STUDIES ON HUMAN PLACENTAL ALKALINE PHOSPHATASE

A thesis submitted for the Degree of

Doctor of Philosophy at the

University of Glasgow

by

NAEL SUDQI ABU-HASAN

Institute of Genetics

Church Street

Glasgow

December 1983

DECLARATION

I declare that this thesis has been composed by myself and has not been accepted in any previous application for a degree. The work is my own except where specifically acknowledged.

ACKNOWLEDGEMENTS

I am profoundly grateful to Dr. Roger Sutcliffe for his tremendous effort, encouragement and constructive advice throughout the course of this research, not only as a supervisor, but as a great personal friend.

I am indebted to An-Najah National University (Nablus, West Bank) for giving me the opportunity to continue my higher education and for their financial support. I gratefully acknowledge the provision of grants by the British Council and the Committee of the Vice-Chancellors and Principals. My sincere thanks to Professor David Sherratt for allowing me to pursue my studies in the Genetics department and for his tireless effort in solving my financial problems. Thanks are due to Dr. P. J. Findlay of the SERC protein sequencing laboratory, Leeds University, Dr. Peter Johnson for a gift of antisera and to Dr. Nabil Afara for proof reading my thesis. I also wish to extend my thanks to Dr. Richard Wilson and Dr. Eldridge Buultjens for their helpful discussions, and all the rest of the academic and technical staff in the institute for their friendship, help and hospitality contributing to an excellent environment during the course of this work. I also thank the staff of the labour suite at the Queen's Mother Hospital, Glasgow, for their time, assistance and gifts of material.

Last, but not least, I wish to thank my wife and my family who supported me morally and financially during the years of my studies.

CONTENTS

SUMMARY

Human placental alkaline phosphatase (PAP) is evolutionarily distinct from that of liver, bone, kidney, and intestine. Although histochemical, immunological and biochemical studies had shown that PAP is a major protein component of the syncytiotrophoblastic microvillous plasma membranes of the placenta, there is little information on the structure of the enzyme purified from these membranes, or the means of its integration into these membranes. Previous work has shown that these microvilli can be sub-fractionated into two distinct fractions. However, the nature and origin of these fractions was not clear.

The purpose of this investigation was to confirm the placental origin of these fractions of microvilli using placental alkaline phosphatase as a marker and to see whether PAP in these preparations is similar to that of the A and B forms of the enzyme extracted from the whole placental tissue.

When butanol extracts of these microvilli and their fractions were analysed on starch gels, a considerable amount of enzyme activity either failed to enter the gel system or caused a smear between the A and B zones of activity. This smearing was resolved into a discrete band of enzyme activity located between the A and B forms of the enzyme after the incorporation of Triton X-100 into the gel system. This band was called the M form of PAP and appears to be largely or wholly specific for microvilli. It was also found at very low concentration relative to the A and B forms in extracts of total placental tissue. The reasons which may account for the failure of previous investigations to observe this form (M-PAP) are their enzyme preparations were obtained from throughly washed placental tissue, which will wash off the microvilli,

and their starch gel systems did not involve the use of Triton X-100, which is essential for this band to enter the gel.

Experiments were included to investigate whether this form of PAP could be generated through artifactual aggregation of either A or B polypeptides or both. A variety of experiments show that the M form is not an artifact of extraction. Serological, heat-stability and genetic studies showed that the A and M forms contain the same enzymatic polypeptide. Gel filtration of butanol extracts of microvilli provided an estimated molecular weight of 725,000; this value was not altered by extracting the enzyme from the placental microvilli in Triton X-100, or by equilibrating filtration columns with detergent.

To study the polypeptide composition of M-PAP in comparison with that of A-PAP, both forms of the enzyme were purified to homogeneity from butanol extracts of syncytiotrophoblastic microvillous membranes using DEAE Sepharose CL-6B chromatography and were subjected to several biochemical investigations. The two isoenzymes had indistinguishable pH optima and Km values. Under reducing conditions in SDS polyacrylamide gels, both forms showed a similar subunit molecular weight of 69,000. In the absence of reduction M-PAP was present in a dimeric form similar to A-PAP. However, the mobility of the dimer of M-PAP corresponded to a molecular weight of 116,000 compared with 130,000 for A-PAP. It was concluded that this difference is due to the A and M dimers being in different conformations. In SDS gels the M form also showed a higher molecular weight species corresponding to a mobility of 180,000, but species corresponding to 725,000 were not found. Under different alkylating conditions the polypeptide subunit of both forms showed similar isoelectric banding patterns. The M-PAP showed some extra bands with relatively high pI values. CNBr fragmentation also yielded very

similar methionine cleaveage patterns in both polypeptides.

