FFAs were released during TG uptake by mammary tissue and changes in the H^3/C^{14} ratio during transfer of the TG from blood into milk indicated that at least 80% of the TG was hydrolysed during uptake with a greater loss of C^{14} glycerol than H^3 palmitic acid during synthesis of milk fat from labelled chylomicron TG.

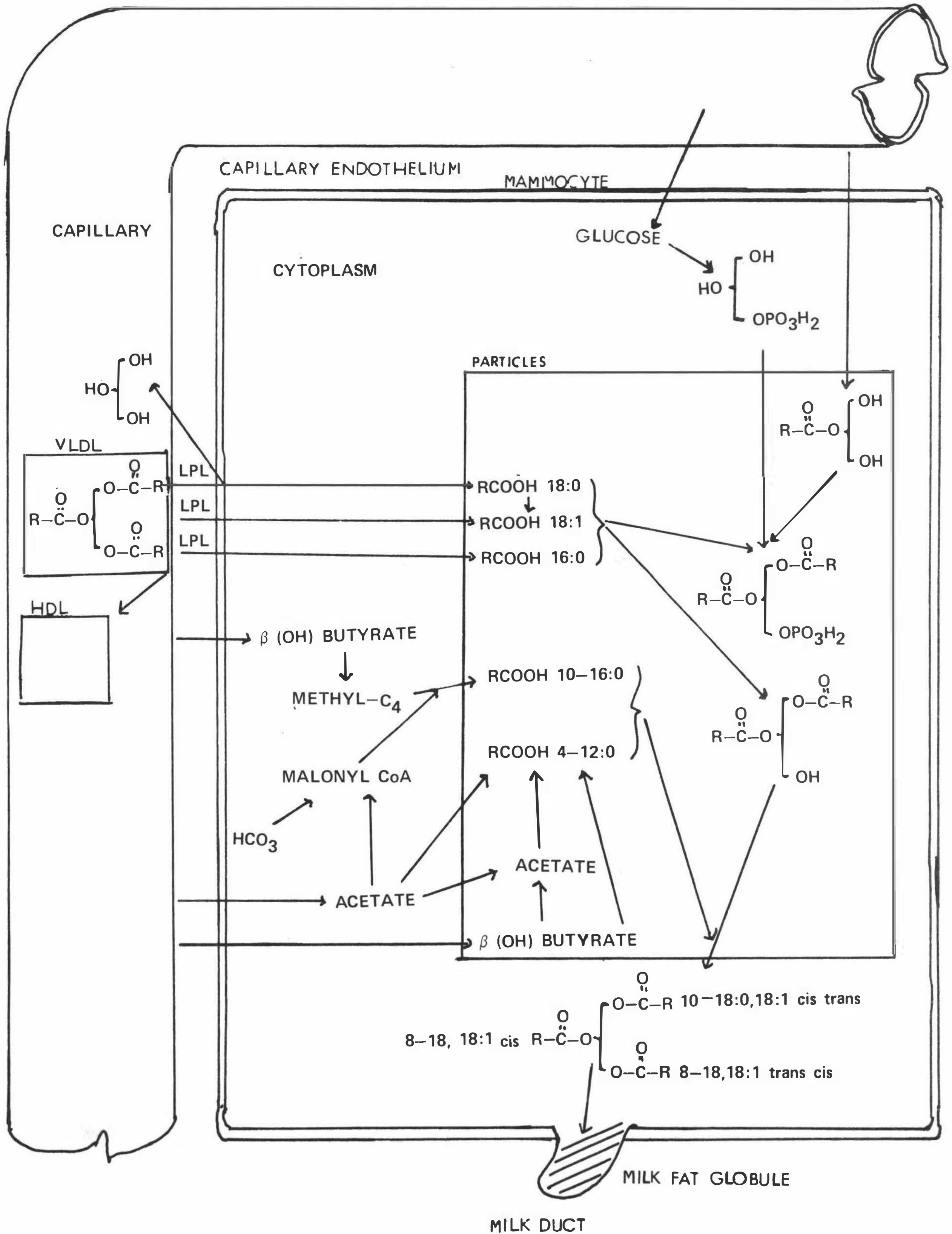
Using perfused rat adipose tissue, Rodbell and Scow (1965) observed that half of the TG in chylomicrons taken up by the adipose tissue was hydrolysed and the FAs re-esterified whereas the other half was retained intact in the tissue and a continual release of C^{14} glycerol after stopping infusion suggested that TG was hydrolysed after being removed from the blood stream.

Scow et al (1972) observed that after infusion of C^{14} palmityl H^3 glycerol TG chylomicrons through rat adipose tissue the FA content of the perfusate decreased 90% whereas glycerol content only decreased 50%. Analysis of adipose tissue lipid extracts after

perfusion gave 9% of the TG as unhydrolysed and 91% appeared as new TG in the distal portion of the adipose tissue whereas in the proximal portion, 55% was unhydrolysed and 45% appeared as new TG. Jones and Havel (1967) by in vivo studies showed that most of the TG taken up by the adipose tissue was hydrolysed to FFA and glycerol.

By observing the molar ratios of glycerol to FFA at various times after infusion of radioactive labelled chylomicron TG into rat adipose tissue in vitro, Scow _et al_ (1972) recorded a decrease in the ratio of glycerol to FFA after 1.5 minutes and assuming a time of 1 minute for perfusion they suggest blood TG is hydrolysed firstly to partial glycerides and FFAs with the immediate release of FFAs to the blood followed by complete hydrolysis to FFA and glycerol 30 seconds later accompanied by the release of glycerol into the blood stream.

Formation of milk TGs within mammocytes according to Emery (1973)



1:3 Site of lipoprotein lipase action

The hydrolysis of chylomicra and LDL TG as blood passes through the mammary gland occurs as in other extra hepatic tissues by the action of LPL acting at a site close to the blood capillary wall. (Robinson (1963) and Schoefl and French (1967)). Chylomicra have been shown to become fused to the endothelium wall where flaps may enclose them and they may eventually be enclosed within vacuoles inside the cytoplasm as demonstrated by Lascelles et al (1964). When the lipid particles become closely associated with the endothelial cell surface, a clear gap is seen between the particles and the endothelial cell surface. The enzyme has been shown to be absorbed on to the chylomicra and the particles of lipid emulsions in vitro by Korn and Quigley (1957) and Robinson, Jeffries and Poole (1955). Schoefl and French (1967) postulate that this absorption might be responsible for the close adhesion of the particles in vivo if the enzyme is normally bound at the endothelial surface. The moulding of the endothelial cytoplasm around the lipid particles would be consistent with this pattern. Measurements of LPL in mammary venous blood of goats are indicative of its release coincident with the uptake of TGFAs of chylomicra and LDL as blood passed through the mammary gland as observed by Barry et al (1963) and Lascelles et al (1964).

Scow et al (1972) after infusing heparin through rat adipose tissue in vitro obtained a release of LPL into the perfusate accompanied by a decrease in chylomicron TG lipolysis in the tissue. After prolonged infusion of heparin, LPL in the perfusate became negligible and TG hydrolysis in adipose tissue was still 70% below that of control tissue. Scow et al (1972) conclude that LPL involved in hydrolysis of blood TG is located in or near the capillary wall.

By histochemical studies of the rat adipose tissue capillary after infusion of chylomicron TG followed by incubation for 1 hour and Pb^{2+} treatment to detect FFAs, Scow et al (1972) observed FFAs and glycerides located in vesicles or surrounding invaginations of the capillary wall indicating that TG was

hydrolysed within the capillary endothelium and between the endothelium and pericyte. Their findings indicated that glycerides cross the endothelial space in a membrane bound system.

They propose a system in which chylomicrons become attached to or are enveloped by the endothelial cell. The hydrolysis of the chylomicron TG is accompanied by the immediate release of FFA into the blood stream. The DG is then taken within the cell in a vacuole microvesicle. Hydrolysis of the DG occurs in transition across the endothelium with the FFA taken up by the fat cell and the final hydrolysis of the MG takes place in the subendothelial space with the release of glycerol into the blood stream and the FFA taken up by the fat cell.

1:4 Mammary Gland levels of LPL

Barry et al (1963) together with McBride and Korn (1964) observed that uptake of blood TG by mammary tissue occurs only during lactation whereas uptake by adipose tissue occurs when the animal is in a positive energy balance as observed by Bragdon and Gorden (1958) together with Havel et al (1962). Since TG uptake is dependent on LPL activity Robinson et al (1970) and Bezman et al (1962) propose that the level of activity in each tissue affects its capacity to remove TG from the blood. Scow et al (1964) and Otway and Robinson (1968) observed that a marked hypertriglycermidemia develops in the rat during the last part of pregnancy and dissappears at parturition. The enzyme activities in heart diaphragm, muscle and lung were unaffected by pregnancy whereas the activity in adipose tissue decreased during the last two days of pregnancy and remained low for three days post partum.

Shirely et al (1971) recorded a 94X increase in bovine mammary gland LPL from 0.0008 μmole FFA released/min/mg to 0.078 μmole FFA released/min/mg while adipose tissue LPL decreased from 0.02 μmole FFA released/min/mg to 0.013 μmole FFA released/min/mg with

the onset of lactation. Glyceride synthesis increased 8X in mammary tissue and decreased 6X in the adipose tissue with the onset of lactation.

Scow _et al_ (1972) studied the effect of pregnancy on rat mammary gland and adipose tissue levels of LPL. Adipose tissue LPL increased during the first 19 days of pregnancy decreased several days before parturition and remained low during lactation whereas mammary gland LPL was low during the first 20 days of pregnancy increased several days before parturition, decreased sharply at parturition and increased again immediately post partum and remained high during lactation. Plasma TG concentrations increased from 0.9 to 3.3mM between the 12th and 10th days of pregnancy, decreased 50% during the next two days, increased at parturition and fell rapidly post partum. These changes were also observed by Hamosh _et al_ (1970).

Otway and Robinson (1968) suggest that the increased lipase activity in the mammary tissue at the end of pregnancy might account for the disappearance of hyperlipemia which is caused by a decreased uptake of TGFAs by adipose tissue due to a decrease in LPL of adipose tissue. Both McBride and Korn (1964) and Hamosh _et al_ (1970) observed a marked decrease in mammary gland LPL if either guinea pigs or rats respectively were not suckled. Non-suckling for 9 hours in rats decreased mammary gland LPL by 70% and increased the plasma TG concentration by 3X to 3mM. Non-suckling for a further 9 hours completely inhibited mammary LPL and increased adipose tissue LPL by 55% of the level of non lactating rats. Hamosh _et al_ (1970) suggest that the relationship between LPL activity in adipose tissue and that in mammary tissue suggests hormonal factors necessary for milk secretion divert dietary FAs to the mammary gland by suppressing LPL activity in adipose tissue and stimulating mammary gland LPL.

1:5 Metabolic and Hormonal Control of lipoprotein lipase

The changes in adipose tissue LPL has been shown to be associated with changes in glucose metabolism and insulin secretion in rats by Robinson (1965). Robinson (1965) has proposed that LPL is part of a regulatory system controlling energy metabolism in the rat since its activity is highest during deposition of FAs in adipose tissue after feeding. Adipose tissue LPL has been shown by Tepperman and Tepperman (1970) to be increased by insulin and inhibited by catecholamines and cyclic 3'5' adenosine monophosphate. FFAs also had an inhibitary effect on LPL and glucose metabolites had a stimulatory effect with insulin and glucose having a co-ordinate response. Insulin may act by causing a decrease in cyclic AMP concentration.

Reichl (1972) using rats adapted to a rhythmic feeding pattern and rats not adapted found that after starvation (low adipose tissue, LPL, low blood glucose and insulin and high concentrations of unesterified FAs) only feeding in the unadapted rats resulted in an increase in insulin and glucose whereas with the adapted rats there was an increase in LPL activity with no corresponding increase in insulin or glucose at the period they would normally be fed if the rats were deprived of food at that period indicating some other influence over the control of LPL activity in the rat epidymal fat pad. Wing et al (1966) and Nestel and Austin (1968) have reported that adrenalin noradrenalin, adrenacorticotrophin, glucagon and thyroid stimulating hormone all inhibit an increase in enzyme activity. Activity in adipose tissue was decreased by fasting and insulin lack and restored to normal by refeeding and insulin administration. Hamosh et al (1970) together with Otway and Robinson (1968) have shown that LPL activity in adipose tissue is suppressed by factors probably the hormones that stimulate milk formation. This suppression of LPL activity occurs even though food intake is increased during lactation. Fasting had no suppressing effect on LPL activity in the rat mammary gland as long as the animal was suckled as shown by McBride and Korn (1963). Non suckling caused suppression of enzyme activities in duct ligated glands with normal activity in other glands indicating the effect of ligation is independent of circulating hormones.

Hamosh *et al* (1970) suggest that the lowering of enzyme activities in mammary tissue when suckling is stopped is caused firstly by local engorgement and then by reduced secretion of hormones needed for milk formation, e.g. prolactin, glucocorticoid.

Hamosh *et al* (1970) suggest that the decrease in activity of LPL in mammary gland immediately after parturition may result from an accumulation of milk in the gland before suckling has been initiated. It is thought that in the parenchymal cells of the adipose tissue the fat cell produces and regulates the level of LPL activity. This view is supported by Scow *et al* (1964), Rodbell (1964), Rodbell and Scow (1965) and Porkajac *et al* (1967). This may also apply for mammary tissue allowing local control of LPL activity by hormones. Fiddler and Falconer (1968) have shown that prolactin released from the pituitary upon parturition and suckling stimulates LPL activity in pseudo pregnant rabbit mammary tissue.

Falconer and Fiddler (1970) reported that prolactin injection gave an increase in LPL in pseudo pregnant rabbits within 24 hours which was prevented by injection of actinomycin D (even if given up to 48 hours after prolactin injection) and cyclohexamide which gave a shorter duration of effect than actinomycin D. They conclude that the prolactin induced increase in LPL in mammary tissue requires the continued production of a short lived messenger RNA and that the enzyme is rapidly inactivated in the tissue once its synthesis is blocked by cyclohexamide. Wing and Robinson (1968) reported that actinomycin, glucose, insulin and heparin are required for a prolonged increase in LPL activity over 9 hours whereas with no actinomycin increase only occurs over 3 hours and to a lesser extent from starved rat adipose tissue extracts. With the omission of glucose, heparin and insulin and the addition of pyruvate, an increase in LPL activity occurs only after a lag of 1 hour whereas with the omission of only heparin no lag is observed and little enzyme appeared in the incubation medium. Puramycin, catecholamines and corticotrophin inhibited the increase in enzyme activity caused by actinomycin. Puramycin addition indicated the enzyme had a ½ life of 1 - 1.5 hours in fed rats and was less stable

in fed rats than starved rats. Wing and Robinson (1968) propose the enzyme exists in two forms, a stable and an unstable form and changes in the relative proportions of active and inactive (stable) forms of the enzyme in adipose tissue explain the increase in enzyme activity occurring during the transformation from the starved into the fed state. The change from starved to fed state involves the appearance of an unstable active LPL. Instability could be due to the conversion of the inactive into the active form in the absence of stablizing factors such as heparin. Eagle and Robinson (1964) propose that the activating effect of actinomycin D might be due to inhibition of synthesis of an inhibiting factor as actinomycin D could act by preventing the formation of a more unstable enzyme which is readily deactivated whereas the stable enzyme will not be affected by actinomycin D and the activation could involve some common constituent (heparin) which is in short supply causing increased activity of the stable enzyme due to no competition from the unstable form for the common factor.

Wing and Robinson (1968) observed that the rise in adipose tissue LPL from starved rats is affected by dibutyryl cyclic AMP if the glucose concentration is 1.3mg/ml or less but has no effect if the glucose concentration is 2.4 mg/ml or above unless caffeine (1mM) - (inhibits cyclic AMP phosphodiesterase) is also present and 5mM caffeine alone can inhibit the rise in a 2.4mg/ml glucose solution. A rise in adipose tissue LPL is associated with a fall in adipose tissue FFA in vitro. In the presence of cyclic AMP this decrease in FFA is prevented. Wing and Robinson (1968) suggest that the concentration of cyclic AMP in adipose tissue may regulate LPL and this regulation may occur through the effects of cyclic AMP on tissue FFA concentrations.

After injecting glucose or insulin intravenously into lactating cows, Rao et al (1973) observed an increase in adipose tissue LPL. In vitro, glucose plus insulin had a greater effect than insulin or glucose alone on increasing adipose tissue LPL.

Shirely and Emery (1973) found that prolactin stimulated formation or activation and release of LPL from explants of rat or bovine mammary tissue incubated for 4 hours in the presence of insulin. Prolactin had little or no effect on adipose tissue LPL in the absence of insulin.

Shirely et al (1971) reported that glyceride synthesis and LPL in adipose tissue was inversely proportional to the milk fat content of milk in response to dietary changes or initiation of lactation.

de Pury and Collins (1972) using one lot of rats fed a diet deficient in essential FAs and another lot on a normal diet observed that FA deficient fed rats had a lower level of VLDL protein, showed a higher level of LPL in their post heparin plasma and there was a lower km value for the VLDL proteins, as substrate for post heparin plasma LPL than with rats with a normal diet of FAs indicating an increased affinity of the LPL for the VLDL from FA deficient rats.

Fielding (1970) observed inhibition of human post heparin plasma LPL by LDL and VLDL. Chylomicrons and HDL give no inhibition and give greater rates of activity than VLDL and LDL. Thus the level and composition of the lipoproteins may act as a controlling mechanism for LPL.

Metz et al (1973) using biopsy samples of subcutaneous tissue of lactating cows observed a reciprocal relationship between the rate of lipolysis and molar ratio of FFA to albumin in the medium.

Lipolysis was gradually inhibited when FFA/albumin ratios were increased from 0.1 to 6.5. When the FFA/ albumin ratio was greater than 4, FFA release was less than glycerol release and resulted in an accumulation of FFA in the adipose tissue. After parturition plasma FFA increases to 0.7 - 1.0 mM corresponding to a FFA/albumin ratio of 1.5 - 2.0. Since these values are in the range where inhibition of lipolysis in vitro is observed, Metz et al (1973) propose that this

FFA accumulation is the controlling mechanism for the rapid changes observed in mammary gland LPL levels at post parturition and at the onset of lactation.

Increases in LPL activity in adipose tissue taken from starved rats in vitro have been shown to be inhibited in the presence of an inhibitor of protein synthesis such as puramycin by Wing et al (1966), Wing and Robinson (1968) and Patten (1970). Cunningham and Robinson (1969) suggest that this increase in enzyme activity is due to activation brought about by the conversion of one form of the enzyme into another which may be due to a decrease in adipose tissue cyclic AMP.

Cryer et al (1973) observed that cyclohexamide had no effect on this increase in total activity during the first 2 hours (but inhibited incorporation of 1 C^{14} leucine into total tissue protein by 90% during this period) but inhibited this increase in adipose tissue LPL thereafter. Cryer et al (1973) suggest that the increase in LPL takes place in two stages. Firstly a conversion of one form of the enzyme into another of higher specific activity and secondly a synthesis of more of the precursor. This hypothesis is supported by Robinson and Wing (1970). Cunningham and Robinson (1969) reported that incubation of intact epidymal adipose tissue from fed rats at 37°C in vitro caused a rapid loss in adipose tissue LPL until a low activity stable to prolonged incubation was attained whereas LPL from intact tissue from starved rats is stable to incubation at 37°C. Collagenase inactivated the LPL from fed rat tissue whereas it had no effect on LPL from unfed rat tissue. This supports the theory of unstable and stable forms of the enzyme proposed by Wing and Robinson (1968).

Askew et al (1971) observed only a small decrease in mammary gland LPL from 0.007 μmole FFA released per min per mg to 0.0063 μmole FFA released per min per mg on feeding high grain restricted roughage rations to lactating dairy cows whereas Baldwin and Emery (1973) recorded a larger decrease in mammary gland LPL from

0.0018 μmole FFA released per min per mg to 0.0033 μmole FFA released per min per mg on feeding high grain restricted roughage diets compared to normal diets. Askew et al (1971) also observed an increase of mammary gland LPL activity from 0.007 μmole FFA released/min/mg to 0.0072 μmole FFA released/min/mg on feeding MgO with the high grain restricted roughage diet compared with the normal diet. Adipose tissue LPL activity increased from 0.0001 μmole FFA released/min/mg to 0.0003 μmole FFA released/min/mg on feeding a high grain restricted roughage diet with a smaller increase from 0.0001 μmole FFA released/min/mg to 0.00026 μmole FFA released/min/mg on feeding a high grain restricted diet plus MgO compared with a normal diet. On feeding a high grain restricted roughage diet Askew et al (1972) recorded a decrease in milk fat percentage with an increase in unsaturated FAs (linoleic and oleic) and a decrease in saturated FAs (stearic and palmitic acids).

1:6 Substrate specificity of lipoprotein lipase

Garneson et al (1971) showed the presence of two distinct lipolytic activities with differential activation of LPL from human post heparin plasma, milk and AT by polypeptides of serum apolipoprotein using Fielding's (1969) method of purification of LPL and preparative polyacrylamide gel electrophoresis. Polypeptides containing glutamate or alanine carboxyl terminal amino acids but not those containing glutamine, serine, threonine, served as activators of adipose tissue and milk LPL. LPL from human post heparin plasma showed a greater activation by R serine polypeptide of apolipoprotein C than by R glutamic acid with R alanine, R threonine or R glutamic polypeptides of apolipoprotein A having no activation. LPL activity required serum or phosphatidylcholine and in the absence of phosphatidylcholine only R serine served as an activator and gave only 10% of the activity with serine apolipoprotein C in the presence of phosphatidylcholine. R glutamate activated LPL from human milk and adipose tissue to a greater extent than human plasma LPL. Dog and rat post heparin LPL gave similar activation studies as with human post heparin plasma LPL.

Havel et al (1973) compared the cofactor activity of the protein components of human VLDL in the hydrolysis of TG by highly purified LPL preparations from human and rat post heparin plasma and cow's milk and by crude preparations of enzyme from cows milk and rat adipose tissue. Carboxyl terminated serine and alanine apo VLDL had slight activity for all of the above sources except from purified milk and at high levels of these apo VLDLs inhibition occurred. Heparin stimulated both the pure and crude preparations of milk LPL and increased the sensitivity of these enzyme preparations to stimulation by carboxyl terminal glutamic acid apo VLDL but had no effect on purified rat post heparin plasma LPL. High concentrations of carboxyl alanine and serine apo VLDL inhibited the stimulatory effect of carboxyl glutamic acid apo VLDL in the presence of heparin with impure cows milk lipase but not with pure preparations from cows milk and rat post heparin plasma. R glutamic acid apo VLDL had a greater stimulatory effect in all cases.

La Rosa et al (1970) studied the effect of HDL apoproteins as activators of rat adipose tissue LPL. Apo LP glu, apo LP thr and apo LP val were inactive as cofactors even in the presence of phospholipid. Apo LP ala was inactive alone but in the presence of phospholipid, stimulated lipase activity by 2 times. Only apo LP glu was able to stimulate LPL in the absence of phospholipid.

Chung and Scanu (1973) observed that VLDL polypeptides of terminal carboxyl glutamic acid and alanine were activator and inhibitor respectively for both skimmed milk and rat adipose tissue LPL. LPL from adipose tissue required ten times greater amount of activator than LPL from skimmed milk. In both cases the kinetics of inactivation of LPL depended on the time of addition of the inhibitor. Chung and Scanu (1973) conclude that the inhibitor acted on the substrate rather than the enzyme itself. Brown and Boginsky (1972) also observed that apo LP glu from human VLDL was stimulatory to milk LPL whereas apo LP ala inhibited the enzyme. Inhibition was not overcome by the addition of phospholipid, apo LP glu or more enzyme. A concentration of 100 mg/ml completely inhibited the enzyme, addition of more substrate reversed the inhibition. Brown and Boginsky (1972)

conclude that inhibition is due to binding of the apoprotein to the substrate preventing enzyme substrate interaction and activation by apo LP ala observed by some workers was due to contamination of the apo LP ala by apo LP glu.

Emery et al (1972) separated lipoproteins with preference for adipose tissue LPL from those with preference for mammary LPL by flotation from blood serum after emulsifying serum in olive oil and sucrose density gradient centrifugation. The less dense lipoproteins were more active with adipose tissue LPL while those of higher density were more active with mammary LPL. Emery et al (1972) propose that the specificity between lipoprotein peptides and LPL from specific organs provides a mechanism for diversion of blood fat between the mammary gland and adipose tissue. Bier and Havel (1970) studied the effect of human serum lipoproteins on milk LPL using a soybean oil emulsion of concentration 1.8 mg/ml. They obtained little or no hydrolysis of the TG in the absence of serum or lipoprotein fractions. Activity showed a maximum at 0.3 ml serum/8 ml total incubation mixture. Serum concentrations greater than this had no greater effect on the activity. HDL showed greater activation than LDL and contributed to most of the activation by serum. VLDL was a potent activator and contributed to 27 - 50% of the activator properties of serum with 3 - 5% being due to LDL. Removing VLDL from serum gave a large decrease in the activation by serum. Removal of HDL and/or LDL had little or no effect on the activation properties of whole serum. Since the VLDL content of serum is less than 5% of HDL, VLDL had a higher concentration of activator per unit of protein. Per unit weight of protein VLDL had 13 times the activity of HDL. Bier and Havel (1970) conclude that a component of apo VLDL which is present as a minor constituent of HDL is required for the activation process.

Fielding (1970) also recorded greater rates of activity for HDL and chylomicrons than for VLDL and LDL for the activation of human post heparin plasma LPL. Addition of LDL to a HDL substrate gave competitive inhibition indicating competition between HDL and LDL as cofactors for LPL. Fielding (1970) proposes that the enzyme activity is dependent upon the absolute concentrations and relative proportions

of lipoproteins present in the serum.

Fielding (1970) showed that LPL from post heparin human plasma was strongly dependent on the ratio of TG to phosphatide in the substrate lipid and the incorporation of unesterified cholesterol into the lipid emulsion at concentrations above 2 - 3 parts per 100 parts TG greatly inhibited the enzyme activity whereas cholesterol esters had a less inhibitory effect. The optimal lipid composition for enzyme activity closely corresponded to that for intact chylomicrons and Fielding (1970) proposes that inhibition by VLDL is due to their high cholesterol content.

Fielding (1970), using post heparin human plasma also observed activity of LPL against both DG and MG emulsions in the absence of lipoprotein but showed no activity against TG in the absence of added lipoprotein. MG hydrolase activity was found to require the presence of either deoxycholate or unesterified FA. Fielding (1970) observed an accumulation of MG throughout the assay period with lipoprotein TG as substrate suggesting the hydrolysis of MG to free glycerol is rate limiting which was dependent on a certain concentration of FFA being reached in the reaction medium. Fielding (1970) suggests that LPL may be a multifunctional complex in which separate hydrolytic sites binding TG and MG together effect the complete degradation of lipoprotein TG.

Using LPL from post heparin human plasma, Nilsson-Ehle <u>et al</u> (1971) found that LPL was specific towards the 1,3 ester bond of the TG substrate with a small but constant amount of DG among the reaction products of which the 1,2(2,3) isomer constituted greater than 94% of the diglyceride fraction and no 1,3 isomers were present. An accumulation of MG also occurred of which the two ester bond constituted 64 - 97% of the MG and greater than 90% of the 1 monoglyceride was formed by isomerization of the two isomer. They conclude that the reaction sequence occurring was: TG $\longrightarrow$ 1,2(2,3) DG $\longrightarrow$ 2MG $\longrightarrow$ 1(3)MG and glycerol is obtained mainly from the hydrolysis of the 1MG formed by isomerization. This is in agreement with the findings of Borgstrom and Carlson (1957), Assman <u>et al</u> (1973) using rat post heparin plasma

and Nilsson-Ehle et al (1973) using purified LPL from bovine milk. Morley and Kuksis (1972), using LPL from cows milk and rat post heparin plasma however, observed a preference for the 1 position followed by positions 2 and 3. They propose that this may act as a controlling mechanism whereby glycerides from hydrolysis by LPL cannot be diverted into phospholipid or TG synthesis since the latter both require 1,2 DGs as an initial substrate.

Bradford and Furman (1968) reported a reduced hydrolysis of TG containing C8 through to C18 FAs with normal hydrolysis of TG containing C4 - C8 FAs in rats with hyperchylomicronemia. They suggest that serum contains two TG lipases, one specific for C8 - C18 FA TGs and the other specific for C4 - C8 FA TGs.

1:7 Characteristics of Lipoprotein Lipase

Bibson and Higgins (1969) showed by chylomicron binding of LPL that the effects of both protamine sulphate and high concentrations of NaCl are on the formation of an enzyme substrate complex rather than on hydrolysis of the TG by the enzyme. Chylomicron binding to LPL was also dependent on pH which showed a maximum at pH 8.7. They suggest that the pH optimum of activity is due to the formation of the enzyme substrate complex rather than hydrolysis of the TG. Brady and Higgins (1967) found a slight variation in Km values between NH_4OH - NH_4Cl extracts of acetone powders of rat heart adipose tissue and lung using chylomicra as substrate with heart LPL having a lower activation energy compared with adipose tissue and lung LPL. All had pH optimum between 8.2 and 8.4. Km values were about 4.5×10^{-4}M for adipose tissue LPL with olive oil chylomicrons.

LPL is often considered to be identical to TG lipase of post heparin plasma but the TG lipase of post heparin plasma is variably and reversibly inhibited by NaCl and protamine sulphate whereas inhibition with TG lipase from adipose tissue,heart and other tissues is irreversible and constant. La Rosa et al (1972) in their comparison

of heparin released TG lipase activity of rat adipose tissue, liver and post heparin plasma found that adipose tissue LPL is completely inhibited by NaCl, sodium pyrophosphate and protamine sulphate whereas plasma TG lipase and liver activity showed no inhibition under the above conditions and also plasma TG lipase and post heparin TG lipase was relatively inactive against lipoprotein substrates compared with adipose tissue LPL. Pyrophosphate or protamine sulphate had no effect on human post heparin plasma TG lipase. Two separate activities were obtained on increasing the concentration of NaCl for rat post heparin plasma. The first peak of activity occurred at 0.15M NaCl and a second peak occurred at 1.2M NaCl. TG lipase from rat adipose tissue only showed one peak of activity at 0.15M NaCl and was inhibited 90% by 1M NaCl, protamine sulphate (300mg/ml) or pyrophosphate (10 umoles/ml). TG lipase from heparin treated liver also showed a bimodel curve of activity on increasing the NaCl strength with protamine sulphate and pyrophosphate giving no inhibition. Heparin treated rat heart, liver, kidney, spleen and intestine TG lipase all contained heparin extractable TG lipase activity with properties identical with those of adipose tissue TG lipase.

Fielding (1972) distinguished pre heparin plasma and heparin released TG lipase from rat liver (hepatic tissue) with that of purified post heparin plasma LPL, post heparin perfusates of extra hepatic tissue and whole post heparin plasma by the former showing lipolytic activities mainly against 1 and 2 MGs and tributyrin substrates with minor TG and DG hydrolase activities and this activity being resistant to inhibition by NaCl and protamine sulphate and sensitive to inhibition by diethylp nitrophenyl phosphate and having a pH optimum of 7.25 - 7.4 whereas the latter was characterized by major lipolytic activity against di and triglycerides and 1MGs, these activities were inhibited by NaCl and protamine sulphate, resistant to inhibition by diethyl p nitrophenyl phosphate and showed a pH optimum of 8 - 8.3 in the presence of serum. Fielding (1972) concludes that the main function of pre heparin plasma and liver lipase is to hydrolyse the 2MG which is the main product of LPL activity.

Vogel et al (1971) observed that human post heparin lipolytic activity was inhibited by 70mM pyrophosphate, 2M NaCl and protamine sulphate. The lipase activity had a pH optimum with Ca^{2+} as FA acceptor of 8.8 and with albumin as acceptor the pH optimum was 9.4. In both cases a rapid decrease in activity was recorded at pH greater than 9.8. The temperature optimum was 34°C in the presence of Ca^{2+} and 38°C using albumin as FA acceptor. Greater activity was observed in the presence of albumin than with Ca^{2+}. MG hydrolase activity was lower than TG hydrolase activity and showed a pH optimum of 9.5.

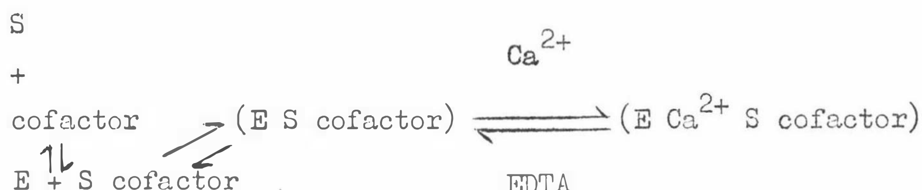
Fielding (1973) observed that LPL from rat post heparin plasma and adipose tissue showed pK values of 6.8 and 9.1 in the absence of serum lipoprotein whereas in the presence of serum lipoprotein pK values were 7.0 and 9.8 indicating a reaction of a group with a pKa of 8.8.

Delipidated VLDL and substrate lipoprotein protein increased the Vmax at all pH values and decreased the Km value of the alkaline pH.

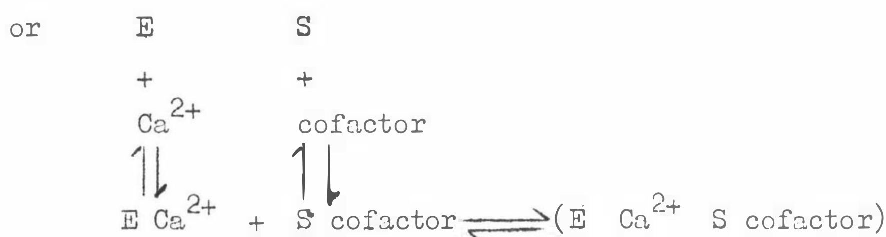
Posner and Monales (1972) studied the effect of Ca^{2+} on the rate of release of FFA using partially purified preparations of rat heart homogenates or acetone powders of adipose tissue. Km for Ca^{2+} was 0.02 to 0.06M.

Preincubation of the substrate by Ca^{2+} was not necessary since rapid activation takes place. The rate of release of FFAs can be increased by increasing the serum concentrations in the incubation medium. 0.5mM Ca^{2+} increases both the Km and Vmax for TG in the presence of serum. Ca^{2+} itself had no effect on the enzyme activity. FA release takes place only in presence of both protein and metal cofactors. Posner and Monales (1972) propose Ca^{2+} acts as an enzyme activator and since 8 - 25% of the metal in the incubation mixture can be bound to the enzyme substrate complex and this complex bound Ca^{2+} can be removed by EDTA the metal binding by the enzyme substrate complex is reversible and therefore maximum activity requires substrate activation by the protein cofactor and enzyme activation by Ca^{2+}.

EDTA inhibited lipase activity but inhibition could be reversed by the addition of excess Ca^{2+}. Posner and Monales (1972) propose that either one of the following reaction mechanisms may take place in the hydrolysis of lipoprotein TG by LPL.



or



Fielding (1970) using post heparin human plasma observed that the temperature optimum of enzyme activity was dependent upon the nature of the lipoprotein cofactor. LDL gave optimum activity at 25°C, HDL gave an optimum at 35°C and whole serum gave an optimum between 25 and 30°C. Fielding (1970) suggests that HDL may serve as the main cofactor of human post heparin plasma LPL. Naito and Felts (1970) observed that when rat livers were perfused with rat blood containing post heparin LPL,the LPL activity disappeared from the filtrate which was not due to non specific inactivation or release of inhibitor by the liver. Addition of heparin blocked the disappearance of LPL activity from the perfusate. Heparin stimulated LPL activity by 200% when added to post heparin serum which had been perfused through the liver but did not restore activity to preperfusion levels indicating a two step inactivation of LPL by the liver. Step I involving a dissociation of the heparin apoenzyme complex and the destruction of heparin and step II involving the removal of the apoenzyme of LPL. No inhibition of LPL activity occurred upon addition of serum plasma which had been perfused through the liver to post heparin samples. Naito and Felts (1970) propose that heparin acts as the prosthetic group for the enzyme which is degraded by heparinase in the first step

and then the apoenzyme is degraded as indicated by an irreversible inactivation on addition of heparin to post heparin plasma perfused through the liver.

Liver, adipose tissue and plasma TG lipase were inactivated at 37°C for 75 minutes. Mayes and Felts (1968) reported that additional heparin added to TG lipase of liver homogenates increased its sensitivity to NaCl inhibition.

Patten and Hollenberg (1969) and Whayne and Felts (1970) postulate that heparin may act to increase enzyme activity by increasing binding of the enzyme to the chylomicron substrate by forming additional binding sites on the enzyme or stimulating the existing binding sites by allosteric activation. Robinson and Wing (1970) postulate that heparin may stimulate LPL by stabalization of LPL whereas Gartner and Vahouny (1966) postulate that heparin may be a prosthetic group for LPL, the inactive apoenzyme requiring heparin for activation.

Stewart and Schotz (1971) using rat epidymal fat pads found that release of LPL activity was partially reduced when either glucose, Ca^{2+} or K^{+} were omitted from the medium and completely prevented in the absence of albumin. 3 times as much LPL was found in the medium compared to intracellular levels which remained constant during incubation for 45 minutes. Cyclohexamide had no effect on both intracellular and release of LPL. Therefore release does not require protein synthesis.

Stewart and Schotz (1971) suggest that LPL is activated prior to or in conjunction with release from the cells, a process which does not require protein synthesis.

Besadoun et al (1974) purified LPL from pig adipose tissue which had a MW of 62,000 - 60,000, was stimulated by heparin (22µg/ml), showed a complete dependence on serum for hydrolysis of triolein emulsions stabalized by gum arabic, had a pH optimum of 8.5, was inhibited by NaCl, required $CaCl_2$, gave a linear release of FFAs with time and only apo LP glu could substitute for serum for its activation

and was inhibited by both apo LP ser and apo LP ala in the presence of serum.

1:8 Types of Lipoprotein lipases

Garfinkel and Schotz (1972) isolated 2 LPLs from NH_4 OH-NH_4Cl extracts of rat adipose tissue acetone powders on sephadex G200 and Bio gel A 1.5M. Both peaks had a pH optimum of 8.0, were stimulated by the presence of serum and heparin, inhibited by NaCl and both showed equal inactivation rates at 37^oC. The high molecular weight enzyme showed greater sensitivity to NaCl than the low molecular weight enzyme.

Rechromatographing each peak separately on sephadex G200, LPL a (eluted at the void volume) still gave a single peak at the void volume whereas LPL b gave two peaks of activity, one at the void volume and another peak where the original peak occurred of MW between 7×10^5 - 1.6×10^5 daltons. Relating the LPLs gave LPL a 5 times the size of LPL b. LPL a had a MW of approximately 1.5×10^6. Garfinkel and Schotz (1972) suggest that LPL b may be a less active precursor or a subunit of LPL a. It is synthesized in the wall and becomes activated on release into the tissues by the addition of heparin, lipid or some unknown factor or activation and inactivation may be due to phosphorylation such as in the glycogen phosphorylase system or they may be different proteins. Garfinkel et al (1967) and Wing and Robinson (1968) postulate LPL b may be a constitutive enzyme whereas LPL a was an inducible enzyme and would account for the increase in LPL in adipose tissue in response to glucose and actinomycin.

Schotz and Garfinkel (1972) using NH_4 OH-NH_4Cl extracts of heart and post heparin plasma acetone powders, separated two peaks of LPL activity from the heart extract which showed elution volumes similar to adipose tissue LPL species and a single peak from plasma which showed a MW similar to that of a higher MW species from adipose tissue.

Using fasted and refed rats Schotz and Garfinkel (1972) observed that refeeding increased the activity of both the two species in adipose tissue and decreased both heart species. The LPL from plasma was unaffected by these nutritional states.

Garfinkel and Schotz (1973) refed starved rats and observed a more rapid increase in the smaller b species of LPL of adipose tissue than of the a species. Continued refeeding caused a decrease in the b form of LPL prior to a rapid increase in the a form. Heparin increased the activity of both species of LPL. Heparin had a less marked effect on the a form than the b form during starvation and refeeding but this response to the b form decreased with longer refeeding times. Since LPL released is in the a form, Garfinkel and Schotz (1973) propose that the appearance of the a form may result from an activation process of the b form located in the adipocytes which was dependent on new protein synthesis.

Guder _et al_ (1969)isolated 3 TG lipases from rat liver, a liposomal bound TG lipase activated by sonication and hypotonic treatment pH optimum 5.0 with triolein as substrate, an alkaline TG lipase sedimenting with the microsomal fraction pH optimum 8.5 which showed a greater substrate specificity for tributyrin than triolein (6 x faster) and a plasma membrane TG lipase pH optimum 7.5, heparin sensitive with an obligate requirement for Ca^{2+} ions. The high pH optimum enzyme showed a lower ratio of FFAs to glyceride than the low pH optimum enzyme indicating a different specificity for DG and MG intermediates. Also heparin gave a stimulatory effect at alkaline and neutral pH but gave an inhibitory effect at an acid pH.

Cooper and Dawney (1971) obtained LPL activity in bovine mammary gland using a tributyrin emulsion which was mainly associated with high MW complexes greater than 20×10^6 MW composed of mammary gland lipases in association with other proteins. The addition of dimethyl formamide dissociated the complexes giving a MW of 6.3×10^5 on sephrose 4B. Cooper and Dawney (1971) obtained 3 peaks of activity on a sephrose 4B column. Two overlapping peaks at or near the void

volume (MW greater than 20 x 10^6) followed by a third lipase peak MW approximately 1.5 x 10^6. The first peak which had little esterase activity they propose represented the intact lipase protein complexes and the other two peaks were intermediate dissociation products which after dimethyl formamide treatment gave a single peak with a MW of 6.3 x 10^5 which had esterase activity . They postulate that the dissociation of the lipase protein complexes may result in an alteration of the substrate specificity of the lipase similar to that observed by Okuda and Fujii (1967, 1968) with rat adipose tissue and liver lipase after acetone treatment.

1:9 Bovine Milk lipases and Lipoprotein Lipases

Lipase activity of milk has been shown to be associated with casein micelles by Harper et al (1956) Tarassuk and Frankel (1957), Saito et al (1958), Saito (1963)and Gaffney et al (1962.)

Harper et al (1956) obtained two lipases of bovine skimmed milk with pH optima of 7.0 and 8.6. Frankel and Tarassuk (1956) have reported a major bovine milk lipase of pH optimum 8.5 - 9.2 and 3 minor lipases of pH optima 6.5, 7.0 and 7.9. Schwartz et al (1956) observed the inhibition of bovine milk lipase by formaldehyde and the appearance of lipases of pH optima 7.0 and 7.5 due to inhibition of the major lipase peak at pH 8.5. Varying the substrate concentrations at different pH values resulted in non linear curves on Michaelis Menton plots at pH values of 6.6, 7.0, 7.5, 7.9, 8.35 and 8.8 suggesting more than one active lipase at these pH values. A Michaelis Menton plot at pH 6.2 gave a straight line suggesting one enzyme at this pH.

Chandon and Shahani (1965) have reported that para chloromercuribenzoate, iodoacetate and formamide disulphide completely inhibited a low MW purified bovine milk lipase whereas N ethyl maleimide (specific for free SH groups) partially inhibited the enzyme. Dialysis or addition of glutathione or mercaptoethanol did not restore the activity of the enzyme and glutathione itself

stimulated the enzyme. Chandon and Shahani (1965) propose that 2 SH groups are required for the lipase activity one of which is exposed and the other is masked.

Shahani and Chandon (1963) observed the addition of either α or β casein inhibited a low MW purified bovine milk lipase whereas K casein or serum albumin increased the lipase activity. $MgSO_4$, $MgCl_2$ and Ca^{2+} salts were all inhibitory.

Chandon and Shahani (1963) purified a bovine milk lipase of MW 7000 by acetone treatment of clarifier slime followed by 50% $(NH_4)_2SO_4$ SO_4 precipitation of the enzyme, solubalization in tris HCl buffer pH 8.5, selective precipitation by $(NH_4)_2SO_4$ (precipitated at 35 - 45% saturation) and sephadex G50 gel filtration. A purification of 2600x was obtained from the skimmed milk representing a 22% yield. The enzyme had a pH optimum of 9.0.

Richter and Randolph (1971) also purified a bovine milk lipase of MW 8500 daltons using acetone powder extracts of skimmed milk, precipitation at 20 - 45% $(NH_4)_2SO_4$ and separation on sephadex G75. The lipase had a pH optimum of 9.2. Fox and Tarassuk (1968) purified a lipase of MW 210,000 from a 1M NaCl extract of the curd of rennet treated skimmed milk by precipitation with ½ saturation $(NH_4)_2SO_4$ SO_4, gradient elution from a DEAE cellulose column in 0.02 M phosphate buffer pH 7.0 (enzyme eluted at 0.5 M NaCl), dialysis in 25% dimethyl formamide and precipitation of contaminating protein by ½ saturation $(NH_4)_2SO_4$, removal of the dimethyl formamide from the ½ saturated $(NH_4)_2SO_4$ supernatant, reprecipitation by ½ saturation $(NH_4)_2SO_4$ and a final separation on sephadex G200. A 500 times purification was obtained representing 10% of the initial activity. Maximum specific activity obtained was 15 µmole FFA released/min/mg. The lipase had a pH optimum of 9.2 and a temperature optimum of 37°C.

Dawney and Andrews (1965) obtained 4 lipase peaks on sephadex G200 from a 0.75 M NaCl extract of the 80,000 g casein precipitate of skimmed milk using tributyrin as substrate at pH 8.5. The activity

peaks had MWs corresponding to 112,000, 75,000, 62,000 daltons and a very low MW peak. Recovery of activity was 40% of the total activity applied to the column. They suggest that the presence of several milk lipases could be due to the binding of the low MW (7000 daltons) lipase to casein forming aggregates but argue that assuming molecular weights of 20,000 - 27,000 for the casein monomers as obtained by McKenzie and Wake (1959) and MWs of 62,000, 75,000 and 112,000, for the milk lipase peaks, then if these forms consist of the low MW enzyme in association with casein then dimeric to pentameric forms of casein would be involved and it is unlikely that casein exists in these limited aggregated forms.

Downey and Andrews (1966) recorded the characteristics of tributyrinase activity of the 0.75M NaCl extract of the 80,000xg casein precipitate of skimmed milk. Activity was activated by NaCl up to a concentration of 0.5 - 0.75 M but inhibition occurred at a concentration greater than 0.75 M. 70% of the activity of skimmed milk was bound to the casein. Initial activity was greater at 37°C than at 25°C but 83% of the activity was lost after 4 hours at 37°C compared with a 9% loss in 6 hours at 25°C. Optimum activity was at a pH of 8.7.