The hydrophobicity of the different forms of PAP were tested on various columns of hydrocarbon-coated agaroses. This study supports the view that M-PAP is hydrophobic relative to the A and B forms, and provided a potentially simple method of purifying M-PAP.

To study the mode of integration of M-PAP into the plasma membrane, the enzyme was extracted from micovilli either by butanol extraction or subtilisin proteolysis. The results indicate that subtilisin cleavage of PAP removes a membrane binding domain of approximately 2,000 molecular weight, leaving the catalytic site intact and the remaining portion of the protein in solution. Sequencing studies on the N-terminal 13 amino acids of both the subtilisin cleaved and uncleaved forms of M-PAP indicated that the enzyme is anchored to the plasma membranes by its carboxy-terminus. The N-terminal 13 amino acids of A-PAP were the same as those of M-PAP. On SDS gels the subtilisin-cleaved M-PAP showed an apparent dimeric molecular size bigger than that of the original uncleaved enzyme, presumably due to the generation of a less compact conformational state. Variations in the electrophoretic mobilities of the different allelic forms of PAP were also observed in these gels. On both gel filtration and ion-exchange chromatography, cleaved M-PAP showed elution patterns similar to that of A-PAP. On starch gels, cleaved M-PAP did not require the presence of Triton X-100 to enter the gel and showed a single zone of enzyme activity with a mobility slightly faster than that of A-PAP.

Trypsin solubilization failed to release M-PAP from these membranes. It appeared to cleave a portion of a molecular weight of about 9K from the amino terminus, leaving an enzymatically active

portion of PAP associated with the membrane.

The available evidence demonstrates the existence of a new form of PAP in association with the microvillous plasma membranes of the placenta. This form of the enzyme seems to share at least part of the same enzymatic polypeptide with the non-membranous form (A-PAP) and appears to differ from it in having a hydrophobic anchoring domain at its carboxy-terminus. The precise relationship of the membrane binding domain to the bilayer is not yet understood. At this stage we can not speculate into the biogenetic relationship between membranous and non-membranous forms of PAP, however, the possibility should be considered that the pathway for the synthesis of secretory and membrane IgM may also be applicable to PAP forms, and therefore have a general biological significance. Direct biogenetic studies will be necessary to obtain complete information on this problem.

ABBREVIATIONS

AP	Alkaline phosphatase
PAP	Placental alkaline phosphatase
α2M	Alpha-2-macroglobulin
BSA	Bovine serum albumin
Alb	Human serum albumin
Tf	Human transferrin
SP3	Pregnancy associated protein 3
PBS	Phosphate-buffered saline: 0.16M NaCl, 5mM potassium phosphate, pH 7.5
SDS	Sodium dodecyl sulphate
CNBr	Cyanogen bromide
pI	Isoeletric point

CHAPTER 1

I N T R O D U C T I O N

1:1 GENERAL INTRODUCTION

The architecture of the chorionic villi is a key factor in the function of the human placenta. In the first trimester of pregnancy the villi are relatively few in number and their outer surface is covered by two distinct layers of epithelium : an outer layer of syncytiotrophoblast and an inner of cytotrophoblast (Langhans' cells). The syncytiotrophoblast is uniform in thickness and has a free surface covered by large microvilli that are normally about 0.5 to 1.2 μm in length and their cytoplasm is continuous with that of the syncytiotrophoblast (Fox, 1978). Between the microvilli, the syncytial surface is often invaginated to form areas which are the precursors of pinocytotic vesicles. The basal plasma membrane of the syncytiotrophoblast shows complex infoldings, resulting in an interrupted extracellular space between the syncytium and cytotrophoblast. Desmosomes are often present where the syncytium is in direct contact with a cytotrophoblastic cell (Fox, 1978). Under the electron microscope, the immature syncytium contains an abundance of rough endoplasmic reticulum, which is usually dilated to give an overall vacuolated appearance to the cytoplasm. Free ribosomes are abundant and tend to occur in clusters or rosettes. Syncytial mitochondria are moderately numerous with distinct lamellated cristae. The Golgi apparatus is usually well developed. The complexity of the syncytiotrophoblast is compounded by the presence of a variety of granules, lipid droplets and complex vesicles. Granular ribosomes, phagolysosomes, multivesicular bodies and occasional myelin bodies are also seen. The syncytial nuclei, with coarsely clumped chromatin and a prominent nucleolus, are regularly distributed. The cytotrophoblastic cells form a complete layer and have a well-marked cell border; their

cytoplasm is less electron dense than that of syncytium and with much fewer subcellular organelles. The endoplasmic reticulum is poorly developed and does not show vacuolation. Free cytoplasmic ribosomes are quite abundant, whilst mitochondria are moderately numerous. There is a well-developed Golgi complex and few secretory granules. Lipid droplets are rarely present and lysosomal structures are sparse or absent.