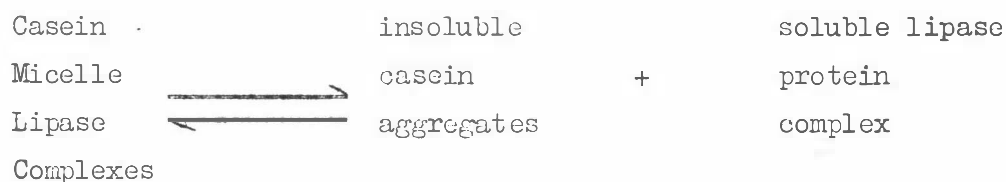
Extraction with 0.75 M NaCl gave 65 - 68% recovery of the activity in the supernatant wash. No loss of activity was recorded for skimmed milk during dialysis in distilled water but dialysis of the NaCl supernatant gave a 10 - 55% loss of activity over 4 - 5 hours which could be prevented by dialysing in solutions of 0.25mM of either $MgCl_2$ or $CaCl_2$. Addition of these salts did not restore the lost activity and EDTA prevented the effect of the salts in restoring the activity. Increasing the sample of protein applied to the sephadex G200 column increased recovery of activity of the 0.75 M NaCl extract of the casein precipitate. Increasing the sample protein applied to the column caused a different elution pattern on sephadex G200 of the NaCl supernatant extract. Increasing protein caused activity to be eluted progressively earlier. The main tributyrinase peak was eluted just after the void volume corresponding to a MW of 350,000 daltons. 3 other peaks appeared and the proportion of activity in each peak

depended on how much protein was applied to the column. The less protein applied the more activity that was eluted in the latter peaks. Elution of the NaCl extracts on a sephadex G200 column equilibrated with increasing salt concentrations from 50mM NaCl to 0.75M NaCl caused a shift in the elution pattern of tributyrinase activity from that eluted with the casein micelles to a second latter peak MW 200,000 but recoveries decreased with increasing ionic strength used. Addition of purified pig pancreatic lipase to the NaCl extracts of the casein gave lipase casein complexes eluted at the void volume.

Dawney and Andrews (1969) using the NaCl extract of the 80,000xg casein precipitate and separation on sephadex G200 showed the substrate specificity of the lipases towards tributyrin, milk fat and triolein emulsions varied. Peaks of MW 150,000 to 180,000 and 35,000 - 40,000 showed greater activity towards triolein emulsions than tributyrin emulsions while peaks corresponding to MWs of 112,000, 75,000 and 62,000 showed greater activity towards tributyrin emulsions. Peaks of MWs corresponding to 150,000 - 180,000 and 112,000 both showed greater activity towards milk fat emulsions compared with tributyrin emulsions while peaks corresponding to MWs of 75,000, 62,000 and 35,000 - 40,000 showed greater activity towards tributyrin emulsions than milk emulsions.

Dawney and Murphy (1970) studied the binding of pancreatic lipase to casein micelles of milk.Up to 70% inhibition occurred when a purified pancreatic lipase preparation was added to either milk, skimmed milk, 0.75M NaCl in skimmed milk, colloidal phosphate free milk or NaCl and distilled water extracts of the 80,000xg casein precipitate. 30% of the lipase activity was bound to the casein micelles on centrifugation. On addition of pancreatic lipase to skimmed milk (2 units/4mls) or colloidal phosphate free milk, over 90% of the eluted lipase activity was eluted with the void volume bound to the casein complexes with a recovery of 25%. On addition of 10mg phosvitin to pancreatic lipase, the enzyme was eluted at the void volume with a recovery of 70%. Elution in synthetic milk serum gave an activity peak corresponding to pure pig pancreatic lipase. On sephrose 2B elution of

pig pancreatic lipase in the presence of skimmed milk or colloidal phosphate free milk gave 3 peaks of activity. The first peak containing casein micelle bound lipase of MW greater than 10^8, the second peak contained lipase activity bound to soluble aggregated casein complexes of MW 2 x 10^6 and a third free lipase peak. Elution in the presence of colloidal phosphate free milk gave much greater activity in the last two peaks than with the skimmed milk where the activity was equally divided between the free and casein micelle bound peaks with a small peak inbetween. Recovery of activity varied from 20 - 33% of that applied. Dawney and Murphy (1970) suggest that 95% of casein of collodial phosphate free milk exists as complexes with MW approximately 2 x 10^6 whereas the majority of the casein in milk exists as a micelle of MW greater than 10^8 daltons. They propose that lipase can bind to both casein micelles (MW greater than 10^8) or soluble complexes (MW 2 x 10^6) which inhibit enzyme activity by steric hinderance. Binding to phosvitin and casein suggests phosphate groups are involved in the binding of the lipase. Dawney and Murphy (1970) suggest the following equilibrium exists in milk.



Murphy et al (1969) obtained by removal of the $Ca_3 PO_4$ from milk a smaller soluble complex which when eluted on sephrose 2B had a MW corresponding to 200,000 containing A_s, B and K casein in aggregated form. They suggest that this is the basic subunit of casein which is bonded together with colloidal Ca_3PO_4 to form micelles. Ca_3PO_4 free milk on a sephrose 4B equilibrated with increasing concentrations of NaCl caused smaller aggregates to be present with increasing NaCl concentrations.

Gaffney et al (1966) studied lipolysis of a water extract of rennet casein which showed an 11 times greater specific activity that the lipase of skimmed milk. Separation on DEAE cellulose by an

eight step gradient in 0.02M sodium phosphate buffer pH 7.0 gave lipase activity in all eight fractions with varying specific activities. Greatest specific activity was for activity eluted at the 0.5M NaCl wash with a 1000 times purification over the skimmed milk. The majority of the protein was eluted off in the 0.14 to 0.23M NaCl washes. A 150 - 180% recovery of activity was obtained. Elution of the water extract of rennet casein on sephadex G25 gave 3 protein peaks, the last of which had the highest specific activity and recovery of activity varied from 124 - 180%.

Gaffney et al (1968) obtained 3 protein peaks on separation of skimmed milk, and frozen thawed skimmed milk on sephadex G25. A large peak at the void volume and two smaller peaks which both contained carbohydrate and had MWs less than 10,000. Freezing increased the relative specific activities of both peaks II and III and decreased the number of protein bands on polyacrylamide gel electrophoresis. Gaffney et al (1968) suggest freezing and thawing may break the bonds similar to the action of rennin and that the different lipases of milk are due to absorption of a single lipase of low MW to different proteins.

Fox et al (1967) studied the effect of milk lipases from k casein using dimethyl formamide and its effect on elution from DEAE cellulose and sephadex G200. Using the 50% sat. $(NH_4)_2 SO_4$ precipitate of skimmed milk (containing 90% of the total milk lipase activity) and prefreezing the sample, elution on DEAE cellulose in 0.02M phosphate buffer pH 7.0 using a NaCl gradient gave one major lipase peak associated with the k casein eluted at about 0.2M NaCl which tailed into the A_s casein component containing some k casein eluted at 0.25M NaCl. Little or no lipase activity was associated with either the gamma casein (not bound to column) or the B casein eluted at about 0.12M NaCl. Gel filtration on sephadex G200 gave one protein peak at the void volume containing k casein having all of the lipase activity and a second protein peak containing A_s and B casein containing no lipase activity. DEAE cellulose gradient elution in the presence of 20% V/V dimethyl formamide and phosphate borate buffer pH 7.8 gave two peaks of activity. One (50 - 60% of the total lipase activity) eluted

at the front edge of the B casein (eluted at 0.1M NaCl) and a second peak eluted at the front edge of the A_s peak.

After the precipitation of the milk by 50% $(NH_4)_2 SO_4$ and dialysis in increasing concentrations of dimethyl formamide (0 - 30%), lipase appeared in the supernatant fraction on reprecipitating by 50% sat. $(NH_4)_2 SO_4$ which increased in both net activity and specific activity on increasing the dimethyl formamide concentration. Minimum concentration of dimethyl formamide required for appearance of lipase in the 50% sat. $(NH_4)_2 SO_4$ supernatant was 15% dimethyl formamide. On starch gel electrophoresis of the ½ sat. $(NH_4)_2 SO_4$ supernatant fractions obtained above, new protein components migrating ahead of the A_s casein appeared on increasing the dimethyl formamide concentration. Fox et al (1967) conclude that lipase is a minor component of k casein which could be separated from the k casein by dimethyl formamide. Yaguchi et al (1964) obtained two major lipase peaks on DEAE cellulose gradient elution of skimmed milk in 0.02M sodium phosphate buffer pH 7.0. These peaks occurred at a position corresponding to where k casein is eluted and just before the elution of the A_s casein. This latter peak contained the majority of the lipase activity eluted. Smaller peaks were recorded at 0.3M NaCl and 0.4M NaCl. Addition of gluthione and cysteine increased the recovery of lipase by 30 to 50% and NaCl concentrations greater than 0.1M inhibited the lipase activity.

Yaguchi and Tarassuk (1963) also obtained a high specific activity peak at 0.5M NaCl from the DEAE cellulose gradient elution of the water extract of rennet casein which corresponded to the first peak eluted from a sephadex G200 column thus confirming the results of Fox et al (1967).

Haynes and Downey (1973) reported a decrease in milk LPL activity in the presence of either NaCl or protamine sulphate against activated tributyrin (5% V/V) or Ediol substrates. 90% of the activity was sedimented with the casein micelles on centrifugation at 38,000 xg for 2 hours and either 1M NaCl or 5 µg/ml heparin solutions gave optimal release of activity into the 38,000 xg supernatant.

Elution of skimmed milk on sephrose 2B columns equilibrated with an increasing concentration of heparin (0, 1 and 5 µg/ml) gave two peaks of activity against both substrates with a larger peak occurring at a MW corresponding to 0.5×10^6 accompanied by a decrease in the activity associated with the casein micelles (MW greater than 10^8) with increasing heparin concentrations. Both peaks gave twice the activity towards Ediol substrates as with tributyrin substrates. Elution of the 1M NaCl extract of the casein micelle precipitate in the presence of 5 µg/ml heparin gave a single peak corresponding to the second peak eluted with the skimmed milk. Elution of the NaCl extract of the casein micelle precipitate in the presence of 5 µg/ml of heparin gave a single peak eluted with the skimmed milk. Elution of the NaCl extract of the casein precipitate on sephadex G200 equilibrated in 0.75M NaCl containing 25mM $MgCl_2$ gave several activity peaks with the dominant species having MWs in the range 60,000 to 120,000 against both substrates.

Preincubation at 4°C for 2 hours in 2M NaCl and 1 mg/ml protamine sulphate gave only a 20% decrease in activity. Activity against either Ediol or tributyrin emulsions had a similar pH optimum of 8.5 - 9.0. Haynes and Dawney (1973) conclude that the lipase activity of bovine milk may not specifically reflect its LPL activity. They suggest lipolytic enzymes process on amphiphile structure causing lipases in milk to assume a radial polarity parallel to k casein which is hydrophobically bound to apolar A_s and B casein micelle complex and are bound by electrostatic forces to the outwardly directed acidic k casein. Addition of NaCl decreases the electrostatic interaction with the negatively charged casein micelles causing the release of lipase. They suggest heparin may act by binding to specific sites on the lipolytic enzymes and enough heparin will eventually cause the release of the enzymes from the casein.

Egelrud and Olivecrona (1973) reported purified milk LPL had activity against emulsified long chain TGs even in the absence of a lipoprotein activator. Using milk LPL purified by the method of Egelrud and Olivecrona (1972), Egelrud and Olivecrona (1973) studied

the effect of TG emulsions in the absence of serum on the activity of LPL. Omission of serum resulted in 5% of the maximum activity for both olive oil emulsions stablized with egg lecithin or Intralipid emulsions. Using olive oil stablized with gum arabic gave 80% of the activity as that with intralipid in the presence of serum. Activity with no serum was 2 times as high as with Intralipid. Therefore activity was influenced by the emulsifier used to stablize the TG. Specific activity was 1 - 7 times higher at 37°C than at 25°C. In the absence of serum, activity showed a gradual decrease with time but the addition of albumin increased activity to a linear rate which was similar to that of the plus albumin assay system. Egelrud and Olivecrona (1973) propose that this levelling off of activity in the absence of albumin is due to inhibition of the lipase by accumulating FFAs in the absence of a FA acceptor. Addition of $CaCl_2$ (36mM) caused an 80% stimulation in activity indicating Ca^{2+} could act as a FA acceptor but was not as effective as albumin. Optimal conditions were pH 8.5, 3.3 mg TG/ml and 36mM $CaCl_2$ in the incubation mixture. Using radioactivity labelled TG, activity in the MG and DG rose initially and then stayed constant indicating hydrolysis of DG and MG took place as well. Using olive oil as substrate optimum activity was at a pH of 8.6. At pH 7.3 initial activity was followed by a rapid levelling off to zero activity after 4 minutes. At pH values of 8.3 to 9.0 activity was linear with time.

In the absence of added emulsifier and using short chain FA TG (tributyrin) the optimum pH was 8 - 8.5. $CaCl_2$ had no effect on the enzyme activity and NaCl (up to 0.1M) was stimulatory in unemulsified tributyrin substrates.

Addition of NaCl caused a decrease in enzyme activity with time. With the addition of heparin as well, NaCl did not have as great an effect on decreasing activity with time. The effect of heparin was more pronounced at 0.7M NaCl. Egelrud and Olivecrona (1973) propose that heparin acts to stablize the enzyme and thus a more linear relationship of activity with time for both activated long chain TG and tributyrate substrates.

Purified milk LPL had a pH optimum of 10.5 with mono-oleate as substrate dispersed with sodium deoxycholate with a linear reaction with time. Activity against tributyrate was 50% of the activity against activated tributyrate substrates. Activity against DG was increased by serum whereas activity against MG was not affected by serum. Egelrud and Olivecrona (1973) conclude that both activities against short chain TG and long chain TG are on the same enzyme.

Brumby (1971) observed that the addition of cholesterol esters, phospholipids and free cholesterol all decreased activity of bovine milk LPL. Cholesterol had a greater effect than phospholipid in decreasing the activity and the percentage decrease was proportional to the concentration of cholesterol or phospholipid. Activity was dependent on the ratio of cholesterol/phospholipid and 15% cholesterol and 30% phospholipid gave the original activity whereas at 5% cholesterol, 20% phospholipid was required for optimal activity but activity was 65% of that of normal activity. Brumby (1971) concludes that the relative composition of plasma lipids is important in the activity of LPL.

Iverius et al (1972) studied the effect of heparin on a crude and purified extract of bovine milk LPL under conditions of different ionic strength and different substrate activators. Using HDL as activator and milk LPL purified by the method of Egelrud and Olivecrona (1972), heparin had little effect on LPL activity at low ionic strengths and increasing heparin concentrations caused inhibition. At high salt concentrations (greater than 0.4M) heparin increased the enzyme activity but not to the levels in low ionic strength solutions. Iverius et al (1972) propose that heparin acts by restoring the lipolytic activity rather than to stimulate the enzyme.

Using HDL as substrate activator in the absence of heparin, activity was linear with time at low ionic strengths (0.05 - 0.16M) but non linear at high ionic strengths at 37°C indicating both inhibition and inactivation was taking place at high ionic strengths in the absence of heparin. The addition of heparin at high ionic

strengths gave a linear plot using HDL as activator and 37°C incubation temperature. Iverius et al (1972) conclude that inactivation as well as inhibition occur at high salt concentrations and both of these processes are impeded by heparin.

At 20°C activity was linear with time both in the presence or absence of heparin at high ionic strengths indicating no inactivation took place when incubated at 20°C. Heparin increased enzyme activity by 2 times at 20°C. When the perfused enzyme was preincubated in the presence or absence of heparin at high ionic strength (0.4M) at various temperatures heparin decreased the inhibition at all of the temperatures.

Heparin had little effect on the purified enzyme in the presence of HDL or serum as activator or the impure skimmed milk enzyme in the presence of HDL or VLDL but increased the activity of the impure enzyme in the presence of serum as activator by 10 times. This was due to an inhibition (approximately 10x) of the impure enzyme by serum in the absence of heparin. The low activity observed in the presence of serum of the impure enzyme was due to inhibition and not inactivation since the activity in both the presence and absence of heparin was linear with time. Iverius et al (1972) conclude that inhibition was due to a factor in serum which was not affected by HDL addition into the serum.

Heparin sulphate and dermatan sulphate also gave an activating effect but 1000x concentrations were required for the same effect as heparin.

Heparin eliminase decreased lipolytic activity of both the skimmed milk and purified enzyme preparations by about 50%. They propose that the heparin like component of the purified preparation was either non essential for the function of LPL or was inaccessible to the eliminase. Since heparin in all cases did not stimulate the enzyme above optimal conditions but prevented the inhibition and inactivation of the enzyme, Iverius et al (1972) propose that heparin

is not involved in the catalytic mechanism of LPL but serves to eliminate the effects of the inhibitor. Strongly bound heparin not available to the action of heparinase may however be required. They propose that heparin is bound to the protein in the ratio of 1:70.

1:10 Purification Techniques used to purify Lipoprotein Lipase

Fielding (1969) obtained a 2800 - 3000 fold purification of post heparin plasma LPL which was 95% homogenous on polyacrylamide gel electrophoresis. Purification was obtained by binding to an Intralipid emulsion and washing the lipid enzyme complex with 0.05M NH_4OH-NH_4Cl buffer pH 8.5 containing 10% sucrose with ultracentrifugation at 30,000 rpm for 1 hour until the protein in the supernatant was about 10mg/ml. 55% of the activity remained bound to the lipid with a 500 fold purification. The enzyme was then eluted off with 0.05M NH_4OH-NH_4Cl buffer pH 8.5 containing 0.5% W/V sodium deoxycholate and 0.5mM potassium oleate and centrifuging at 35,000 rpm for 1 hour . 80% of the bound activity was recovered in the aqueous layer. Another washing with the above buffer gave 15% of the activity recovered in the aquous layer. Further purification was obtained by the addition of $\frac{1}{4}$ volume of 0.2M Ca_3PO_4 gel to the enzyme supernatant from above, centrifugation of the enzyme Ca_3PO_4 complex and washing the gel with 2 volumes of 0.05M NH_4OH-NH_4Cl buffer pH 8.5 containing 0.5% W/V sodium deoxycholate, 0.5mM potassium oleate and 0.1M sodium oxalate and finally washing the gel with $\frac{1}{4}$ volume NH_4OH-NH_4Cl buffer pH 8.5 containing 0.05M sodium citrate. 65% of the bound enzyme activity was recovered in the sodium citrate wash. 80% of the protein eluted with the sodium citrate was precipitated between 33% and 50% V/V acetone. Maximum specific activity obtained was 40 µmole FFA released min/mg protein representing a 30% yield. The MW was 72,600.

Greten et al (1972) purified a TG lipase from human post heparin plasma which was activated by a NaCl concentration up to 0.75M but was slightly less active thereafter on increasing the NaCl

concentration. By twice binding to a heparin sephrose column they obtained a purification of 9160 fold representing a 10% yield of the original activity. The enzyme was eluted off at a concentration of about 0.6M NaCl. VLDL and LDL were firstly removed from the plasma by adjusting the density to 1.21gm/ml by the addition of KBr and centrifugation at 60,000 rpm for 1 hour before being applied to the heparin columns. The enzyme did not require serum for activity.

Greten and Walter (1973) obtained a 1500 fold purification of LPL by binding of an acetone powder extract of rat adipose tissue to a heparin sephrose column. Elution was by a stepwise method of increasing NaCl concentrations in 0.005M sodium barbital buffer pH 7.4. NaCl concentrations of 0.4M, 0.75M and 1.2M NaCl were used to elute the enzyme from the heparin sephrose. Maximum specific activity obtained was 60 μmole FFA released/min/mg representing a yield of 17% in the 1.2M NaCl wash. 22% of the total activity was bound to the heparin sephrose column and total recovery was 47% of the activity eluted onto the column. Isoelectric focusing gave a single band of activity at a pI of 4.2. The presence of 2M NaCl inhibited the enzyme by 10 times. Activity showed an optimum at 2.5 μg/ml of heparin but decreased thereafter on increasing the heparin concentration. The enzyme required serum for full activity.

Assman et al (1973) partially purified rat liver plasma membrane TG lipase by binding to a heparin Bio gel column and eluting with high salt concentrations from 0 to 5M NaCl in glycine NaOH buffer pH 9.4. No binding of the heparin released activity occurred and only activity that was released in the absence of heparin bound to the column. 50% of this activity was eluted off as a peak at 32 mmho resulting in a 15 - 20 fold purification. This activity was not activated by the presence of serum in the incubation medium and represented a yield of 0.5%. Maximum specific activity obtained was 1.1 μmole FFA released/min/mg representing a 363 fold purification from the liver homogenate.

Egelrud (1973) observed a binding of hen adipose tissue LPL to a heparin substituted gel at 0.5M NaCl in 0.005M sodium veronal buffer pH 7.4. 55% of the applied enzyme was eluted at 1.16M NaCl obtaining an 80 fold purification. The enzyme was dependent on serum for maximum activity, inhibited by NaCl and was inhibited by heparin both before and after elution from the heparin sephrose column.

Olivecrona et al (1971) obtained a 2000 fold purification of skimmed milk LPL by binding onto a heparin sephrose column. Recovery of activity eluted from the column was 50% and the enzyme was eluted at an ionic strength of 0.83M NaCl.

Egelrud and Olivecrona (1972) purified bovine milk LPL 5000 - 7000 times from skimmed milk by an initial extraction of a rennet curd with 1.16M NaCl followed by precipitation by 30% W/V $(NH_4)_2SO_4$, dialysis of the precipitate in 0.154M NaCl, precipitation of the dialysate by the addition of 1/3 volume of acetone, redispersing the precipitate and reprecipitating with acetone and a final wash with diethyl ether. The acetone ether powder was redissolved in 5mM veronal buffer pH 7.4 containing 0.16M NaCl. After these steps a 10 fold purification was obtained representing 9.5% of the initial activity started with. Binding to heparin sephrose equilibrated in 0.16M NaCl in 0.5mM sodium veronal buffer pH 7.4 and elution with a linear salt gradient from 0.16 to 1.5M NaCl gave a maximum purification of 7000 fold representing 67% of the activity eluted onto the column. Maximum specific activity obtained was 28,000 μ equiv FFA released/hr/mg. By polyacrylamide gel electrophoresis the enzyme was 80% pure with a yield of 5 - 10%. The MW of the enzyme determined by polyacrylamide gel electrophoresis in sodium dodecyl sulphate was 62,000 - 66,000. Inactivation occurred at the rennet precipitation step and the heparin sephrose step.

Havel et al (1973) used the method of Egelrud and Olivecrona (1972) with additional purification by gel chromatography on sephadex G100 in 1.5M NaCl in 0.005M sodium veronal buffer pH 7.4 to purify skimmed milk LPL.

Besadoun *et al* (1974) purified LPL from acetone powders of pig adipose tissue by extraction with 1.2M NaCl in 0.005M sodium barbital buffer pH 7.4 to obtain 85% of the total activity in the supernatant extract which was 6 times as effective as 0.025M NH_4OH-NH_4Cl buffer pH 8.6 for extraction into the supernatant, binding to heparin sephrose 4B to obtain a 600 times purification followed by binding to concanavalin A covalently linked to sephrose 4B and elution with 0.2M L methyl D mannoside, 1.0M NaCl and 0.005M sodium barbital pH 7.0 to obtain a purification of 2100 fold. The purified enzyme gave a single band on polyacrylamide gel electrophoresis.

Lipoprotein lipase is physiologically important in the regulation of the lipoprotein TG concentration in the blood and in the uptake of FA by the tissue especially in the adipose tissue in times of storage and in the mammary gland during lactation.

The control of its activity is affected by hormones and by proteins in the blood some stimulating the enzyme while others inhibiting it. Overall the fine detail of its regulation is not properly understood. Also there may be more than one enzyme, each being specific in its inhibition, activation, substrate specificity etc and each contributing to the overall control of lipoprotein TG in the blood and the uptake of plasma TG by specific tissues and these enzymic subunits or forms may interact to form complexes and thus may account for the different LPL species. The comparison of LPL and lipases in milk and mammary gland has not been studied and it is questionable whether these are the same enzymes or not due to them being synthesized in different cell organelles and different cell types.

Purification procedures have been hindered by the small amount of soluble enzyme present at any one time, low recoveries of activity obtained due mainly to the high ionic strengths required to elute it from heparin sephrose columns and low binding to artificial TG emulsions.

This study was carried out in order to determine a better method of purification which would give better recoveries than what have been obtained by previous workers, to investigate the enzyme or enzymic forms, to try and determine the physical and chemical characteristics of the enzymic forms and see if any interrelationships exist between the different forms, if any control mechanisms exist within the enzyme itself and the relative functions of the lipoprotein lipases and to compare the mammary gland enzymes with those of milk to give an insight into their synthesis and secretion from the mammary cell and to determine if any changes in the structural and chemical characteristics occur on secretion from the mammary gell.

Chapter II

MATERIALS AND METHODS

SECTION I. Mammary gland lipoprotein lipases.

2:1 Enzyme source

Bovine mammary gland was obtained from the local freezing works immediately after culling and approximately 15 minutes before homogenizing in ice cold 10^{-3} M E.D.T.A. in 0.25M sucrose solution. The cows were in full lactation. Adipose tissue as well as some of the very fibrous tissue was removed before homogenization. Tissue was obtained from all four quarters in the lower and mid regions of the udder. Total weight of the udders varied from 10 to 20kg.

Extraction using prefrozen mammary glands gave no apparent soluble enzyme activity with greatly reduced insoluble activity.

2:2. Homogenization and Extraction

The udder was cut into about 100gm pieces and passed through an electrical mincer. The minced udder extract was then homogenized in 5 times the volume of ice cold 10^{-3} M E.D.T.A. in 0.25M sucrose solution at top speed in a waring blender for 5 minutes with intermittant stops. The above solution was centrifuged at 16,500 xg for 30 minutes at 0-2°C in a sorvall RC2B model centrifuge using a G-S3 head. Three layers separated - a top cream layer, a middle supernatant layer and a bottom precipitate. The middle supernatant layer was removed by gently tipping the centrifuge tube allowing the supernatant to pour into a beaker and retaining the top cream layer in the tube by the use of tissue paper placed at the mouth of the centrifuge tube. The gelatenous precipitate was resuspended in 5 times volume of ice cold 10^{-3} M E.D.T.A. in 0.25M sucrose and again homogenized in a waring blender. The homogenized extract was centrifuged and the middle supernatant solution extracted as above. The above was repeated a third time giving three tissue extracts.

2:3. Preparation of Triglyceride emulsion and enzyme binding

A 20% V/V triglyceride emulsion of olive oil was prepared by dissolving 10gms of gum arabic in 80mls of distilled water and to this was added 20mls of olive oil and the mixture homogenized using a high speed homogenizer for approximately 3 minutes. To the middle supernatant solution obtained as in section 2:2 was added 1/40 volume of the 20% olive oil emulsion and the mixture incubated in a water bath at $37^{O}C$ for 10 minutes with constant stirring allowing time for the solution to reach $37^{O}C$ required for activation. The solution was then placed in an ice bath and the temperature allowed to fall to $2^{O}C$ with intermittant stirring. After allowing the solution to settle for 5 minutes at $2^{O}C$ the top lipid layer was removed by gently pipetting the surface layer off.

2:3:1 Preparative ultracentrifugation and extraction of TG bound enzyme

The top lipid layer from above was centrifuged at 105,000g in a Beckman model L preparative ultracentrifuge for 1 hour at $2^{O}C$ using a 30 head. All following centrifugations were carried out at 105,000xg at $2^{O}C$ for 1 hour using a 30 head. The lower aqueous supernatant layer was removed by gently sucking through the top lipid layer using a long pasteur pipette without disturbing the top layer. The top lipid layer was then transferred to a flask and homogenized in 0.05M NH_4OH-NH_4Cl buffer pH 8.4. The above homogenate was again centrifuged at 105,000g for 1 hour. The bottom supernatant was removed as above and the top lipid layer was homogenized in 0.05M NH_4OH-NH_4Cl buffer pH 8.4 and recentrifuged and the supernatant solution removed as before. This was repeated several times until most of the activity was eluted off. In some cases the top TG extract was sonified in 0.05M NH_4OH-NH_4Cl buffer at $2^{O}C$ and then centrifuged at 105,000g.

2:4 Purification using calcium phosphate gel and silica gel

2:4:1 Preparation of gel: Equal volumes of 0.25M $CaCl_2 2H_2O$ and 0.167M Na_3PO_4 $12H_2O$ were mixed and the pH adjusted to 7.5 using acetic acid. The Ca_3PO_4 gel was then poured into a filter funnel containing

Whatman No.I filter paper and the gel washed with distilled water until the pH of the elutant was about 7.0. The gel was also washed by the addition of large volumes of distilled water and decanting the water off after the gel had settled.

The 16,500g supernatant from section 2:2 was added to the gel slurry in the ratio 100:1 V/V supernatant: slurry and filtered using a large buchner funnel containing Whatman No.I filter paper with a vaccum applied. A hard pan of Ca_3PO_4 gel was prepared by pouring the slurry into a buchner funnel containing Whatman No.I filter paper and applying a vaccum until the excess water was removed but not permitting the gel to run dry. Large volumes of distilled water were then eluted through until a firm layer was obtained on top of the filter paper. In some instances the Ca_3PO_4 gel slurry had to be homogenized in a high speed blender to form fine gel particles.

The filtrate from the first Ca_3PO_4 gel filtration was then applied to the top of the gel without disturbing the surface and filtered through using a vaccum. In some cases the supernatant was centrifuged at 105,000g for 1 hour at 2^oC before being applied to the gel. Following the elution of the enzyme solution through the gel the gel was washed with 0.05M NH_4OH-NH_4Cl buffer pH 8.4 followed by 0.1M potassium oxalate and then 0.05M trisodium citrate.

2:4:2 Silica gel filtration: A slurry of silica gel was prepared by the addition of distilled water to silicic acid and shaking. The slurry was then poured onto a buchner funnel containing Whatman No.I filter paper and vaccum applied until a firm surface was obtained and washing with several volumes of distilled water. The 16,500xg supernatant was then poured gently on to the top of the gel and vaccum applied.

2:5 Purification using viscose sulphate

2:5:1 Preparation of viscose sulphate: Viscose sulphate was prepared according to a method by Dr. Ayres. 20gms of cross linked viscose was dried overnight in an oven at 70^oC and then soaked in 75ml formamide for 3 hours degassing for half an hour at the start.

A second solution was prepared by adding 17mls conc. chlorosulphonic acid slowly to 150ml formamide at 0° C in an enclosed flask connected to a drying tube with the dropping funnel containing the chlorosulphonic acid also protected with a drying tube containing $CaCl_2$. The above solution was added to the viscose formamide slurry and stirred intermittently for 48 hours at room temperature. 500ml of ice was then added followed by an ice cold solution of 22gms NaOH in 200mls distilled water. The resin was then filtered and washed with distilled water in a buchner funnel.

2:5:2. Binding and stepwise elution from viscose sulphate. The viscose sulphate was equilibrated with 0.05M $MgCl_2$ in 0.05M Tris HCl buffer pH 8.0. One litre of the enzyme extract II obtained as from section 2:2 was made up to a 0.05M $MgCl_2$ in 0.05M Tris HCl solution by the addition of solid $MgCl_2$ and 1.5M Tris HCl pH 8.0. 20 gms of viscose sulphate was added and the solution centrifuged at 16,500g for 20 minutes. The precipitate was redispersed in 1 litre of 0.5M NaCl in 0.05M Tris HCl pH 8.0 and again centrifuged. The precipitate was redispersed in a 0.75M NaCl solution in 0.05M Tris HCl pH 8.0 and again centrifuged. This was repeated using 500mls of a 0.9M NaCl solution and 250mls of a 1.2M NaCl solution in .05M Tris HCl pH 8.0. The supernatants in each case were dialysed against 0.05M NH_4OH-NH_4Cl buffer pH 8.4 before being assayed.

2:5:3 Gradient elution from viscose sulphate. 20gms of resin was poured into a 15 x 3.2cm column and equilibrated with 0.05M $MgCl_2$ in 0.05M Tris HCl pH 8.0.

1.8 litres of the 105,000g supernatant of extract Iafter triglyceride binding as in section 2:3:2 was made up to a 0.05M $MgCl_2$ solution in 0.05M Tris HCl pH 8.0 by the addition of solid $MgCl_2$ and 1.5M Tris HCl pH 8.0 and eluted on to the column. Excess protein was washed off with 0.05M $MgCl_2$ in 0.05M Tris HCl pH 8.0. The protein was then eluted off using a 0 to 2M NaCl gradient in 0.05M Tris HCl pH 8.0. 4 ml fractions were collected and the fractions dialysed against 0.05M NH_4OH-NH_4Cl buffer pH 8.4 before being assayed. The sulphate polysaccharide was regenerated by firstly washing the NaCl

out with distilled water then drying the slurry in an oven at 70°C overnight followed by reswelling of the resin in 0.05M $MgCl_2$ in 0.05M Tris HCl pH 8.0.

2:6 Purification using sodium dextran sulphate

2:6:1 Method I. Stepwise elution with $CaCl_2$. 2mls of a 10% sodium dextran sulphate solution was added to 100mls of enzyme solution and the dextran sulphate precipitated by the addition of 10mls 1M $CaCl_2$. The suspension was centrifuged at 16,500g for 20 minutes and the precipitate resuspended in 1M $CaCl_2$ and recentrifuged. This was repeated using a 2M $CaCl_2$ solution and the suspension recentrifuged. The $CaCl_2$ supernatants were dialysed against 0.05M NH_4OH-NH_4Cl buffer pH 8.4 before being assayed.

2:6:2 Method II. Precipitation with $MnCl_2$. The following procedure was carried out as for the isolation of high density lipoproteins by Burstein *et al* (1970) with modifications. The enzyme solution used was the filtrate after elution through Ca_3PO_4 gel as described in section 2:4.

0.5gms of sodium dextran sulphate was dissolved in 1 litre of enzyme solution and 10gms of $MnCl_2$ slowly added keeping the temperature approximately 2°C. The precipitate formed was collected by centrifugation at 16,500g for 20 minutes. The precipitate was then dissolved in 30mls 10% $NaHCO_3$ solution and the $MnCO_3$ precipitate formed was removed by centrifugation at 17,200g for 20 minutes. The supernatant was then dispersed in 1 litre of 0.02M Tris HCl pH 7.7 and the dextran sulphate reprecipitated by the addition of 10gms of $MgCl_2$. The precipitate was collected by centrifugation at 16,500g for 20 minutes and dissolved in 20mls 5% NaCl. The above solution was then suspended in 1 litre of 0.05% NaCl in 0.02M Tris HCl pH 7.7 and the dextran sulphate reprecipitated with the addition of 10gms of $MgCl_2$. The precipitate was collected by centrifugation at 16,500g for 20 minutes and the last step repeated as above. The final precipitate was dissolved in 12.5mls of 10% Na citrate and the solution dialysed for 24 hours against 1%

NaCl in 0.02M Tris HCl pH 7.7 to remove citrate and Mg^{2+} ions. The above solution was then dialysed against 1% $BaCl_2$ in 1% NaCl for 24 hours and the insoluble Ba salt of dextran sulphate removed by centrifugation at 17,200g for 20 minutes. The supernatant from above was then dialysed against 0.9% NaCl to remove the Ba^{2+} ions. All of the above steps were carried out at $2^{o}C$.

The supernatant was then eluted through a sephadex G200 column (2.3 x 39.5cm) with 0.05M NH_4OH-NH_4Cl buffer pH 8.4. The large protein peak was then eluted onto a DEAE cellulose column (1.4 x 20cm) after dialysis in 0.005M KH_2PO_4 buffer pH 8.4 and eluted with a gradient of 1.5 mmho to 20 mmho collecting 2ml fractions (see section 2:7).

2:7 Purification using Diethyl amino ethyl cellulose (DEAE cellulose)

2:7:1 Preparation and equilibration of DEAE cellulose. The dry DEAE cellulose was left in 15 vols by gm weight of cellulose of 0.5M HCl for 30 minutes and then poured into a column and distilled deionized water eluted through it until the effluent had attained a pH of 4.0. The DEAE cellulose was then poured into 15 vols by gm weight of dry cellulose of 0.5M NaOH and left for 30 minutes. The cellulose slurry was then poured into a column and deionized distilled water eluted through it until the effluent had attained a pH of 7.0. A volume of concentrated KH_2PO_4 solution was stirred into the DEAE cellulose until the pH of the slurry was less than 5 and was then titrated with NaOH until the required pH (7.6 or 8.4) was obtained. The slurry was then degassed and poured into a column and starting buffer eluted through until the effluent had the same pH and ionic strength as the starting buffer. After each run the high salt buffer was eluted through using starting buffer and the above procedure repeated omitting the first HCl wash step. All elutions were carried out at $4^{o}C$ in a cold room and all buffer solutions were degassed before being eluted through the column. Deionized distilled water was used for all buffer solutions.

2:7:2. Stepwise elution from DEAE cellulose in 0.005M KH_2PO_4 buffer pH 8.4. The supernatant enzyme extract as obtained in section 2:2 was centrifuged at 105,000g for 1 hour at $2^{o}C$ and the 105,000g supernatant dialysed against starting 0.005M KH_2PO_4 buffer pH 8.4 until the

conductivity and pH was that of the starting buffer solution. About 200mls of the 105,000g supernatant was eluted on to the column. . 0.005M KH_2PO_4 buffer pH 8.4 was then eluted through until no protein was detectable in the effluent. 0.8M NaCl in 0.005M KH_2PO_4 buffer pH 8.4 was used as the final buffer solution. The NaCl gradient was eluted through the column using two equilibration flasks. 10ml fractions were collected and conductivity readings were read when the elutent solutions had attained room temperature. OD 280 readings were read against starting buffer solution and one in every four fractions assayed.

Solutions of the required conductivity were made up by the addition of NaCl to 0.005M KH_2PO_4 buffer pH 8.4 using a Copenhagen conductivity meter. The solutions were made up to the conductivity corresponding to that of the activity peaks obtained through gradient elution. These were at conductivities of about 2, 5, 8, 10, 13, 15, 18, 21 and 24 mmho.

After elution of the enzyme extract on to the column starting buffer solution was eluted through until no protein was detectable in the effluent. A solution of conductivity 2 mmho was then eluted through and 10ml fractions collected until no protein was detectable in the effluent. This required about 1 litre of buffer. The fractions with OD 280 readings greater than 0.05 were pooled, and the rest discarded. Buffer of conductivity 5 mmho was then eluted through the column and 10ml fractions collected until no protein was detectable in the effluent and the fractions whose OD 280 readings were greater than 0.05 were pooled and the rest discarded. This was repeated for buffer solutions of conductivities of 8, 10, 13, 15, 18, 21 and 24 mmho. The column was then regenerated and re-equilibrated as described in section 2:7:1. A second 200ml of enzyme solution was eluted on to the column and the above stepwise elution procedure carried out and elutants combined with the corresponding buffer conductivities obtained as above.

<u>2:7:3. Gradient elution from DEAE cellulose in 0.005M KH_2PO_4 buffer pH 8.4</u>. The pooled fractions from above (section 2:7:2) were dialysed against .005M KH_2PO_4 buffer pH 8.4. These were then eluted on to a smaller column (15 x 2.3cm). For the pooled fractions eluted off at

conductivities of 18 and 21 mmho, the initial starting buffer solution had a conductivity of 15 mmho with a gradient elution of conductivity 15 to 25 mmho collecting 2 ml fractions. Activity eluted off at a peak of conductivity 24 mmho from the above two gradient elutions was combined and dialysed against 0.005M KH_2PO_4 buffer pH 8.4 and eluted on to a DEAE cellulose column. Starting buffer of conductivity 15 mmho in 0.005M KH_2PO_4 buffer pH 8.4 was eluted through the column followed by a gradient elution of conductivity 15 to 25 mmho.

The activity eluted off at conductivity 15 mmho (starting buffer) from the above gradient elutions was combined, dialysed against 0.005M KH_2PO_4 buffer pH 8.4, eluted on to the column and a gradient run from conductivity 0.5 to 25 mmho in 0.005M KH_2PO_4 buffer pH 8.4.

The second protein peak (4th activity peak) at conductivity 15 mmho from the above column run was then dialysed against 0.005M KH_2PO_4 buffer pH 8.4, eluted on to a DEAE cellulose column and a gradient run from conductivity 0.5 to 25 mmho. The extracts from the stepwise elution of conductivity 13 and 15 mmho elutants were dialysed against .005M KH_2PO_4 buffer pH 8.4. Starting buffer of conductivity 10 mmho in 0.005M KH_2PO_4 buffer pH 8.4 was then eluted through followed by a gradient from conductivity 10 to 25 mmho collecting 2 ml fractions.

Activity eluted off at the starting buffer (conductivity 10 mmho) from the two gradient elutions from above was combined and dialysed against 0.005M KH_2PO_4 buffer pH 8.4, eluted on to a DEAE cellulose column and a gradient run from conductivity 0.5 to 25 mmho.

2:7:4. Purification using DEAE cellulose in 0.005M KH_2PO_4 buffer pH 7.6

A gradient elution of the 105,000g supernatant of homogenized mammary gland extract was carried out as in section 2:7:2 except the pH of the 0.005M KH_2PO_4 buffer used was 7.6. Stepwise elutions were then carried out as described in section 2:7:2 except that 0.005M KH_2PO_4 buffer of pH 7.6 was used instead of pH 8.4 and conductivities of the eluting buffers were 2, 10, 15, 21 and 24 mmho.

Fractions from conductivity 2 mmho were combined and dialysed against 0.005M KH_2PO_4 buffer pH 7.6 and eluted on to a second DEAE cellulose column (15 x 2.6cm) equilibrated in 0.005M KH_2PO_4 buffer pH 7.6 and a gradient of 0 to 0.8M NaCl in 0.005M KH_2PO_4 buffer pH 7.6 run through the column collecting 10ml fractions. The above was also carried out as for fractions from conductivities of 21 and 24 mmho except 2ml instead of 10ml fractions were collected. Gradient elutions of fractions from conductivities of 10 mmho and 15 mmho were also carried out as above except that a larger column (3.2 x 30cm) was used and 10ml fractions were collected.

2:8 Concentration and Gel filtration.

Samples were concentrated using a Biolab diaflow membrane and concentrator at 50lb/in^2 nitrogen pressure. Gel filtration was carried out on a sephadex G200 (separation limits 5000-400,000 daltons) column (2.3 x 39.5cm) equilibrated with 0.05M NH_4OH-NH_4Cl buffer pH 8.4 at 2^oC in a cold room. Flow rate was about 2mls per hour and about 2ml fractions were collected. Elution volumes were measured volumetrically. Samples were dialysed against equilibrating buffer before being eluted through the column. 1ml samples were applied to the column. All sephadex G200 columns were eluted with 0.05M NH_4OH-NH_4Cl buffer pH 8.4 at 2^oC in a cold room.

2:8:1. Calibration of gel filtration column.

Proteins of known molecular weight were eluted through the column, their elution volumes recorded and used to calculate their distribution coefficients. Dextran blue was used to determine the void volume and Cu_2SO_4 to determine the totally included volume.

2:9 Polyacrylamide gel electrophoresis.

All gels were of 7.5% acrylamide composition run in 0.025M Tris glycine buffer pH 8.5 with constant current of 3.7mA per gel in a Vokam disc gel electrophoresis apparatus. A few drops of 0.001% bromophenol blue was used as a marker for the progress of the electrophoretic front. Gels were also run in 0.02M KH_2PO_4 buffer pH 7.5 for determination of purity of samples.

Gels were prepared by mixing the following stock solutions in the ratio 1:1:2 a:b:c composed of

(a) 48 mls 1 N HCl
36.3gms Trishydroxylmethylamino-methane
0.23ml temed (dimethyl amino-propionitril).
The above mixture was made up to 100mls with distilled water.

(b) 30gm acrylamide plus
0.735gm N N^1 methyl bis acrylamide per 100 ml

(c) 0.4gm ammonium persulphate per 100ml.

Solution (a) was deairated and solutions were mixed in a 10ml syringe before injecting into 6cm x 0.4cm tubes within 0.5cm of the top of the tube held erect in plasticine and a drop of water applied to the top of the gel and the tubes allowed to polymerize for 1 hour at room temperature. 0.25 - 0.5ml of protein sample containing sucrose was applied to each gel in the disc gel electrophoresis apparatus layered over with buffer using a microsyringe. Gels took 3 - 6 hours to run at room temperature. Gels were removed from the tubes by injecting water down the sides of the gel and applying pressure to the ends of the gel.

2:9:1. Staining.

Gels were stained in a solution of 0.6% amido black in 7% acetic acid for 30 minutes and destained by placing in a bath of 7% acetic acid for 2 days.

2:10. Determination of protein.

Protein for assay purposes was determined by the method of Lowry _et al_ (1951).

Bovine serum albumin (fraction V) was used as a protein standard. Protein from column fractionations was estimated by reading absorbance at OD 280nm on an Hitachi 101 spectrophotometer against elution buffer.

2:11. Substrate preparation.

2:11:1. Serum.

Bovine blood was obtained at the local freezing works from freshly culled animals and centrifuged at 16,500g for 20 minutes in a Sorvall RC2B model centrifuge using a GS3 head at $2^{\circ}C$. The serum was stored at $2^{\circ}C$ and was used up to 2 weeks after extraction.

2:11:2. Triglyceride emulsion.

Beef tallow was obtained from the local dairy. By GLC analysis of methyl esters of the TG composition of the FA residues was Myristic acid (14:0) 3.3%, Palmitic acid (16:0) 22.4%, Palmitoleic acid (16:1) 4.1%, stearic acid (18:0) 14.1%, oleic acid (18:1) 48.8%, linoleic acid (18:2) 6.4%. By TLC it had about a 95% TG content.

Olive oil by GLC analysis of the methyl esters of the TG gave a FA composition of Palmitic acid (16:0) 10.3%, Palmitoleic acid (16:1) 0.9%, Stearic acid (18:0) 3.3%, oleic acid (18:1) 79% and linoleic acid (18:2) 6.5%.

A 12.5% W/V TG emulsion was prepared by sonifying 2gm of tallow in 16mls of gum arabic solution of 1gm/16mls distilled water using an MSE ultra sonicator at 8 microns peak to peak for 10 minutes. The emulsion was then centrifuged at 3000rpm for 5 minutes on a bench centrifuge to remove large unemulsified particles. Final concentration of TG was 0.138M.

An 11% V/V olive oil emulsion was prepared by sonifying or homogenizing 2mls of olive oil in 16mls of a solution of 1gm gum arabic /16mls distilled water. Final conc. of TG was 0.11M.

2:11:3. Activated TG substrate.

TG serum substrates were prepared by mixing the TG emulsion prepared as in section 2:9:2 with serum in the ratio 1:1 and incubating in a water bath at $37^{\circ}C$ for 20 minutes with constant shaking.

2:12. Assay procedure and determination of FFAs released.

Incubations were carried out in 10ml vials stoppered with either a screw on top or press on top in a constantly shaking water bath at $32^{\circ}C$. The incubation mixture was composed of 1ml preincubated TG serum (1:1) prepared as in sections 2:9:1, 2:9:2 and 2:9:3, 0.5ml 1M Tris HCl buffer pH 8.5, 0.05mls 22% $CaCl_2$. These were added to the vials and allowed to equilibrate at $32^{\circ}C$ for 10 minutes before the addition of 0.5ml enzyme solution.

The extraction of the FFAs released was according to the method of Dole et al (1955). Immediately after the addition of 0.5mls of enzyme solution, the vial was shaken (with the stopper on) and 0.5ml withdrawn and pipetted into 2.5ml heptane:isopropanol:1 N H_2SO_4 (10:40:1) mixture in 20ml B_{14} stoppered glass tubes and the stopper replaced. The top was placed back on the vial and the vial put back into the shaking water bath. After the incubation time had elapsed the vial was withdrawn, shaken vigorously and 0.5ml withdrawn and pipetted into a second tube containing 2.5ml heptane: isopropanol: 1 N H_2SO_4 (10:40:1) and the B_{14} stopper replaced. With large numbers of fractions to assay (as occurs with column runs) about 15 assays could be carried out simultaneously allowing one minute for the addition of enzyme, shaking of the vial, withdrawing 0.5ml and pipetting into the extraction mixture, stopper the incubation vial and tube and place back into the water bath. Incubation time was usually 20 minutes but was varied according to the activity when it went beyond the limitations of the assay. In each case

a control with the addition of distilled water instead of enzyme was set up.

FFAs were extracted by the addition of 1ml of distilled water to the extraction mixture followed by 1.2mls of heptane giving a top heptane layer of 1.7ml. The tubes were shaken vigorously for 1 minute on a mechanical shaker and allowed to settle for 10 minutes. A top heptane layer separated out.

FFA released was determined colourmetrically by the method of Mosinger (1965). A stock solution consisted of 1% phenol red in 0.12M sodium barbitol (0.25gm per 10ml). This was diluted before use by the addition of 1ml of stock buffer solution (as above) to 300ml of an absolute ethanol:heptane (1:2) mixture and shaken vigorously. 0.9mls of the above diluted stock buffer solution was added to 0.6mls of extracted FFA in heptane (as above) in a curvette and shaken until both solutions were dispersed and the colouration formed was read at 560nm in an Hitachi 101 spectrophotometer using a micro cell adaptation against pure heptane: absolute ethanol (4:1). After each reading the curvettes were rinsed with acetone followed by distilled water and finally dried with acetone and left to stand until dried. Palmitic acid (99% pure) was used as a standard for calebration of FFAs giving a linear relationship up to 0.4 umoles palmitic acid. From the standard graph a relationship of 0.5137µmole FFA/ml/OD unit was obtained. Limits of accuracy for the colouration was ± 0.002 µmole/ml. A colour change of red to yellow occurred with increasing FFA concentration. The change in OD 560 readings at 0 and 20 minutes was recorded, the final reading (yellow colouration) was subtracted from the first reading (red colouration) and taking into account dilution factors and a conversion factor of 0.514 with time gives activity in µmole FFA released/min/ml enzyme solution.

2:13. Determination of amino acid composition.

The protein was hydrolysed with 6N HCl in evacuated sealed tubes at 100°C for 24 hours. Hydrolysates were evaporated to dryness on a Buchi rotary evaporator, redissolved in 2cm^3 water and evaporated to dryness again. The analysis was carried out on a Beckman 120C amino acid analyser which had been calibrated with a known mixture of amino acids. $\frac{1}{4}$mg was applied to the column.

Definition of unit:

1 unit = amount of enzyme required to release 1 μmole FFA per min. Enzyme activity is in μmole FFA released per min per mg of protein.

SECTION II. Milk Lipoprotein Lipase.

2:14 Enzyme source:

Fresh milk was obtained from the local Massey University dairy farm (predominantly Fresian cows) immediately after milking. The milk was then centrifuged at 16,500g for 20 minutes in a Sorvall RC 2B refrigerated centrifuge at 2°C to remove lipid which formed a solid layer on top of the milk. The skimmed milk was then poured into a flask.

2:15. Purification of milk lipoprotein lipase by binding to and elution from calcium phosphate gel.

50mls of calcium phosphate gel slurry, prepared as in section 2:4:1 was poured into 250ml of skimmed milk and the solution filtered through a buchner funnel using Whatman No I filter paper. The filtrate from above was then filtered through a hard pan of

Ca_3PO_4 gel on Whatman No I filter paper in a buchner funnel prepared as in section 2:4:1. The gel was then washed with 0.05M NH_4OH-NH_4Cl buffer pH 8.4 until protein was no longer detectable in the filtrate. The gel was then washed with solutions of 0.1M potassium oxalate followed by 0.05M sodium citrate, solutions of 1%, 2%, 3%, 4% and 5% NaCl in 0.05M NH_4OH-NH_4Cl buffer pH 8.4 and 5% NaCl in 25% and 50% dimethyl formamide in 0.05M NH_4OH-NH_4Cl buffer pH 8.4. The gel was also washed with solutions of 0.5M Na_2CO_3 buffer pH 8.4, 0.5M Na_2SO_4 in 0.05M NH_4OH-NH_4Cl buffer pH 8.4 and 0.5M and 1M KH_2PO_4 buffer pH 8.4.

Each of the above washings were carried out on separate gels. The filtrates were dialysed against 0.05M NH_4OH-NH_4Cl buffer pH 8.4.

2:16. Purification by ultracentrifugation and sodium chloride extraction of the casein precipitate.

800ml of skimmed milk was centrifuged at 105,000g in a Beckman model L preparative ultracentrifuge using a 30 rotor head at 2°C for 1 hour. The casein precipitate was then redispersed in 360ml ice cold deionized distilled water and the casein solution centrifuged at 105,000g for 1 hour as above. The casein precipitate was washed a second time with 360ml ice cold deionized distilled water and the 105,000g casein precipitate collected. The casein precipitate was then homogenized in 360mls ice cold 1.2M NaCl in 0.05M NH_4OH-NH_4Cl buffer pH 8.4 and the casein solution again centrifuged at 105,000g for 1 hour as above. The casein precipitate from above was homogenized in a second volume of ice cold 1.2M NaCl in 0.05M NH_4OH-NH_4Cl buffer pH8.4 and the solution centrifuged at 105,000g as above.

2:17. DEAE cellulose gradient elution in 0.005M KH_2PO_4 buffer pH 7.6 of the 1.2M NaCl extract of the 105,000g casein precipitate.

The sodium chloride extract of the casein precipitate was dialysed against 0.005M KH_2PO_4 buffer pH 7.6 and eluted on to a DEAE cellulose column (3.2 x 30cm) equilibrated as described in section 2:7.

Starting buffer was eluted through followed by a sodium chloride gradient of 0 to 0.8M NaCl in 0.005M KH_2PO_4 buffer pH 7.6 collecting 10ml fractions.

Chapter III.

EXPERIMENTAL AND RESULTS

SECTION I. Mammary gland lipoprotein lipases.

3:1. Effect of pH on lipase activity of a crude enzyme extract in the presence and absence of serum.

Assay was as in section 2:12 except assay time was 40 minutes and the following buffers used in the range of pH;

pH 5-7 0.5M KH_2PO_4
pH 7-10 0.5M Tris HCl
pH 10-11.5 0.5M NH_4OH-NH_4Cl

The assay in the absence of serum was carried out as above except that serum was replaced by an equal volume of distilled water. Enzyme source was the 105,000g supernatant of the homogenized udder extract obtained as in section 2:2.

In the absence of serum activity peaks were recorded at pH's of 6.0, 7.5, 8.25, 9.0, 9.5, 10.25, 11.0 and 11.5.

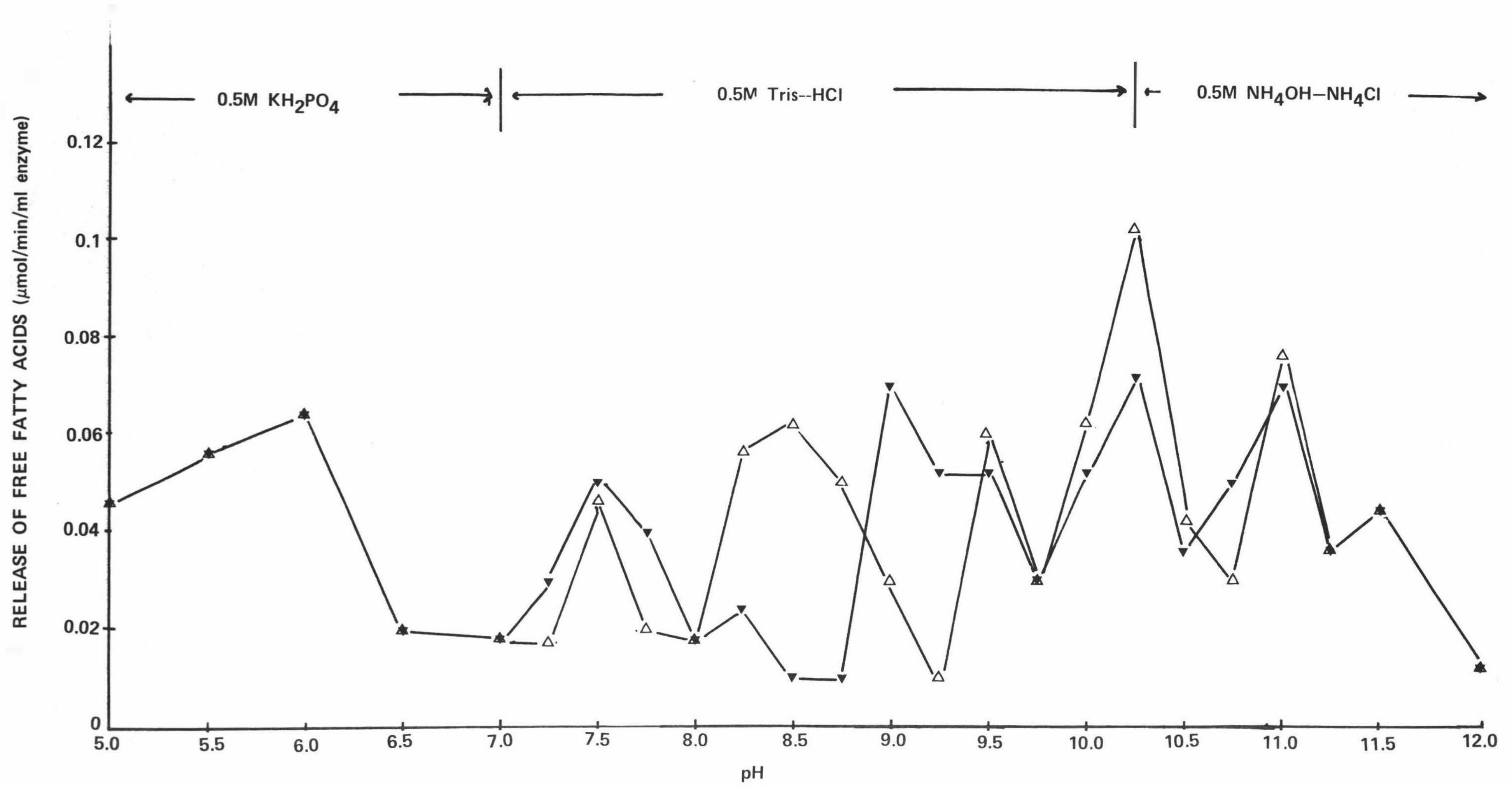
Activity peaks in the presence of serum were recorded at the same pH's as in the absence of serum except there was no peak at pH 9.0 and a peak appeared at pH 8.5. Serum had a stimulatory effect on lipases with pH optima of 8.25, 9.5, 10.25 and 11.0. Serum had a slight inhibitory effect on the lipases of pH optimum 7.5 and 9.0, and had an inhibitory effect at a pH of 10.25. Serum increased lipase activity by 500% at a pH of 8.5 and increased lipase activity by 43% at pH 10.25. (See graph I). Two assays were carried out at each pH and the average was taken. Activity was within ± 2% in each case.

GRAPH 1 Effect of pH on lipase activity of a crude mammary gland extract in the presence and absence of serum.

Key: △——△ with serum

▼——▼ without serum

See text for details of assay conditions



3:2. Effect of tallow and olive oil substrates on the activity of the 16,500g fractions of homogenized cows udder.

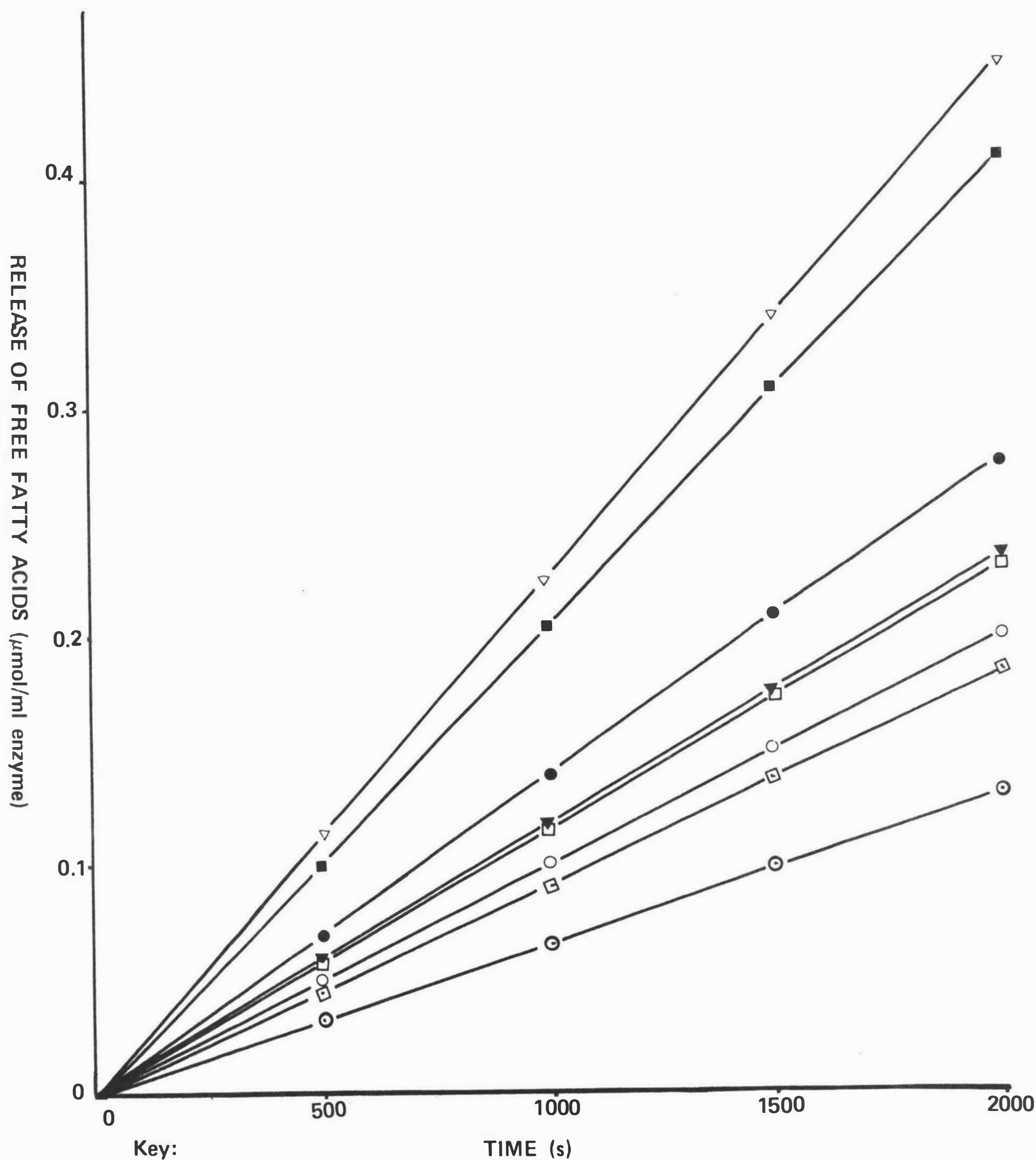
Assay was as in section 2:12 except that twice columes were used i.e. 2 mls serum:TG, 1 ml 1M Tris HCl buffer pH 8.5, 0.01 ml 22% $CaCl_2$ and 1 ml enzyme solution added at zero time and 0.5mls was withdrawn at 0, 500, 1000, 1500 and 2000 secs. Olive oil and tallow activated substrates were prepared as in sections 2:9:1, 2:9:2 and 2:9:3. The tallow emulsion was diluted 11/14 to make it equivalent to the olive oil emulsion. Final concentration of triglyceride in the incubation vials was 0.027M. Enzyme sources used were the total udder homogenate and the 16,500g supernatant, precipitate and top fractions.

All fractions gave linear relationships of activity with time within the time recorded (33 minutes) and all fractions showed greater activity with tallow as substrate rather than olive oil. The total extract showed the greatest percentage increase in activity with tallow over olive oil of 125%. This was followed by the 16,500g supernatant fraction with a 115% increase in activity of tallow over olive oil. The precipitate fraction had the next greatest increase of 74% with the top cream layer having only a 27% increase in activity with tallow over olive oil. See Graph II.

3:3. Effect of varying serum to triglyceride (tallow) ratios on lipase activity of crude enzyme extract.

Enzyme source was the 16,500g supernatant fraction of homogenized cow's udder prepared as in section 2:2. The reaction mixture consisted of 1.5ml preincubated substrate, 0.75ml 0.1M Tris HCl pH 8.5, 0.075mls 22% $CaCl_2$ and 0.75mls enzyme solution. 0.5 mls was withdrawn at 0 and 30 minutes and analysed as in section 2:12. The following substrate solution mixtures (tables I and II) were prepared as in sections 2:9:1 and 2:9:2 with tallow as triglyceride substrate and preincubated as described in section 2:9:3.

GRAPH 2 Effect of tallow and olive oil substrates on the lipoprotein lipase activity of the 16,500g fractions of homogenized cows udder extracts



Key:

- ▽———▽ Total extract with tallow as substrate
- ○———○ Total extract with olive oil as substrate
- ■———■ 16,500g precipitate fraction with tallow as substrate
- □———□ 16,500g precipitate fraction with olive oil as substrate
- ●———● 16,500g supernatant fraction with tallow as substrate
- ⊙———⊙ 16,500g supernatant fraction with olive oil as substrate
- ▼———▼ 16,500g top extract with tallow as substrate
- ◲———◲ 16,500g top extract with olive oil as substrate

See text for details of assay conditions

TABLE I.

The effect of varying the concentration of bovine serum on the activity of LPL.

Mls of serum	Mls of TG	Mls of distilled water	Activity µmole FFA released/ 30 min/ml enzyme
0	0.5	1.0	0.035
0.1	0.5	0.9	0.075
0.2	0.5	0.8	0.13
0.3	0.5	0.7	0.19
0.4	0.5	0.6	0.23
0.5	0.5	0.5	0.25
0.6	0.5	0.4	0.23
0.7	0.5	0.3	0.19
0.8	0.5	0.2	0.135
0.9	0.5	0.1	0.09
1.0	0.5	0	0.065

GRAPH 3

The effect of varying serum: TG ratios on the activity of lipoprotein lipase

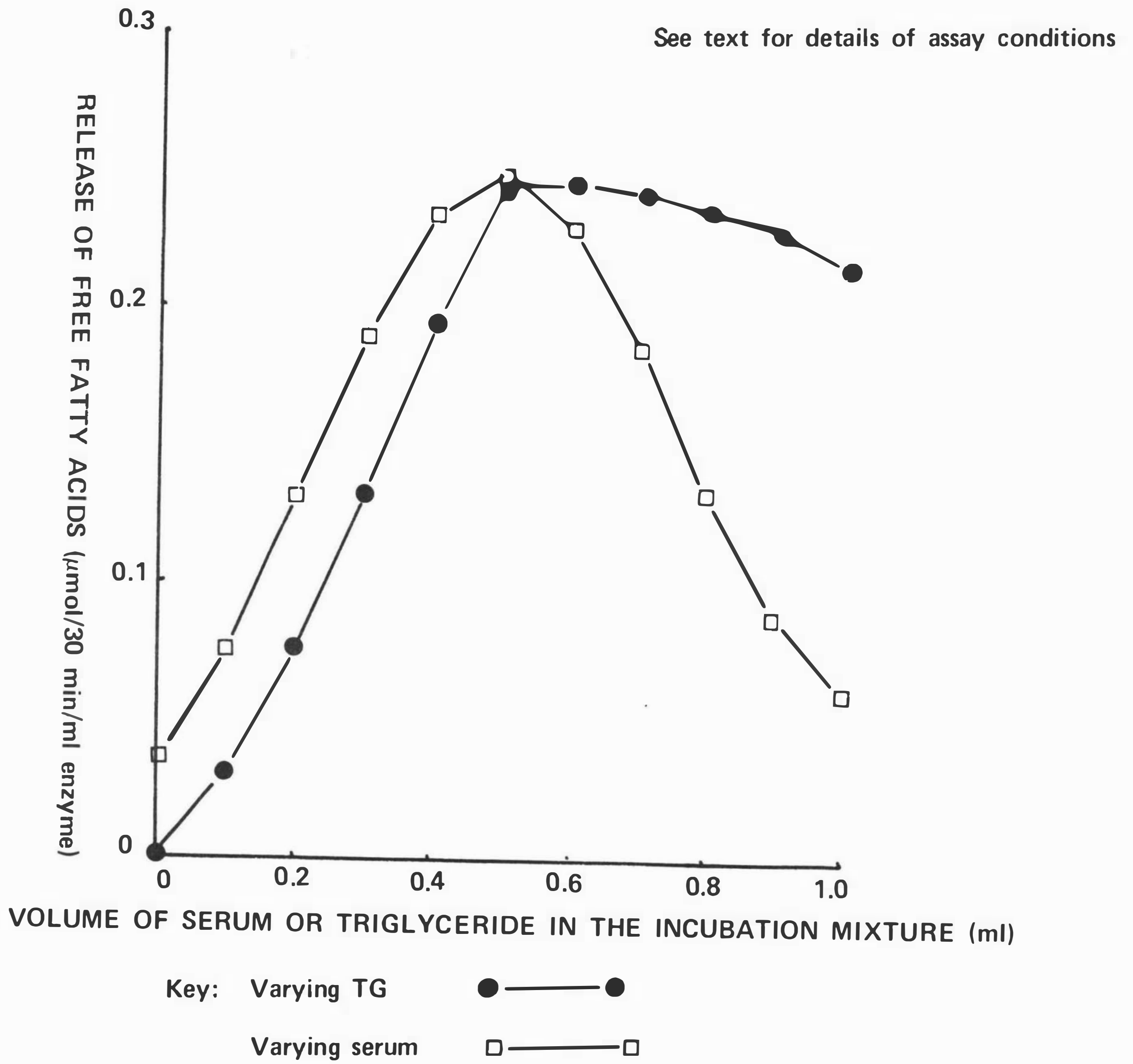


TABLE II.

The effect of varying the concentration of TG on the activity of LPL

Mls of serum	Mls of TG	Mls of distilled water	Activity µmole FFA released / 30 min/ml enzyme
0.5	0	1.0	0
0.5	0.1	0.9	0.03
0.5	0.2	0.8	0.075
0.5	0.3	0.7	0.13
0.5	0.4	0.6	0.195
0.5	0.5	0.5	0.25
0.5	0.6	0.4	0.25
0.5	0.7	0.3	0.24
0.5	0.8	0.2	0.235
0.5	0.9	0.1	0.23
0.5	1.0	0	0.215

Reaction rate was dependent on the ratio of TG to serum. Maximum activity occurring at a TG:serum ratio of 1:1. Increasing serum levels above this ratio gave rapid inhibition. Slight activity (13%) was observed in the absence of serum. See graph III.

3:4. Comparisons of methods of purification of lipoprotein lipase.

Several methods were used in an attempt to purify LPL from mammary gland extracts. These included binding to triglyceride (olive oil) sodium dextran sulphate, viscose sulphate, calcium phosphate and DEAE cellulose as initial stages of purification. 105,000g centrifugation and filtration through silica gel and Ca_3PO_4 gel were used to clarify the udder homogenates. 1650gm of mammary tissue was homogenized in two 5 litre and one 5.5 litre volumes of ice cold 0.25M sucrose containing 10^{-3}M EDTA described in section 2:2 and the fractions assayed as in section 2:12.

TABLE III. Activity of mammary gland extracts

Enzyme Extract used	Volume mls	Activity Units/ml	Total Activity Units	Protein Conc. mg/ml	Total Protein mg	Specific Activity Units/mg	% recovery
Total Homogenate	6000	0.134	804	111	666,000	0.0012	100
16,500g Top Lipid Layer	800	0.10	80	131	104,800	0.0008	10
16,500g Precipitate	700 gm	0.671/gm	468	757mg/gm	530,000	0.00088	58.5
16,500g Supernatant Extract I	4400	0.046	198	7.15	31,460	0.0063	25
16,500g Supernatant Extract II	4500	0.012	54	6.1	27,950	0.002	6.75
16,500g Supernatant Extract III	5000	0.0128	64	1.2	6,000	0.01	8

25% of the total activity was extracted into the first supernatant extract. Of that still bound to large particles, 25% was extracted into the second two supernatant extracts giving a total of 40% of the total activity in the supernatant fractions.

3:4:1. Triglyceride binding.

Binding to an olive oil triglyceride emulsion was carried out as in section 2:3:1 and 2:3:2. Assay was as in section 2:12. Enzyme solutions used were the 16,500g supernatant extracts obtained as above. (See Table III)

TABLE IV. Binding and elution from a TG emulsion of the 16,500g supernatant Extract I using an homogenized TG emulsion.

Enzyme Extract	Volume mls	Activity Units/ml	Total Activity Units	Protein Conc. mg/ml	Total Protein mg	Specific Activity Units/mg	% recovery
Extract I	4400	0.046	198	7.15	31,460	0.0063	100
Addition of TG emulsion. Activation Top TG Layer	500	0.0384	19.2	6.0	3,000	0.0064	9
Supernatant solution	4000	0.0451	180	7.12	28,500	0.0062	91
105,000g Supernatant + Sucrose Wash	710	0.0125	8.87	3.4	2,414	0.0037	4.4
105,000g Precipitate	40	0.041	1.64	4.1	164	0.01	0.82
Top TG Layer in 0.05M NH_4OH-NH_4Cl buffer	400	0.015	6.	1.05	420	0.015	3
105,000g Supernatant of Top TG Layer	385	0.018	6.9	0.5	192	0.036	3.4
Top Lipid Extract in ammonia buffer	360	0.015	5.4	0.65	234	0.023	2.7
105,000g Supernatant of above Lipid Extract	340	0.019	6.46	0.079	26.86	0.24	3.23
Top TG Layer in buffer	360	0.0144	5.184	0.575	207	0.025	2.1
105,000g Supernatant of above Lipid Extract	340	0.012	4.08	0.04	13.6	0.30	2

TABLE IV (Continued)

Enzyme Extract	Volume mls	Activity Units/ml	Total Activity Units	Protein Conc. mg/ml	Total Protein mg	Specific Activity Units/mg	% recovery
Top TG Layer in buffer	360	0.0168	6.048	0.536	193	0.031	3.02
105,000g Supernatant of above TG Layer	340	0.0017	0.578	0.02	6.8	0.085	0.29
Top TG Extract from above in buffer	360	0.0144	5.184	0.52	187	0.027	2.92
Sonification and 105,000g Centrifugation Supernatant	340	0.0216	7.34	0.05	17	0.43	3.67
Top TG Extract in buffer	100	0.0072	0.72	1.7	170	0.0042	0.36
105,000g Supernatant of above Lipid Layer	90	0.007	0.63	0.03	2.7	0.23	0.31
Top TG Extract of above	100	0.006	0.6	1.67	167	0.0036	0.3

TABLE V. Second TG extraction of the 16,500g supernatant of Extract I using a sonified TG emulsion.

Enzyme Extract	Volume mls	Activity Units/ml	Total Activity Units	Protein Conc. mg/ml	Total Protein mg	Specific Activity Units/mg	% recovery
Extract I after initial TG binding	4000	0.045	180	7.12	28,500	0.0062	100
Addition of TG emulsion, activation, centrifugation. 105,000g Supernatant	3600	0.0168	67.2	5.1	21,400	0.0033	37.3
105,000g Precipitate	60	0.1	6	38.5	2,310	0.0026	3.3
Top TG Layer	500	0.0312	15.6	9.6	4,800	0.0032	8.6
105,000g centrifugation of Top TG Layer Supernatant	450	0.0288	12.96	5.4	2,430	0.0052	7.2
Top TG Layer in ammonia buffer	360	0.033	11.88	3.8	1,368	0.0086	6.6
Above TG solution centrifuged 105,000g Supernatant	340	0.012	4.08	1.5	510	0.008	2.2
Addition of ammonia buffer to Top TG Layer from above, centrifuged 105,000g Supernatant	340	0.04	13.6	0.5	170	0.08	7.5

TABLE V (Continued)

Enzyme Extract	Volume mls	Activity Units/ml	Total Activity Units	Protein Conc. mg/ml	Total Protein mg	Specific Activity Units/mg	% recovery
Combined 105,000g Precipitates from above	192	0.0288	5.472	6.25	1,200	0.046	3
Top TG Extract in buffer	360	0.0216	7.776	1.35	486	0.016	4.3
105,000g Supernatant of above Top Lipid Extract	340	0.0088	3	0.43	146	0.02	1.6
105,000g Top TG Layer in buffer	360	0.0088	3.168	0.944	340	0.0093	1.7
105,000g Supernatant of above Top Lipid Extract	340	0.012	4.08	0.075	25.5	0.16	2.2
Top Lipid Layer in buffer	360	0.0092	3.312	0.857	315	0.01	1.8
Top Lipid Layer from above sonified, centrifuged 105,000g Supernatant	340	0.036	12.34	0.033	11.22	1.08	6.8
105,000g Top Lipid Layer from above in buffer	100	0.0264	2.64	3	300	0.0086	1.4

TABLE V. (Continued)

Enzyme Extract	Volume mls	Activity Units/ml	Total Activity Units	Protein Conc. mg/ml	Total Protein mg	Specific Activity Units/mg	% recovery
105,000g Precipitate	20	0.018	0.36	0.85	17	0.021	0.2
Top TG solution centrifuged 105,000g Supernatant	90	0.0192	1.79	0.4	36	0.0475	1
Top Lipid Layer in buffer	100	0.0108	1.08	1.9	190	0.0057	0.6
Precipitate	20	0.0024	0.048	2.85	57	0.0008	0.02

TABLE VI. Binding and elution from a TG emulsion of the 16,500g supernatant Extract II.

Enzyme Extract	Volume mls	Activity Units/ml	Total Activity Units	Protein Conc. mg/ml	Total Protein mg	Specific Activity Units/mg	% recovery
Extract II	4500	0.012	54	6.1	27,950	0.002	100
Addition of TG emulsion, activation. Top TG Layer	500	0.015	8.5	8	4,000	0.0019	16
105,000g Supernatant of Top Lipid Extract	450	0.008	3.5	5	2,250	0.0016	7
105,000g Precipitate	50	0.043	2.15	7.2	360	0.006	4
Top Lipid Extract in ammonia buffer	450	0.0126	5.67	3.33	1,498	0.0038	10.5
Top Lipid Extract above centrifuged 105,000g Supernatant	400	0	0	3.1	1,240	0	0
Top Lipid from above washed with a second volume of ammonia buffer Supernatant	400	0.018	7.2	0.32	128	0.056	14

TABLE VI (Continued)

Enzyme Extract	Volume mls	Activity Units/ml	Total Activity Units	Protein Conc. mg/ml	Total Protein mg	Specific Activity Units/mg	% recovery
Third washing of Top Lipid Extract Supernatant	400	0	0	0.114	44.8	0	0
Fourth washing of Top Lipid Extract Supernatant	400	0.0144	5.6	0.06	24	0.23	10
Fifth washing of Top Lipid Extract Supernatant	400	0.006	2.4	0.03	12	0.2	4.4
Top Lipid Extract left	100	0.018	1.8	0.5	50	0.036	3

TABLE VII. Binding and elution from a TG emulsion of the 16,500g supernatant Extract III.

Enzyme Extract	Volume mls	Activity Units/ml	Total Activity Units	Protein Conc. mg/ml	Total Protein mg	Specific Activity Units/mg	% recovery
Extract III	5000	0.0128	64	1.2	6,000	0.01	100
Addition of TG emulsion, activation Top Lipid Extract	400	0.0145	5.8	1.5	600	0.01	10
105,000g Supernatant of Top Lipid Extract	350	0.005	1.75	0.8	280	0.0062	2.7
105,000g Precipitate	60	0.021	1.26	3.1	186	0.007	2
Top Lipid Extract in ammonia buffer	450	0.011	4.95	0.288	130	0.039	7.7
105,000g centrifugation of Top Lipid Extract Supernatant	400	0.026	10.4	0.2	80	0.13	17
Top Lipid Layer from above washed with a second volume of ammonia buffer. Centrifuged 105,000g Supernatant	400	0.0064	2.586	0.024	9.6	0.27	4
Third washing of Top Lipid Extract	400	0.0131	5.24	0.034	13.6	0.39	8
Top Lipid Extract	150	0.012	1.8	0.18	27	0.06	3

Binding to the olive oil triglyceride emulsion varied from 3.8% (for extract I first binding) to 10.5% for extract II. Binding of extract I (second binding) and extract III was 6.0% and 7.7% respectively. Recovery thereafter of the TG bound enzyme was 443%, 384%, 300% and 400% for extracts I (first binding), I (second binding), II and III respectively. Overall recoveries of protein in the buffer washings of the TG bound enzyme were 0.8%, 3%, 5% and 1.6% for extract I (first binding), extract I (second binding), extract II and extract III respectively.

Maximum purifications obtained were 66 fold, 174 fold, 115 fold and 39 fold representing 3.7%, 6.8%, 10% and 8% of the total activity of extract I (first binding), extract I (second binding), extract IIand extract III respectively. Overall recovery of protein in these extracts of maximum purification were 0.05%, 0.04%, 0.08% and 0.02% of the total initial protein of extracts I (first binding), I (second binding,) II and III respectively.

Sonifying the TG emulsion increased protein binding by 224% and increased enzyme binding by 130%. Total recovery of enzyme activity was 110% for extract I (first binding) and 73% for extract I (second binding).

Maximum purification from the original homogenized mammary gland extract was 900 fold representing 1.5% of the total activity obtained from the fifth ammonia buffer washing of the TG layer of extract I, second TG binding.

3:4:2. Calcium phosphate gel filtration.

Preparation and elution from calcium phosphate gel was carried out as in section 2:4:1.

TABLVE VIII. Calcium phosphate gel filtration of the 105,000g supernatant of Extract I after TG binding.

Enzyme Extract	Volume mls	Activity Units/ml	Total Activity Units	Protein Conc. mg/ml	Total Protein mg	Specific Activity Units/mg	% recovery
105,000g Supernatant of Extract I	1000	0.0168	16.8	5.1	5,100	0.0033	100
Addition of Ca_3PO_4 gel Filtered Filtrate	1000	0.0448	44.8	4.9	4,900	0.009	266
Filtrate from above eluted through Ca_3PO_4 gel Filtrate	1000	0.0644	64.4	4.3	4,300	0.015	384
Ammonia Buffer wash of Ca_3PO_4 gel	100	0.003	0.3	3	300	0.01	1.8
Potassium oxalate wash of Ca_3PO_4 gel	100	0.001	0.1	0.4	40	0.0025	0.6
Sodium citrate wash of Ca_3PO_4 gel	100	0.026	2.6	2.1	210	0.011	12.5
Ca_3PO_4 gel	50	0.006	0.30	1.2	60	0.005	7.1

An overall recovery of activity of 405% was obtained. Maximum purification was 4.5 fold obtained in the Ca_3PO_4 gel filtrate. 5% of the total activity recovered was bound to the gel. The gel filtrate was a red clear solution.

TABLE IX. Calcium phosphate gel filtration of supernatant Extract II after TG binding.

Enzyme Extract	Volume mls	Activity Units/ml	Total Activity Units	Protein Conc. mg/ml	Total Protein mg	Specific Activity Units/mg	% recovery
Extract II after TG Binding	1000	0.011	11	6.05	6,050	0.002	100
Gel filtrate after twice elution through Ca_3PO_4 gel	1000	0.013	13	3.95	3,950	0.0032	118
0.05M NH_4OH-NH_4Cl buffer pH 8.4 wash of gel Filtrate	100	0.0135	1.35	3.2	320	0.0042	12
Potassium oxalate plus sodium citrate wash of gel Filtrate	100	0.009	0.9	2.35	235	0.004	8
Ca_3PO_4 gel in ammonia buffer	100	0.022	2.2	15.5	1,550	0.0014	20

Total recovery of enzyme activity was 158%. 10% was bound to the gel and of this 50% could not be eluted from the gel. Maximum purification obtained was 2.1 fold in the 0.05M NH_4OH-NH_4Cl wash of the gel.

3:4:3. Silica gel filtration

Filtration through a silica gel slurry was carried out as in section 2:4:2.

TABLE X. Silica gel filtration of the 105,000g supernatant of Extract I after TG binding.

Enzyme Extract	Volume mls	Activity Units/ml	Total Activity Units	Protein Conc. mg/ml	Total Protein mg	Specific Activity Units/mg	% recovery
105,000g Supernatant of Extract I	1000	0.0168	16.8	5.1	5,100	0.0033	100
Filtrate from silica gel	1000	0.0196	19.6	0.45	450	0.043	116
Silica gel extract in ammonia buffer	200	0.034	6.8	23.2	4,640	0.0014	40

Total recovery of activity was 156%, with a 13 fold purification obtained in the filtrate representing 116% of the activity recovered (Ca_3PO_4 gel filtrated 4.5 fold purification representing 384% of the activity recovered). The supernatant was a yellow clear solution.

3:4:4. Binding and elution from viscose sulphate

Viscose sulphate was prepared as in section 2:5:1.

(a) Stepwise elution.

Binding and stepwise elution from viscose sulphate was carried out as in section 2:5:2. Enzyme solution was the 16,500g supernatant of Extract II after TG binding.

TABLE XI. Binding and stepwise elution from viscose sulphate.

Enzyme Extract	Volume mls	Activity Units/ml	Total Activity Units	Protein Conc. mg/ml	Total Protein mg	Specific Activity Units/mg	% recovery
16,500g Supernatant of Extract II	1000	0.011	11.0	6.05	6,050	0.0018	100
Addition of viscose sulphate. Centrifuged 16,500g Supt.	1000	0	0	?	?	?	0
Precipitate from above re-dispersed in 0.5M NaCl in 0.05M Tris HCl pH 8.0. Centrifuged 16,500g Supt.	1000	0.0048	4.8	?	?	?	42
Precipitate from above re-dispersed in 0.75M NaCl in 0.05M Tris HCl buffer pH 8.0. Centrifuged 16,500g Supt.	1000	0.0033	3.3	2.5	2,500	0.0013	30
Precipitate from above re-dispersed in 0.9M NaCl in 0.05M Tris HCl buffer pH 8.0. Centrifuged 16,500g Supt.	500	0.0028	1.43	0.134	67	0.0021	13

TABLE XI (Continued)

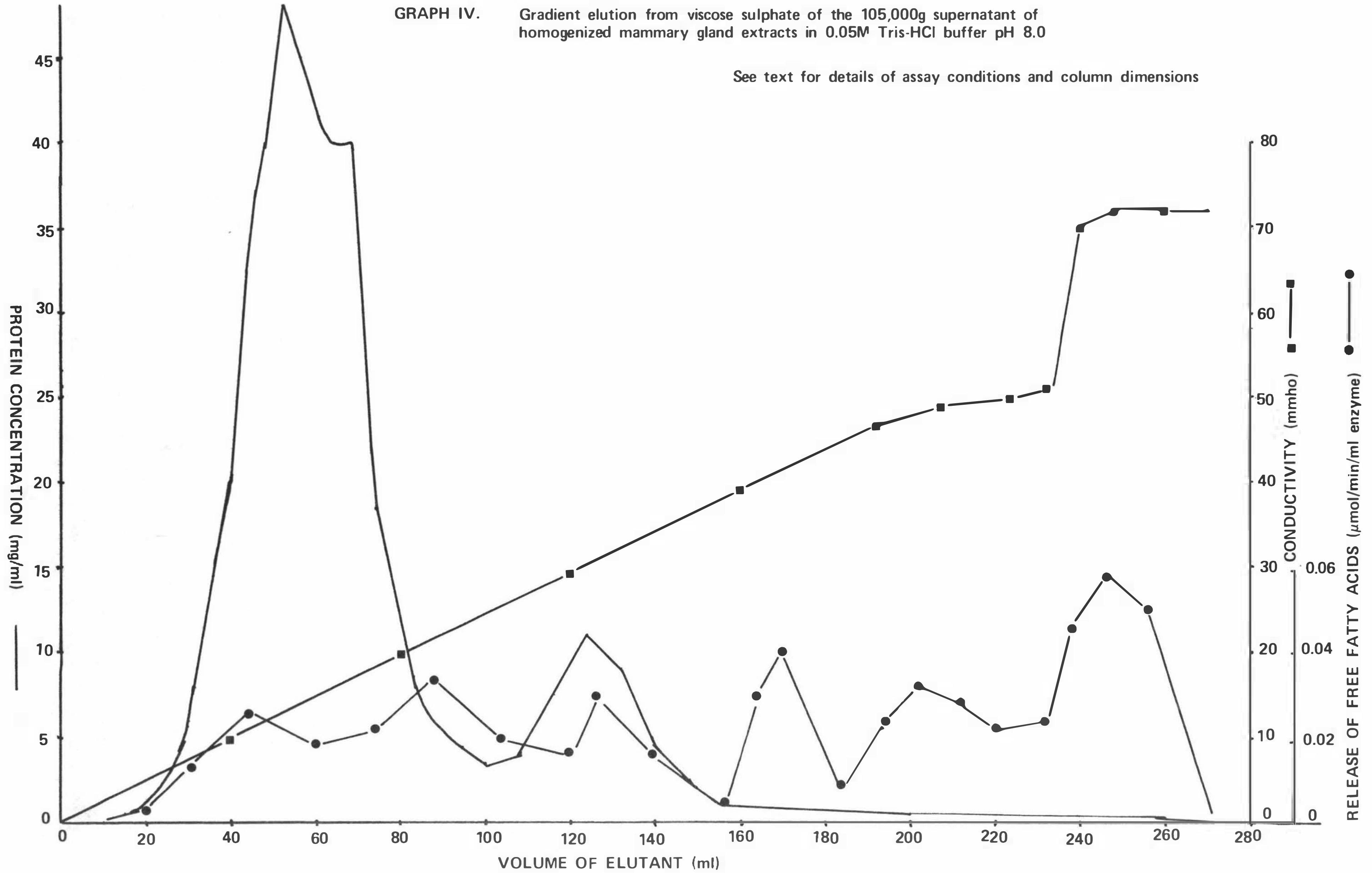
Enzyme Extract	Volume mls	Activity Units/ml	Total Activity Units	Protein Conc. mg/ml	Total Protein mg	Specific Activity Units/mg	% recovery
Precipitate from above re-dispersed in 1.2M NaCl in 0.05M Tris HCl buffer pH 8.0. Centrifuged 16,500g Supt.	250	0.0067	1.67	0.17	42	0.04	15
Precipitate dispersed in 0.05M Tris HCl buffer pH 8.0.	100	0.002	0.2	0.13	13	0.014	1.8

Complete binding to the viscose sulphate occurred. Total recovery of activity was 100%. Maximum purification obtained was 20 fold representing 15% of the total activity bound to the viscose sulphate. Protein concentrations could not be determined with the supernatant and 0.5M NaCl wash due to the interference of Mg^{2+} ions with the protein determination.

(b) Gradient elution from viscose sulphate.

Gradient elution was carried out as in section 2:5:3. Enzyme solution was the 105,000g supernatant of Extract I after TG binding (see Table V). 1.1 litres was eluted on to the column, i.e. total activity eluted on was 19.5 units. Protein binding capacity of the sulphate resin was 7.8 gm per 20 gm resin.

Fractions were assayed as in section 2:12. Total activity recovered was 5.5 units, i.e. percentage recovery was 28% (see Graph IV). Activity was distributed into 6 peaks. 60% of the total activity was eluted at a conductivity greater than 38 mmho. Maximum specific activity obtained was in the final peak with an activity of 0.0122 units/mg.



GRAPH IV. Gradient elution from viscose sulphate of the 105,000g supernatant of homogenized mammary gland extracts in 0.05M Tris-HCl buffer pH 8.0
See text for details of assay conditions and column dimensions
PROTEIN CONCENTRATION (mg/ml)
CONDUCTIVITY (mmho)
RELEASE OF FREE FATTY ACIDS (μmol/min/ml enzyme)
VOLUME OF ELUTANT (ml)
0
5
10
15
20
25
30
35
40
45
20
40
60
80
100
120
140
160
180
200
220
240
260
280
50
70
0.02
0.04
0.06

3:4:5. Binding and elution from sodium dextran sulphate.

Method I.

Stepwise elution with $CaCl_2$ was carried out as in section 2:6:1. Enzyme solution used was 100 mls of the sodium citrate extract of the calcium phosphate gel filtration of the 105,000g supernatant of Extract I after TG binding (See Table VIII).

TABLE XII. Binding and elution of LPL from sodium dextran sulphate with calcium chloride.