The ultrastructure of the mature villous trophoblast differs from that of the immature villi quantitatively rather than qualitatively, and its general morphology remains essentially unaltered (Fox, 1978). The syncytium is irregularly thinned and the nuclei are often aggregated. The microvilli are fewer in number and tend to be shorter and blunter than those seen in the immature villi. The syncytial cytoplasm has a less complex structure; the endoplasmic reticulum is less abundant, whilst lipid droplets, secretory granules, ribosomes and mitochondria are less frequently seen. The cytotrophoblastic cells are much less prominent and their ultrastructural characteristics are much the same as in the first trimester.

The connective tissue core of the mature villi contains collagen fibres, fibroblasts, Hofbauer cells and capillaries. The fibroblasts often have elongated processes and their cytoplasm contains an abundance of both rough and smooth endoplasmic reticulum, many free ribosomes and well-developed Golgi apparatii. The Hofbauer cells are less abundant than in the immature villi and have the typical morphological, histochemical and functional characteristics of macrophages (Fox and Kharkonger, 1969). In the initial stages of gestation the villi are avascular and the vessels usually appear by the end of the second month of gestation. The lumen of the fetal capillaries is lined by a single layer of endothelial cells.

The multiple functions of the chorionic villi, including transportation and synthesis, is clearly reflected in the morphology of the syncytiotrophoblast. Getzowa and Sadowsky (1960), Burgos and Rodriguez (1966), and Jones and Fox (1976) were among the first workers to assign functions to structurally differentiated areas within the syncytiotrophoblast. Experimental evidence which supports the synthetic role of the syncytium has come from immunohistochemical studies (Horne, Towler, Thomson, Bohn and Pugh-Humphreys, 1976; Lin and Halbert, 1976; Currie, Beck, Ellis, and Read, 1966).

The total area of the trophoblast surface membrane is clearly an important factor in the rate of exchange of materials between the placenta and the maternal circulation. This membrane is normally characterized by "turf" microvilli (Hamilton and Hamilton, 1977) which must massively increase the effective area of transfer. Conversion of the microvillar surface to a relatively smooth surface, which is a feature of some pathological processes (Fox, 1967) would much reduce the surface area and hence the potential for exchange between the placenta and the maternal circulation.

1:2 The Syncytiotrophoblastic Microvillous Plasma Membranes

The microvillous surface membranes of the human syncytiotrophoblast represent the major site of contact between the fetus and the maternal circulation. It participates in many diverse functions which serve to maintain a suitable fetal environment. Nutrients and metabolites are exchanged across this surface membrane and it is also involved in hormonal and immunological interactions between the mother and the fetus. This barrier has long been believed to be involved in the failure of maternal rejection of the fetal allograft. While the mechanisms of immunological acceptance of the fetus by the maternal host during pregnancy remains unclear, many hypotheses involving syncytiotrophoblast membrane components have been presented (Billington, 1979; Wegmann, Singh and Carlson, 1979). Several studies have shown that human syncytiotrophoblastic components are sufficiently immunogenic when injected into animals to induce the production of antibodies showing a high degree of tissue specificity (Beer, Billingham and Yang, 1972; Behrman, Yoshida, Amano and Paine, 1974). In addition, there seems to be a general agreement that these membranes do not express the histocompatibility (HLA) antigens, on the basis of failure to detect such antigens by immunohistochemistry (Goodfellow, Barnstable, Bodmer, Snary and Crumpton, 1976; Faulk and Temple, 1976; Faulk and Johnson, 1977; McIntyre and Faulk 1979). However, non-villous trophoblast is reported to express HLA A, B, and C antigens, but not the HLA D antigen(s) (Sunderland, Redman, and Stirrat, 1981). In addition, the villous stroma were found to have HLA-DR positive cells (for review see, Redman, 1983). The lack of defined antigens on syncytiotrophoblast support the notion that it may be an effective barrier to the maternal

immune system. Several workers (Carlson, Wada and Sussman, 1976; Ruzycki Smith, Nelson, King, Donohue and Kelley, 1977; Ogbimi, Johnson, Brown and Fox, 1979; Ogbimi and Johnson, 1980; Davies, Parry and Sutcliffe, 1981) have demonstrated that numerous components can be isolated from solubilized trophoblast membrane preparations and differences in immunological properties reported (Whyte and Loke, 1979). In these studies up to 26 major polypeptide bands from a membrane preparation were identified by SDS-polyacrylamide gels of which 16 were major proteins and 10 glycoproteins. The differences in the number and intensity of the bands reported by those investigators are more likely to be due to the different solubilization protocols used. Using a variety of experimental approaches these proteins can be classified into groups according to their sialic acid content, exposure on the external surface, tightness of binding to the membrane lipid, and relation to membrane structure (Kelley, King, Johnson and Smith, 1979).

These studies on placental microvillous plasma membranes were stimulated by the discovery of Smith, Brush and Luckett (1974), that a preparation of microvilli could be obtained by differential centrifugation of a saline extract of whole placental tissue. Briefly, a fresh term placenta, from which the cord and membranes had been removed, was cut into small pieces and washed rapidly in ice-cold 0.11M calcium chloride followed by ice-cold physiological saline. This removes much of the blood from the intervillous spaces. The washed tissue was placed in saline and agitated for 30 min at 4°C using a magnetic stirrer. During this procedure, the chorionic villi spread out so that their surfaces are well irrigated. The saline was then removed and centrifuged at 800g for 10 min to remove fragments of tissue and red blood cells. The resulting supernatant was re-centrifuged, first at

10,000g for 5 min to remove intracellular debris and then at 100,000g for 60 min. The resulting pellet, of placental villous surface membrane was re-suspended using a tight-fitting glass homogenizer, and washed in phosphate buffer saline (PBS) by centrifugation at 100,000g for 30min. The justification of this technique was based on electron microscope studies, which indicated that the chorionic villous surface was almost entirely free of the microvilli after the washing procedure and that little or no cellular damage was visible. Using this method, Davies, Parry and Sutcliffe (1981) found that the microvilli could be separated by sucrose density gradient centrifugation into two populations, which showed similar polypeptide patterns on SDS polyacrylamide gels, but showed different binding properties for labelled insulin, transferrin and IgG. Truman, Wakefield, and Ford (1981) have also reported that two fractions of microvilli can be prepared in a similar way. Although these two studies involved the use of several membrane markers such as 5`nucleotidase, placental alkaline phosphatase, IgG receptors, insulin receptors and transferrin receptors, the origin of these two fractions was not clear. In order to confirm the placental origin of these fractions I have used placental alkaline phosphatase as a membrane marker.

These studies on placental microvillous plasma membranes provide a base of information which can serve as a reference for further investigations into the correlation of the functions of the microvillus plasma membrane of the human placenta with protein and glycoprotein subunit structure .

1:3 Alkaline Phosphatases

Alkaline phosphatases [orthophosphoric-monoester phosphohydrolase (alkaline optimum), EC 3.1.3.1] have been studied more than any other group of enzymes. A book on alkaline phosphatases (McComb, et al, 1979) lists more than 6,000 references. In man, alkaline phosphatases (APs) are enzymes which hydrolyse a wide range of monophosphates and show a peculiarly high pH optimum-around 10-10.5. They are present in most tissues but have been particularly well studied in liver, bone, kidney, intestine and placenta where they show the greatest activities. They are glycoproteins which are membrane associated and have been detected as cell surface antigens. As we shall see there are at least three gene loci coding for the protein moieties of these enzymes. In spite of their abundant distribution in nature, including bacteria, plants, and animals, the physiological functions of these enzymes remain a mystery.

1:3.1 Genetic and Biochemical Distinctions Between Human Alkaline Phosphatase Isoenzymes

The human alkaline phosphatases (APs) constitute a system of multiple molecular forms of enzymes in which heterogeneity is partly due to genetic factors and partly to post-translational modifications. The number of structural gene loci that code for the different molecular forms is unknown. However, at least three gene loci seem to be involved in determining the various forms of alkaline phosphatase that occur in human tissues : one coding for the placental form, at least one coding for intestinal forms (adult and fetal), and at least one coding for the liver, bone, and kidney forms (Harris, Hopkinson and Robson, 1974). These three classes of APs can be distinguished from one another by

their behaviour with certain inhibitors, as well as their electrophoretic, immunochemical and thermostability properties (Boyer, 1963; Robson and Harris, 1967; Stinson and Seargeant, 1981; Seargeant and Stinson, 1979; Goldstine, Rogers and Harris, 1980; Lehman, 1975). More evidence for the presence of these different classes has come from studies on their primary structures (Green and Sussman, 1973; Badger and Sussman, 1976).

Using these criteria the alkaline phosphatases occuring in liver, kidney, bone, intestine and placenta from a series of mammals were compared with those occuring in human tissues. It was found that in each of the animal species studied the liver, bone, kidney and placental alkaline phosphatases resembled each other and were distinct from that occuring in the intestine. They were also similar to human liver, bone and kidney alkaline phosphatases (Goldstine, et al, 1980; Goldstine and Harris, 1979) . These results suggest the presence of two distinct forms of alkaline phosphatase in mammalian species, a liver, bone, kidney and placental form which corresponds to human bone, kidney and liver isoenzymes and an intestinal form which corresponds to human intestinal enzyme. These are presumably coded by two separate gene loci. In humans a third distinct form occurs, human placental alkaline phosphatase (PAP), coded by an additional gene locus. This was evident from starch gel electrophoresis in which PAP was found to be highly polymorphic with more than 18 different allelic variants (Donald and Robson, 1973). Similar electrophoretic surveys have been carried out on the intestinal and other tissue alkaline phosphatases but no such allelic variants have been found (Harris, et al, 1974). This implies that the mutations affecting the locus for PAP do not affect the other

APs which are therefore presumably coded by other loci.