Enzyme Extract	Volume mls	Activity Units/ml	Total Activity Units	Protein Conc. mg/ml	Total Protein mg	Specific Activity Units/mg	% recovery
Extract used	100	0.026	2.6	2.1	210	0.011	100
Addition of 10% dextran sulphate. Precipitate with $CaCl_2$ Centrifuged 16,500g Supt.	100	0.0076	0.76	1.74	174	0.0045	30
Precipitate dispersed in 1M $CaCl_2$	40	0.0456	1.8	0.9	36	0.05	70
Above Precipitate extract centrifuged 16,500g Supt.	40	0.03	1.2	0,17	6.8	0.18	43
Precipitate from above dispersed in 2M $CaCl_2$ solution. Centrifuged 16,500g Supt.	40	0.012	0.48	0.13	5.2	0.092	17
Precipitate from above	15	0.018	0.27	1.4	21	0.013	10

A 15 fold purification was obtained from the 1M $CaCl_2$ wash. 70% of the total activity was bound to the sulphate polysaccharide. Of this 66% and 26% was eluted in the 1M and 2M $CaCl_2$ washes respectively.

Method II.

Binding on to sodium dextran sulphate precipitated with divalent cations $MgCl_2$ and $MnCl_2$.

Enzyme solution was the Ca_3PO_4 filtrate of the 105,000g supernatant of Extract I after TG binding (see Table VIII). Procedure was carried out as in section 2:6:2. Assay is as in section 2:12.

TABLE XIII. Binding of LPL to sodium dextran sulphate precipitated with $MnCl_2$.

Enzyme Extract	Volume mls	Activity Units/ml	Total Activity Units	Protein Conc. mg/ml	Total Protein mg	Specific Activity Units/mg	% recovery
Ca_3PO_4 filtrate of the 105,000g Supernatant of Extract I	1000	0.0644	64.4	4.3	4,300	0.014	100
Addition of dextran sulphate Precipitate with $MnCl_2$. Centrifuged 165,000g Supt.	1000	0.008	8.0	2.65	2,650	0.003	12.5
Precipitate redispersed in 10% $NaHCO_3$	30	0.015	4.5	55	1,650	0.0003	7
Above Precipitate solution centrifuged 17,200g $MnCO_3$ Precipitate in buffer	30	0.012	0.36	0.093	2.8	0.013	0.5
$NaHCO_3$ Supernatant dispersed in 0.02M Tris HCl buffer pH 7.7, reprecipitated with $MgCl_2$, centrifuged 16,500 Supt.	800	0.011	8.8	1.47	1,176	0.0075	13.6
Precipitate from above in 5% NaCl	30	0.046	1.38	16.0	480	0.003	2.1

TABLE XIII (Continued)

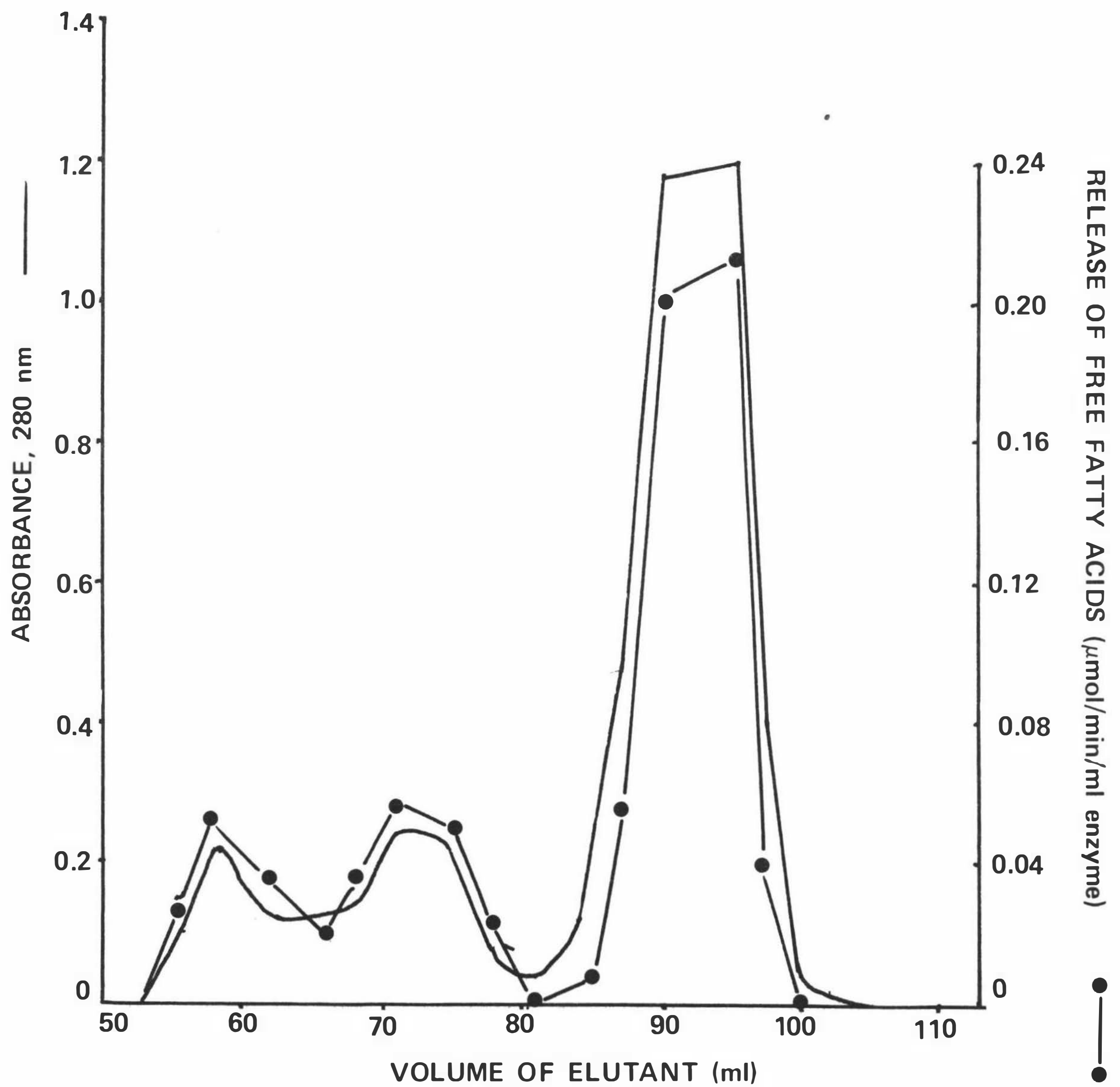
Enzyme Extract	Volume mls	Activity Units/ml	Total Activity Units	Protein Conc. mg/ml	Total Protein mg	Specific Activity Units/mg	% recovery
Precipitate solution dispersed in 0.5% NaCl in 0.02M Tris HCl buffer pH 7.7, reprecipitated with $MgCl_2$, centrifuged 16,500g Supernatant.	800	0.037	29.6	0.43	344	0.09	46
Precipitate from above in 5% NaCl in 0.02M Tris HCl pH 7.7	30	0.053	1.59	4.3	129	0.012	2.46
Precipitate solution dispersed in 0.5% NaCl in 0.02M Tris HCl buffer pH 7.7, reprecipitated with $MgCl_2$, centrifuged 16,500g Supernatant.	800	0.014	11.2	0.141	112	0.1	17
Precipitate dissolved in 10% sodium citrate	15	0.126	1.89	1.14	17.1	0.11	3
Precipitate solution dialysed against 1% NaCl in 0.02M Tris HCl pH 7.7 followed by dialysis in 1% $BaCl_2$ 1% NaCl solution, centrifuged 17,200g Supernatant dialysed in 0.9% NaCl and concentrated	5	0.72	3.6	3.0	15	0.24	5.6

87.5% of the total activity was bound to the sulphate polysaccharide. Almost complete recovery of the bound activity was obtained on elution with the buffer solutions although inhibition occurred when the enzyme was bound to the sulphate polysaccharide. A 17 fold purification was obtained in the final extract and this represented 5.6% of the original activity of the Ca_3PO_4 gel filtrate started with. From the original homogenized udder extract a 200 fold purification was obtained. This represents 2.6% of the activity of the homogenized cows udder extract.

3:5. Sephadex G200 gel filtration of the final supernatant solution obtained from Method II of the sodium dextran sulphate precipitation of LPL.

Gel filtration was carried out as in Section 2:8:1 and the fractions assayed as in Section 2:12. Total recovery of activity was 98%. Activity was eluted off in 3 peaks, 63% of the total activity was in the third peak. See Graph V.

GRAPH V. Sephadex G200 gel chromatography of the final solution from sodium dextran sulphate precipitation method II.

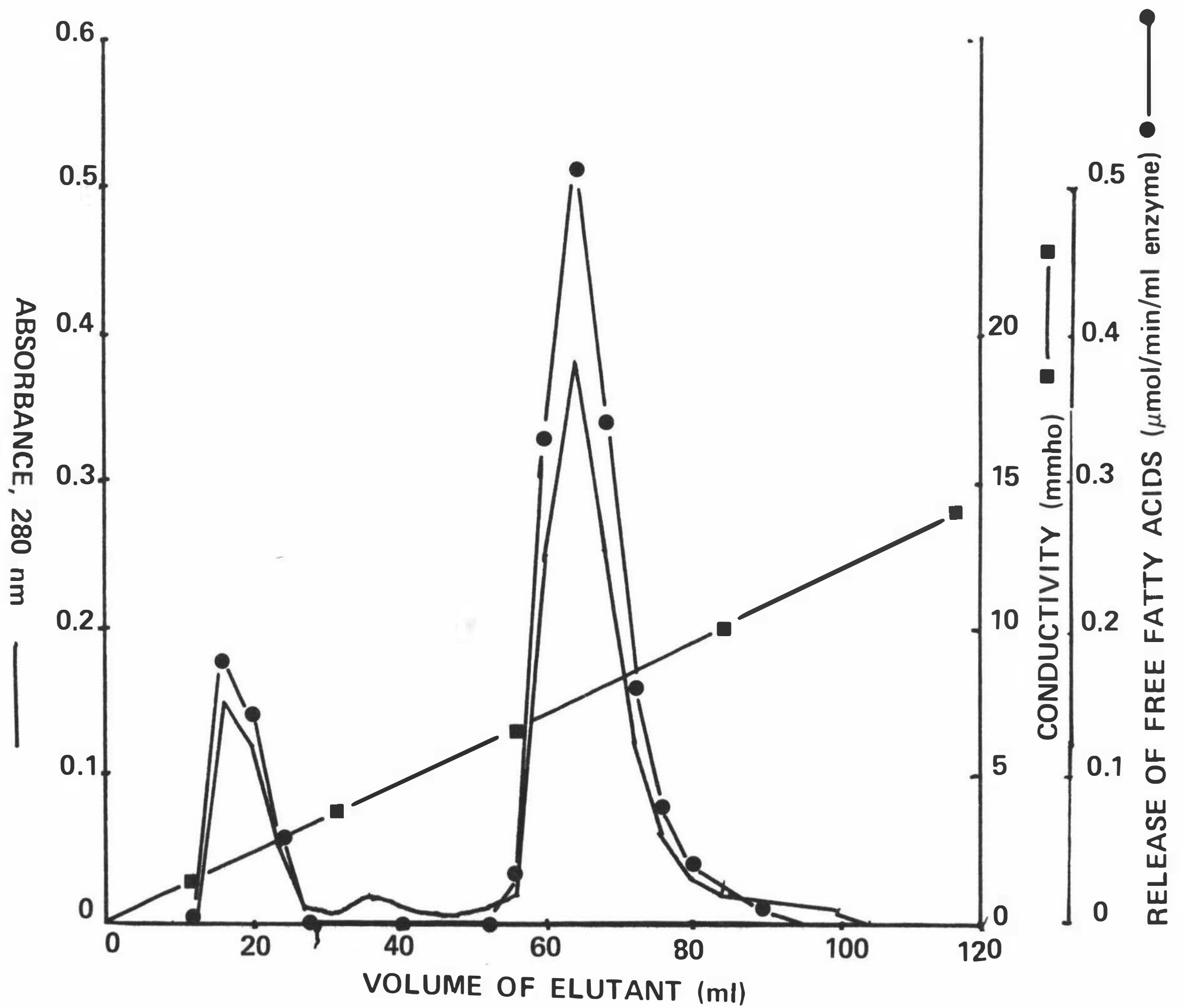


3:6. DEAE cellulose elution of peak 3 from sephadex G200 gel filtration of the final extract from sodium dextran sulphate precipitation Method II.

DEAE cellulose equilibrated in .005M KH_2PO_4 buffer pH 8.4 as described in Section 2:7:1 was poured into a column (1.4 x 20cm) and the combined extracts of Peak III eluted on to the column after being dialysed in .005M KH_2PO_4 buffer pH 8.4. A gradient of 0 to 0.7M NaCl in .005M KH_2PO_4 buffer pH 8.4 was eluted through the column collecting 2ml fractions. Fractions were assayed as in Section 2:12, OD 280 readings of fractions taken and conductivity of fractions was read using a Copenhagen conductivity meter at room temperature.

Elution gave two peaks of activity, one occurring at a conductivity of 2.5 mmho containing 19% of the total activity and a larger peak at a conductivity of 7.5 mmho containing 81% of the total activity. A 440% total recovery over the activity eluted on was obtained. Specific activity of the major peak was 1.3 units/mg which represents a 1084X purification from the homogenized cows' udder extract. See Graph VI.

GRAPH VI. DEAE cellulose gradient elution of peak III from sephadex G200 gel filtration of the final solution from sodium dextran sulphate precipitation method II.



Polyacrylamide gel electrophoresis at Peak II as described in section 2:9 gave a single protein band when the gels were run in both tris glycine buffer pH 8.4 and KH_2PO_4 buffer pH 7.5. See Figure I.

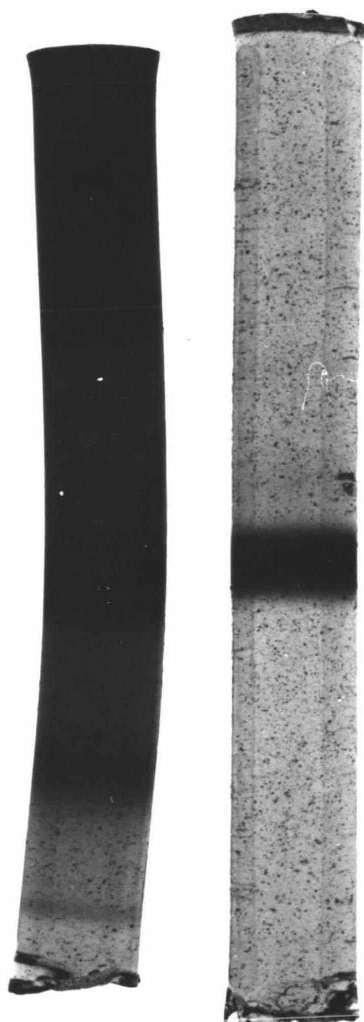


Figure I. Polyacrylamide gel electrophoresis of Peak II from DEAE cellulose elution of Peak III of final enzyme extract from sodium dextran sulphate precipitation Method II on sephadex G200.

The initial total homogenate extract is on the left photograph and LPL on the right.

3:7. Purification of LPL using DEAE cellulose.

3:7:1. Preparation of enzyme extract.

1000 gm of mammary tissue was homogenized and extracted in 9 litres of ice cold 10^{-3}M EDTA in 0.25M sucrose as described in section 2:2 and the 16,500g supernatant extract recentrifuged at 105,000g for one hour at 2°C.

TABLE XIV. Activity of mammary gland extracts. Assays were carried out as described in section 3:12.

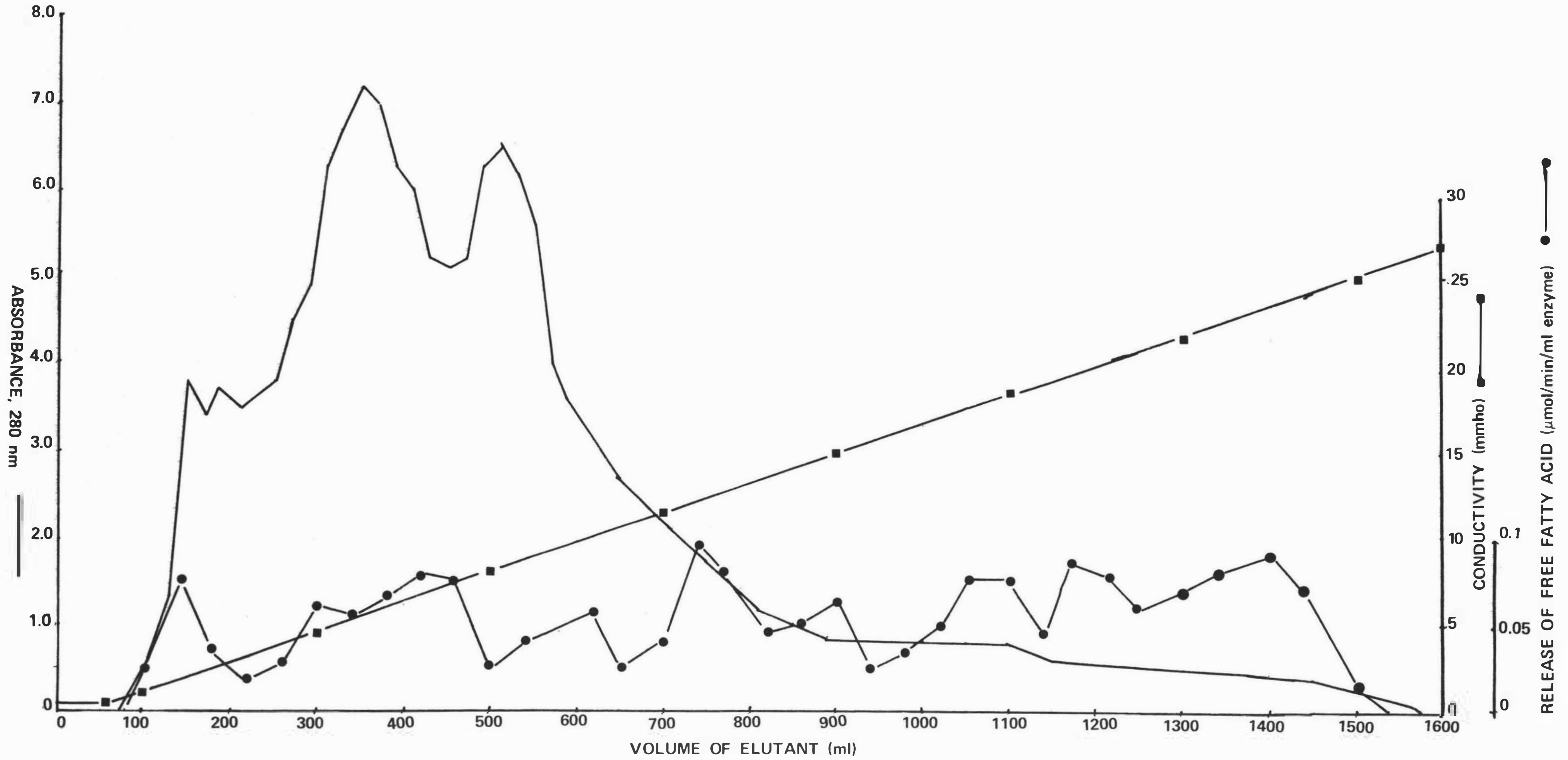
Enzyme Extract	Volume mls (or wet weight of tissue gms)	Activity Units/ml (Units/gm)	Total Activity Units	Protein Conc. mg/ml(mg/gm)	Total Protein mg	Specific Activity Units/mg	% recovery
Homogenized mammary gland extract	10000	0.046	460	68	680,000	0.0007	100
16,500g Precipitate and Top Lipid Fractions	860gms	0.386/gm	326	700/gm	602,000	0.00055	70.8
16,500g Supernatant Fraction	9000	0.0158	142	9.0	81,000	0.0017	30.8
Supernatant Fraction centrifuged 105,000g Precipitate	440	0.0188	8.5	70	30,800	0.00027	1.84
105,000g Supernatant	9000	0.0142	126	5.55	50,000	0.0025	27.4

3:7:2. DEAE cellulose gradient elution in 0.005M KH_2PO_4 buffer pH 8.4.

DEAE cellulose was equilibrated in 0.005M KH_2PO_4 buffer pH 8.4 as described in section 2:7:1 and poured into a column with dimensions 3.2 x 30 cm. 200 mls of enzyme solution (105,000g

GRAPH VII. DEAE cellulose gradient elution of the 105,000g supernatant fraction of homogenized mammary gland extracts in 0.005M KH_2PO_4 buffer pH 8.4

See text for details of assay and column dimensions



supernatant fraction from above) was dialysed in equilibrating buffer and eluted on to the column. A gradient from 0 to 0.8M NaCl in 0.005M KH_2PO_4 buffer pH 8.4 was eluted through the column as described in section 2:7:2 collecting 10ml fractions. OD 280 and conductivity readings were taken and fractions were assayed as in section 2:12. See Graph VII.

Elution gave 9 peaks of activity at conductivities of 1.5, 4.5, 6.5, 10, 12.5 15, 18, 19.5 and 23.5 mmho. 2.8 units of activity were eluted on to the column and 81.2 units were recovered giving a 2930% recovery. Approximate percentage of activity occurring at each peak of conductivity are 1.5 mmho (5.5%); 4.5 mmho (8.1%); 6.5 mmho (12.3%); 10 mmho (7.3%); 12.5 mmho (13.5%); 15 mmho (7%); 18 mmho (16.8%); 19.5 mmho (9.6%) and 23.5 mmho (19.5%)

3:7:3. Stepwise elution followed by gradient elution from DEAE cellulose in 0.005M KH_2PO_4 buffer pH 8.4.

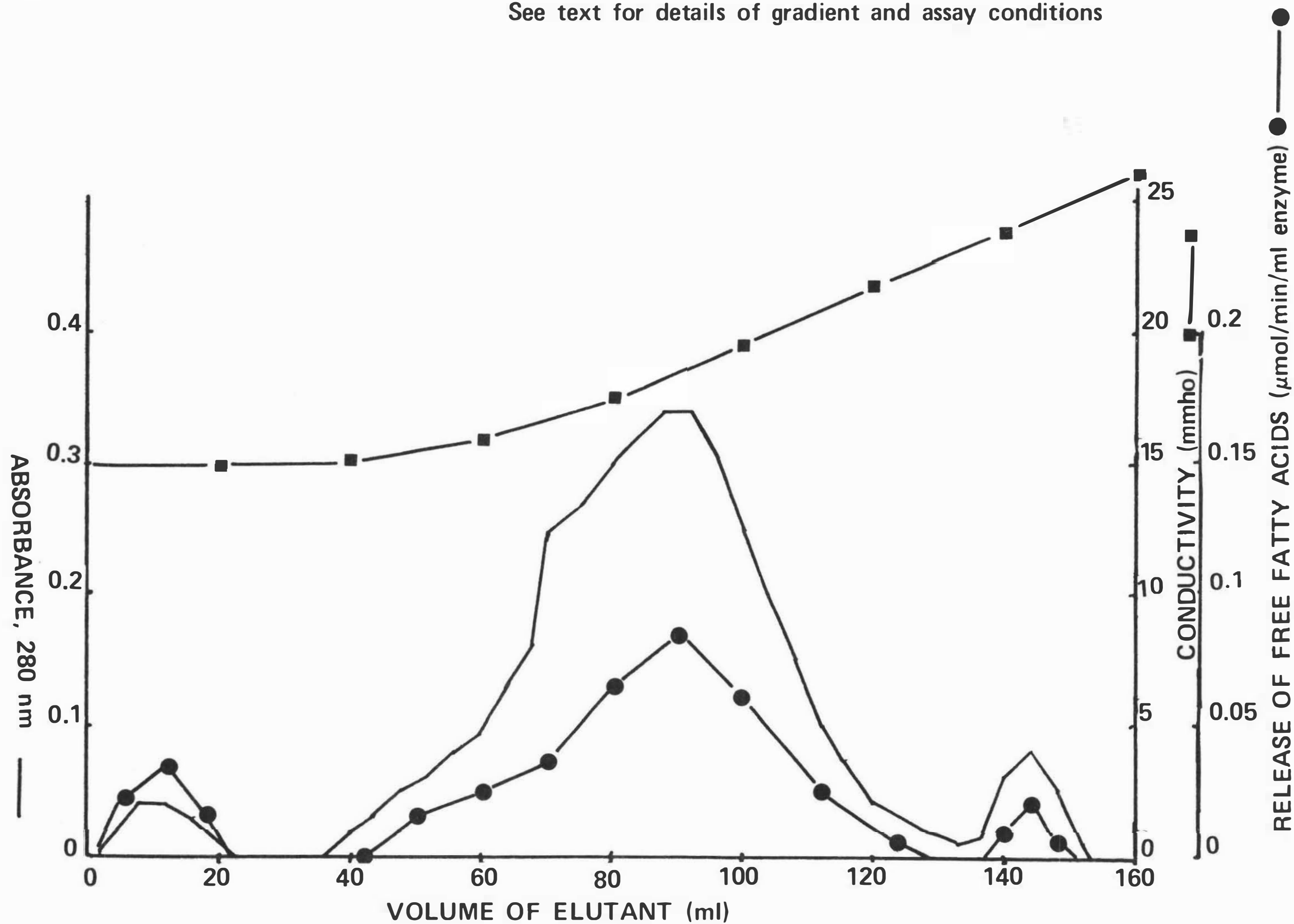
Separation of the peaks obtained from DEAE cellulose by stepwise elution was carried out as described in section 2:7:2. These fractions were then dialysed against 0.005M KH_2PO_4 buffer pH 8.4, eluted on to a DEAE cellulose column and eluted off using a sodium chloride gradient in 0.005M KH_2PO_4 buffer pH 8.4 as described in section 2:7:3. The fractions were assayed as in section 2:12. OD 280 and conductivity readings were also taken.

(a) Gradient elution of pooled extracts of conductivity 21 mmho on DEAE cellulose with a gradient from conductivity 15 mmho to 30 mmho in 0.005M KH_2PO_4 buffer pH 8.4.

Elution gave a small activity peak at the starting buffer (7% of total activity), a larger peak at a conductivity of 18.5 mmho comprising 90% of the total activity, and a third smaller peak at a conductivity of 24 mmho (3% of total activity). No protein or activity peaks occurred at a conductivity of 21 mmho.
See Graph VIII.

GRAPH VIII. Gradient elution from a DEAE cellulose colum (14 x 20 cm) of the conductivity 21 mmho extracts obtained from stepwise elution in 0.005M KH_2PO_4 buffer pH 8.4.

See text for details of gradient and assay conditions

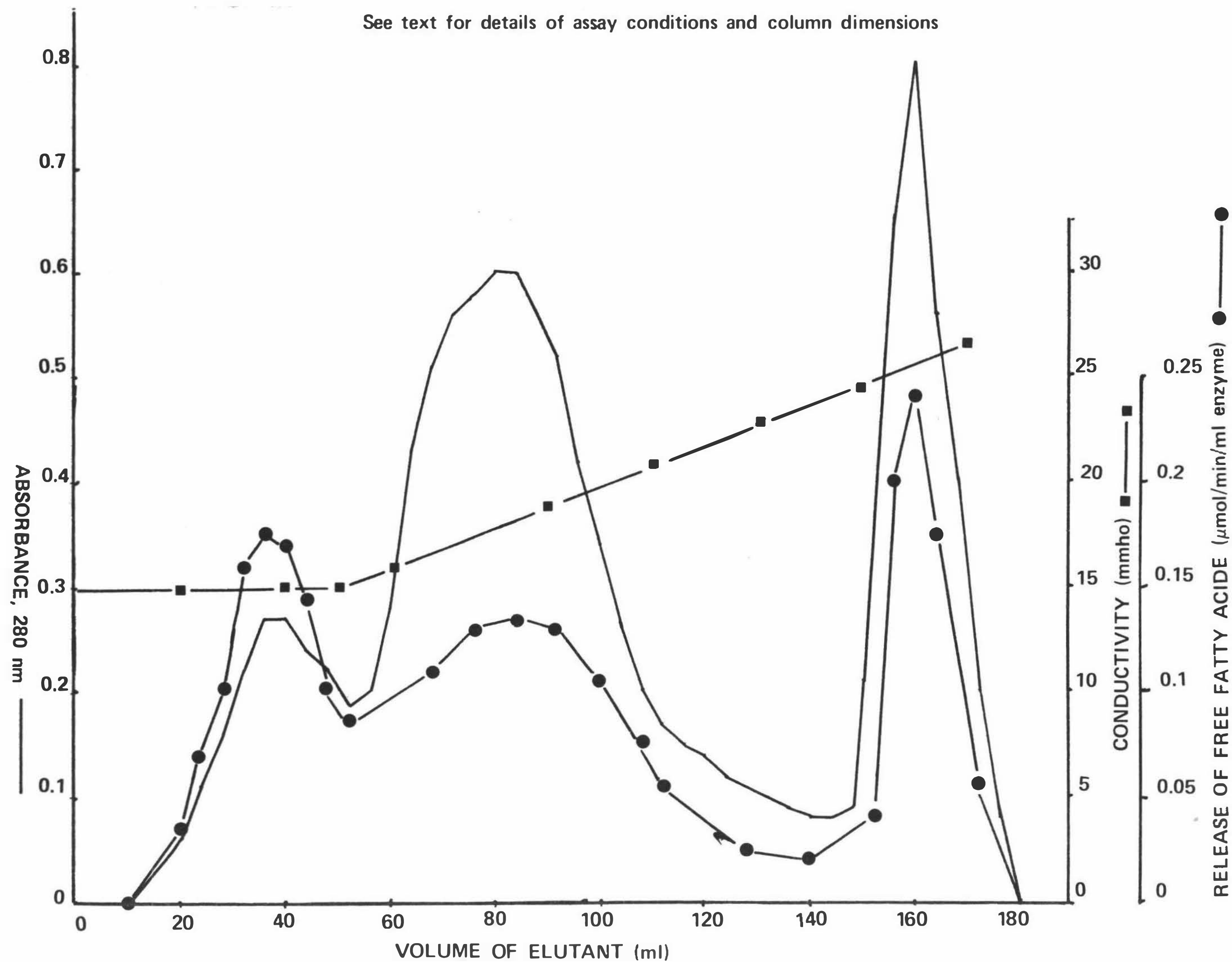


(b) Gradient elution of pooled extracts of conductivity 18 mmho on DEAE cellulose with a gradient from conductivity 15 mmho to 30 mmho in 0.005M KH_2PO_4 buffer pH 8.4.

Elution gave 3 peaks of activity, one at the starting buffer (17% of total activity), a second peak at conductivity of 18 mmho (61%) and a third peak at a conductivity of 24.5 mmho (21%). See Graph IX.

Specific activity was highest for the activity peak eluted at the starting buffer with an activity of 0.63 units/mg with lower specific activities for the peaks at conductivities of 18 mmho (0.23 units/mg) and 24.5 mmho (0.3 units/mg). Column dimensions were 2.3 x 20cm. Flow rate was 1 ml/5 min. and 2 ml fractions were collected.

GRAPH IX. Gradient elution from DEAE cellulose of conductivity 18 mmho extracts obtained from stepwise elution from DEAE cellulose in 0.005M KH_2PO_4 buffer pH 8.4.



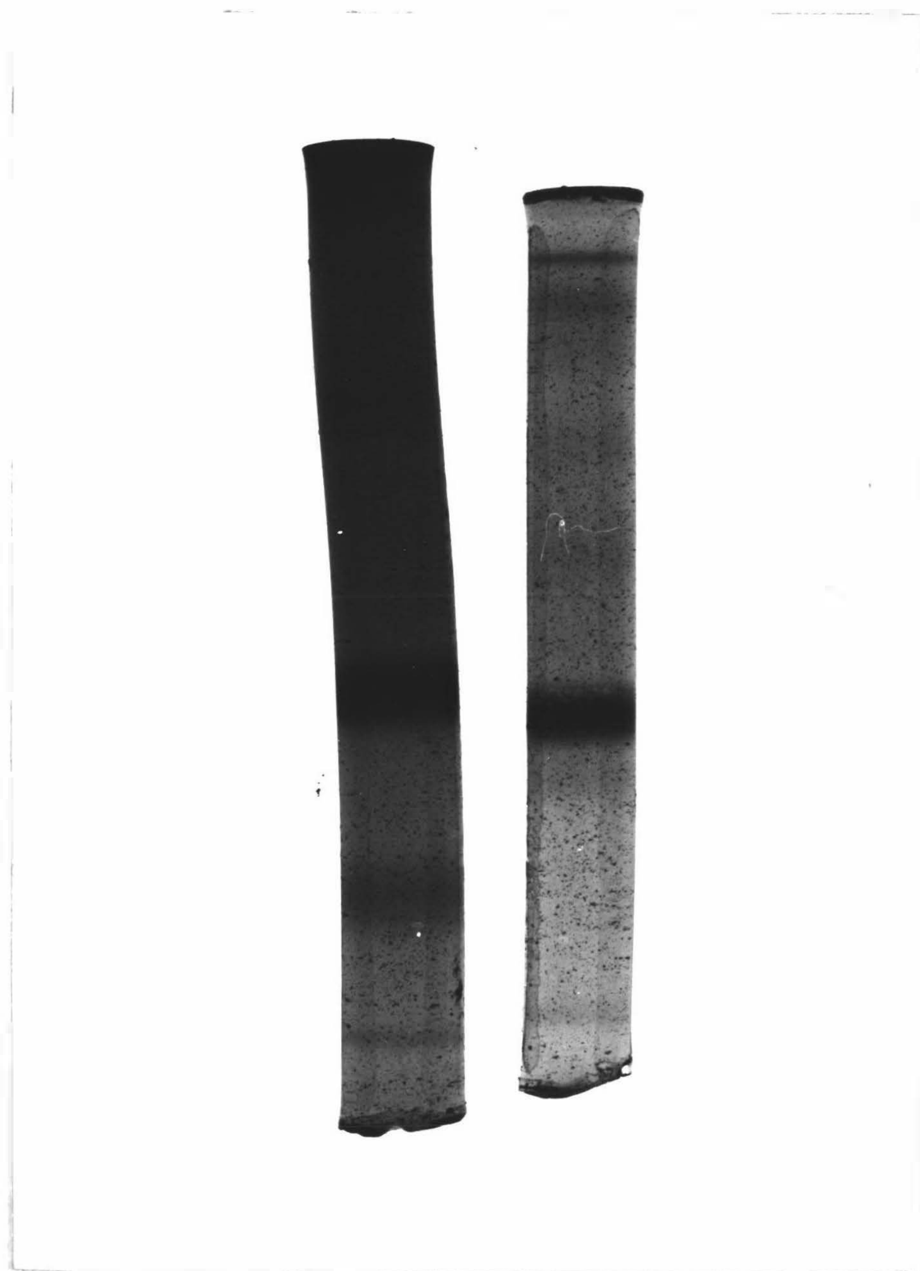


Figure II. Polyacrylamide gel electrophoresis of the peak eluted at conductivity 25 mmho from the gradient elution in 0.005M KH_2PO_4 buffer pH 8.4 of the combined extracts eluted at conductivity 18 mmho (right photograph) and the combined extracts eluted from the stepwise elution of conductivity 5 mmho from DEAE cellulose in 0.005M KH_2PO_4 buffer pH 8.4 of the 105,000g supernatant fraction of homogenized mammary gland extracts.

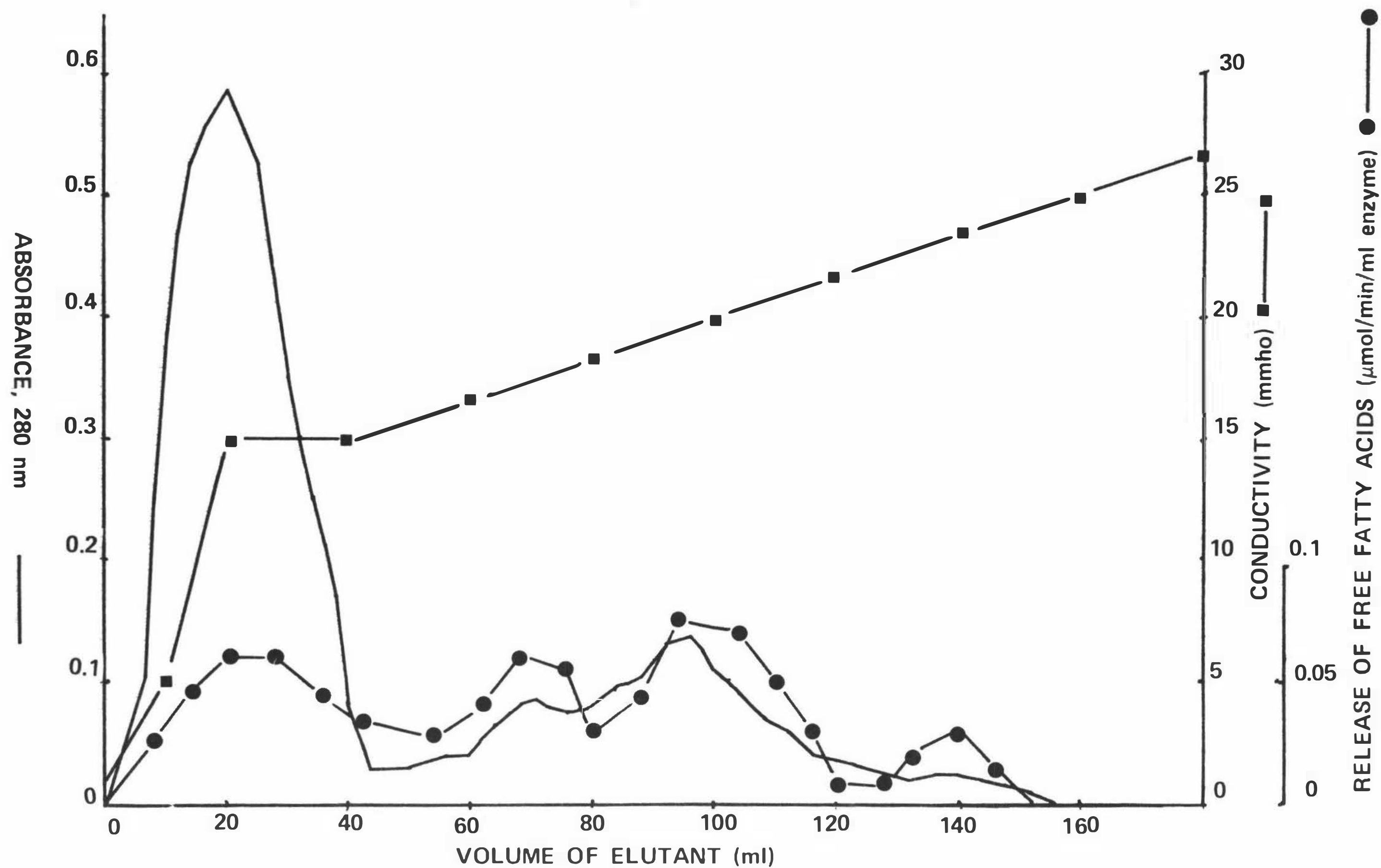
(c) Gradient elution of conductivity 24 mmho peak from gradient elutions of conductivity 18 mmho and 21 mmho extracts obtained by stepwise elution.

The gradient was run from conductivity 15 mmho to conductivity 24 mmho in 0.005M KH_2PO_4 buffer pH 8.4.

Elution gave 4 peaks of activity, one at the starting buffer (38% of total activity), another peak at a conductivity of 17 mmho (21% of total activity), a third at a conductivity of 19.5 mmho (34% of total activity) and a final small peak at a conductivity of 23.5 mmho (6.3% of total activity). The peak eluted at the starting buffer had a specific activity of 0.1 units/mg, whereas the latter 3 peaks all had higher specific activities of between 0.6 units/mg to 0.75 units/mg. Four units of activity were eluted on with 4.9 units being recovered in the 4 peaks, i.e. a 122% recovery of activity was obtained. 50% of the total protein was eluted off at the starting buffer. See Graph X. Column dimensions were 1.4 x 20cm 1 ml/5 min. and 2 ml fractions were collected.

GRAPH X. Gradient elution from DEAE cellulose of the combined conductivity 24 mmho peaks from the gradient elutions of conductivity 18 mmho and 21 mmho extracts from stepwise elution on DEAE cellulose in 0.005M KH_2PO_4 buffer pH 8.4.

See text for details of assay conditions, elution gradient, column dimensions and flow rate

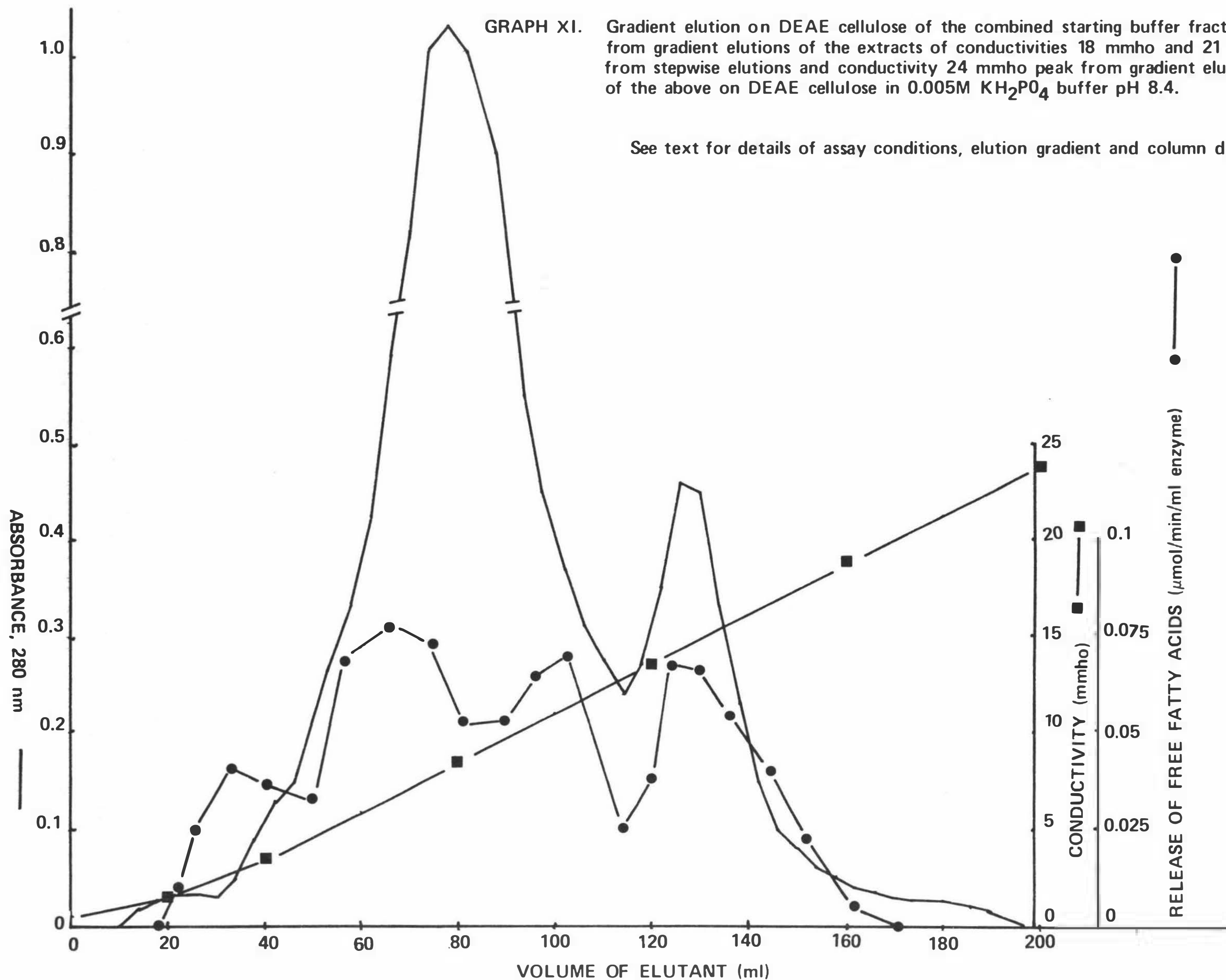


(d) <u>Gradient elution on DEAE cellulose of the combined starting buffer fractions from gradient elutions of conductivities 18 mmho and 21 mmho from stepwise elution and conductivity 24 mmho from gradient elutions of the above.</u> (See sections 3:7:3 (a), (b) and (c) and Graphs VIII, IX and X.)

Gradient elution was from 0 to 0.7M NaCl in 0.005M KH_2PO_4 buffer pH 8.4.

Elution gave 4 peaks of activity, a small peak at a conductivity of 2.5 mmho (15% of total activity) and 3 larger peaks at conductivities of 6.8 mmho (30% of total activity), 11 mmho (24.8% of total activity) and 14 mmho (30% of total activity).

Total activity eluted on was 5.38 units. Total activity recovered was 5.48 units, i.e. percentage recovery 102%. Highest specific activity obtained was for Peak I with an activity of 1 unit/mg, with Peaks II, III and IV having lower specific activities of 0.13 units/mg, 0.18 units/mg and 0.16 units/mg respectively. See Graph XI. Column dimensions were 2.3 x 20cm and two ml fractions were collected.



GRAPH XI. Gradient elution on DEAE cellulose of the combined starting buffer fractions from gradient elutions of the extracts of conductivities 18 mmho and 21 mmho from stepwise elutions and conductivity 24 mmho peak from gradient elutions of the above on DEAE cellulose in 0.005M KH_2PO_4 buffer pH 8.4.

See text for details of assay conditions, elution gradient and column dimensions

(e) Gradient elution from DEAE cellulose of the conductivity 15 mmho peak from gradient elution in section (d) above.
(See Graph XI.)

Elution from 0 to 0.7M NaCl in 0.005M KH_2PO_4 buffer pH 8.4 gave 5 activity peaks, one at a conductivity of 2.5 mmho (10.7% of total activity), two larger peaks at conductivities of 9 mmho (44% of total activity) and 15 mmho (25% of total activity), and two smaller peaks at conductivities of 18.5 mmho and 21 mmho both containing 10% of the total activity.

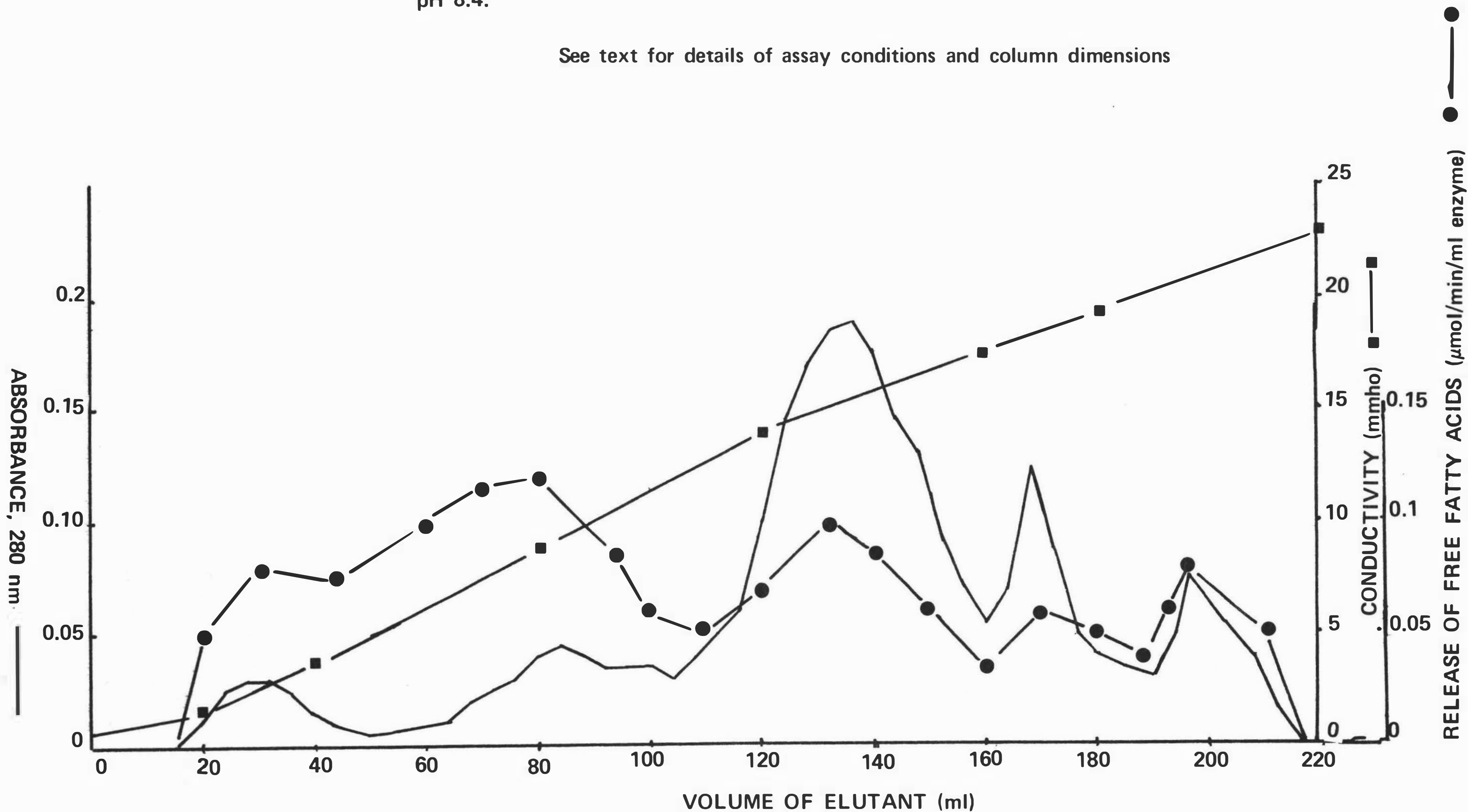
1.62 units of activity were eluted on and 13.05 units recovered representing a 812% recovery of enzyme activity. A large variation in specific activities occurred between the 5 peaks. Peaks I and II had specific activities of 2.66 units/mg and 3.0 units/mg respectively. Peaks III and IV both had specific activities of 0.5 units/mg and Peak V had a specific activity of 1.0 unit/mg. (See Graph XII).

A purification of 3800X and 4286X was obtained for Peaks I and II respectively over the total udder homogenate.

Column dimensions were 1.4 x 20cm and 2 ml fractions were collected.

GRAPH XII. DEAE cellulose gradient elution of peak 2 (conductivity 15 mmho) from the gradient elution on DEAE cellulose of the combined starting buffer fractions from gradient elutions of the extracts of conductivities 18 mmho and 21 mmho from stepwise elutions and the conductivity 24 mmho peak from gradient elutions of the above on DEAE cellulose in 0.005M KH_2PO_4 buffer pH 8.4.

See text for details of assay conditions and column dimensions



(f) Gradient elution from DEAE cellulose of combined extracts of conductivity 15 mmho from stepwise elution on DEAE cellulose in 0.005M KH_2PO_4 buffer pH 8.4.

A gradient of conductivity 10 mmho to 30 mmho in 0.005M KH_2PO_4 buffer pH 8.4 gave 6 peaks of activity. Little separation of the activity peaks was obtained due to the high activities of the peaks. Activity peaks were eluted off at the starting buffer and at conductivities of 10, 13, 15.5, 18.5 and 20 mmho. Activity was about equally distributed between all of the peaks. All of the peaks had relatively high specific activities. These were as follows:

1.6 units/mg and 11.3 units/mg for peaks of activity 10, 13, 15.3, 18.5 and 20 mmho respectively. (See Graph XIII.)

Column dimensions were 2.3 x 20cm and 2 ml fractions were collected.

(g) Gradient elution from DEAE cellulose of combined extracts of conductivity 13 mmho from stepwise elution on DEAE cellulose in 0.005M KH_2PO_4 buffer pH 8.4.

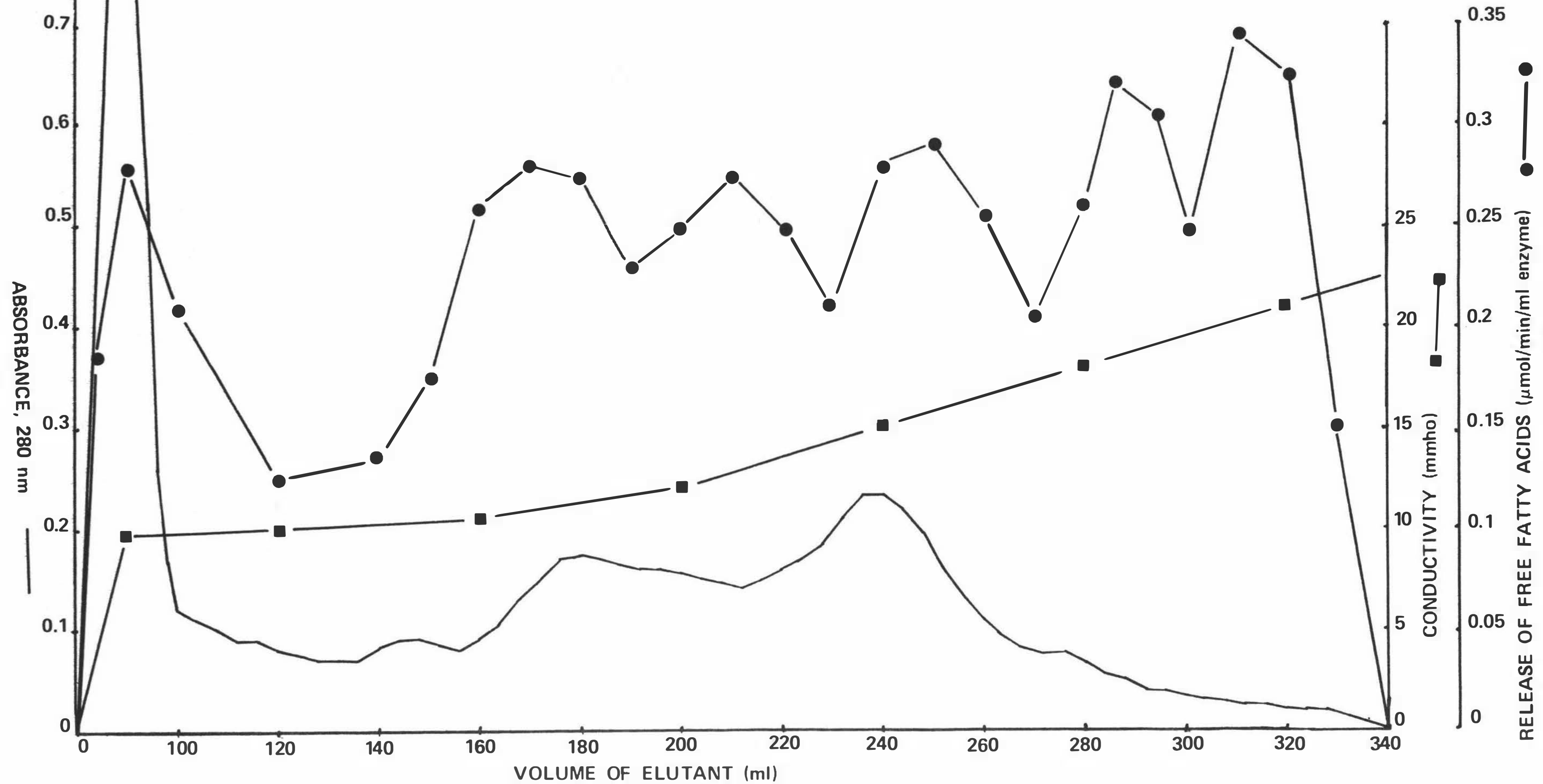
A gradient of conductivity 10 mmho to 30 mmho in 0.005M KH_2PO_4 buffer pH 8.4 gave 4 peaks of activity, one at the starting buffer and at conductivities of 10, 15 and 18 mmho. No activity peak was recorded at a conductivity of 13 mmho although there was a large protein peak present. Little separation of the activity peaks was obtained due to the high activities of the peaks. Activity was approximately equally distributed between all of the peaks. Specific activities are as follows:

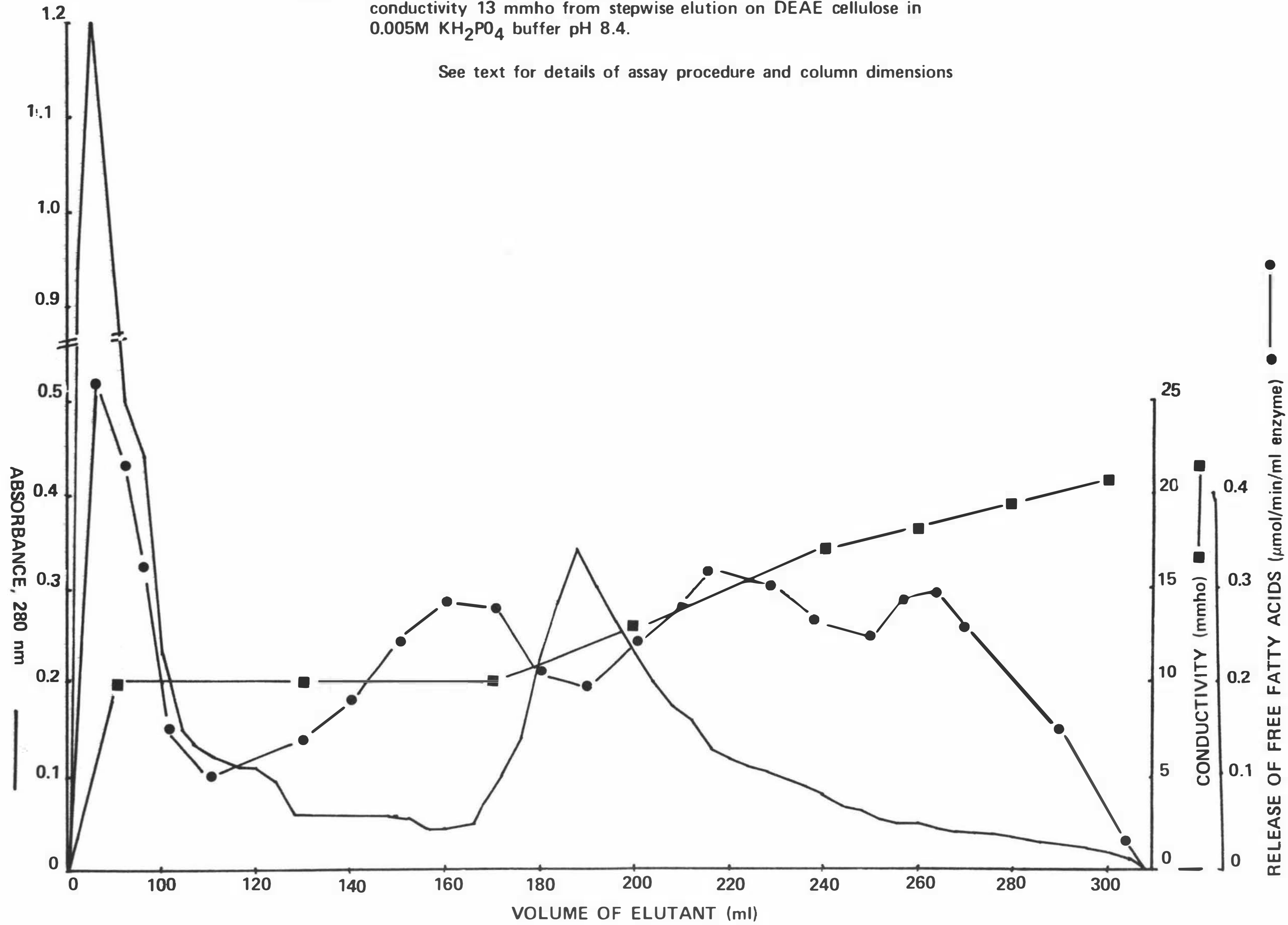
7.0 units/mg, 2.4 units/mg and 5.8 units/mg for peaks of conductivity 10, 15 and 18 mmho respectively. (See Graph XIV.)

Column dimensions were 2.3 x 20cm and 2 ml fractions were collected.

GRAPH XIII. Gradient elution from DEAE cellulose of the conductivity 15 mmho combined extracts from stepwise elution from DEAE cellulose in 0.005M KH_2PO_4 buffer pH 8.4.

See text for details of assay conditions and column dimensions





GRAPH XIV. Gradient elution from DEAE cellulose of the combined extracts of conductivity 13 mmho from stepwise elution on DEAE cellulose in 0.005M KH_2PO_4 buffer pH 8.4.

See text for details of assay procedure and column dimensions

(h) <u>Gradient elution on DEAE cellulose of activity eluted in the starting buffer of gradient elutions of conductivity 13 mmho and 15 mmho pooled fractions obtained from stepwise elution on DEAE cellulose in 0.005M KH_2PO_4 buffer pH 8.4.</u> (See section 4:7:3 (f) and (g) and Graphs XIII and XIV.)

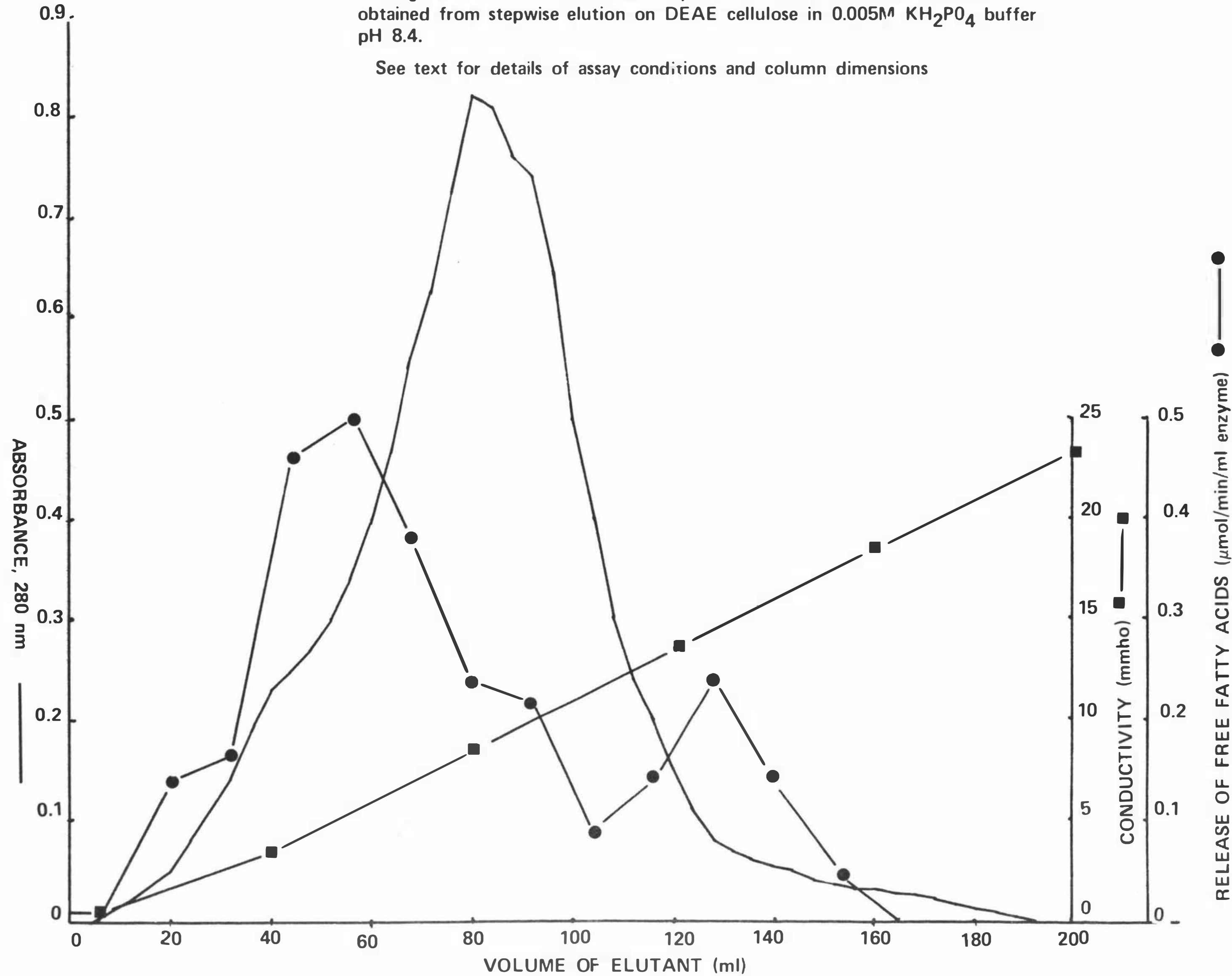
Elution from 0 to 0.8M NaCl in 0.005M KH_2PO_4 buffer pH 8.4 gave two major peaks of activity, one large peak at a conductivity of 5 mmho (containing approximately 53% of the total activity) with two shoulder peaks at conductivities of 1.5 mmho (10% of total activity) and 10 mmho (12% of total activity), and a small peak at a conductivity of 14.5 mmho (24% of total activity). Specific activity of the major peak was 1.8 units/mg and that of the smaller peak at conductivity 14.8 mmho was 3 units/mg.

A 95% recovery of activity was obtained (see Graph XV).

Colum dimensions were 2.3 x 20cm and 2 ml fractions were collected.

GRAPH XV. Gradient elution on DEAE cellulose of activity eluted in the starting buffer from gradient elutions of conductivity 13 mmho and 15 mmho extracts obtained from stepwise elution on DEAE cellulose in 0.005M KH_2PO_4 buffer pH 8.4.

See text for details of assay conditions and column dimensions



3:8. Sephadex G200 gel chromatography.

Gel chromatography was carried out on a sephadex G200 column (2.3 x 39.5cm) as described in section 2:8:1. Assays were carried out as described in section 2:12 and OD 280 readings taken as an estimation of the protein concentration of fractions. The void volume (Vo) of the column was 57 mls and the totally included volume (Vt) was 171 mls.

3:8:1. Sephadex G200 gel chromatography of conductivity 5 mmho peak from gradient elution on DEAE cellulose in 0.005M KH_2PO_4 buffer pH 8.4 of starting buffer fractions from gradient elutions of conductivity 15 mmho and 13 mmho fractions obtained by stepwise elution from DEAE cellulose. (See section 3:7:3.(f), (g) and (h) and Graph XV.)

6 peaks of activity were obtained, one peak near the void volume (10% of total activity) followed by another smaller peak (0.6% of total activity), two large peaks in close proximity to one another (21.8% and 30% of total activity), a smaller peak (15% of total activity) and finally a broad peak near the totally included volume (19% of total activity).

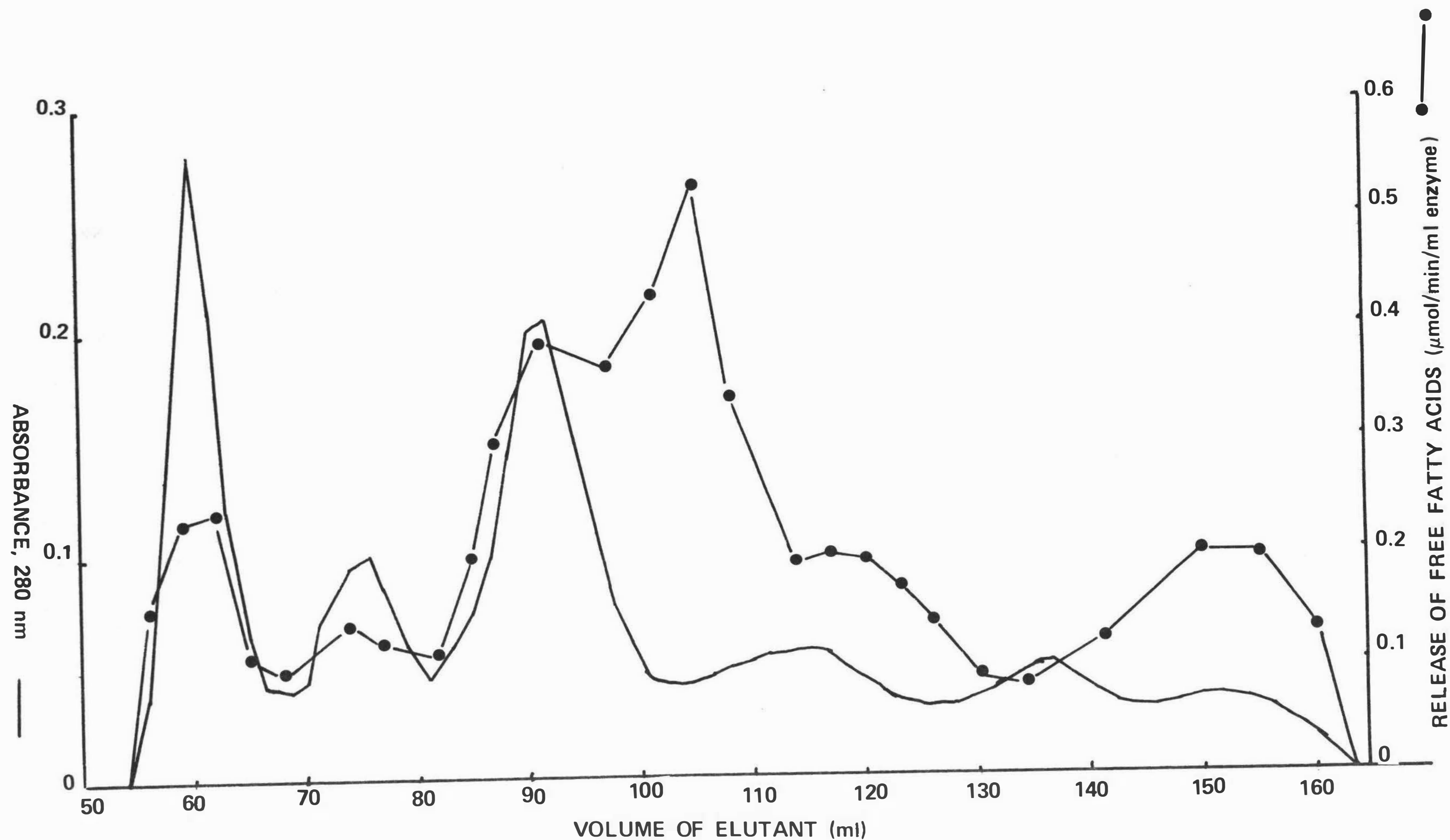
Activity put on column = 18.6 units
Activity eluted off = 20.62 units
i.e. % recovery = 110%

Peaks I and II both had low specific activities of 0.85 units/mg and 0.7 units/mg respectively. Peak III had a higher specific activity of 2 units/mg followed by Peaks V, VII and IV with specific activities of 3.6, 5.7 and 13.2 units/mg respectively. (See Graph XVI)

Peak IV represents a purification of 18,857X over the homogenized udder extract.

GRAPH XVI. Sephadex G200 gel chromatography of the conductivity 5 mmho peak from gradient elutions on DEAE cellulose in 0.005M KH_2PO_4 buffer pH 8.4 of starting buffer fractions from gradient elutions of conductivity 15 mmho and 13 mmho extracts obtained by stepwise elution from DEAE cellulose

See text for details of incubation and column dimensions



3:8:2. Sephadex G200 gel chromatography of pooled fractions of conductivity 2 mmho from stepwise elution from DEAE cellulose in 0.005M KH_2PO_4 buffer pH 8.4.

Enzyme sample was obtained as described in section 2:7:2, concentrated and separated on a sephadex G200 column (2.3 x 39.5cm) as described in section 2:8:1. Assays were carried out as in section 2:12.

Gel filtration gave 6 peaks of activity. A small peak at the void volume (specific activity 0.09 units/mg) followed by two larger peaks of lower specific activity (0.067 units/mg and 0.065 units/mg respectively), a smaller peak (specific activity 0.17 units/mg) and two larger peaks of higher specific activity (0.37 units/mg and 0.66 units/mg respectively).

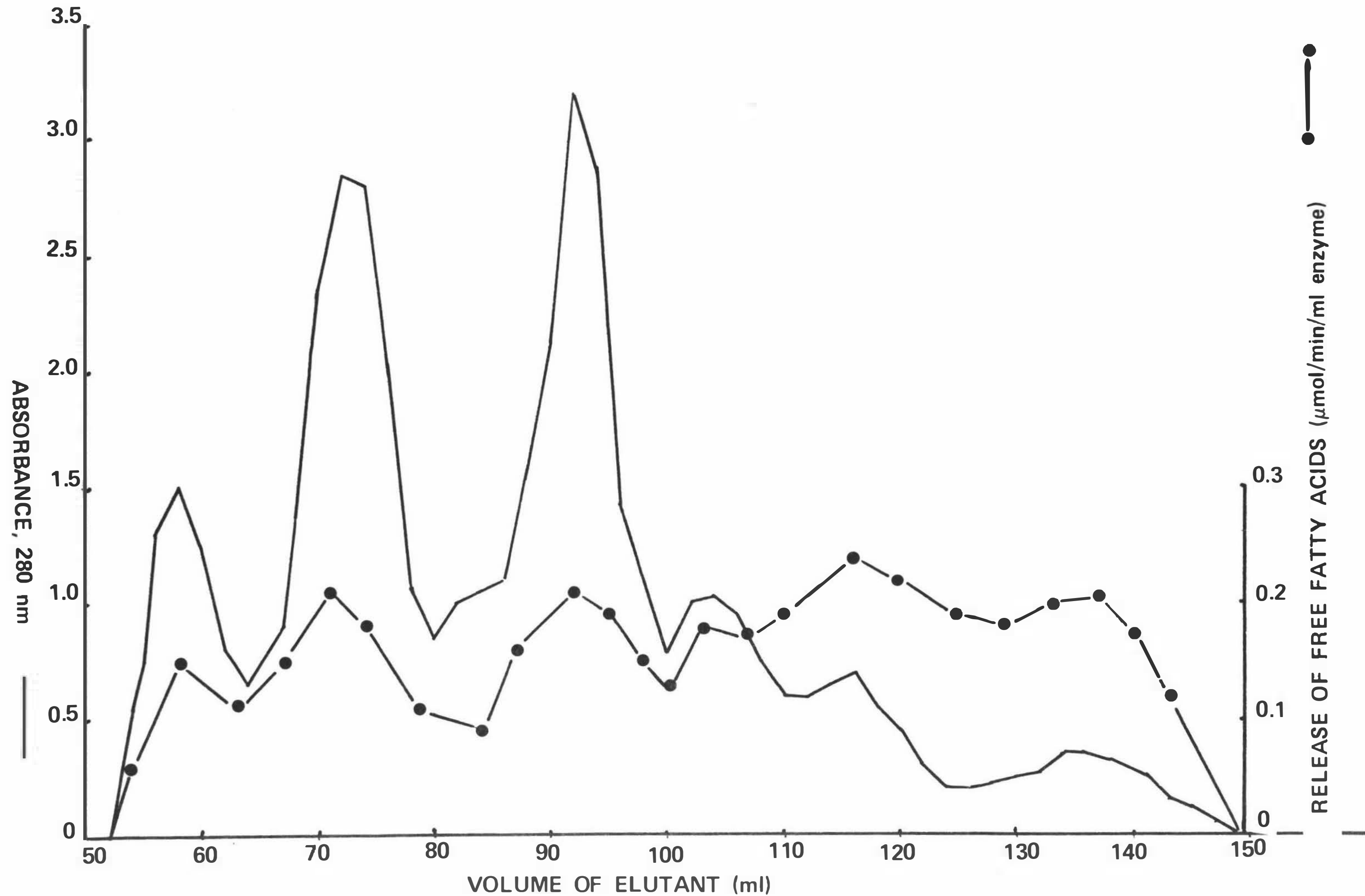
Approximate percentages of the total activity in each peak are:

Peak I 8%; Peak II 20%; Peak III 18%; Peak IV 8.6%; Peak V 25.8%; and Peak VII 19.3%.

See Graph XVII.

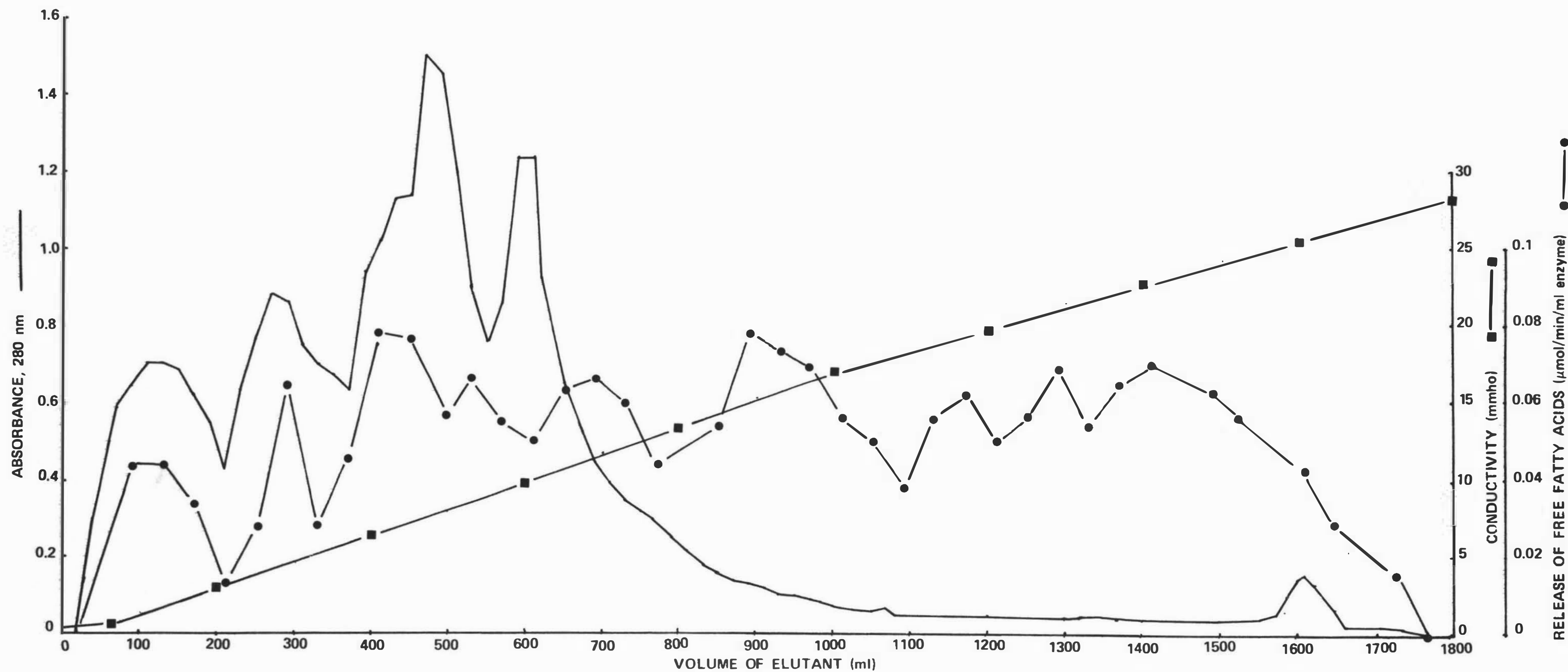
GRAPH XVII. Sephadex G200 gel chromatography of extracts of conductivity 2 mmho obtained from stepwise elution of the 105,000g supernatant extracts of homogenized mammary gland on DEAE cellulose in 0.005M KH_2PO_4 buffer pH 8.4.

See text for details of assay procedure and elution conditions



GRAPH XVIII. DEAE cellulose gradient elution of the 105,000g supernatant extract of homogenized mammary gland in 0.005M KH_2PO_4 buffer pH 7.6.

See text for details of assay procedure and elution conditions



3:9. Purification using DEAE cellulose in 0.005M KH_2PO_4 buffer pH 7.6.

3:9:1. Gradient elution from a DEAE cellulose column (3.2 x 30cm) of the 105,000g supernatant obtained as in section 2:7:4 using a gradient elution from 0 to 0.8M NaCl in 0.005M KH_2PO_4 buffer pH 7.6.

Protein was estimated by OD 280 readings, conductivity of samples read at room temperature and samples assayed as in section 2:12, after dialysis in 0.05M NH_4OH-NH_4Cl buffer pH 8.4.

Elution gave 9 peaks of activity at conductivities of 2, 4.5, 7, 9, 11.5, 15, 19.5, 21.5 and 23 mmho. See Graph XVIII.

3:9:2. Stepwise elution from DEAE cellulose in 0.005M KH_2PO_4 buffer pH 7.6.

Stepwise elution was carried out as in section 2:7:4 using buffer solutions of conductivities of 2, 10, 15, 21 and 24 mmho. The 10ml fractions of each conductivity were pooled, dialysed against 0.005M KH_2PO_4 buffer pH 7.6 and reeluted on to another DEAE column and an NaCl gradient run in 0.005M KH_2PO_4 buffer pH 7.6.

3:9:3. Gradient elution from DEAE cellulose in 0.005M KH_2PO_4 buffer pH 7.6.

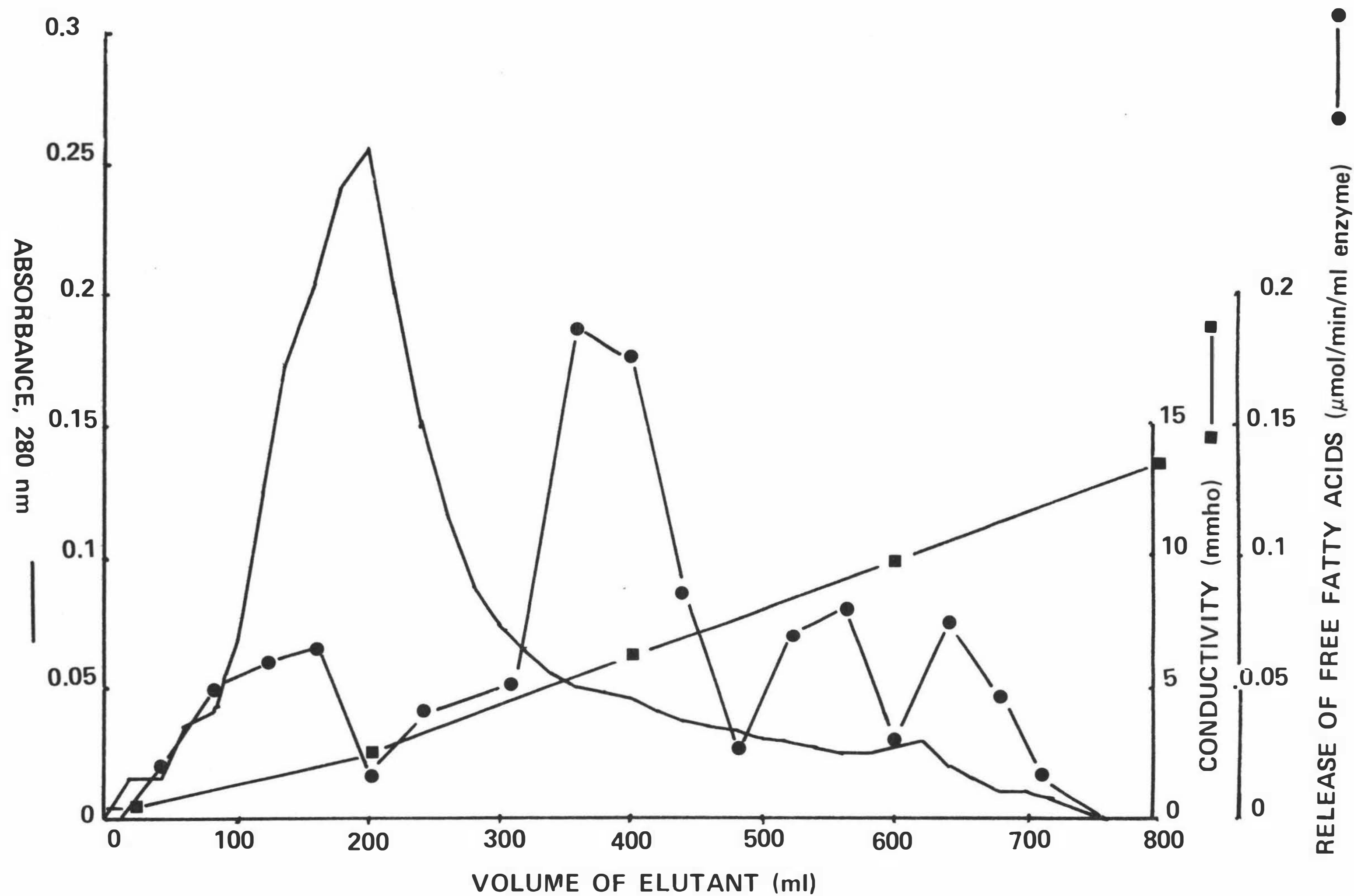
This was carried out as in section 2:7:4.

(a) Gradient elution from 0 to 0.8M NaCl in 0.005M KH_2PO_4 buffer pH 7.6 of conductivity 2 mmho pooled fractions from stepwise elution on DEAE cellulose.

Elution gave one major peak at conductivity 5.5 mmho (53% of total activity) and 3 minor peaks at conductivities of 2 mmho (19% of total activity), 9 mmho (15% of total activity) and 10.5 mmho (14% of total activity).

GRAPH XIX. DEAE cellulose gradient elution of conductivity 2 mmho extracts obtained from stepwise elution of the 105,000g supernatant extract of homogenized mammary gland from DEAE cellulose in 0.005M KH_2PO_4 buffer pH 7.6.

See text for details of assay procedure and conditions of elution



Peaks II and III both had specific activities of 3.6 units/mg and Peak IV had a specific activity of 3.7 units/mg. See Graph XIX.

Column dimensions were 2.3 x 20cm and 10ml fractions were collected.

(b) DEAE cellulose gradient elution from 0 to 0.8M NaCl in 0.005M KH_2PO_4 buffer pH 7.6 of conductivity 10 mmho pooled fractions from stepwise elution on DEAE cellulose.

Gradient elution gave 9 peaks of activity at conductivities of 2, 5, 8, 10, 13, 15, 17.8, 20 and 23 mmho. Approximate percentages of the total activity in each peak are as follows:

2 mmho 8.6%; 5 mmho 18.4%; 8 mmho 12.1%; 10 mmho 12.7%; 13 mmho 7.9%; 15 mmho 11.8%; 17.8 mmho 8.4%; 20 mmho 5.5% and 23 mmho 14.6%.

Specific activity was low for the initial peaks but increased for the latter peaks. Specific activities for the peaks are as follows:

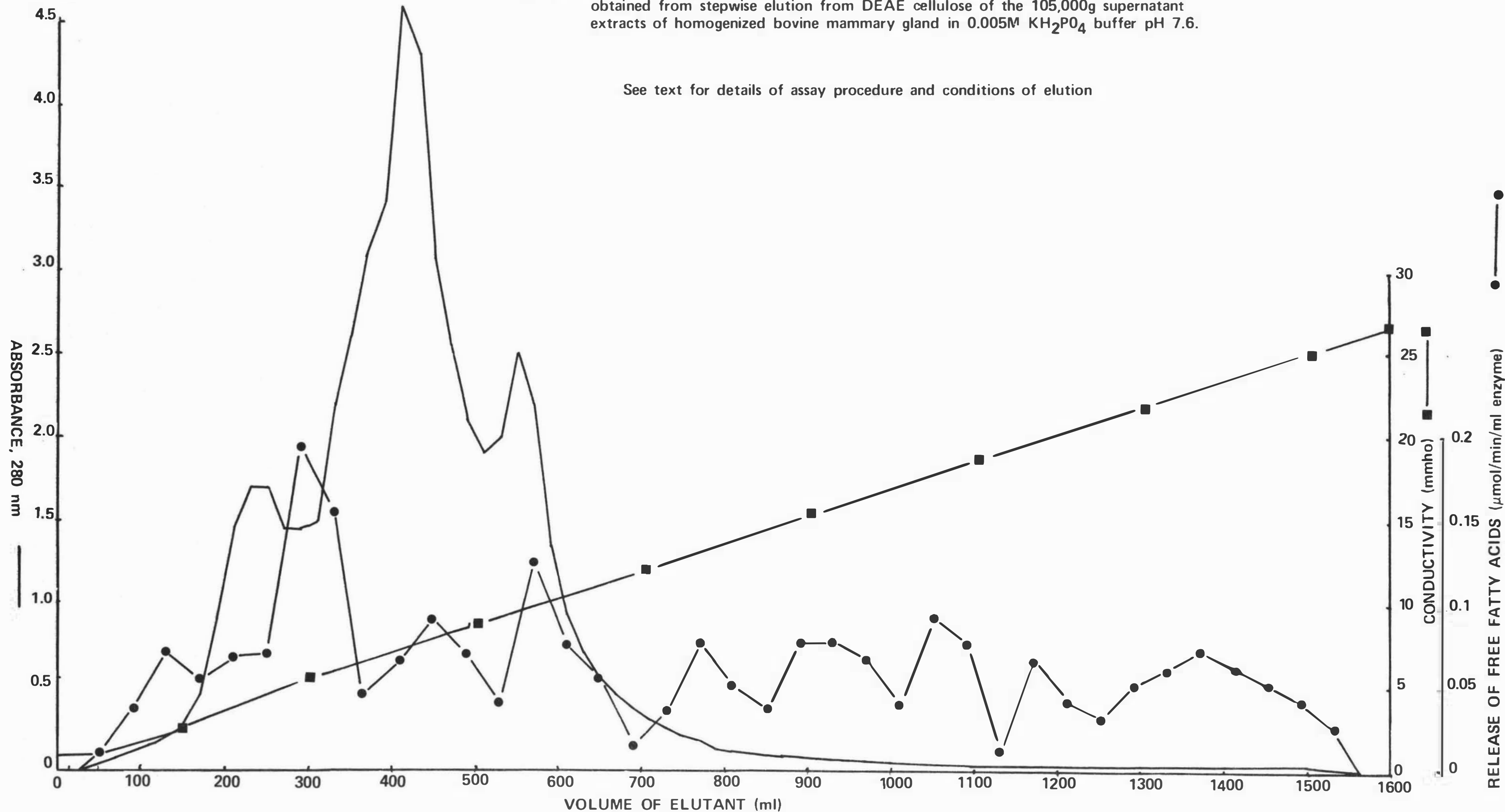
2 mmho 0.35 units/mg; 5 mmho 0.13 units/mg; 8 mmho 0.03 units/mg; 10 mmho 0.05 units/mg; 13 mmho 0.45 units/mg; 15 mmho 1.3 units/mg; 18 mmho 2.57 units/mg; 20 mmho 2.7 units/mg and 23 mmho 3.5 units/mg.

See Graph XX.

Column dimensions were 3.2 x 30cm, 10ml fractions were collected.

GRAPH XX. Gradient elution from DEAE cellulose of conducticity 2–10 mmho extracts obtained from stepwise elution from DEAE cellulose of the 105,000g supernatant extracts of homogenized bovine mammary gland in 0.005M KH_2PO_4 buffer pH 7.6.

See text for details of assay procedure and conditions of elution



(c) DEAE cellulose gradient elution from 0 to 0.8M NaCl in 0.005M KH_2PO_4 buffer pH 7.6 of conductivity 15 mmho pooled fractions from stepwise elution on DEAE cellulose in 0.005M KH_2PO_4 buffer pH 7.6.