Some direct evidence for differences in primary structure between placental and liver isoenzymes has come from the studies of Green and Sussman, (1973) and Badger and Sussman, (1976), in which differences were found between their respective N-terminal amino acid sequences, two-dimensional tryptic-peptide maps, and their amino acid compositions. Differences in the N-terminal amino acid sequences of the liver, intestine, and placental isoenzymes were also reported (Badger and Sussman, 1976; Komoda, Sakagishi and Sekine, 1981). More structural studies were carried out on radioiodionated tryptic peptide maps of purified preparations of the enzyme forms from liver, kidney, intestine, placenta, and serum from a patient with Paget's disease of the bone by Seargeant and Stinson (1979). In this study, the fingerprint patterns obtaind from liver, bone, and kidney were very similar and can be distinguished from those of the intestinal and placental isoenzymes which also differ from each other.

The genetic origin of the alkaline phosphatases that constitute the non-placental, non-intestinal group i.e. those from such tissues as liver, bone, and kidney remain unclear. These alkaline phosphatases differ in electrophoretic mobility and are less heat resistant than intestinal and placental alkaline phosphatases (Boyer, 1965; Mulivor, Hannig and Harris, 1978; Goldstine and Harris, 1979; Goldstine, et al, 1980). Their electrophoretic mobilities varied between tissues, but the differences in mobility may be accounted for by differences in the carbohydrate side chains of the enzymes, as their de-sialated forms have identical isoelectric points and similar migration patterns on agar gels (Stinson and Seargent, 1981). Furthermore, the phosphatases in this group do not differ significantly in catalytic properties, nor in

antigenic specificity, as might be expected of the products of distinct structural genes. Therefore, a comparison of their properties suggests (but does not prove) that bone, liver, and kidney alkaline phosphatases are encoded by a single distinct locus and that some tissue-specific post-translational variations are introduced during the expression of this locus in different types of cells. Evidence from the rare bone disease, hypophosphatasia, which is inherited as an autosomal recessive (see Harris, 1982), also support the suggestion of a single structural gene for these three phosphatases. In one such case, AP activities of bone, liver, and kidney were markedly subnormal, whereas activity in the small intestine was normal.

The three main classes of human APs determined by these different loci can be sharply distinguished from each other by a variety of methods including thermostability, inhibition, immunological, and electrophoretic studies. Placental alkaline phosphatase has remarkable thermostability (Neale, Clubb, Hotchkis and Posen, 1965; Fishman and Ghosh, 1967; Goldstine and Harris, 1979; Stinson and Seargent, 1981). It can be heated at 65°C for an hour or more without any loss of activity. The other APs are destroyed under these conditions. At 56°C intestinal AP is seen to be much more stable than liver, bone, and kidney APs. Table 1 shows the time required to produce 50% inactivation at 56 and 65°C.

Differential inhibition studies on human alkaline phosphatases by certain amino acids and other low molecular weight substances also gives good discrimination among these enzymes. The most commonly used inhibitors are L-phenylalanine, L-homoarginine , L-phenylalanylglycyl-glycine , L-leucine, and levamisol. Several reports showed that L-

phenylalanine strongly inhibits placental and intestinal AP under conditions in which liver and bone APs are only slightly inhibited (Mulivor, Plotkin and Harris, 1978; Goldstine, et al, 1980; Stinson and Seargent, 1981). In contrast, L-homoarginine was found to strongly inhibits liver and bone APs but placental and intestinal APs are hardly inhibited. Table 2 shows the concentrations of different inhibitors required to produce 50% inhibition in the different isoenzymes.

These isoenzymes were also found to be immunologically distinct. Antisera raised against purified PAP usually cross react with intestinal AP but show a reaction of only partial identity on Ouchterlony double diffusion plates (Boyer, 1965; Lehmann, 1975; 1980). The cross reacting antibodies can be absorbed out with intestinal AP leaving antiserum monospecific for PAP. These antisera do not cross-react with liver, bone, or kidney APs, so one may infer that intestinal AP is more closely related immunologically to PAP than is liver, bone, and kidney isoenzymes.