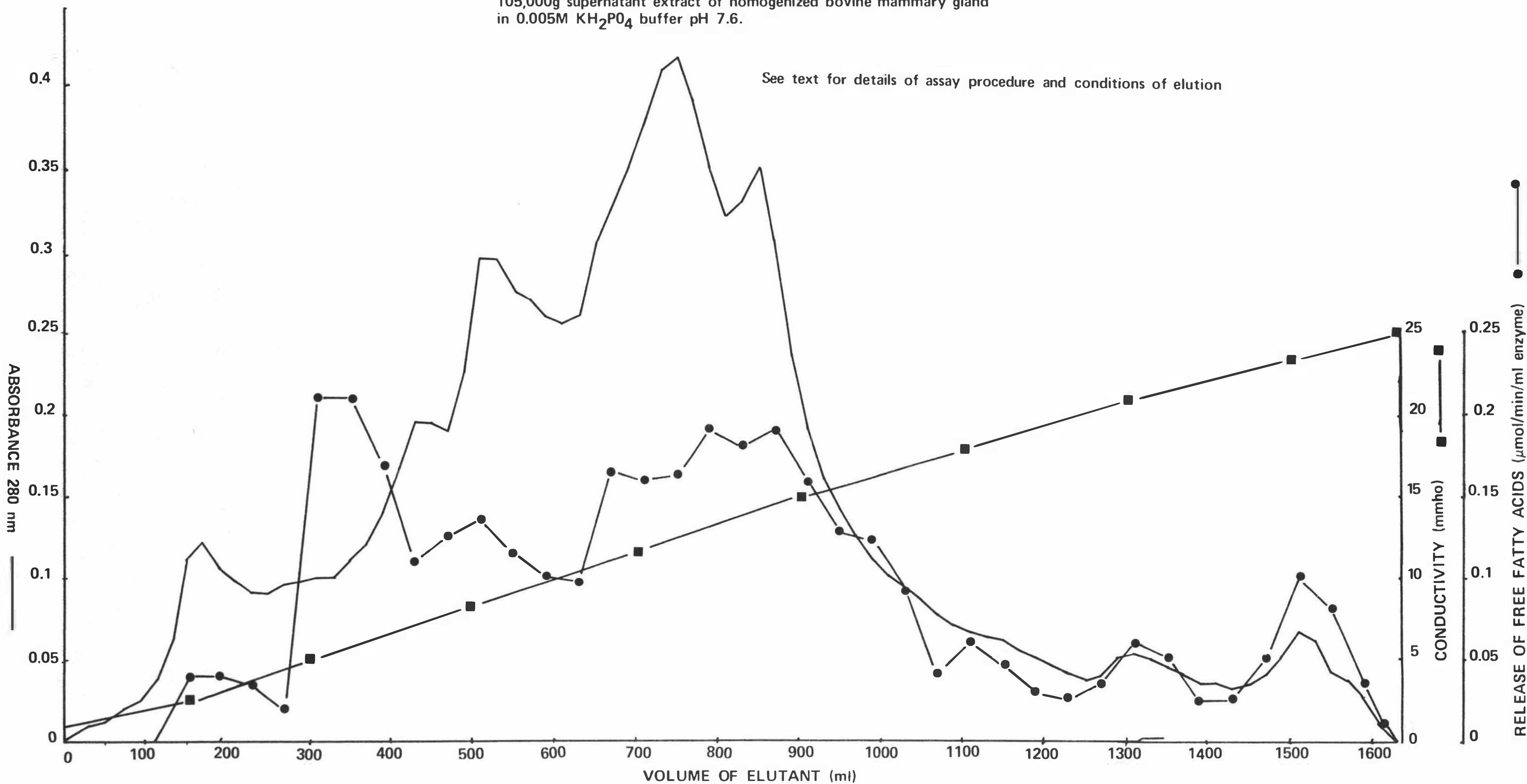
Gradient elution gave 9 peaks of activity. A small peak at a conductivity of 3 mmho (specific activity 0.33 units/mg) was followed by a larger peak at a conductivity of 5.5 mmho (specific activity 2.1 units/mg). A smaller peak appeared at a conductivity of 8.5 mmho with 3 larger less defined peaks at conductivities of 11 mmho, 13 mmho and 14.5 mmho. Specific activity of these latter 4 peaks was low (0.5 units/mg, 0.5 units/mg, 0.53 units/mg and 0.53 units/mg for peaks of conductivities 8.5, 11, 13 and 14.5 mmho respectively). Three smaller peaks at conductivities of 18, 20 and 23 mmho had higher specific activities of 0.9 units/mg, 1.1 units/mg and 1.5 units/mg. respectively. Approximate percentages of the total activity appearing in each peak was 3%, 16.9%, 14.3%, 3%, 4.1% and 8% for peaks of conductivities 3, 5.5, 8.5, 18, 20 and 23 mmho respectively. The other 52% of the activity was approximately evenly distributed between the three peaks at conductivities of 11, 13 and 14.5 mmho (see Graph XXI).

Column dimensions were 3.2 x 30cm 10ml fractions were collected.

(d) Gradient elution on DEAE cellulose from 0 to 0.8M NaCl in 0.005M KH_2PO_4 buffer pH 7.6 of pooled fractions at conductivity 21 mmho obtained from stepwise elution of the 105,000g supernatant of mammary gland extract on DEAE cellulose.

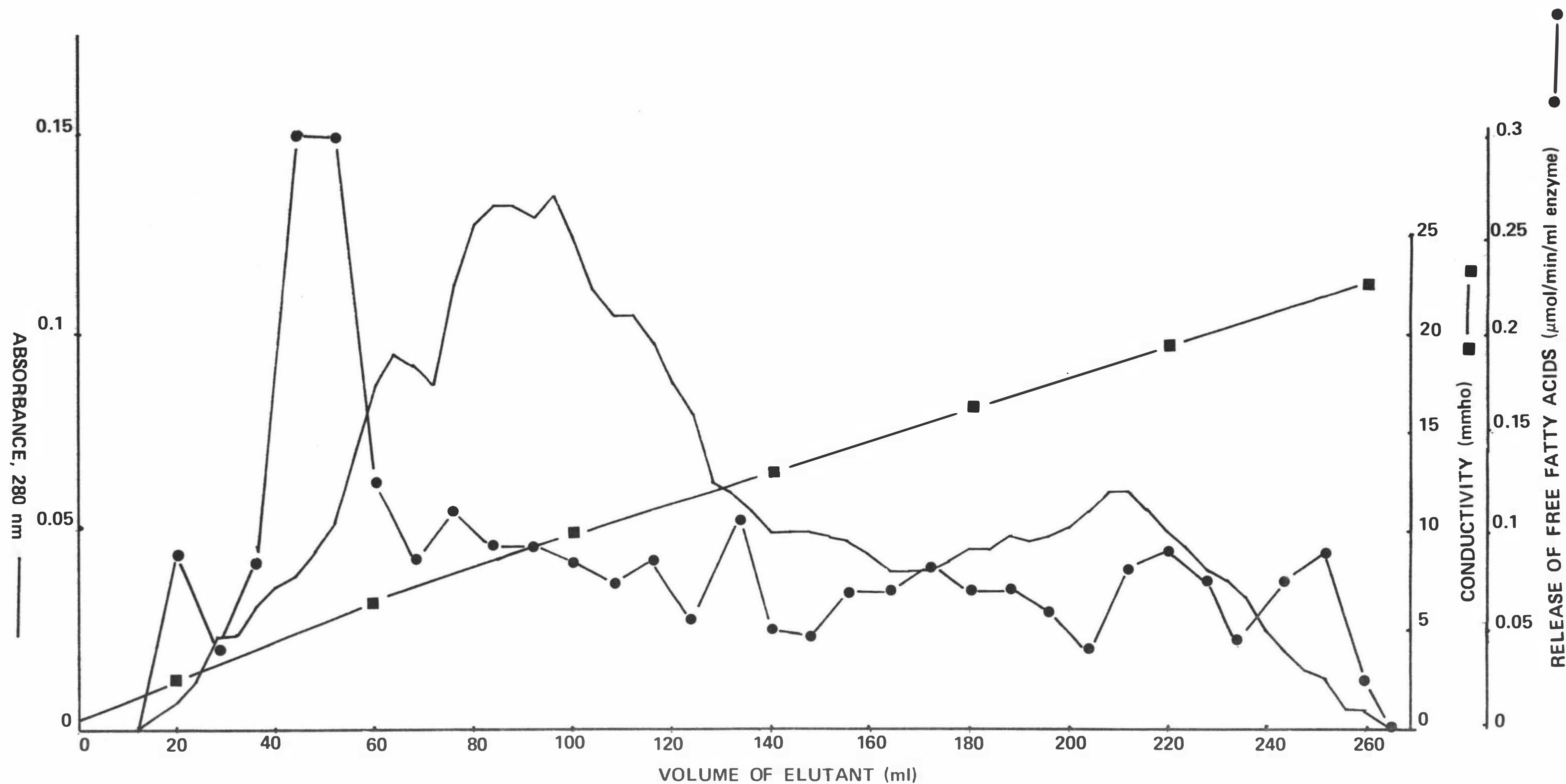
Elution gave a small activity peak at a conductivity of 2 mmho followed by a large peak at conductivity 5.5 mmho comprising 33.6% of the total activity recovered and smaller less defined peaks at conductivities of 8, 11, 12.5, 15, 19 and 22 mmho. Maximum specific activity was obtained by the activity peaks at conductivities of 2 and 22 mmho with activities of 13 units/mg, with the next highest specific activity being that of the major peak at conductivity of 5.5 mmho (7.5 units/mg). In order of decreasing specific activity

GRAPH XXI. Gradient elution from DEAE cellulose of conductivity 10–15 mmho extracts obtained from stepwise elution from DEAE cellulose of the 105,000g supernatant extract of homogenized bovine mammary gland in 0.005M KH_2PO_4 buffer pH 7.6.



GRAPH XXII. Gradient elution from DEAE cellulose of conductivity 15–21 mmho extracts obtained from stepwise elution from DEAE cellulose of the 105,000g supernatant extract of homogenised bovine mammary gland in 0.005M KH_2PO_4 buffer pH 7.6.

See text for details of assay procedure and conditions of elution



the order of remaining activity peaks are:

15 mmho 2.0 units/mg; 19 mmho 1.8 units/mg;
12.5 mmho 1.6 units/mg; 8 mmho 1.units/mg and
11 mmho 0.85 units/mg.
(See Graph XXII.)

Column dimensions were 2.3 x 20cm, 2ml fractions were collected and the gradient was from 0 to 0.8M NaCl.

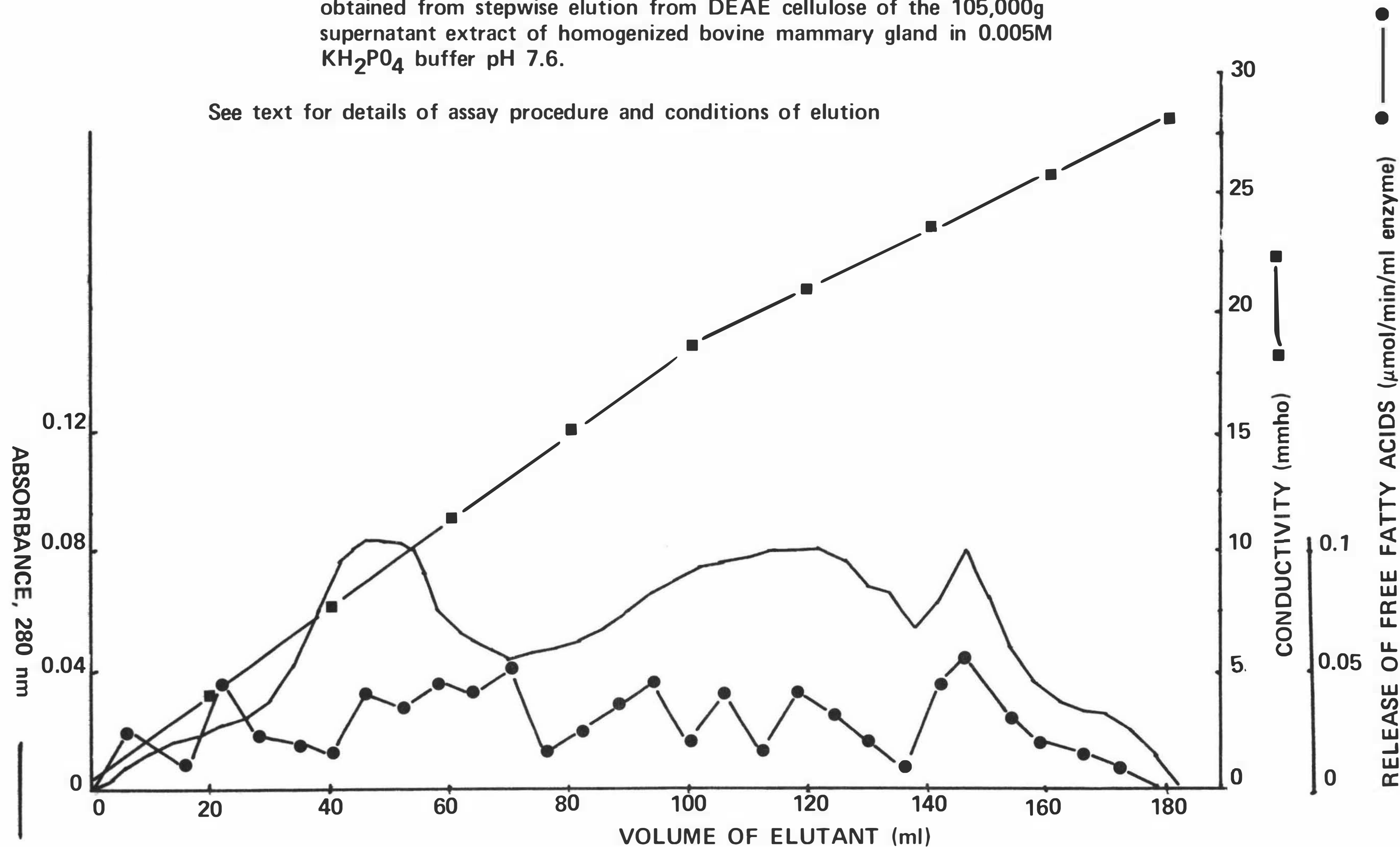
(e) DEAE gradient elution from 0 to 0.8M NaCl in 0.005M KH_2PO_4 buffer pH 7.6 of pooled fractions of conductivity 24 mmho from stepwise elution of the 105,000g supernatant of mammary gland extracts on DEAE cellulose in 0.005M KH_2PO_4 buffer pH 7.6.

Elution gave 9 peaks of activity at conductivities of 1.5, 4.5, 8.5, 11, 13, 17, 19.5, 21 and 24 mmho, comprising 4.3%, 12.7%, 8%, 10.4%, 11.1%, 11.7%, 5%, 10.4% and 26% of the total activity recovered respectively. Specific activity of the peaks varied from a maximum of 2.5 units/mg for Peak I at a conductivity of 1.5 mmho, to a minimum of 0.47 units/mg for Peak III at a conductivity of 8.5 mmho. (See Graph XXIII.)

Column dimensions were 1.4 x 20cm, 2ml fractions were collected and the gradient was from 0 to 0.8M NaCl.

GRAPH XXIII. Gradient elution from DEAE cellulose of conductivity 21–24 mmho extracts obtained from stepwise elution from DEAE cellulose of the 105,000g supernatant extract of homogenized bovine mammary gland in 0.005M KH_2PO_4 buffer pH 7.6.

See text for details of assay procedure and conditions of elution



3:10. Sephadex G200 gel chromatography of elution peaks from DEAE cellulose.

Column dimensions and techniques are as in section 2:8. All assays were carried out as described in section 2:12.

(a) Sephadex G200 gel chromatography of the activity peak eluted at conductivity 5 mmho from gradient elution on DEAE cellulose in 0.005M KH_2PO_4 buffer pH 7.6 of pooled extracts of conductivity 15 mmho obtained through stepwise elution of the 105,000g supernatant of homogenized mammary gland on DEAE cellulose. (See sections 3:9:2 and 3:9:3 (c)).

Gel filtration gave 7 peaks of activity, a small peak at the void volume (5.9% of total activity), followed by 3 larger peaks (comprising 20.4%, 20.2% and 18.8% of the total activity respectively) and then 3 smaller peaks comprising 10.9%, 14.8% and 8.1% of the total activity respectively. 14 units of activity was put on the column and 15.41 units recovered giving an overall recovery of 110%.

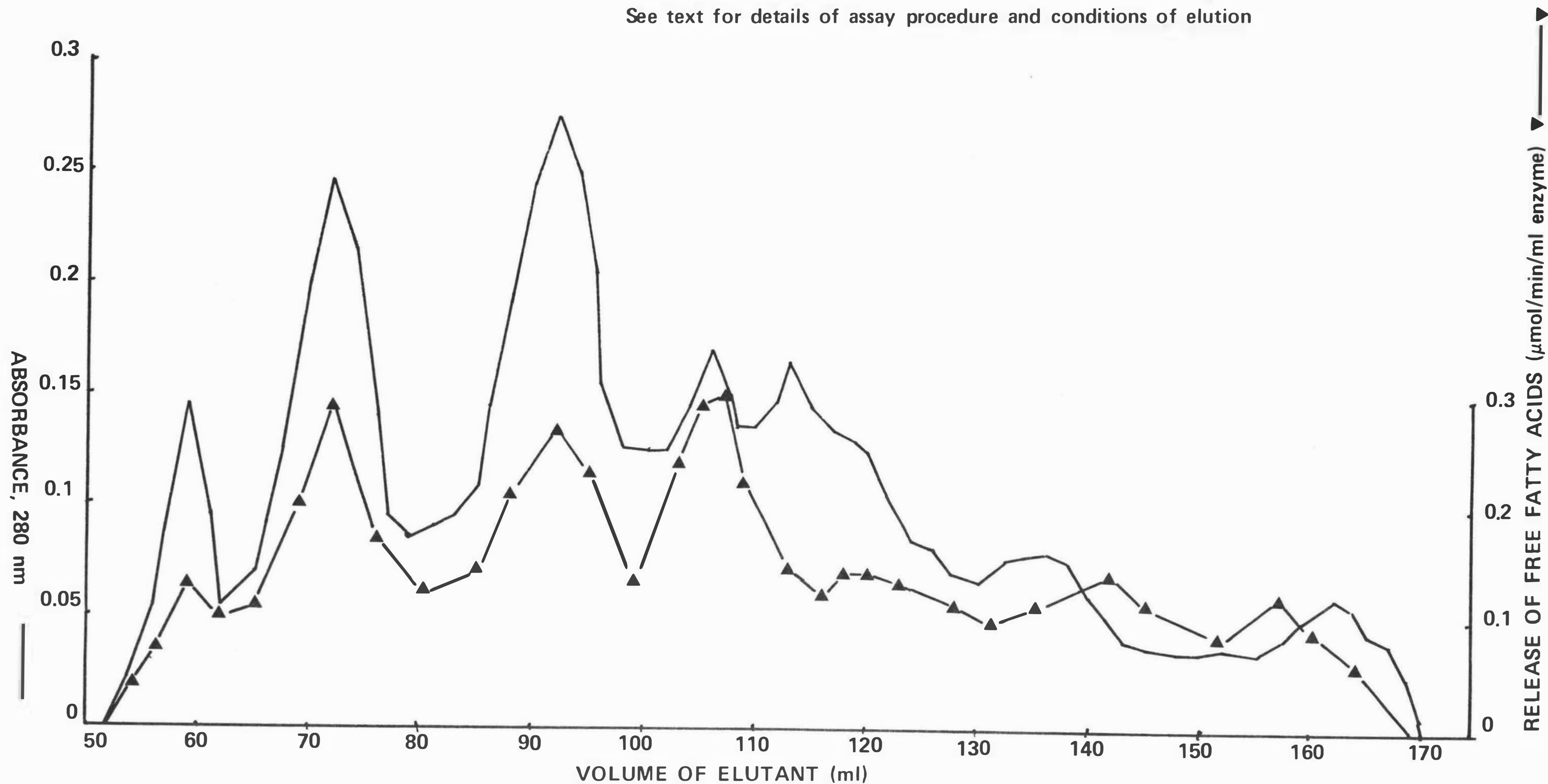
Highest specific activity obtained were for peaks VII and VIII with activities of 2.6 units/mg and 2.4 units/mg respectively with lower specific activities of 0.9 units/mg, 1.2 units/mg, 1 unit/mg, 1.7 units/mg and 1.1 units/mg recorded respectively for Peaks I, II, III, IV and V. (See Graph XXIV).

(b) Sephadex G200 gel chromatography of the activity peak eluted at conductivity 15 mmho from gradient elution on DEAE cellulose in 0.005M KH_2PO_4 buffer pH 7.6 of pooled extracts of conductivity 10 mmho obtained through stepwise elution of the 105,000g supernatant of homogenized mammary gland on DEAE cellulose. (See sections 3:9:2 and 3:9:3 (b)).

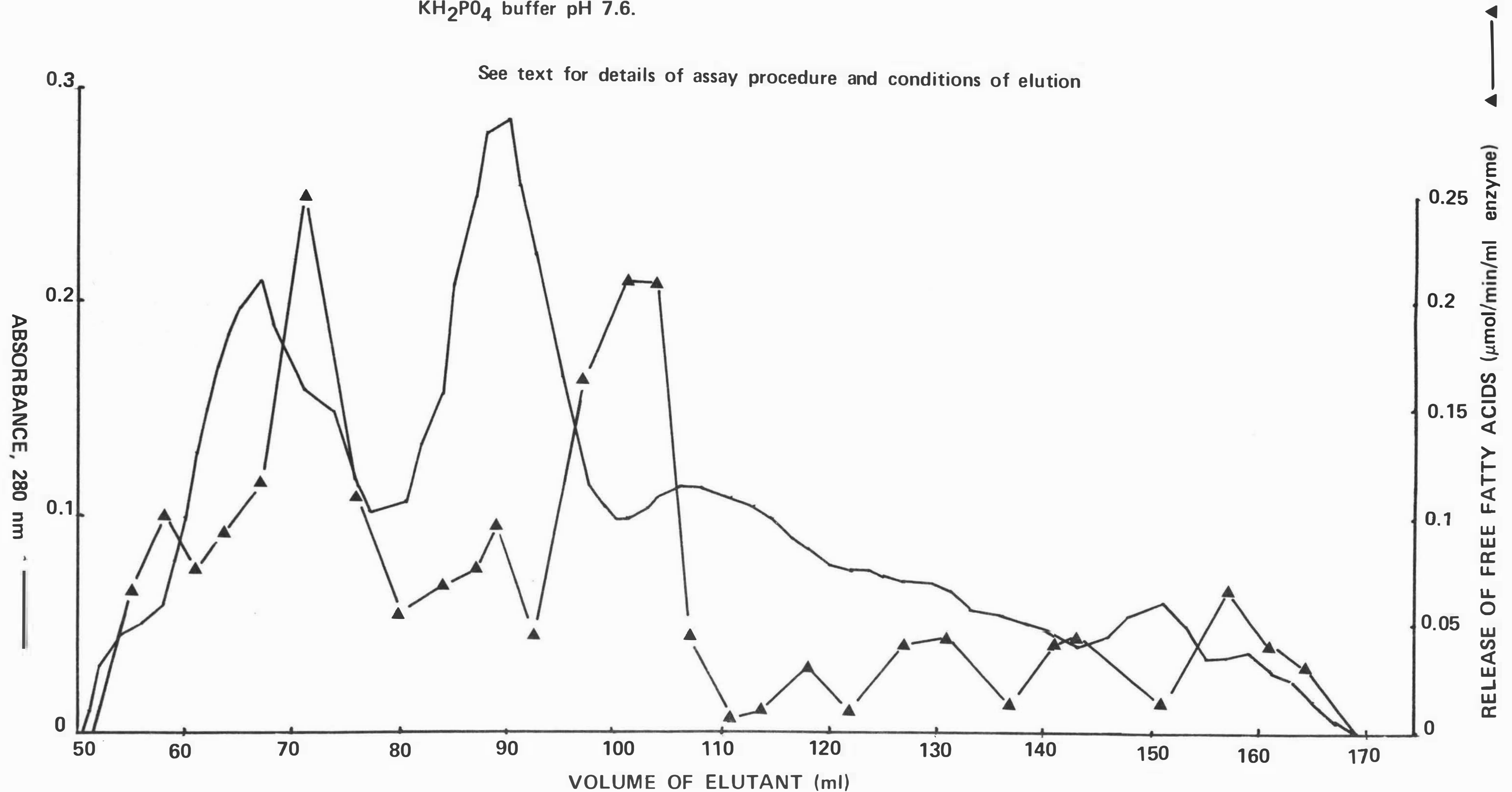
Activity was distributed into 8 peaks, a small peak at the void volume (7.8% of total activity) followed by a large peak comprising 32.5% of the total activity, then a small peak (11.5% of total activity), followed by another large peak (27% of total activity)

GRAPH XXIV. Sephadex G200 gel chromatography of the conductivity 5 mmho eluted peak from gradient elution on DEAE cellulose in 0.005M KH_2PO_4 buffer pH 7.6 of the conductivity 10–15 mmho extract obtained from stepwise elution from DEAE cellulose of the 105,000g supernatant extract of homogenized bovine mammary gland in 0.005M KH_2PO_4 buffer pH 7.6.

See text for details of assay procedure and conditions of elution



GRAPH XXV. Sephadex G200 gel chromatography of the activity peak eluted at conductivity 15 mmho from gradient elution on DEAE cellulose in 0.005M KH_2PO_4 buffer pH 7.6 of the conductivity 2–10 mmho extract obtained from stepwise elution from DEAE cellulose of the 105,000g supernatant extract of homogenized bovine mammary gland in 0.005M KH_2PO_4 buffer pH 7.6.



and 4 smaller peaks comprising 2.7%, 5.4%, 4.9% and 7.9% of the total activity respectively in order of elution from the column.

Maximum specific activity obtained was for Peak IV with an activity of 2 units/mg with activities of 1.6, 1.6, 0.3, 0.35, 0.63, 1 and 1.8 units/mg for Peaks I, II, III, V, VI, VII, VIII respectively. 79% of the activity eluted on to the column was recovered. (See Graph XXV.)

(c) Sephadex G200 gel chromatography of pooled extracts of conductivity 24 mmho obtained through stepwise elution of the 105,000g supernatant of homogenized mammary gland extract on DEAE cellulose in 0.005M KH_2PO_4 buffer pH 7.6.

After gradient elution on DEAE cellulose of fractions of conductivity 24 mmho, all fractions were combined, dialysed in 0.05M NH_4OH-NH_4Cl buffer pH 8.4, concentrated and applied to the sephadex G200 column as described in section 2:8.

Activity was distributed into 8 peaks. Approximate percentages of the total activity occurring in each peak in order of elution from the column are:

10%, 8%, 21.4%, 7.5%, 20%, 10.8%, 9.1% and 11.9%.

Specific activities of the peaks in the order as above are:

1.06, 1.8, 2.2, 0.95, 1.7, 1.5, 2. and 1.75 units/mg.

4.595 units were put on the column and 5.56 units recovered, this representing a 128% recovery of activity. (See Graph XXVI.)

GRAPH XXVI. Sephadex G200 gel chromatography of the conductivity 21–24 mmho eluted fractions from stepwise elution combined after gradient elution on DEAE cellulose in 0.005M KH_2PO_4 buffer pH 7.6 of the 105,000g supernatant extract of homogenized bovine mammary gland.

See text for details of assay procedure and conditions of elution

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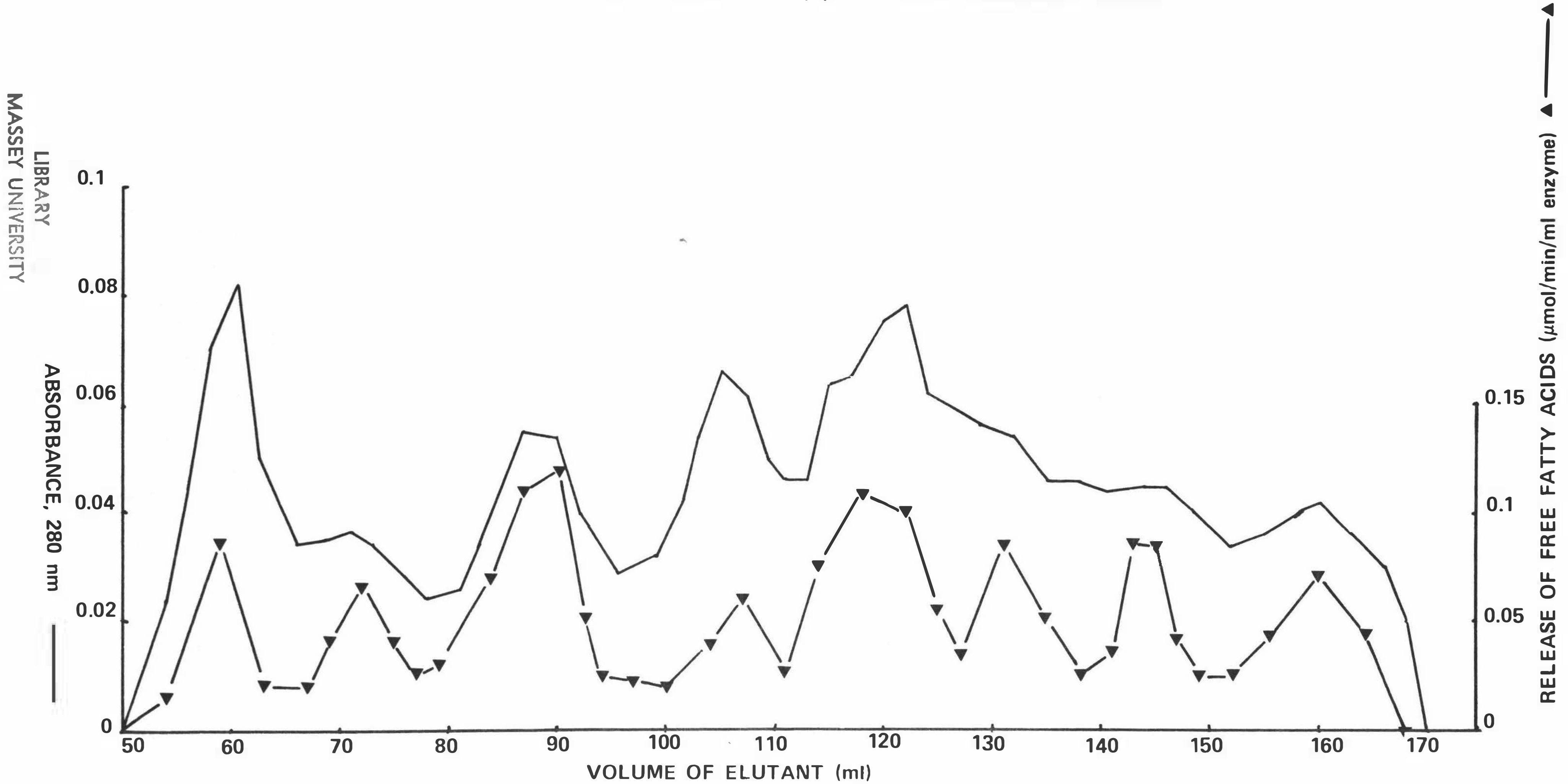


3:11. Sephadex G200 gel chromatography of the 105,000g supernatant of homogenized bovine mammary gland extract.

The 105,000g supernatant enzyme extract was that obtained as in section 3:7:1 (See Table XIV). The extract was dialysed in 0.05M NH_4OH-NH_4Cl buffer pH 8.4, concentrated and eluted on a sephadex G200 column as described in section 2:8:1.

Assays were carried out as described in section 2:12 and OD 280 readings taken of the fractions as an indication of protein concentrations.

Total activity eluted on the column was 0.09 units. Total activity recovered was 3.7 units, i.e. a 4100% recovery was obtained.

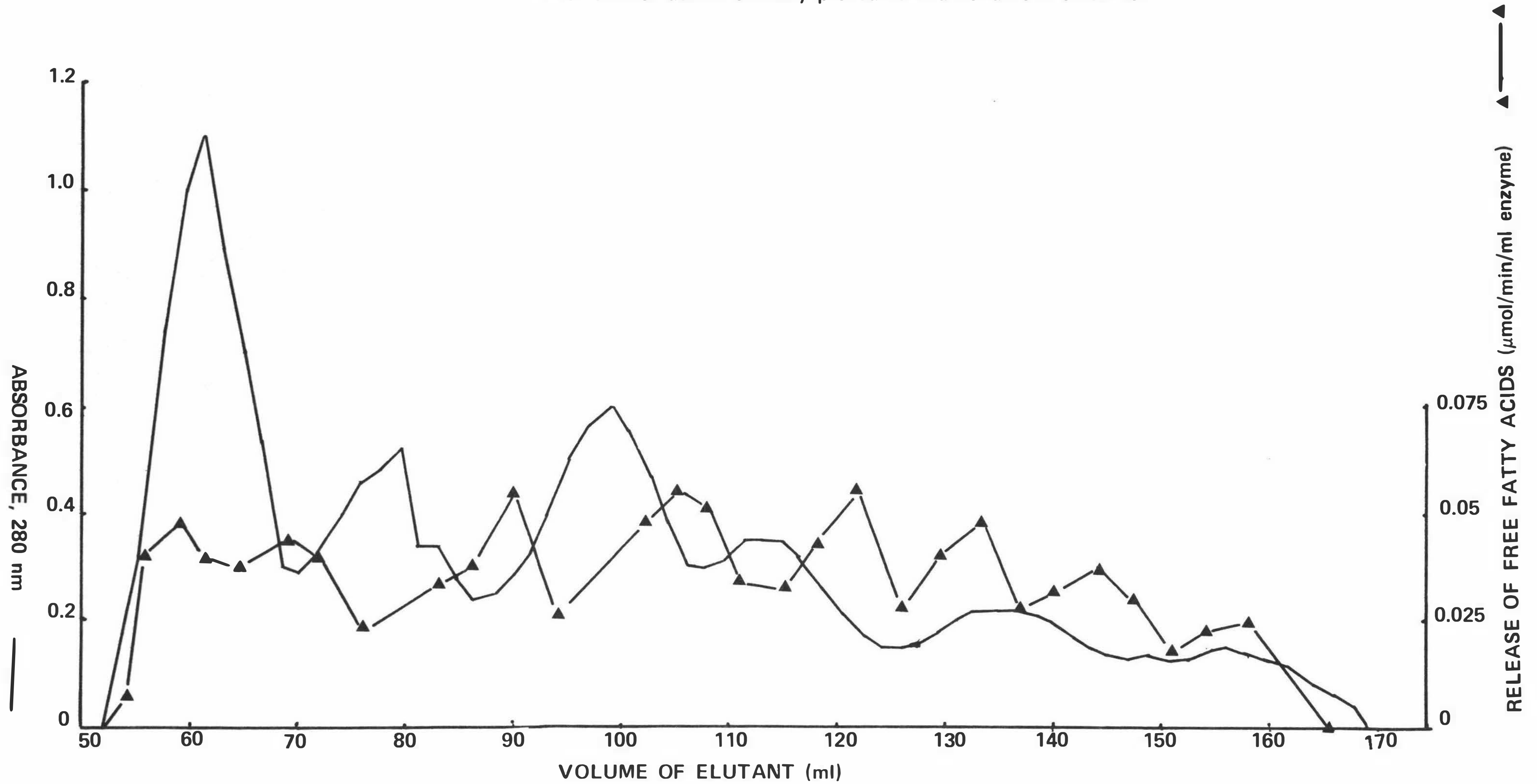
Activity was distributed into eight peaks. Approximate percentages of the total activity occurring in each peak in order of elution from the column are:

12.1%, 11%, 16%, 19%, 11.9%, 10.4%, 10.4% and 7%.
(See Graph XXVII)

By correlation of the distribution coefficients of the activity peaks with those of standard proteins on Graph XXVIII, the peaks had molecular weights corresponding to 263,000, 186,000, 89,000, 52,000, 27,000, 19,000, 13,000 and 8,000 daltons.

GRAPH XXVII. Sephadex G200 gel chromatography of the 105,000g supernatant extract of homogenized mammary gland

See text for details of assay procedure and conditions of elution



3:12. Calebration of the sephadex G200 gel column.

Standard proteins of known molecular weight were eluted through the sephadex G200 column (2.3 x 39.5cm) collecting 2ml fractions. Their elution volumes (Ve) were recorded and their distribution coefficients $\frac{Ve-Vo}{Vt-Vo}$ plotted against the log of their molecular weight to obtain a linear relationship.

The void volume (Vo), determined by elution of dextran blue through the column was 55mls and the total included volume (Vt) determined by elution of copper sulphate through the column was 171mls.

TABLE XV. Molecular weights and distribution coefficients of standard proteins eluted from a sephadex G200 column (2.3 x 39.5cm) with 0.05M NH_4OH-NH_4Cl buffer pH 8.4.

Protein	Molecular Weight Daltons	$\frac{Ve-Vo}{Vt-Vo}$
Catalase (Beef liver)	250000	0.04
Alpha globulin	160000	0.16
Aldolase (Rabbit muscle)	142000	0.19
Bovine serum albumin	66500	0.36
Ovalbumen	45000	0.48
Chymotrypsin	21600	0.65
Myoglobin	17000	0.71
Cytochrome C	12400	0.77

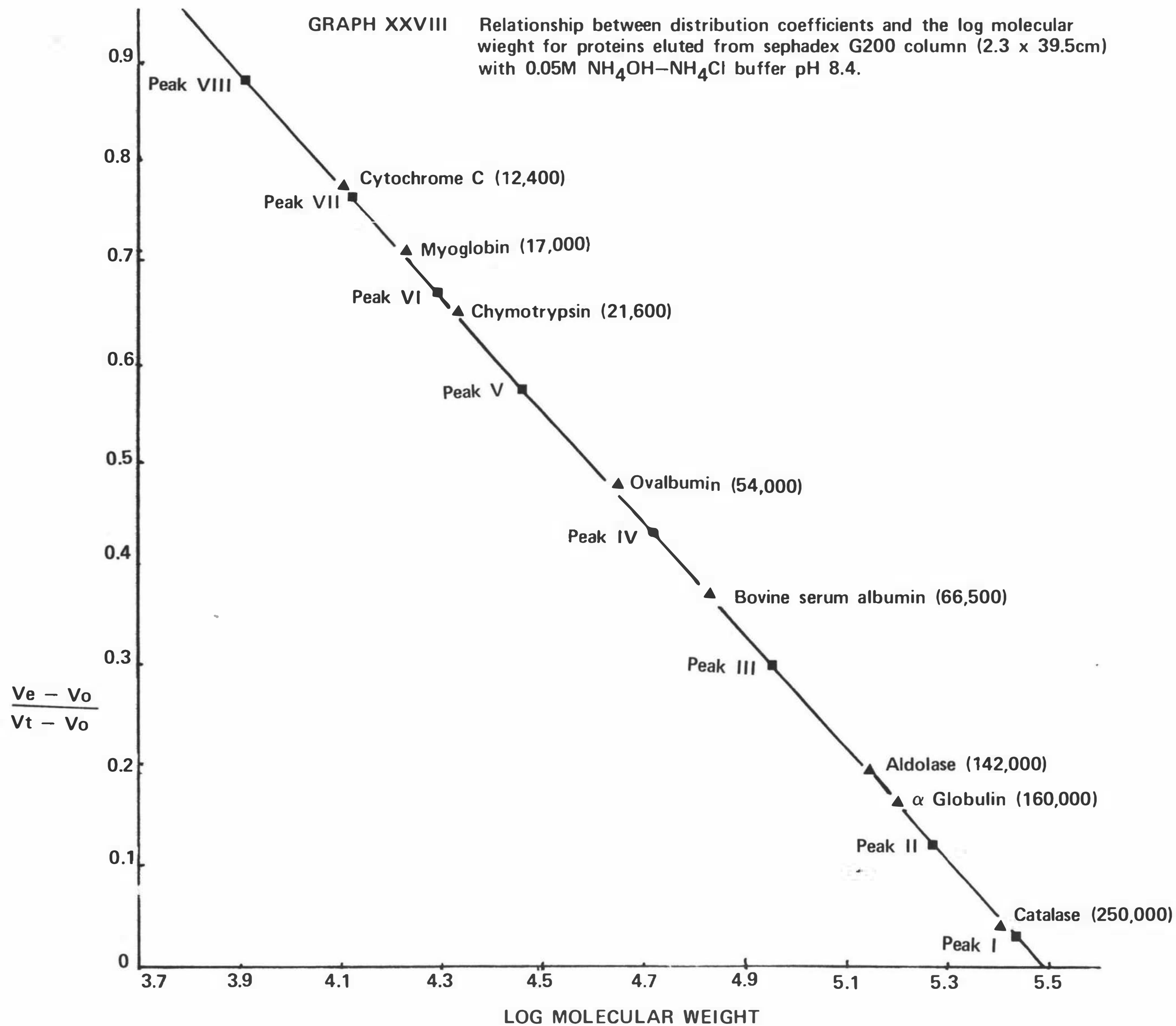

GRAPH XXVIII Relationship between distribution coefficients and the log molecular wieght for proteins eluted from sephadex G200 column (2.3 x 39.5cm) with 0.05M NH_4OH-NH_4Cl buffer pH 8.4.
0.9
0.8
0.7
0.6
0.5
0.4
0.3
0.2
0.1
0
$\frac{Ve - Vo}{Vt - Vo}$
3.7
3.9
4.1
4.3
4.5
4.7
4.9
5.1
5.3
5.5
LOG MOLECULAR WEIGHT
Peak VIII
Cytochrome C (12,400)
Peak VII
Myoglobin (17,000)
Peak VI
Chymotrypsin (21,600)
Peak V
Ovalbumin (54,000)
Peak IV
Bovine serum albumin (66,500)
Peak III
Aldolase (142,000)
α Globulin (160,000)
Peak II
Catalase (250,000)
Peak I

TABLE XVI. Summary table of the molecular weights of the activity peaks eluted from a sephadex G200 column (2.3 x 29.5cm) of activity peaks from elutions on DEAE cellulose in 0.005M KH_2PO_4 buffer pH 8.4 and 7.6 and of the 105,000g supernatant of the total udder extract.

Peak No.	Conductivity of elution peaks from DEAE cellulose in - 0.005M KH_2PO_4 buffer pH 8.4: 2 mmho	5 mmho	0.005M KH_2PO_4 buffer 7.6: 5 mmho	15 mmho	24 mmho	Total Extract	Average Molecular Weight Daltons
I	257000	237000	263000	275000	263000	263000	260000
II	173000	158000	169000	173000	169000	186000	171000
III	82000	86000	82000	93000	89000	89000	87000
IV	57000	52000	50000	57000	48000	52000	53000
V	36000	34000	31000	33000	33000	27000	32000
VI				22000	21000	19000	20000
VII	15000		14000	13000	13000	13000	14000
VIII		9000	8000	8000	8000	8000	8000

TABLE XVII. Summary table of the percentages of the total activity in each of the peaks eluted from sephadex G200 of the activity peaks eluted from DEAE cellulose in 0.005M KH_2PO_4 buffer pH 7.6 and 8.4

Peak No.	Average Molecular Weight Daltons	Conductivity of elution peaks from DEAE cellulose in - 0.005M KH_2PO_4 buffer pH 8.4: 2 mmho	5 mmho	0.005M KH_2PO_4 buffer 7.6: 5 mmho	15 mmho	24 mmho	Total Extract
I	260000	8	10	5.9	7.8	10	12.1
II	171000	20	0.6	20.4	32.5	8	11
III	87000	18	21.8	20.2	11.5	21.4	16
IV	53000	8.6	30	18.8	27	7.5	19
V	32000	25.8	15	10.9	2.7	20	11.9
VI	20000				5.4	19.8	10.4
VII	14000	19.3		14.8	4.9	9.1	10.4
VIII	8000		19	8.1	7.9	11.9	7

3:13. Serum requirement of the activity peaks eluted from DEAE cellulose and sephadex G200 gel chromatography columns.

Assays were carried out as in section 2:12 except that the serum was replaced by distilled water in the assays without serum.

(a) Effect of serum on the activity peaks eluted from DEAE cellulose in 0.005M KH_2PO_4 buffer pH 7.6

A stepwise elution from DEAE cellulose of the 105,000g supernatant fraction of homogenized mammary gland in 0.005M KH_2PO_4 buffer pH 7.6 was carried out as described in section 2:7:4, using buffer solutions of conductivity 2, 5, 8, 10, 13, 15, 18, 21 and 24 mmho. Fractions of each conductivity were collected, pooled and dialysed in 0.005M NH_4OH-NH_4Cl buffer pH 8.4 and concentrated before being assayed.

TABLE XVIII. Effect of serum on the lipase activity peaks eluted from DEAE cellulose in 0.005M KH_2PO_4 buffer pH 7.6.

Peak No.	Conductivity of elution buffer mmho	NaCl conc. of elution buffer Molar	Activity µmoles FFA released/min/ml		Percentage increase (or decrease) $\frac{+serum}{-serum}$
			+serum	-serum	
I	2	0.023	0.1288	0.0045	2762
II	5	0.07	0.0252	0.0353	-40
III	8	0.125	0.0868	0.0493	76
IV	10	0.162	0.0756	0.0409	82
V	13	0.22	0.042	0.0095	342
VI	15	0.252	0.0308	0.028	10
VII	18	0.31	0.014	0.0941	-577
VIII	21	0.375	0.0308	0.0442	-43
IX	24	0.44	0.0252	0.0156	50

Peak I showed greatest activation by serum with an overall percentage increase in activity in the presence of serum over the activity in the absence of serum of 2762%. A 40% inhibition by serum was recorded

for Peak II followed by a progressive increase in activation by serum from Peak III to Peak V. Peak VI showed the least activation by serum of 10% and Peaks VII and VIII both showed inhibitions by serum with decreasing inhibition from Peak VII to Peak VIII with the final peak showing a 50% activation by serum.

(b) Effect of serum on the activity peaks eluted from a sephadex G200 column with 0.05M NH_4OH-NH_4Cl buffer pH 8.4.

Enzyme solutions used were the activity peaks eluted from the sephadex G200 column in section 3:11 of the 105,000g supernatant fraction of homogenized mammary gland extract.

TABLE XIX. Effect of serum on the lipase activity peaks eluted from a sephadex G200 column (2.3 x 39.5cm).

Peak No.	Molecular Weight Daltons	Activity µmoles FFA released/min/ml +serum	-serum	% increase (or decrease)
I	263000	0.0475	0.04	19
II	186000	0.045	0.011	309
III	89000	0.055	0.007	685
IV	52000	0.055	0.325	-482
V	27000	0.055	0.0015	3633
VI	19000	0.0475	0.032	50
VII	13000	0.0375	0.035	7
VIII	8000	0.025	0.023	8

Peak V showed the largest activation by serum of 3633% increase in activity in the presence of serum over the activity in the absence of serum. This was followed by Peaks III, II, VI, I, VIII and VII in order of decreasing activation by serum. Peak IV showed a decrease of 482% in the presence of serum

3:14. Effect of sodium chloride and protamine sulphate on lipase activity peaks eluted from sephadex G200.

Enzyme solutions were the activity peaks eluted from a sephadex G200 column in section 3:11 of the 105,000g supernatant fraction of mammary gland extracts.

Assays were carried out as in section 2:12 except that in the + NaCl assays the buffer consisted of a solution of 5.6M NaCl in 1M tris HCl buffer pH 8.5 to give a final concentration of 1.4M NaCl in the incubation mixture and in the + protamine sulphate assays the buffer contained 400 µg/ml protamine sulphate to give a final concentration of 100 µg/ml of protamine sulphate in the incubation mixture.

TABLE XX. Effect of NaCl and protamine sulphate on the lipase activity peaks separated on a sephadex G200 column (2.3 x 39.5cm) of the 105,000g supernatant fraction of mammary gland extracts.