Starch gel electrophoresis of extracts from various tissues have shown that all APs have two groups of components. One moves relatively rapidly toward the anode and represents the dimeric forms of the enzyme. The other migrates relatively slowly. These slow moving components have higher molecular weights. The origin of such heterogeneity has been discussed mainly in terms of post-translational modification; for example, differences in carbohydrate content or formation of enzyme lipid complex (Debroe, Borgers, Wieme, 1975; Doellgast, Spiegel Gunther and Fishman, 1977). Recently, other types of heterogeneity have been reported such as the occurence of soluble and membrane-bound forms (Komoda, et al, 1981; Colbeau and Maroux, 1978). Treatment with neuraminidase results in considerable retardation of the liver, bone,

Table 1 Time in minutes required to give 50% inactivation of different human APs at 56°C (T^{56}) and at 65°C (T^{65}).

Human alkaline phosphatase	T^{56} (minutes)	T^{65} (minutes)
Liver/bone/kidney	7.4	1.0
Intestinal	>60.0	6.5
Placental	-	>60.0

(Adapted from Harris, 1980)

Table 2 Concentrations of various inhibitors required to produce 50% inhibition ($[I_{50}]$ mM), of different human alkaline phosphatases under standarized conditions*.

	Phe	Har	Pgg	Leva	Leu
Liver/bone/kidney (AP)	31.0	2.7	30.6	0.03	13.1
Intestinal (AP)	0.8	40	3.7	6.8	3.6
Placental (AP)	1.1	>50	0.1	1.7	5.7

*The inhibitors are L-phenylalanine (Phe), L-Homoarginine (Har), levamisol (Lev), L-phenylalanylglycylglycine (Pgg) and L-leucine (Leu).

(Adapted from Harris, 1980)

and kidney APs. After the treatment their mobilities are essentially the same, so the mobility differences they exhibit in the untreated state can be largely attributed to differences in sialic acid residues (Robinson and Pierce, 1964; Butterworth and Moss, 1966; Moss, Eaton, Smith and Whitby, 1966; Mulivor, et al, 1978; Harris 1980). PAP is also retarded after neuraminidase treatment though to a lesser extent than liver AP. After treatment the various PAP phenotypes remain clearly distinct, so the allelic variations are not attributed to the numbers of sialic acid residues. The mobility of the adult intestinal AP is not altered by neuraminidase. It evidently contains no accessible sialic acid residues. In contrast, fetal intestinal AP, which has a greater mobility than the adult form, is some what retarded by neuraminidase (Komoda and Sakagishi, 1978).

There is now some evidence that there might be a fourth locus in man expressed at low levels in testis and thymus, coding for trace amounts of heat stable placental-like AP with marked sensitivity to inhibition with L-Leucine (Chang, Angellis and Fishman, 1980; Harris, 1980; Millan, Eriksson and Stigbrand, 1982).

1:3.2 Placental Alkaline Phosphatase Polymorphism

The existence of a unique gene locus for placental alkaline phosphatase (PAP) is evident from the marked allelic variation exhibited by this isoenzyme, variation that is not present in other tissue alkaline phosphatases. The electrophoretic variations in the enzyme extracted from different placentae were first reported by Boyer (1961). He described three types of electrophoretic patterns and suggested on the basis of their population frequencies that the differences were

genetically determined. He also reported the existence of a further component, migrating slower than his three bands, which he termed the D-band. This slow migrating variant was also reported in the studies of Beckman and Beckman (1968,1969), as a rare variant, and found to be inhibited by L-leucine (Doellgast and Fishman, 1976). Using placentae from single and dizygotic twin pairs in the English, Negro and Indian populations, Robson and Harris (1965; 1967) confirmed Boyer's findings and described six common phenotypes, refered to as F1, F1I1, I1, S1I1, F1S1, and S1, as well as nine other rare phenotypes, usually in combination with one of the common alleles. There is now evidence for more than 15 rare alleles (gene frequencies <0.005) beside the three common ones (Donald and Robson, 1973; Harris, Hopkinson and Robson, 1974). In addition, based on population data from different investigators, the existence of a "null" allele, $\underline{Pl}^{0}$ has been proposed (Beckman, Beckman, Christodoulou, and Ifekwunigwe, 1967). The nomenclature for the alleles at the AP locus was changed to a simple numbering system by Donald and Robson (1973). The former S became 1, F became 2 and I became 3. Thus the FS is now 2-1, SI is 3-1 and FI is 3-2. Rare alleles were numbered from 4 to 18. The frequencies of these common phenotypes agreed with the hypothesis that they are determined by three common alleles, $\underline{Pl}^{s}$ (slow mobility), $\underline{Pl}^{f}$ (fast mobility), and $\underline{Pl}^{i}$ (intermediate mobility). The electrophoretic patterns, in starch gels, support this idea since the postulated homozygotes have only one principal component whereas the heterozygotes all have three components, two in the positions of the product made by the corresponding homozygotes, and a third, intermediate in position, which is presumably a hybrid molecule since PAP is a homodimer. Gene frequencies of these alleles showed a marked variations among various ethnic groups. The $\underline{Pl}^{2}$