Peak No.	Molecular Weight Daltons	Activity µmoles FFA released/min/ml -NaCl -protamine sulphate	+NaCl	+protamine sulphate	% inhibition +NaCl	% inhibition +protamine sulphate
I	263000	0.0475	0	0	100	100
II	186000	0.045	0	0	100	100
III	89000	0.055	0	0	100	100
IV	52000	0.055	0	0	100	100
V	27000	0.055	0	0	100	100
VI	19000	0.0475	0.015	0.009	68.4	81
VII	13000	0.0375	0.023	0.007	38.7	81.4
VIII	8000	0.025	0.021	0.013	16	48

Peaks I to V all showed complete inhibition by both 1.4M NaCl and 100 µg/ml protamine sulphate in the incubation mixture. Peaks VI to VIII showed decreasing inhibition by NaCl from Peak VI to Peak VIII. Both Peaks VI and VII showed equal inhibitions by protamine sulphate with Peak VIII least affected by the presence of protamine sulphate.

3:14:1. Effect of inhibitors iodoacetate, N ethylmaleimide, 5 5'dithio bis (2 Nitro benzoic acid) and 2,4 dinitrophenol on the activity of mammary gland lipoprotein lipase.

Assays were carried out as in section 2:12 except the buffers containing either 0.4mM iodoacetate, 0.4mM N ethylmaleimide, 0.4mM 5 5'dithio bis (2 Nitro benzoic acid) or 0.4mM 2,4 dinitrophenol were used for the standard assay. Final concentration of the inhibitors in the assay medium after enzyme addition was 0.1mM. Enzyme solution was a diluted solution of the purified enzyme extract obtained as in section 4:6.

Final concentration of enzyme in the incubation medium was 0.0035 mg/ml.

TABLE XXI. Effect of 0.1mM iodoacetate, N ethylmaleimide, 5 5'dithio bis-(2 nitro benzoic acid) and 2, 4 dinitrophenol on purified LPL from lactating bovine mammary gland.

Inhibitor	Activity µmoles FFA released/min/ml	% inhibition
No inhibitor	0.02496	0
2 4 dinitrophenol	0	100
N ethylmaleimide	0.00672	75
iodoacetate	0.01288	50
5 5'dithio bis (2 nitro benzoic acid)	0.01848	29

0.01mM 2, 4 dinitrophenol completely inhibited the enzyme with N ethylmaleimide (mainly specific for free SH groups) partially inhibiting the enzyme.

3:15. Amino acid composition.

The amino acid composition of Peak III (MW 87,000) on sephadex G200 purified as in sections 2:4:5 Method II, 3:5 and 3:6 was carried out as in section 2:13.

TABLE XXII. Amino acid composition of Peak III lipoprotein lipase from sephadex G200 of lactating bovine mammary gland. Values are for 24 hour hydrolysis.

Amino acid	Micromoles	Moles/10^3moles
Lysine	0.5456	97.46
Histidine	0.1654	29.54
Arginine	0.21178	37.83
Aspartic Acid	0.5339	95.37
Threonine	0.30711	54.86
Serine	0.28343	50.63
Glutamic Acid	0.7838	140.01
Methionine	0	0
Proline	0.3253	58.11
Glycine	0.18333	32.75
Alanine	0.45194	80.73
Half Cystine	0.31435	56.15
Valine	0.35789	63.93
Isoleucine	0.13293	23.74
Tyrosine	0.17651	31.53
Phenylalaline	0.24849	44.39
Leucine	0.57644	102.97

SECTION II: Milk Lipoprotein lipase.

3:16. Binding and elution of milk lipoprotein lipase to calcium phosphate gel.

Binding and elution from a hard pan of calcium phosphate gel was carried out as in section 2:4. Assays were carried out as in section 2:12.

TABLE XXIII. Effect of potassium oxalate and sodium citrate on the elution of lipoprotein lipase from Ca_3PO_4 gel.

Enzyme Extract	Volume mls	Activity Units/ml	Total Activity Units	Protein Conc. mg/ml	Total Protein mg	Specific Activity Units/mg	% recovery
Milk	250	0.3217	80.5	45	10,250	0.0071	100
Addition of Ca_3PO_4 gel Filtered Filtrate	250	0.3125	78.05	39.6	9,900	0.0079	97
Ca_3PO_4 gel	100	0.025	2.5	3.5	350	0.0071	3.1
Filtrate from above filtered through Ca_3PO_4 gel Filtrate	250	0.0168	4.225	0.066	16.5	0.24	5.2
Ca_3PO_4 gel washed with 0.1M potassium oxalate Filtrate	100	0.01875	1.875	0.058	7.8	0.32	2.3
Ca_3PO_4 gel washed with 0.05M sodium citrate Filtrate	100	0.02625	2.625	0.043	4.3	0.61	3.3
Ca_3PO_4 gel washed with 0.05M NH_4OH-NH_4Cl buffer pH 8.4 Filtrate	100	0.00375	0.375	0.012	1.2	0.312	0.4
Ca_3PO_4 gel	200	0.355	71.0	49.25	9,850	0.0072	88.2

TABLE XXIV. Effect of sodium chloride and dimethyl formamide (DMF) on the elution of lipoprotein lipase from Ca_3PO_4 gel.

Enzyme Extract	Volume mls	Activity Units/ml	Total Activity Units	Protein Conc. mg/ml	Total Protein mg	Specific Activity Units/mg	% recovery
Milk	60	0.3115	18.7	42	2,520	0.0074	100
Milk filtered through Ca_3PO_4 gel Filtrate	60	0.0094	0.564	0.054	3.24	0.173	3
0.05M NH_4OH-NH_4Cl wash of gel Filtrate	50	0.015	0.75	0.078	3.9	0.19	4
1% NaCl wash of gel Filtrate	50	0.015	0.75	0.034	1.7	0.44	4
2% NaCl wash of gel Filtrate	50	0.0127	0.635	0.026	1.3	0.5	3.4
3% NaCl wash of gel Filtrate	50	0.0112	0.56	0.021	1.05	0.53	3
4% NaCl wash of gel Filtrate	25	0.0187	0.467	0.032	0.8	0.580	2.4
5% NaCl wash of gel Filtrate	25	0.012	0.287	0.018	0.45	0.62	1.5
5% NaCl + 25% DMF wash of gel Filtrate	25	0.01	0.25	0.15	3.75	0.066	1.3
5% NaCl + 50% DMF wash of gel Filtrate	25	0.003	0.075	0.19	4.75	0.015	0.4
Ca_3PO_4 gel in 0.05M NH_4OH-NH_4Cl buffer pH 8.4	100	0.103	10.3	25	2,500	0.004	55

TABLE XXV. Effect of sodium carbonate, sodium sulphate, sodium gluconate and potassium dihydrogen phosphate on the elution of lipoprotein lipase from Ca_3PO_4 gel.

Enzyme extract	Volume mls	Activity Units/ml	Total Activity Units	Protein conc. mg/ml	Total Protein mg	Specific Activity Units/ml	% recovery
Milk	60	0.3115	18.7	42	2520	0.0074	100
Milk filtered through Ca_3PO_4 gel Filtrate	60	0.013	0.78	0.058	3.48	0.22	4.1
0.05M NH_4OH-NH_4Cl buffer pH 8.4 wash of gel Filtrate	50	0.016	0.8	0.062	3.1	0.25	4.2
0.5M Na_2CO_3 wash of gel Filtrate	50	0.0196	9.98	0.043	2.15	0.44	5.2
0.5M Na_2SO_4 wash of gel Filtrate	50	0.0356	1.78	0.08	4.05	0.42	9.5
0.05M sodium gluconate wash of gel Filtrate	50	0.0375	1.875	0.102	5.1	0.37	10
0.5M sodium gluconate wash of gel Filtrate	50	0.0487	2.435	0.095	4.75	0.51	13
0.5M KH_2PO_4 wash of gel Filtrate	50	0	0	0.02	1.0	0	0
1M KH_2PO_4 wash of gel Filtrate	50	0	0	0.03	1.5	0	0

Binding of milk lipoprotein lipase to Ca_3PO_4 gel varied from 95 to 97% of the total activity with 99.7% of the total protein binding to the gel after it had been washed with buffer. 0.1M potassium oxalate followed by 0.05M sodium citrate extracted 5.6% of the bound activity with a maximum purification of 86X obtained in the 0.05M sodium citrate wash. Extraction with sodium chloride gave a total elution of 14% of the bound activity with a maximum purification of 83X obtained in the 5% NaCl wash, representing 1.5% of the total activity.

Total protein eluted was 0.36% of the total bound protein. The addition of dimethyl formamide increased the elution of protein to 0.71% of the total bound protein but decreased the specific activity by about 10X of that of the 5% NaCl wash.

Maximum elution of enzyme was obtained with the sodium gluconate washes with a total extraction of 24% of the bound enzyme in the two washes.

Lower recoveries of 5.4% and 9.3% of the total bound activity were obtained for the Na_2CO_3 and Na_2SO_4 washes respectively. No activity was eluted with the KH_2PO_4 washes. Maximum purification obtained was 70X obtained in the 0.5M sodium gluconate wash. Protein elution was 0.08%, 0.16%, 0.4% and 0.1% of the total bound protein in the Na_2CO_3, Na_2SO_4, sodium gluconate and KH_2PO_4 washes respectively.

3:17. Ultracentrifugation and NaCl extraction of lipoprotein lipase from casein micelles.

Casein was collected by centrifigation of skimmed milk at 105,000g for one hour and the washed precipitate extracted with 1.2M NaCl as described in section 2:17. Assays were carried out as in section 2:12.

TABLE XXVI. Ultracentrifugation and NaCl extraction of milk casein bound lipoprotein lipase.

Enzyme extract	Volume mls	Activity Units/ml	Total Activity Units	Protein conc. mg/ml	Total Protein mg	Specific Activity Units/mg	% recovery
Milk	800	0.23	184	43	34400	0.00535	100
105,000g supernatant	800	0.056	44	27	21600	0.001	24
105,000g ppt. in distilled water	400	0.342	136.8	32	12800	0.0107	74.4
Above ppt. centrifuged 105,000g supernatant fraction	340	0.033	11.22	1.86	632	0.017	6.1
Ppt. fraction in distilled water	360	0.34	122	34	12200	0.01	66.3
Above ppt. centrifuged 105,000g supernatant fraction	340	0.009	3.06	0.32	109	0.027	1.6
Ppt. fraction in NaCl 1.2M	360	0.34	122	33.6	12096	0.0101	66.3
Above ppt. centrifuged 105,000g supernatant fraction	340	0.184	62.56	1.14	387.6	0.161	33.4
Ppt. in 1.2M NaCl	360	0.324	116.8	32.5	11700	0.01	63.4
Above ppt. centrifuged 105,000g supernatant fraction	340	0.127	43.78	0.5	170	0.254	23.7
Ppt. in NaCl	360	0.302	108.7	32	11520	0.0094	59

24% of the total lipase activity of milk was associated with the 105,000g supernatant fraction, i.e. was in a soluble form. The remaining activity was associated with the casein micelles. Total recovery of activity from the casein micelles was 175% with the two sodium chloride washes and of this 88% was still bound to the casein.

Thus a recovery of 86% was obtained in the NaCl washes with a loss of 11.4% of the casein bound activity was obtained.

Maximum purification obtained was 48X from the original skimmed milk in the second NaCl wash.

3:18: Effect of pH on the solublized lipase activity from casein in the presence and absence of serum.

Assays were carried out as in section 2:12 except assay time was 10 minutes and the following buffers used in the range of pH:

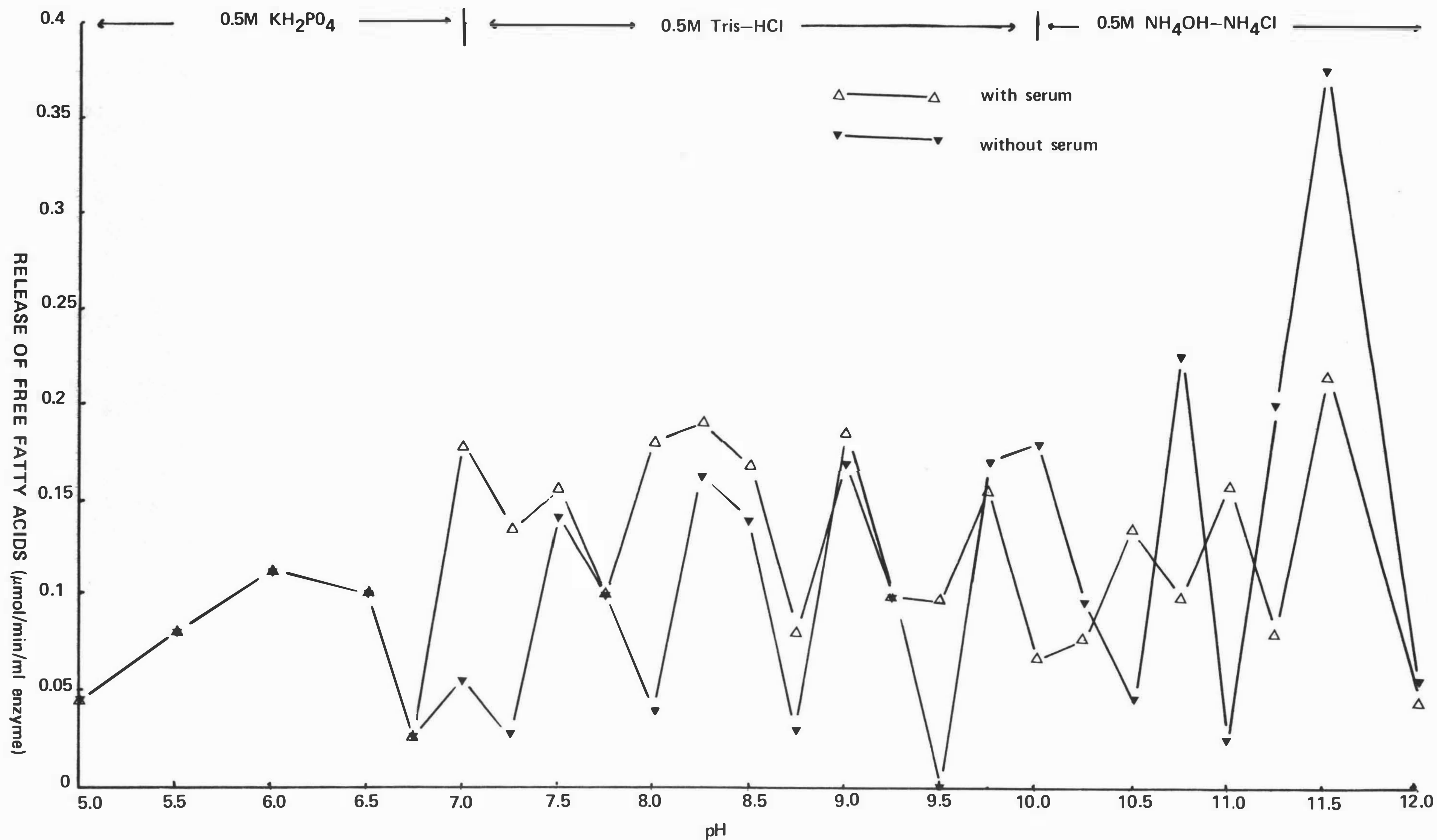
pH 5 - 7	0.5M KH_2PO_4
pH 7 - 10	0.5M Tris HCl
pH 10 - 12	0.5M NH_4OH-NH_4Cl

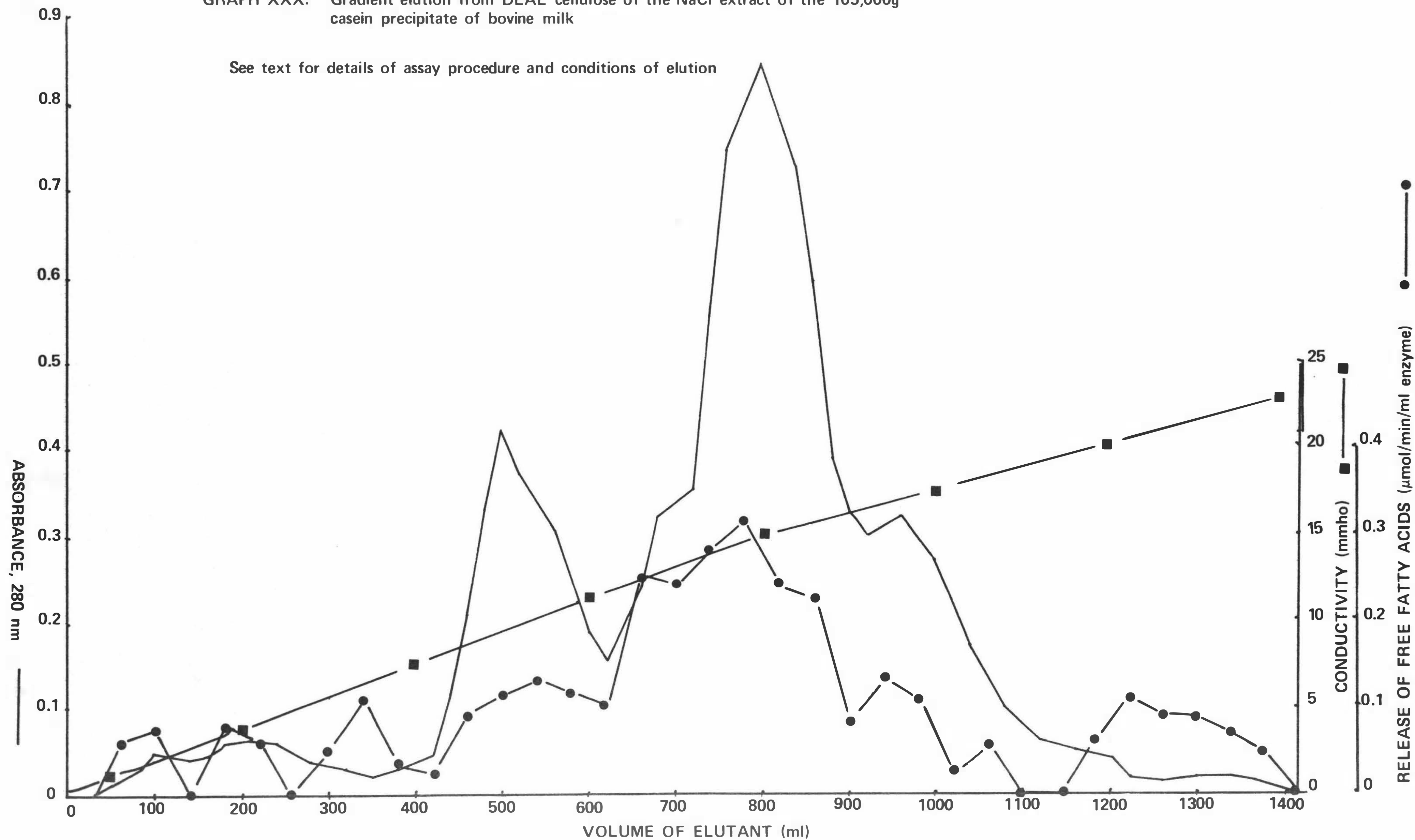
The assay in the absence of serum was carried out as above except that serum was replaced by distilled water. Enzyme source was the first NaCl extract of the 105,000g casein precipitate of skimmed milk obtained as in section 3:17.

In the presence of serum 9 peaks of activity were recorded at pH's of 6, 7, 7.5, 8.25, 9, 9.75, 10.5, 11 and 11.5. In the absence of serum 8 peaks of activity occurred at pH's of 6, 7, 7.5, 8.25, 9, 10, 10.75 and 11.5. Serum had an activating effect on lipases of pH optimums 7 (263.1% increase), 7.5 (7% increase) and 8.25 (18% increase). Both peaks at pH 10.5 and 11 had an obligate requirement for serum. Serum inhibited lipases of pH optimums 9 (5% inhibition), 9.75 (6% inhibition), 10 (61% inhibition), 10.75 (55.5% inhibition) and 11.5 (43% inhibition). See Graph XXIX. The figures taken were the average of two assays which were $\pm$ 3% of each other.

GRAPH XXIX. Effect of pH on lipase activity of the NaCl extract of the 105,000g casein precipitate of bovine milk in the presence and absence of serum

See text for details of assay conditions





GRAPH XXX. Gradient elution from DEAE cellulose of the NaCl extract of the 105,000g casein precipitate of bovine milk
See text for details of assay procedure and conditions of elution
ABSORBANCE, 280 nm
0.9
0.8
0.7
0.6
0.5
0.4
0.3
0.2
0.1
0
0
100
200
300
400
500
600
700
800
900
1000
1100
1200
1300
1400
VOLUME OF ELUTANT (ml)
CONDUCTIVITY (mmho)
25
20
15
10
5
0
RELEASE OF FREE FATTY ACIDS (µmol/min/ml enzyme)
0.4
0.3
0.2
0.1
0

3:19. DEAE cellulose gradient elution of the NaCl extract of the 105,000g casein precipitate.

A DEAE cellulose column gradient of the NaCl extract of the casein precipitate after dialysis in 0.005M KH_2PO_4 buffer pH 7.6 was carried out as in section 2:17.

Assays were carried out as in section 2:12. 10ml fractions were collected.

230mls of NaCl extract I were eluted on to the column representing 43.7 units of activity. Total activity recovered was 131.7 units, i.e. a 300% recovery of activity was obtained.

Activity was distributed into 9 peaks eluted at conductivities of 2, 3.5, 6.5, 10, 12.5, 15, 17, 18.5 and 20.5 mmho. Approximate percentages of the total activity occurring in each peak are as follows:

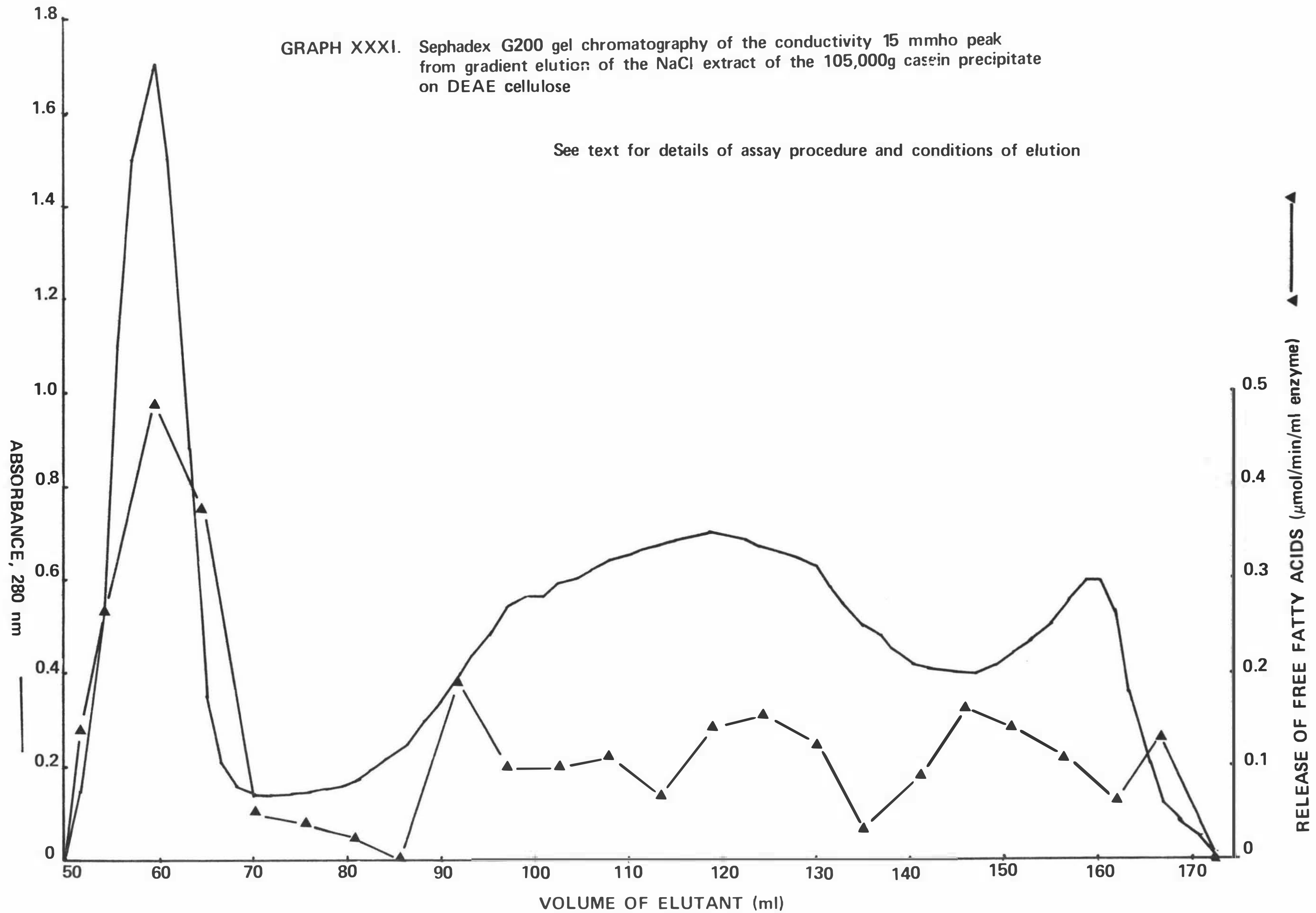
> 3.7%, 3.6%, 5.9%, 14.7%, 12.6%, 35.3%, 8.4%, 2% and 13.4% for peaks eluted at conductivities of 2, 3.5, 6.5, 10, 12.5, 15, 17, 18.5 and 20.5 mmho respectively.

Maximum specific activity was 4.4 units/mg obtained for both peaks eluted at conductivities of 2 and 6 mmho representing a 880X purification over the skimmed milk.

Column dimensions were 3.2 x 30cm.

3:20. Sephadex G200 gel chromatography of conductivity, 15 mmho activity peak from a gradient elution on DEAE cellulose of the NaCl extract of the 105,000g casein precipitate.

Gel filtration was carried out on a sephadex G200 column (2.3 x 39.5cm) equilibrated with 0.05M NH_4OH-NH_4Cl buffer pH 8.4 at 2°C. The sample was dialysed in 0.05M NH_4OH-NH_4Cl buffer pH 8.4 and concentrated to 1ml before being applied to the column. 2ml


GRAPH XXXI. Sephadex G200 gel chromatography of the conductivity 15 mmho peak from gradient elution of the NaCl extract of the 105,000g casein precipitate on DEAE cellulose
See text for details of assay procedure and conditions of elution
ABSORBANCE, 280 nm
RELEASE OF FREE FATTY ACIDS (μmol/min/ml enzyme)
VOLUME OF ELUTANT (ml)
1.8
1.6
1.4
1.2
1.0
0.8
0.6
0.4
0.2
0
0.5
0.4
0.3
0.2
0.1
0
50
60
70
80
90
100
110
120
130
140
150
160
170

fractions were collected. Assays were carried out as in section 2:12. Total activity applied to the column was 25.2 units. Total activity recovered was 14 units, i.e. a 55.5% recovery of activity was obtained.

Activity was distributed into 6 peaks. A large peak, near the void volume comprising 39% of the total activity recovered and 5 other peaks comprising 10%, 9.4%, 16.4%, 17.3% and 4.2% of the total activity recovered respectively in order of elution from the column. By comparing their distribution coefficients with those of the standards obtained as in section 3:12, approximate molecular weights of the peaks were 263,000, 82,000, 48,000, 28,000, 12,000 and 8,000 daltons.

3:21. Sephadex G200 gel chromatography of the Ca_3PO_4 gel filtrate of skimmed milk.

11.4mls of the Ca_3PO_4 gel filtrate of skimmed milk obtained as in section 3:16 (Table XXIII) was dialysed in 0.05M NH_4OH-NH_4Cl buffer pH 8.4, concentrated to 1ml and applied to the sephadex G200 column (2.3 x 39.5cm) and eluted with 0.05M NH_4OH NH_4Cl buffer pH 8.4 collecting 2ml fractions. Assays were carried out as in section 2:12.

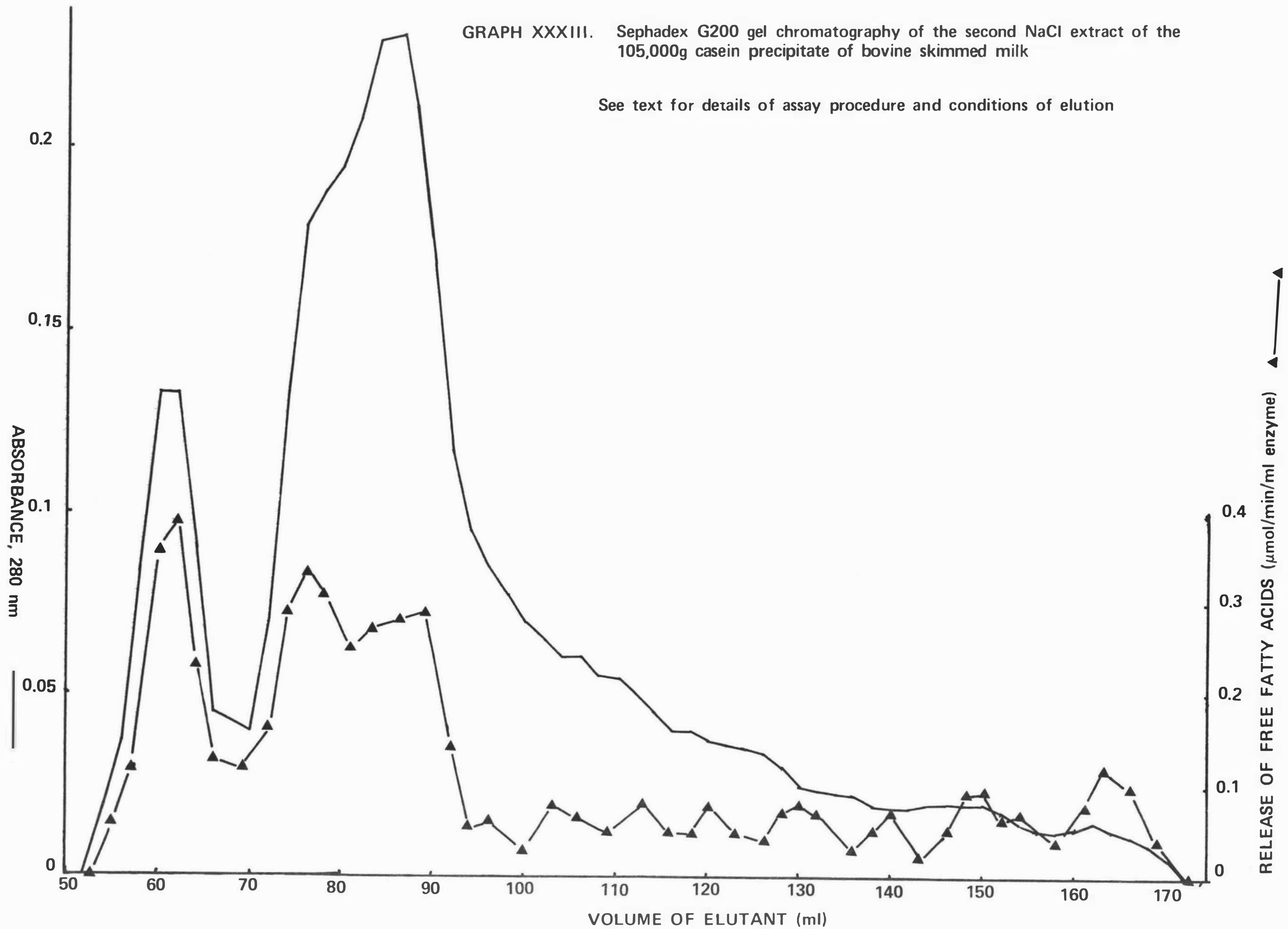
Total activity applied to the column was 1.94 units and 1.8 units were recovered, i.e. % recovery = 92.7%.

Activity was distributed into 13 peaks. Approximate percentages of the total activity occurring in each peak, in order of elution from the column are:

14.4%, 8.8%, 5.6%, 7.3%, 2.7%, 9.1%, 8.2%, 10%, 5.2%, 3.1%, 7%, 4.6% and 13.2%.

By comparing the distribution coefficients of the activity with those of the standards obtained as in as in section 3:12, approximate molecular weights of the activity peaks are:

240,000, 148,000, 120,000, 89,000, 71,500, 57,000, 41,000, 31,000, 22,000, 15,000, 11,000, 9,000 and 8,000 daltons.



GRAPH XXXIII. Sephadex G200 gel chromatography of the second NaCl extract of the 105,000g casein precipitate of bovine skimmed milk

See text for details of assay procedure and conditions of elution

ABSORBANCE, 280 nm
0.2
0.15
0.1
0.05
0
RELEASE OF FREE FATTY ACIDS (μmol/min/ml enzyme)
0.4
0.3
0.2
0.1
0
50
60
70
80
90
100
110
120
130
140
150
160
170
VOLUME OF ELUTANT (ml)

3:22. Sephadex G200 gel chromatography of the 1.2M NaCl extract of the 105,000g casein precipitate.

Gel filtration was carried out on a sephadex G200 column (2.3 x 39.5cm) equilibrated with 0.05M NH_4OH-NH_4Cl buffer pH 8.4 at 2°C. The sample was dialysed in eluting buffer and concentrated to 1ml before being applied to the column. 2ml fractions were collected. Assays were carried out as in section 2:12.

22mls of the second NaCl supernatant extract of the 105,000g casein precipitate obtained as in section 3:17 was dialysed and concentrated to 1ml and applied to the column.

Total activity eluted on was 3 units. Total activity recovered was 12.46 units representing a recovery of 415%.

Activity was distributed into 12 peaks. A large peak near the void volume containing 20.8% of the total activity recovered followed by another two large overlapping peaks containing 22.1% and 23% of the total activity recovered respectively in order of elution from the column. The remaining 34% of the recovered activity was distributed into the remaining 9 smaller peaks. The 3 large peaks had molecular weights corresponding to 340,000, 148,000 and 89,000 daltons respectively. See Graph XXXIII.

3:23. DEAE cellulose gradient elution of Peaks II and III eluted from a sephadex G200 column of the 1.2M NaCl supernatant extract of the 105,000g casein precipitate.

A DEAE cellulose column gradient elution in 0.005M KH_2PO_4 buffer pH 7.6 was carried out as in section 2:18 using a gradient from 0 to 0.8M NaCl collecting 2ml fractions. The column dimensions were 1.4 x 20cm. Assays were carried out as in section 2:12.

Enzyme solution was the combined fractions from the sephadex G200 gel filtration of the second NaCl supernatant extract of the

105,000g casein precipitate in section 3:22 eluted between 72 and 93mls. The sample was dialysed against starting buffer before being applied to the column.

Total activity eluted on was 5.65 units. Total activity recovered was 15.97 units, representing a recovery of 282.6%.

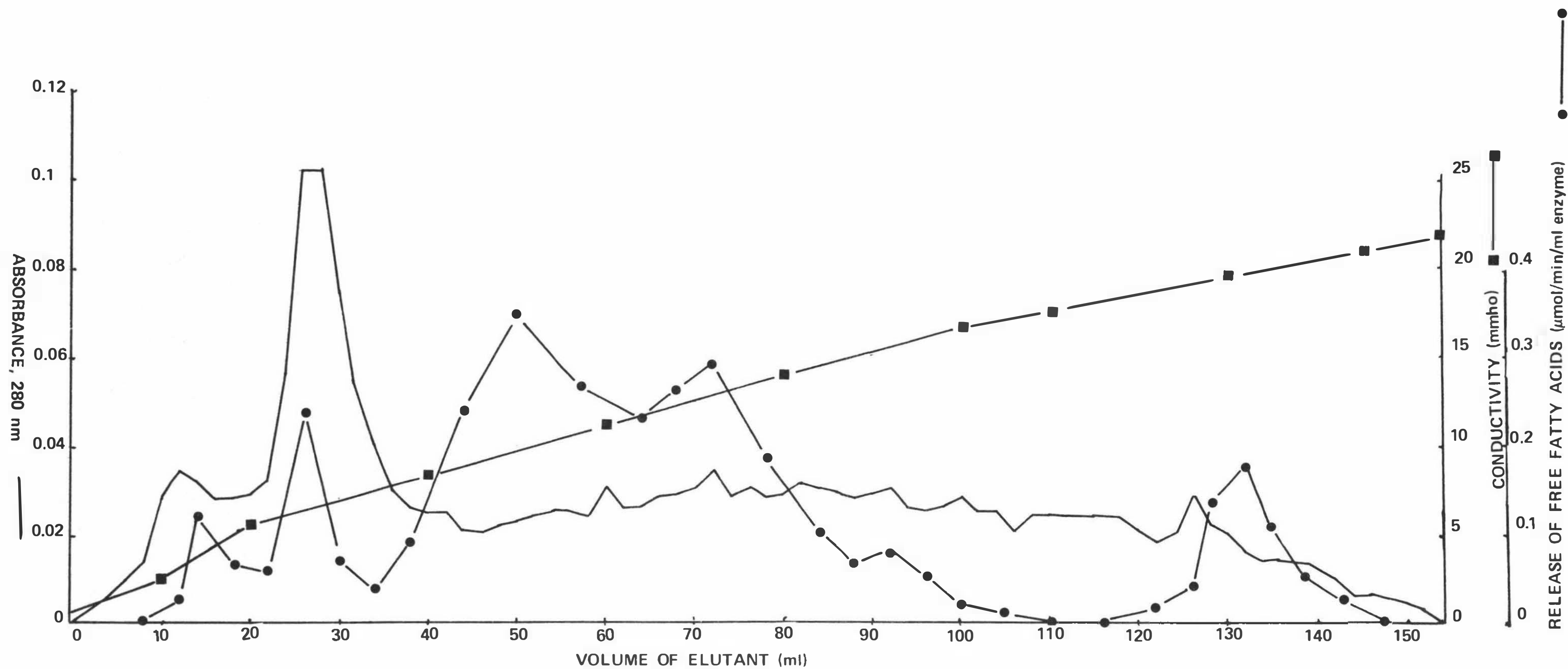
Activity was distributed into 6 peaks. The activity peaks were eluted at conductivities of 3.5, 6.5, 10, 12.5, 15 and 20 mmho. Approximate percentages of the total activity recovered occurring in each peak are:

> 4.2%, 9%, 42.3%, 29.9%, 4.2% and 10.6% of the activity peaks of conductivity 3.5, 6.5, 10, 12.5, 15 and 20 mmho respectively.

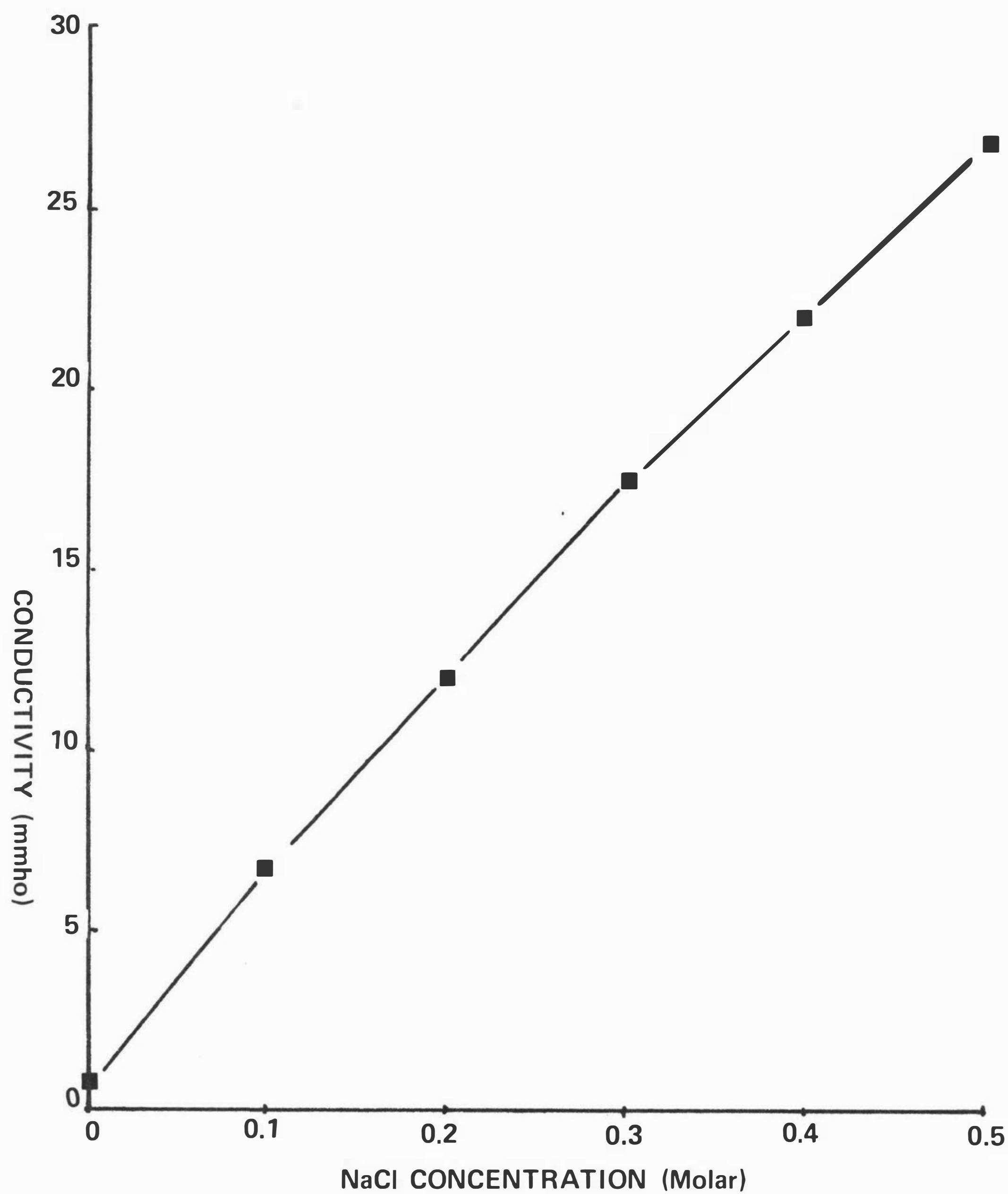
Maximum specific activity obtained was 16 units/mg, representing a purification of 3018X from the skimmed milk. See Graph XXXIV.

GRAPH XXXIV. DEAE cellulose gradient elution of peaks II and III eluted from a sephadex G200 column of the second NaCl supernatant extract of the 105,000g casein precipitate of skimmed milk

See text for details of assay procedure and conditions of elution



GRAPH XXXV. Relationship between conductivity and molarity of NaCl in 0.005M KH_2PO_4 buffer pH 7.6.



CHAPTER IV

DISCUSSION OF RESULTS

DISCUSSION.

Homogenates of mammary gland extracts were characterized by low levels of LPL in the 16,500g supernatant fraction which would bind to TG (3.8 - 10%), a high percentage in the 16,500g particulate fraction (60%) and a high percentage in the top cream layer (10%) which may indicate a constant release of enzyme from the tissue and thus a constant loss of LPL from the mammary gland, and is probably due to epithelial cell breakdown during milk formation as suggested by Korn (1962). Both soluble and insoluble forms of LPL may indicate that LPL is located both at the surface of the epithelium in a readily releasable form and also bound tightly to the particulate fraction and appears to be largely inextractable. The soluble enzyme fraction may be present at the epithelial cell surface where most of the lipolysis of plasma TG takes place. The particulate enzyme may be a store of LPL inside the cell for release to the surface on stimulation by some factor such as heparin where it is continually being lost into the blood stream as according to Robinson (1963), or it may be involved in the lipolysis of lipid emulsions which have formed vacuoles inside the cell as observed by Schoelf and French (1968) and Scow et al (1972).

Askew et al (1970) obtained 20% of the total activity of the bovine mammary gland in the soluble fraction and in agreement with the high proportion of enzyme in the particulate fraction of rat heart as shown by Gartner and Vahouny (1966) and in adipose tissue as shown by Hallet and Auditore (1967). Askew et al (1970) recorded 10% of the total lipolytic activity of the udder was from the milk fraction which is in agreement with my finding of 10% of the total udder LPL in the cream layer.

An activity of 0.0012 µmoles FFA released/min/mg for the total mammary gland homogenate, is in close agreement with the activity of

0.0018 µmoles FFA released/min/mg observed by Baldwin and Emery (1973). Askew et al (1971) obtained a higher figure of 0.007 µmole FFA released/min/mg for total udder homogenates and Baldwin and Emery (1973) obtained an activity of 0.0033 µmole FFA released/min/mg for cows fed a restricted roughage high grain diet, indicating a wide variation in activity can occur from cow to cow depending on diet and period of lactation.

An activity of 0.007 µmoles FFA released/min/mg in the second udder extract also verifies this wide variation observed in LPL activity of total udder homogenates.

Many pH optima in mammary gland extracts suggests a complex lipase system whereby TG is hydrolysed over a wide range of pH values. pH optima of 9, 8.6, 7.9, 7 and 6.5 have been reported by Frankel and Tarassuk (1956), Schwartz et al (1956) and Harper et al (1956) from cows' milk which could apply for mammary gland lipases which are synthesized in the mammary gland and secreted into the milk. The variation in activation or inhibition caused by serum over the different pH values may suggest different lipases at these pH values, as suggested by Schwartz et al (1956) for cows' milk lipase, one requiring serum for activity and the other not, and the relative composition of the two lipases which have the same pH optimum may determine the net serum requirement at that particular pH. Only at a pH of 8.5 was there a complete serum requirement for the activity peak and thus a true LPL , or this could be due to a shift in pH optimum of the peak at pH 9 to 8.5 in the presence of serum since the serum or lipoprotein activator which, according to Brady and Higgins (1967), determines the pH optimum of the TG hydrolyse activity by their pH dependence on binding to the enzyme. This is unlikely as inhibition occurred in the presence of serum at pH 9. This inhibition is probably due to an inhibitor present in serum as postulated by Askew et al (1970), Robinson (1963) and Iverius et al (1972) which inhibits the enzyme with this pH optimum. Thus LPL had an alkaline pH optimum between 8.2 and 8.5 which is in agreement with the findings of Korn (1959) for adipose tissue LPL, Brady and Higgins (1967) for rat heart, and Askew et al (1970) for bovine mammary gland LPL. Lipase activity has been shown to be active over a wide

range of pH values which are present in various parts of the cell and may vary from tissue to tissue by Assman et al (1973). The function of these lipases with pH optimum extremes is not known. Thus the measurement of LPL activity is best undertaken at pH 8.5 as pH values either above or below this would measure other lipase activity as well.

All of the 16,500g fractions of homogenized mammary gland extracts showed greater activity in the presence of tallow rather than olive oil. The small difference in the activity of the top cream layer in the presence of tallow rather than olive oil could be due to the presence of cream in the enzyme extract and thus competition between the two substrates for the active site. LPL activity has been shown to be decreased in cows fed restricted roughage high grain diets which also has been shown to decrease the net total milk content (partially dependent on LPL activity) and also increase the unsaturated FA content and decreased the saturated FA content by Askew et al (1971). Thus LPL having a greater specificity for long chain saturated FA TGs rather than long chain unsaturated FA TGs explains the results of Askew et al (1971) whereby feeding restricted roughage high grain diets increase the unsaturated FA TG level of the blood relative to the saturated FA TG level, and thus less conversion of blood fat TGs into milk fat would occur and these blood fat TGs would be diverted elsewhere, probably to the adipose tissue for storage.

Lipoprotein lipase required a specific ratio of serum to TG and gave an optimum at 50% serum for a serum tallow substrate and the crude enzyme extract gave 13% of the maximum activity in the absence of serum, indicating some other hydrolase activity not requiring serum as cofactor such as MG hydrolase activity, or could be due to the presence of other lipases other than LPL as postulated by Askew et al (1970), or could be due to LPL which has protein tightly bound to the enzyme. Askew et al (1970) also obtained maximum activity at 50% serum with 90% of the activity occurring at 25% serum using Ediol as TG substrate containing a 50% TG emulsion of coconut oil. The inhibition of activity at high serum concentrations could be due to an inhibitor present in the serum as postulated by Askew et al (1970)

and Robinson (1963) who also obtained inhibition at high serum concentrations.

The low binding to the TG emulsion suggests a small percentage of the LPL is in a soluble form. Sonification of the TG emulsion increased enzyme binding from 3% to 6.6% of the use of an homogenized TG emulsion and thus the use of a more stable TG emulsion gives better binding. Inhibition occurred at the TG binding stage using a sonified TG emulsion but not using an homogenized TG emulsion. This could probably be due to greater binding to the sonified TG emulsion and thus a greater inhibition. In all cases a high recovery of enzyme activity was obtained from the TG bound enzyme suggesting a release of inhibition on elution of the enzyme from the TG which could be due to either the presence of the olive oil emulsion acting competitively with the tallow substrate since the enzyme is more active against tallow rather than olive oil substrates, or due to an inhibitor which is also TG binding and may thus explain the 440 - 300% recovery of activity when the enzyme was eluted from the TG emulsion.

The greater than 100% recovery of activity when the mammary gland extracts were eluted through Ca_3PO_4 gel suggest the removal of an inhibitor which is bound to the Ca_3PO_4 gel. Thus the inhibition caused by the binding to the olive oil TG emulsion was not due to the presence of an olive oil substrate contamination which showed lower activity than tallow substrate, but due to a specific inhibitor of the enzyme. The low activation of enzyme activity of Extract II compared with Extract I after elution through Ca_3PO_4 gel was probably due to a lower concentration of inhibitor in the enzyme extract and thus the inhibitor appears to be readily extracted into the supernatant layer, or could be due to variation in the proportion of enyzmic types, one of which is inhibited by the inhibitor to a greater extent than the other and thus the more sensitive enzyme is more readily extracted into the supernatant extract than the less sensitive enzyme. The greater percentage of activity bound to the Ca_3PO_4 gel in Extract II compared with Extract I could be due to LPL enyzmic types which are bound more strongly to the epithelial cell membrane and thus may bind more strongly to the Ca_3PO_4 gel.

The 2.1X and 4.5X purification obtained for Extracts II and I respectively was mainly due to relief of inhibition by an inhibitor binding to the Ca_3PO_4 gel rather than removal of contaminating protein. Clear supernatant fractions were obtained, thus the removal of high molecular weight particles present in the udder extract can be carried out on a large scale compared to the limited volumes that can be used with ultracentrifugation to remove the large precipitable particles.

Filtration on silica gel also gave activation of activity indicating relief of inhibition. A greater purification was obtained compared with the Ca_3PO_4 gel filtration method due mainly to the removal of contaminating protein rather than relief of inhibition caused by an inhibitor present in the extracts. A clear yellow supernatant was obtained thus removing haemoglobin pigments and high MW sedimentable particles and again large scale preparations can be carried out to remove suspended particles compared to ultracentrifugation.

Binding and stepwise elution from viscose sulphate gave complete binding and recovery of activity with no activation occurring. The elution of activity over a wide range of ionic strengths required to elute the enzyme may cause irreversible inactivation caused by structural changes to the enzyme as suggested by Korn (1955).

Gradient elution from viscose sulphate gave very low recoveries of activity (28%), most probably due to the high ionic strengths required to elute the enzyme from the sulphate polysaccharide causing irreversible inactivation. Several activity peaks eluted suggests the binding of several different LPLs which bind to varying degrees to the viscose sulphate or the degree of binding may depend on the amount of heparin already bound to the enzyme binding site. Lack of binding specificity of the sulphate for LPL makes purification by this method only on a limited scale due to saturation of the sulphate binding groups by contaminating protein. Binding on to sodium dextran sulphate and precipitation with $CaCl_2$ gave incomplete binding of LPL activity to the sulphate polysaccharide and of this bound activity complete recovery of activity was obtained indicating no inhibition

had occurred by the $CaCl_2$ required to elute the enzyme. A high recovery of activity in the high specific activity fraction, together with a more selective binding of LPL activity makes this method a better alternative than viscose sulphate binding for initial stages of purification. Elution of the enzyme extract through Ca_3PO_4 gel would remove the lipoproteins which also bind strongly to sulphate groups as shown by Burstein et al (1970).

Binding to sodium dextran sulphate and precipitation by divalent cations by the method of Burstein et al (1970) gave a wide distribution of activity in all of the supernatant washes, a low recovery of enzyme activity in the high specific activity fraction (5.6%) and inhibition occurred while bound to the sulphate polysaccharide probably due to steric hinderance caused by the sulphate polysaccharide binding close to the active site. Activity lost in the supernatant fractions was probably due to weak binding of the enzyme or different enzymes which bind to varying degrees to the sodium dextran sulphate as occurs with the viscose sulphate. The use of large scale techniques makes this method practical for the isolation and purification of LPL from udder homogenates where large volumes of enzyme extract are involved.

Preelution of the enzyme extract through Ca_3PO_4 gel would remove lipoproteins which also bind to sulphate groups probably by the same mechanism as LPL.

Elution of the final extract on sephadex G200 gave three activity peaks corresponding to three protein peaks, of which the main activity peak corresponded to a molecular weight of 89,000 daltons. No increase in specific activity was obtained indicating no activation occurred in elution on sephadex G200.

On elution of the major peak from sephadex G200 on DEAE cellulose, two peaks of activity occurred accompanied by a 44% recovery of activity applied to the column. This could be due to dissociation

of the enzyme into two different subunits which have different activities and on dissociation activation may take place which could be due to steric hinderance or competition by the subunits for the substrate when the subunits existed as a complex, or this could be due to two different LPL active proteins with similar molecular weights which are separated on DEAE cellulose which act competitively for the substrate. Since substrate is not rate limiting it would imply that these two proteins exist as a complex which when bound together give a competition for the substrate, whereas if they existed as a separate enzymes on sephadex G200, this competition for substrate would not occur and thus on elution on DEAE cellulose the large activation occurring would not take place. Thus these two active LPL proteins associate to form a complex which is less active than the net activity obtained from the free single active protein units or subunits.

Egelrud and Olivecrond (1972), Greten and Walter (1973), Greten et al (1972) and Havel et al (1973) all used binding to a heparin sephrose column to purify LPL from various sources and all obtained a single lipase peak. In all cases high ionic strength was required to elute the lipase from the heparin sephrose columns and all gave low recoveries of activities on elution, probably due to the high salt concentrations required to elute the enzyme.

The nine peaks eluted from DEAE cellulose could be due to variable binding of a single enzyme which can exist in several conformations and binding would depend on whether or not specific groups were exposed such as may be the case with the elution from the viscose sulphate columns, or could be due to an enzyme complex which is composed of different subunits and each subunit may be active by itself. The large percentage recovery could be due to the relief of an inhibition by the separation of an inhibitor from the enzyme or could be due to transformation of a less active form of the enzyme into another form or forms of higher specific activity which could be due to dissociation of an enzyme complex which is

composed of different active subunits and thus dissociation would increase the net overall activity since steric hinderance or competition for the substrate may exist when the enzyme exists in a complex rather than separate subunits as mentioned previously, and as implied by Wing and Robinson (1968) and Garfinkel and Schotz (1973) to explain the changes in activity from the starved to the fed state of rat adipose tissue LPL. The highest specific activity fraction was obtained at 0.44M NaCl and Gaffney et al (1966) obtained the highest specific activity at 0.5M NaCl using a rennet extract of skimmed milk on DEAE cellulose.

Reelution of the extracts of conductivity 21 and 18 mmho on DEAE cellulose in 0.005M KH_2PO_4 buffer pH 8.4 gave three protein and activity peaks and in both cases specific activity was highest for the peak eluted at the starting buffer with a lower specific activity for the latter eluted peaks, suggesting a conversion to a form of high specific activity and a form of lower specific activity. The disappearance of a peak eluted at a conductivity of 21 mmho could be due to an unstable form of enzyme or an intermediate form of enzyme which readily dissociates into more stable forms and could be due to the effect of the NaCl required to elute it from the DEAE cellulose.

The reappearance of a peak at a conductivity of 21 mmho on elution of conductivity 24 mmho extracts indicates that this enzyme peak may be an intermediate form of enzyme and the appearance of a large protein peak at the starting buffer accompanied by an increase in total activity could be due to tightly bound protein which is dissociated from the enzyme on reelution on DEAE cellulose. On removal of this protein from the enzyme complex, dissociation into forms of higher specific activity occurs and an increase in total recovered activity may imply that this contaminating protein may inhibit the enzyme probably by causing steric hinderance for the enzyme to act on the substrate and thus may act as a controlling mechanism.

A large protein peak eluted off at a conductivity of 8.5 mmho on reelution of the starting buffer extracts of the above gradient elutions may indicate a universal protein which binds to all of the

LPL enzymes and the appearance of activity peaks eluted off at conductivities corresponding to the first four peaks eluted with whole homogenates may indicate complete dissociation into all nine peaks can occur from either one of the peaks but the disappearance of the peaks eluted at conductivities greater than 15 mmho and the reappearance of these peaks on reelution of the conductivity 15 mmho activity peak may imply a limited initial dissociation occurs depending on the ionic strength of elution of the initial extract. The large total recovery of 812% indicates a relief of inhibition, probably due to steric hinderance caused by bound contaminating proteins and the dissociation of active subunits which when in the form of a complex, are not as active as when in a dissociated form. The reappearance of the latter eluted peaks may be due to the removal of these protein components which associate with the enzymes causing them to be eluted earlier from DEAE cellulose. The presence of two high specific activity peaks eluted at conductivities of 2.5 and 9 mmho and lower specific activity peaks eluted at conductivities of 18 and 21 mmho again indicate the presence of different 'subunit' species of the enzyme which have different specific activities.

Gradient elution of conductivity 13 and 15 mmho extracts also gave a shift of protein into the starting buffer elutant as occurred with conductivity 18 and 21 mmho extracts on reelution on DEAE cellulose at pH 8.4 which suggests a bound protein which is common to all enzymic forms. The absence of a peak at conductivity 13 mmho on reelution of these extracts on DEAE cellulose would suggest that this enzyme may also be an unstable transition form of enzyme as occurs with the peak eluted at a conductivity of 21 mmho.

Dissociation into a peak at conductivity 21 mmho occurred on reelution of conductivity 15 mmho extracts whereas no dissociation into this peak occurred on reelution of conductivity 13 mmho extracts which may imply that limited dissociation occurs depending on the ionic strength the initial extract was eluted at. However complete dissociation occurs on reelution of extracts eluted at high ionic strength and thus equilibrium conditions may favour the formation of the enzymic peaks eluted at the low ionic strengths. This could be due to the

dissociating effect of the high salt concentrations and the effect of pH since elution of conductivity 10 mmho extracts on DEAE cellulose at a pH of 7.6 gave complete dissociation into all nine peaks. This could explain the variations in percentage activity in the peaks of conductivity 5 mmho extracts when eluted on sephadex G200. The extracts eluted at a pH of 8.4 activity was divided mainly up into five peaks of different molecular weight whereas elution of conductivity 5 mmho extracts obtained from DEAE cellulose at pH 7.6 showed seven major peaks. Thus decreasing the pH to below optimum for enzyme activity (7.6) causes greater association into a peak of molecular weight 171,000 daltons which had a lower specific activity than the lower molecular weight peaks. This may explain the higher activity of the enzyme of pH 8.4 than at 7.6 whereby greater aggregation at pH 7.6 into higher MW forms causing a net inhibition.

Reelution of the starting buffer extracts from conductivity 15 and 13 mmho elutions on DEAE cellulose pH 8.4 gave a large protein peak at a conductivity of 8.5 mmho as was observed on reelution of conductivity 18 and 21 mmho starting buffer extracts, indicating a common protein component may be bound to all of the enzymic types and again dissociation beyond a peak at conductivity 15 mmho did not occur, implying bound proteins may effect elution characteristics and only a limited initial dissociation occurs.

A preference for association into a peak of conductivity 5 mmho on elution of starting buffer extracts from elution of conductivity 13 and 15 mmho extracts compared with a more even distribution of activity into peaks of conductivity 2, 7, 11 and 14 mmho on elution of starting buffer extracts from elution of conductivity 18 and 21 mmho extracts, may imply that the relative proportions of different enzymic forms depends on the ionic strength at which the original extract was eluted at.

The presence of 6 different molecular weight species of lipase ranging in MW from 270,000 to 8,000 daltons may imply the enzyme can exist as 6 different MW species, probably due to aggregation of subunits which may be of different MW.

The absence of a low MW component of 9,000 daltons and the appearance of a component of MW approximately 15,000 daltons on elution of conductivity 2 mmho extracts compared with the elution of conductivity 5 mmho extracts on sephadex G200, may imply that these are non specific lipases which associate with the lipoprotein lipase complexes. The 5 higher MW forms all had MWs corresponding to those of the conductivity 5 mmho extracts indicating common species in the two extracts.

Gradient elution of the 105,000g supernatant mammary gland extract on DEAE cellulose in 0.005M KH_2PO_4 buffer pH 7.6 gave a similar elution as at pH 8.4 and thus decreasing the pH of elution had no effect on the ionic strength at which the peaks were eluted but increased the binding capacity of the DEAE cellulose.

Gradient elution on DEAE cellulose pH 7.6 of the various mammary gland extracts obtained from stepwise elution on DEAE cellulose gave distinct elution patterns depending on what conductivity the original extract was eluted at with a predominance of a peak eluted at conductivity 5 mmho, indicating a form probably of high stability and equilibria conditions favouring the formation of this enzymic form.

The limited degree of dissociation only as far as a peak eluted at conductivity 10 mmho on elution of conductivity 2 mmho extracts also complies with the elution of conductivity 5 mmho extracts eluted at pH 8.4 where an initial dissociation only occurred as far as a peak eluted at a conductivity of 15 mmho with complete dissociation occurring on reelution of extracts eluted at high ionic strengths at pH 7.6 and thus supporting the idea that the degree of dissociation depends on the ionic strength at which the original extract was eluted at.

The degree of shift of activity into a peak eluted at conductivity 5 mmho appeared to be related to the degree of a shift of protein into a peak eluted at about 9 mmho for extracts eluted above 2 mmho. This shift in activity was more marked on reelution of conductivity 21 mmho extracts where a higher net transfer of protein

occurred to a peak eluted at about 9 mmho and thus the degree of association into a peak eluted at conductivity 5 mmho is probably related to the degree of dissociation of bound protein from the enzyme complex.

The variable percentages of the different MW species present on elution of the DEAE extracts on sephadex G200 could be due to the variation in the relative proportions of these different subunit species being present and the relative affinity of the subunit species for each other, and thus the elution pattern on sephadex G200 depends on the point of elution from DEAE cellulose. The different proportions of enzymic species could also be due to a pH effect whereby decreasing the pH of elution on DEAE cellulose causes greater association of subunits as explained earlier. The large percentage of the peak eluted at a MW corresponding to 171,000 daltons from conductivity 2 mmho extracts compared with conductivity 5 mmho extracts when eluted from DEAE cellulose at pH 8.4 could be due to the low ionic strengths at which the conductivity 2 mmho extracts were eluted at (0.023M NaCl) from DEAE cellulose compared with the conductivity 5 mmho extracts which were initially eluted at a conductivity of 13 to 15 mmho (0.22 - 0.25M NaCl). Thus both pH and NaCl concentration may affect the dissociation of the enzyme. This greater dissociation of conductivity 5 mmho extracts could also be due to the greater number of elutions carried out on DEAE cellulose with this extract compared with the conductivity 2 mmho extract (3 and 1 respectively).

Separation of the total extract on sephadex G200 gave eight peaks of activity with each activity peak correlating in MW to those eluted from sephadex G200 of the DEAE cellulose extracts. In all cases peaks occurred at MWs corresponding to 260,000, 170,000, 87,000, 53,000 and 32,000 in the gel filtrations of the activity fractions eluted on DEAE cellulose, suggesting LPL can exist in all of these different MW forms or these forms are composed of different active subunits which are bound to form complexes. Thus the MW of the enzyme complex would depend on the relative composition of its subunits. The higher MW enzyme could be due to complex formation of lower MW forms or subunit structures, i.e. 3 units of Peak IV

would form Peak II and 3 units of Peak III would form Peak I. Thus the enzyme may bind to form a trimer of the subunits or Peak II could be a dimer of Peak III units and Peak I could be an octamer of Peak V subunits. Thus the enzyme may consist of three different MW forms of about 87,000, 53,000 and 32,000 daltons and these may bind in only specific ways to form an enzyme of particular MW and form. By their specific orientation they can only form specific conformations. There may be several enzymes of approximately the same MW but composed of different subunits and they would have different substrate specificities depending on the relative composition of its subunit structure. The varying percentages of each MW form eluted on sephadex G200 of the elution peaks from DEAE cellulose could support this theory due to dissociation of the subunits to varying degrees to form the different MW forms which are separated on DEAE cellulose. Elution on DEAE cellulose suggests also the binding of non active protein subunits to the enzyme causing different chemical properties such as strength of binding to DEAE cellulose and this may account for the different MW units on sephadex G200. The presence of the five different MW forms on sephadex G200 of the enzyme from all of the DEAE cellulose activity peaks suggests that the enzyme is in a constant state of flux between the different forms and can exist in any one form in any one time, or the enzyme can dissociate or reassociate to give both a change in MW and a change in elution pattern on DEAE cellulose. Thus any one of the forms on DEAE cellulose can exist in all the five forms eluted from sephadex G200. The absence of forms of MW 20,000, 14,000 and 8,000 in all of the elutions could suggest that these are non specific lipases which are in association with the LPL complex.

The differences in elution patterns and the percentages in each peak on sephadex G200 of the DEAE cellulose eluted fractions would suggest a preference of some forms to dissociate or associate to form a particular MW form of enzyme or could be due to the fact that the enzyme is composed largely of particular forms of subunits. The ability of the enzyme to change to any one of the other forms on DEAE cellulose suggests that they have similar subunits, but it is the relative composition of the subunits composing that particular

enzyme which determine its characteristics. The variation in recoveries on elution on sephadex G200 (79 - 128%) could imply the enzymes' different molecular forms have different activities depending on the subunits involved in the formation of that particular form of enzyme. Extracts of conductivity 5 mmho from both the DEAE elutions at pH 8.4 and 7.6 when eluted on sephadex G200 both gave a 110% recovery of activity indicating that even though both may dissociate to form different proportions of the different MW forms, the relative net enzyme activity remains the same, and thus it is the relative proportions of subunits and not the particular manner in which they associate to form the different enzyme complexes that determines the net total recovery of activity when the DEAE peaks are eluted on sephadex G200.

The effect of serum on the particular enzyme extract depended on its position it was eluted from DEAE cellulose and serum gave a large activation of the activity eluted firstly from the column at low ionic strengths followed by inactivation of extracts eluted at 5 mmho and gradual activation of the activity eluted thereafter, and then a rapid inactivation of the extracts eluted at a conductivity of 18 mmho and 21 mmho, followed by activation of the extracts eluted at a conductivity of 24 mmho. Thus there may be distinct forms of the enzyme, some activated and some inactivated by serum, depending on the state or composition of the enzyme, i.e. the relative composition of its subunits or distinct enzymic forms could be involved.

On sephadex G200 serum gave variable activation of the peaks and activation appeared to increase with decreasing MWs for the 260,000, 186,000, 89,000 and 27,000 MW forms of the enzyme. This could be due to an activator lipoprotein already bound to the enzyme and the enzyme thus does not require serum for activation. Serum had little effect on the very low MW forms of lipase and thus may be due to a general activation effect of serum as observed by Shahani and Chandon (1963) for a low MW milk enzyme. The large inhibition by serum of the lipase peak of MW 52,000 (Peak IV on sephadex G200) could be due to inhibition by a specific apolipoprotein such as apolipoprotein ala as observed by Brown and Baginsky (1972) and

Chung and Scanu (1973) which inhibits bovine milk and rat adipose tissue LPL or due to a specific inhibitor present in serum as postulated by Iverius (1972) for milk LPL or an inhibitor of MW 38,000 isolated by previous work on LPL of mammary gland extracts (Deane, Dip. Sci. thesis). A good correlation exists between the effect of serum on the peaks separated by sephadex G200 and those separated by DEAE cellulose and the percentages of the activity peaks eluted from sephadex G200 of the activity peaks from DEAE cellulose.

A high percentage of Peak IV having a MW of approximately 53,000 from sephadex G200, eluted at conductivities of 5 and 15 mmho, could account for the low serum requirement and inhibition observed with activities eluted at these conductivities. The high percentages of Peaks V (MW 32,000), III (MW 87,000) and II (MW 171,000) on sephadex G200 which have a high requirement for serum together with a low percentage of Peak IV (MW 53,000) which is inhibited by serum on sephadex G200 of the conductivity 2 mmho extracts, explains the large net requirement for serum of the conductivity 2 mmho extracts.

The high percentage of Peak IV (MW 53,000) which is inhibited by serum and the lower percentage of Peak V (MW 32,000) which is activated by serum, explains the net inhibitory effect of serum on conductivity 5 mmho extracts. This is even more pronounced with conductivity 15 mmho extracts where a high percentage of Peak IV (MW 53,000) (27%) combined with a very low percentage of Peak V (MW 32,000) (2.7%) gives a net very small activation by serum. The large inhibition by conductivity 18 mmho extracts could be due to a larger proportion of Peak IV (MW 53,000) since both conductivity 15 mmho and 5 mmho extracts are derived from conductivity 18 mmho and 21 mmho extracts on reelution of these extracts on DEAE cellulose, and therefore must contain a high proportion of Peak IV activity which is inhibited by serum.

Conductivity 24 mmho extracts gave a low percentage of Peak IV (MW 53,000) activity combined with a high percentage of Peak V (MW 32,000) and Peak III (MW 87,000), but a low percentage of Peak II (MW 171,000) activity, all of which are activated by

serum to give a net activation effect by serum but not as high as conductivity 2 mmho extracts due to a lower proportion of Peak V (MW 32,000), and Peak II (MW 171,000) activities. Thus the relative proportion of the different units or subunits separated on sephadex G200 determines both the serum requirement and the position the enzyme complexes or subunits are eluted from DEAE cellulose. Thus a high proportion of Peak IV (MW 53,000) gives inhibition by serum, which on initial elution appears at conductivities 18 - 21 mmho, and on reelution appears at conductivities of 5 and 25 mmho. The effects of Peak IV (MW53,000) inhibition by serum can be overcome, depending on the amount of the other units or subunits present to make up the enzyme complex. Thus inhibitions at conductivities of 18 and 21 mmho are greater than those at 5 mmho due to the presence of serum activating enzyme subunits at conductivity 5 mmho, and since a greater conversion of conductivity 18 mmho and 21 mmho to conductivity 5 mmho extracts other than to conductivity 15 mmho extracts, inhibition occurs at conductivity 5 mmho extracts and only slight activation (10%) is observed at conductivity 15 mmho extracts.

In all cases the high MW form (260,000) comprised a low percentage (6 - 10%) of the total activity, which could be due to dissociation into the lower molecular weight forms. Its low activation by serum (19%) could be due to the presence of Peak IV (MW 53,000) subunits which are inhibited by serum, combined with other subunits which are activated by serum to give a net low activation effect by serum. Thus there appears to be distinct enzymes or subunits which can combine to form an enzyme complex whose characteristics depend on the relative composition of subunits composing it, i.e. the activity of the enzyme depends on the relative composition of its subunits.

The inhibition by serum of Peak IV (MW 53,000) lipase from sephadex G200 could be due to the presence of the lipase of pH optimum of 9 which also showed inhibition by serum. This inhibition could be similar to that observed by Iverius et al (1972) for bovine milk LPL which was overcome by the addition of heparin. Heparin was not used in the assay mixture since it had no effect on the enzyme activity of mammary gland LPL as shown in previous studies (Dip. Sci.

thesis 1972) which was also in agreement with the findings of McBride and Korn (1964) who also obtained no activation by heparin of LPL from guinea pig mammary gland homogenates, and Askew et al (1970) who obtained a variable stimulation of bovine mammary gland homogenates ranging from 3 - 25%, depending on the type of extraction. Thus the inhibition of one of the structural forms or subunits of LPL by serum which probably could be due to apo LP ala, could explain these results and thus may act as a controlling mechanism whereby if one of the subunits is inhibited by serum, then the amount of this enzyme present, either in free form or composing a complex, would control the overall LPL activity of the mammary gland and also the quantity of this inhibitor present in serum could act as a secondary control mechanism. The different serum requirement of the peaks could be due to the alteration of the substrate specificity of the lipase on dissociation from protein complexes as postulated by Cooper and Dawney (1971).

The presence of different subunits, some activated by serum to varying degrees, and one inhibited by serum, probably due to some protein factor in the serum whose synthesis may be controlled by hormones such as prolactin, could explain the rapid changes occurring in LPL activity in mammary tissue at post partum and at early lactation as observed by Hamosh et al (1970) and Scow et al (1972) for rat mammary gland, and the gradual decrease in milk fat synthesis at the termination of lactation whereby there is an increase in the enzyme subunit which is inhibited by this factor in serum occurring on termination of the lactation, and a decrease in the synthesis of this subunit enzyme at the onset of lactation. Thus the cells themselves may regulate the level of LPL as proposed by Scow (1964), Rodbell (1966), Rodbell and Scow (1965) and Porkajae (1967) for adipose tissue LPL. The presence of subunits which are synthesized and then form a high MW complex, supports the findings of Garfinkel and Schotz (1973) who observed a decrease in a low MW LPL which was accompanied by an increase in a high MW LPL of rat adipose tissue after 360 minutes of refeeding after an initial starvation period which they propose is due to the conversion of the low MW enzyme into the high MW enzyme.

The activation occurring on dissociation of the complexes may explain the results of Wing et al (1966), Wing and Robinson (1968) and Patten (1970) who observed an increase in LPL activity taken from starved rats in vitro which, according to Cryer et al (1973), was not affected by cyclohexamide during the first two hours, and supports the theory of Cunningham and Robinson (1969) who propose that this increase is due to activation brought about by the conversion of one form of the enzyme into another. Thus, after starvation, less synthesis would occur of the subunits which, after a period, may dissociate and since there are less subunits synthesized, less complexing would occur and thus increased activity would take place due to the smaller number of less active complexes, and a greater number of the more active free subunit structures being present.

Both 1.4M NaCl and 100μg/ml protamine sulphate completely inhibited the five highest MW LPLs separated on sephadex G200 which is in agreement with the findings of La Rosa et al (1972) using rat adipose tissue LPL, and Askew et al (1972) using bovine mammary gland LPL. Thus they are true LPLs. The lower molecular weight lipases (MW 19,000 - 8,000) were not completely inhibited by 1.4M NaCl or 100 μg/ml protamine sulphate and inhibition of these lower MW lipases by NaCl was proportional to their MW, i.e. the higher the MW the greater the inhibition by NaCl. Also incomplete inhibition by protamine sulphate suggests these lower MW forms are not true LPLs but may be lipases whose activation by serum may be by stabilizing the enzyme and not as a substrate cofactor as is the case with LPL. Lower concentrations of NaCl were not used to determine the relative sensitivity of the five higher MW enzymes to NaCl inhibition, but Garfinkel and Schotz (1972) in agreement with my findings previously (Dip. Sci. thesis), observed greater sensitivity of the high MW LPLs to NaCl inhibition than the low MW LPLs, suggesting NaCl acts on the enzyme by probably dissociating the subunits or dissociating some bound activator protein and thus may account for the dissociation occurring on DEAE cellulose. Korn (1955) observed that the effect of NaCl was irreversible and Askew et al (1970) observed that at a constant NaCl concentration and with increasing substrate concentration, no reversal of inhibition occurred, indicating an effect on the enzyme itself, most probably affecting protein structure causing some conformational change.

The purified LPL was completely inhibited by 2, 4 dinitrophenol, suggesting sulphydryl groups are involved in the catalytic process or the binding of 2, 4 dinitrophenol may have caused some structural change to the enzyme or blocked the active site of the enzyme.

N ethylmaleimide which is partially specific for free SH groups gave a 75% inhibition, indicating free SH groups are involved in the catalytic process as suggested by Chandan and Shahani (1965) using a purified low MW milk lipase. The free SH groups could be involved in the binding of the substrate to the enzyme. The variable inhibitions caused by iodoacetate and 5 5'dithio bis-(2 Nitro benzoic acid) could be due to the low reactivities of these compounds to the sulphydryl groups at the pH used, or due to inaccessibility to the SH groups. Chandan and Shahani (1965) obtained complete inhibition by iodoacetate on a low MW milk lipase although with a higher MW lipase the lower inhibition observed could be due to inaccessibility of the SH groups to be inhibited by iodoacetate. The greater inhibition caused by N ethylmaleimide (75%) over that observed by Chandan and Shahani (1965) for a low MW milk lipase suggests a greater function of the free SH groups in the activity of the high MW enzyme. Also certain sulphydryl groups react to different degrees depending upon their location in the 3 dimensional structure of the protein, neighbouring groups causing steric interference of different polar or apolar environments, or the sulphydryl group may be involved in direct interaction with functional groups through hydrogen bonds or reversible covalent bond formation and thus different sulphydryl may react to different degrees to the sulphydryl reagents as according to Vallee and Roirdan (1969).

Striking characteristics of the amino acid composition is the high half cysteine content, the excess of acidic groups over basic groups (1.43:1) and the complete lack of methionine residues. The high cysteine content supports the idea of its function in enzyme substrate binding whereby a large number of active sites would be available for substrate binding.

Milk LPL activity was characterized by its almost complete binding to Ca_3PO_4 gel which could not be eluted to any large extent by potassium oxalate, sodium citrate, NaCl, NaCl in the presence of dimethyl formamide, sodium carbonate, sodium sulphate, sodium gluconate or KH_2PO_4. This is in strong comparison to mammary gland LPL which was eluted from the Ca_3PO_4 gel and did not bind to any large extent. The strong binding to Ca_3PO_4 gel was probably due to the strong binding of the casein bound enzyme activity to the Ca_3PO_4 gel by Ca_3PO_4 bonds as postulated to occur by Dawney and Murphy (1970). The zero activity eluted when KH_2PO_4 was eluted through the gel supports the theory of a Ca_3PO_4 binding of the lipase to the gel and thus to casein.

Activity appearing in the Ca_3PO_4 gel filtrate of the skimmed milk suggests free forms of lipase also exist which are not associated with casein either in micelle form or as high MW complexes as suggested by Dawney and Murphy (1970). Dimethyl formamide increased protein eluted from the Ca_3PO_4 gel but decreased activity suggesting an inhibiting effect of dimethyl formamide on the enzyme. In all cases very little protein and a low percentage of the bound activity was eluted from the gel and thus was not a good starting method in the purification of milk LPL. The majority of the activity appeared to be irreversibly bound to the Ca_3PO_4 gel.

76% of the total lipase activity of skimmed milk was associated with the casein micelles which is in agreement with the findings of Dawney and Andrews (1966) who obtained 70% bound to the casein micelles. Also 3 - 5% appeared to be in a free form which was not bound to the casein in either the high MW soluble complex form of MW approximately 200,000 as suggested by Dawney et al (1969), or the casein micelles. The remaining 20% was probably bound to the high MW soluble casein complexes as proposed by Dawney and Murphy (1970).

Extraction with NaCl of the free casein gave a large recovery of enzyme activity in the 105,000g supernatant extract whereas NaCl had little effect on the extraction of activity from the Ca_3PO_4 bound casein. Thus the enzyme appeared to be bound

more strongly in the presence of Ca_3PO_4 gel and is unaffected by the high ionic strengths which normally are used to dissociate the lipase from the casein, thus supporting Dawney and Murphy's theory (1970) of Ca_3PO_4 involved in the binding of the casein lipase complexes to the casein micelles. Ca_3PO_4 probably acts by complexing the casein micelles together and thus prevents NaCl from decreasing the electrostatic interaction with the negatively charged casein micelles causing the release of the lipase, as suggested by Haynes and Dawney (1973).

The activation resulting from the release of the lipase when extracted from the casein micelles by NaCl could be due to the inhibitory effect of A_s and B casein components as observed by Shahani and Chandan (1963), and thus an increased activity is observed on release of the lipase from the casein.

Activity peaks with pH optima of 6, 7, 7.5, 8.25 and 9 are in agreement with the findings of Schwartz et al (1956), Harper et al (1956) and Frankel and Tarassuk (1956) for bovine skimmed milk lipase. The differences in activity in the presence of serum compared with the absence of serum at the various pH values could be due to different lipases or LPLs with similar pH optima as suggested by Schwartz et al (1956). In comparison of the pH optima profile with that of mammary gland extracts, no enzyme with a pH optimum of 7 appeared in mammary gland extracts, whereas an enzyme or enzymes with a pH optimum of 7 appeared in NaCl extracts of skimmed milk.

The appearance of an enzyme or enzymes of pH optimum 7 in milk but not in mammary gland extracts suggests this enzyme is secreted only into the mammary milk ducts and is not present on the epithelial cell surface, or could be due to a structural change occurring on secretion of one of the other forms of enzyme present in mammary gland extracts, and thus causing a change in its pH optimum, and thus the enzyme appears only in milk and was not detectable in the mammary gland extracts, or it could be due to the effect of binding of one of the enzymic forms in the mammary gland extract to casein causing a change in its pH optimum.

Both mammary gland extracts and NaCl extracts of skimmed milk showed the presence of a lipase or lipases of pH optimum 7.5 with serum having little or no effect on activity at this pH optimum in both cases. This could probably be due to the presence of milk in the mammary gland extracts. The absence of an enzyme peak in the absence of serum with a pH optimum of 8.5 for mammary gland extracts and the presence of an activity peak with this pH optimum in the absence of serum for milk, could be due to a different enzyme being present in milk which shows activity in both the presence or absence of serum. Thus this enzyme in the mammary gland may be secreted into the blood and is not present in milk, or it could be secreted into the milk whereby a structural change may take place such as the addition of a cofactor protein, and thus the enzyme in milk does not require serum for its activity but the mammary gland enzyme requires the presence of this cofactor protein which is present in serum, i.e. a change in its specific requirement for serum may take place or it could be due to an entirely different enzyme.

Bothextracts showed pH optima at 7.5, 8.25, 9 and 11.5 in the absence of serum. The presence of enzymes with similar pH optima in both milk and mammary gland extracts suggests that the enzyme remains essentially unchanged when secreted into the milk, whereas the presence of new pH optima suggests a change in conformation of the enzyme on secretion into the milk and thus a change in chemical and physical properties which could be due to binding to casein micelles. Thus the appearance of new enzyme peaks with pH optima of 7, 9.75, 10, 10.5 and 10.25.

The disappearance of lipases of pH optima 9.5 and 10.25 in NaCl extracts of milk compared to mammary gland extracts also could be due to a structural change in the lipase as it is secreted into the mammary duct, thus changing its pH optimum, or could be due to these enzymes being only present in mammary gland extracts and are not secreted into the milk.

The differences in the serum requirement for the lipase peaks of pH optima 8.25, 9, 11 and 11.5 between the two extracts could

also be due to a transformation occurring on secretion from the mammary gland, or could be due to entirely different enzymes, one requiring serum and the other not requiring serum which are present in variable amounts in the milk and mammary tissue, thus suggesting selective secretion of lipases into the milk.

On DEAE cellulose gradient elution of the NaCl extracts of the casein precipitate gave activity peaks eluted off at similar ionic strengths as those of the mammary gland extracts suggesting similarities in the two lipase systems. Protein was eluted off mainly in two major peaks, one at an ionic strength of 0.16M corresponding to B casein, and another eluted at 0.25M NaCl corresponding to A_s casein. Lipase activity was eluted off at ionic strengths corresponding to the elution of B, K and A_s caseins although most activity was eluted off at a position corresponding to K casein and overlapping into the A_s casein component as observed also by Fox et al (1967) and Yaguchi et al (1964).

The NaCl extraction of the casein appeared to have a similar effect as dimethyl formamide as used by Fox et al (1967). A 300% recovery of activity on elution on DEAE cellulose of the NaCl extracts of the casein precipitate is in partial agreement with the 150 - 180% recovery of activity Gaffney et al (1966) obtained on elution of a water extract of rennet casein on DEAE cellulose, probably due to separation from the inhibitory effects of A_s and B casein.

Elution of the major activity peak from DEAE cellulose on sephadex G200 gave a large activity peak at the void volume which could be due to lipases still bound to the soluble casein complex. Five latter peaks suggest free forms of lipases which have dissociated from the casein. Peaks of MWs corresponding to 82,000, 48,000, 28,000, 12,000 and 8,000 daltons have similar MWs to Peaks III (87,000), IV (53,000), V (32,000), VII (14,000) and VIII (8,000) of the mammary gland extracts, suggesting that these may be the same enzymes present in both mammary gland extracts and milk. The low recovery of activity (55%) could be due to inhibition caused by the B and A_s casein of the activity eluted near the void volume and is in agreement with

the findings of Dawney and Murphy (1970) who obtained a 25% recovery of activity on elution of purified pig pancreatic lipase with skimmed milk with over 90% of the activity eluted at the void volume, suggesting complexing of the lipase to the casein which is accompanied by inhibition.

On elution of the Ca_3PO_4 gel supernatant extracts of skimmed milk on sephadex G200, thirteen peaks of activity appeared, suggesting all of these forms can exist in a free unbound form and are distinct enzymes in themselves and not a single low MW enzyme complexed to K casein as suggested by Dawney and Andrews (1965) since if they were casein bound they would bind strongly to the Ca_3PO_4 gel. These lipases however can bind to casein forming complexes.

The presence of forms of enzymes with different MWs than those of the mammary gland extracts of MWs corresponding to 148,000, 120,000, 71,000, 41,000 and 11,000 daltons could be due to a structural change occurring when the enzyme is secreted into the milk enabling new complexes to be formed by association or dissociation of active subunits. The forms with MW corresponding to 240,000 - 270,000, 89,000, 57,000, 31,000, 22,000, 15,000 and 9,000 which appear in both mammary gland extracts and Ca_3PO_4 supernatant extracts of skimmed milk suggests these forms are secreted into the milk in an essentially unchanged form, or could be due to the presence of milk extracts in the mammary gland extract. The form of MW corresponding to 171,000 daltons was present only in mammary gland extracts, suggesting that this enzyme is not secreted into the milk and perhaps is secreted in this form only into the blood, or is retained within the mammary cell, or it could undergo modification in structure by the addition or removal of subunits or bound protein before it is secreted into the milk, and thus forming one or several of the new enzyme types which are present only in milk.

Sephadex G200 gel filtration of the NaCl extract of the casein precipitate gave most of the activity eluted in three peaks; one corresponding to an MW of 360,000 which was probably due to

casein bound enzyme activity, or high MW enzyme as eluted from mammary gland extracts, and two peaks eluted at MWs corresponding to 150,000 - 85,000 which is in partial agreement with the findings of Haynes and Dawney (1973) who observed the dominant species of the NaCl extracts of the casein precipitates was in the range 60,000 - 120,000 daltons. Activity was also distributed into all of the other peaks of MWs mentioned previously.

The appearance of a low MW lipase of 8,000 daltons is in agreement with the findings of Chandon and Shahani (1963) and Richter and Randolph (1971) who also observed a low MW lipase of MW 7,000 - 8,500 daltons. This enzyme appears to be in both the mammary gland and milk lipase systems. Also a lipase of MW 9,000 is in agreement with the findings of Gaffney et al (1968) who obtained two lipase peaks of MW less than 10,000 daltons from bovine milk. Dawney and Andrews (1965, 1969) observed lipase peaks of MW corresponding to 150,000 - 180,000, 112,000, 75,000, 62,000, 35,000 - 40,000 and a very low MW lipase from NaCl extracts of bovine milk casein which may correspond to lipases of MWs of approximately 148,000, 120,000, 71,000, 57,000, 41,000 and 8,000 daltons separated on sephadex G200 of the NaCl extracts of the NaCl extracts of the casein precipitate. The lipase of MW 210,000 purified by Fox and Tarassuk (1968) from bovine milk could correspond to the lipase obtained of an MW of 240,000 daltons.

On elution of the activity peaks of MW 85,000 - 150,000 of the NaCl extracts of the casein precipitate obtained from sephadex G200 on DEAE cellulose, six activity peaks appeared. The majority of the activity was eluted in two peaks between 0.13 and 0.25M NaCl with no distinct protein peaks eluted between these points. Thus this indicates that the lipase activity was free of casein and thus is eluted at this point whether bound or free from the casein. The large protein peak eluted corresponded to a position where B casein is normally eluted at 0.11M NaCl which is in agreement with the findings of Fox et al (1967) who observed that B casein is eluted after the K casein on sephadex G200. Thus complete dissociation from the K casein (eluted at about 0.2M NaCl) occurs on NaCl extraction. The

greater than 100% recovery of activity on elution of the NaCl supernatant extracts of the casein precipitate on both sephadex G200 and DEAE cellulose supports the idea of Shahani and Chandah (1963) that binding to casein inhibits the enzyme probably by causing steric hinderance.

Thus both changes may occur to the lipase enzyme itself on secretion from the cell into the milk duct and differential secretion of lipases may occur, probably due to the different lipases or LPLs being synthesized in different parts of the cell such as the rough endoplasmic reticulum producing only secretory lipases, and the smooth endoplasmic reticulum producing lipases which are retained within the cell. Also different types of lipases in the mammary gland and milk extracts could be due to the different types of lipases or LPLs being synthesized in different cell types. The endothelial cells producing the LPL which is secreted into the blood and the mammocyte cells producing LPLs and lipases which are secreted into the milk. Thus a comprehensive study of the lipase and LPL systems of the mammary gland, blood and milk would give a good insight into the secretory functions of both different cell types and different cell organelles of the mammary gland.

CHAPTER V

CONCLUSION

LPL in mammary gland extracts exists as five different molecular weight forms of MW approximately 260,000, 171,000, 87,000, 53,000 and 32,000 daltons, each having a different serum requirement which was related to their MW with the species of MW 52,000 daltons being inhibited by serum, and these five different molecular weight forms are in a constant state of flux, probably due to the three lower MW forms being the basic subunits and these can associate or dissociate to form nine different forms on elution from DEAE cellulose, each of which can associate or dissociate to form the various forms on reelution on DEAE cellulose, the degree of dissociation and relative proportions of the different species formed depending on the ionic strength at which the original extract was eluted at on DEAE cellulose. Also the serum requirements of the peaks eluted from DEAE cellulose depended on the relative proportions of the five MW forms present and on dissociation activation takes place either by dissociation of subunits which when bound together as a complex are less active than the net activity of the separate active subunits, or by dissociation of some bound contaminating protein.

Three other lipase forms of MWs 20,000, 14,000 and 8,000 daltons were also present in the mammary gland extracts and these may also bind to the LPL complexes. LPL of the mammary gland extracts exhibited a pH optimum of 8.5, was dependent on serum for maximum activity, required a specific ratio serum to TG of 1:1, was more active against tallow than olive oil substrates, was inhibited by protamine sulphate and NaCl, was present mainly in the insoluble fractions of mammary gland homogenates, and was inhibited by a substance in mammary gland extracts which bound to TG and Ca_3PO_4 gel. The purified enzymic form of MW 87,000 was inhibited by SH reagents, showed an excess amino acid acidic to basic ratio, and had a high cysteine content with free SH groups being involved in the activity of the enzyme probably involved in subunit and substrate binding.

Purification was best obtained by binding to sodium dextran sulphate after preelution of the extracts through Ca_3PO_4 gel and precipitation by divalent cations to give the three high MW species. Purification by binding to TG is limited by the low binding of the enzyme to the TG, and purification by binding to viscose sulphate is impaired by the low recoveries of activity obtained due to the high salt concentrations required to elute the enzyme and the lack of specificity of LPL binding by the viscose sulphate. Clarified extracts are best obtained by elution through Ca_3PO_4 gel or silica gel for large scale preparations to remove both lipoproteins and high MW sedimentable suspended particles and ultracentrifugation is only of limited use due to the large volumes of enzyme extract obtained.

Milk LPL was characterized by being largely associated with the casein in both micelle and soluble casein complexes which could be extracted from the casein by NaCl when the casein was in the free form, but not when bound to Ca_3PO_4, thus the involvement of Ca_3PO_4 in casein lipase complexes. NaCl extracts of the casein precipitate gave a similar elution pattern of activity on DEAE cellulose as with mammary gland extracts, showed some differences in the pH profile, and showed new MW lipase species corresponding to MWs of 148,000, 120,000, 71,000, 41,000, 11,000 and 9,000 daltons with the absence of the LPL species of MW 171,000 present only in mammary gland extracts and all the lipase species present existed as free lipases and not as a single lipase or lipases complexed to casein.

APPENDIX

A.T.P.	Adenosine triphosphate
Cyclic AMP	Cyclic 3' 5' adenosine monophosphate
LDL	Low density lipoprotein
TG	Triglyceride
FFA	Free fatty acid
RNA	Ribonucleic acid
DG	Diglyceride
VLDL	Very low density lipoprotein
HDL	High density lipoprotein
MG	Monoglyceride
MW	Molecular weight
DEAE	Diethyl amino ethyl
TGFAs	Triglyceride fatty acids
TGL	Triglyceride lipase
LPL	Lipoprotein lipase

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