allele has its highest frequencies (24-34%) in Caucasian populations, whereas lower frequences are found in Asiatic mongoloid groups (2-8%) and African Negroes (2-5%). On the other hand, the $\underline{Pl}^3$ is comparatively common in Chinese and Japanese (20-24%) but low in Caucasians and Negroes (4-9%). The $\underline{Pl}^1$ is the most common in all ethnic groups (Beckman and Beckman, 1969; Donald and Robson, 1974). It was proposed that these variants are most likely controlled by a series of multiple alleles. Twin studies showed that the placental alkaline phosphatase is determined by the fetal genotype and not by that of the mother (Robson and Harris, 1965).

There are no differences between these enzyme variants with respect to pH-optima, inhibition by L-phenylalanine and L-leucine, and Km values towards the tested substrates. However, variations in the thermostability between these variants were observed (Holmgren and Stigbrand, 1976). Antigenic specificity for the different allelic forms was also observed by Wei and Doellgast (1980) using allozyme-absorbed antisera.

The structural differences between the products of the different alleles have not been elucidated. Simple amino acid substitutions may account for the alleles of PAP, as they do for the Adh-f and Adh-s electromorphs of Drosophila alcohol dehydrogenase (Kreitman, 1983). As substitutions might involve charged and uncharged amino acids, one would not expect the electrophoretic analysis to represent all the possibly existing variants. Some evidence for this has come from investigations on the potential value of various monoclonal antibodies for the detection of the allelic variants of PAP, in which allelic differences not detectable electrophoretically have been observed (Slaughter, et al, 1981; Millan, et al, 1982).

To determine the species specificity of PAP, Doellgast and Benirschke (1979), used the criteria which distinguish the human placental isoenzyme to compare the enzymes in species closely related to man. In this study only the chimpanzee and orangutan were found to have an isoenzyme resembling that in humans, thus suggesting that the occurrence of this isoenzyme is a relatively late event in primate evolution. The absence of a similar isoenzyme in lowland gorillas in this study was surprising, as there is no evidence for a divergence of this species from the line of man's descent after the orangutan. Further studies (see Harris, 1980) confirmed these observations and suggested that PAP is a relatively recent newcomer on the evolutionary scene and appeared subsequent to the divergence of the evolutionary lineages leading to man and the great apes from the lineages to most other mammalian species including lower apes (e.g. gibbon).

1:3.3 Synthesis and Molecular Heterogeneity of Placental Alkaline Phosphatase During Pregnancy

Placental alkaline phosphatase becomes detectable in the serum of pregnant women between the 16th and the 20th weeks of pregnancy. Its levels follow an exponential course, with the highest level occurring at delivery in most pregnancies and disappearing within three to six days of the delivery. The level during the last trimester of pregnancy amounts to 200-300 ng/ml (Holmgren, Stigbrand and Damber, 1978). No correlation has been found between term values of placental isoenzyme and placental weight, weight or the sex of the baby, maternal or newborn blood type, age or parity of the mother, or the number of the previous children (Fishman, et al, 1972). The amount of PAP in serum is often increased during complications of pregnancy such as hypertension, pre-

eclampsia, eclampsia and toxaemia (Aleem, 1972; Hunter, et al, 1970). Later studies, on serum levels of PAP, by Holmgren, et al (1978) were contradictary to the previous observations. Using a radioimmunoassay, their study has shown values of PAP significantly lower than that of the mean value for normal pregnancies which were associated with pre-eclampsia, low weight of the feto-placental unit and intra uterine growth retardation.

In 1976 Fishman, Miyayama, Driscoll and Fishman described isoenzymes of alkaline phosphatase in placental tissue that seemed confined only to the first 6-10 weeks of pregnancy. These isoenzymes have been called developmental phase-specific alkaline phosphatases, early-type, or chorionic type phosphatases. Following electrophoresis, early-type phosphatases have been shown to consist of two heat sensitive, L-homoarginine-inhibited bands, the slower band possessing antigenic determinants shared with bone and liver phosphatases, whereas the faster migrating band lacks any of the known alkaline phosphatase antigenic determinants. Phase 2 (11 to 13 weeks) is a mixture of phase 1 and 3 isozyme components, on starch gels, the latter exhibiting two isozyme bands with the characteristics of term PAP, which is now corresponding to the A and B forms of PAP. The A form is more anodal and constitutes the major zone of enzyme activity in pregnancy serum as well as in placental extracts. The B form does not move far from the origin and is considered to be a minor component of PAP. Both forms are sialoglycoproteins and when treated with neuraminidase showed retarded zones of activity on starch gels (Robson and Harris, 1965). The similar heat stability and the expression of the same polymorphism in the A and B forms of PAP indicates that they contain the same enzymatic

polypeptide. The structural basis of the difference between the two forms is not known, though the B form appears to be of higher molecular weight than the A form. Studies on purified preparations of both forms by Ghosh and Fishman (1968) gave molecular weight estimates of 70,000 and 200,000 for the A and B forms respectively, on the bases of sucrose-density-gradient ultracentrifugation. The two forms were also reported to be interconvertible by storage and dilution, so B-PAP was considered to be a molecular aggregate of the A form. In further studies the two forms were found to have different isoelectric focusing points and behaved differently on acrylamide gel electrophoresis and on ion exchange chromatography. The differences in the properties of these two enzyme forms were attributed to the association of the B form with hydrophobic and basic residues and not to the polymerization of subunits alone. On this basis the B form was assumed to be a membrane associated form of PAP (Doellgast, et al, 1977). Genetic and structural studies on the A form of PAP have shown that the enzyme is a homodimer composed of two identical polypeptides. Molecular weight estimates between 63,000-70,000 were reported for the fully processed form of PAP monomer (Ghosh and Fishman, 1968; Holmgren and Stigbrand, 1976; Kelley, et al, 1979; Ogbimi, et al, 1979), while an estimate of 61,500 for its precursor, synthesized by choricarcinoma cells in vivo, was reported by Ito and Chou (1983).

1:3.4 Tumour-related Alkaline Phosphatases

It is perhaps significant that fetal proteins are often expressed in tumours. The term "oncodevelopmental gene products" has been coined for such proteins (Fishman, et al, 1968). The existence of oncodevelopmental gene products such as alphafetoprotein of hepatoma, carcino-embryonic antigens of colonic carcinoma as well as several other proteins (Wahren, et al, 1979), suggests that malignancy may involve disturbances in the regulation of the expression of genes normally active in the embryo and the fetus. Whether most of these proteins are tumour-specific antigens or not has yet to be clearly elucidated, as detectable levels of these proteins are found in normal human sera and in several other tissues (Usategui-Gomez, et al, 1974; Ruoslahti and Seppala, 1972; Millan, Eriksson and Stigbrand, 1982).

Placental alkaline phosphatase was the first enzyme described as an oncofetal or carcinoplacental antigen by Fishman, Inglis, Stolbach, and Kraut (1968). It was found in the serum of a patient (Mr. Peter Regan) with squamous cell carcinoma of the lung. The identity of this enzyme with respect to the placental form has not been completely resolved but, with regard to immunological cross reactivity and similar catalytic properties, they seem to be structurally similar (Fishman, et al, 1968; Lehman, 1975). Several years later in Japan, another tumour-related form of alkaline phosphatase was found in a patient (Nagao) with pleural carcinomatosis (Nakayama, et al, 1970). Although the Nagao isoenzyme is indistinguishable from placental isoenzymes immunologically and in some biochemical respects (heat inactivation, L-phenylalanine inhibition), it is distinct from placental and Regan isoenzymes in terms of its sensitivity to L-leucine and EDTA inhibition. With respect to L-leucine

inhibition, Inglis, Kirley, Stolbach and Fishman (1973) re-examined 39 sera known by electrophoretic and catalytic methods to contain the ectopically produced Regan isoenzyme, and observed a high incidence of sera which exhibited a high degree of L-leucine inhibition, in addition, migrated on starch gels to the position of the D-variant placental phenotype. On this basis they suggested identity between the rare D-variant of PAP and the Nagao isoenzyme. This postulation, however, has been contested on technical and genetic grounds by Beckman and Beckman (1975). Most recent work consider the Nagao isoenzyme to represent re-expression of a gene structurallty related to but distinct from the placental locus (Wei and Doellgast, 1981; Millan, Stigbrand, Ruoslahti and Fishman, 1982).

Placental AP is not the only enzyme expressed during malignant transformation : the intestinal type as well as other alkaline phosphatases are known to be ectopically expressed in malignant cells (Timperley, et al, 1971; Benham, et al, 1978). One such tumour enzyme was identified in a patient with hepatoma (Warnock and Reisman, 1969) and later named the Kasahara variant. The available data suggest that the Kasahara isozyme represents the ectopic expression of the intestinal alkaline phosphatase gene (Higashino, Hashinotsume, Yang, Takahashi[a] and Yamamura, 1972; Higashino, Otani, Kudo and Yamamura 1977; Higashino, Kudo, and Yamamura, 1974). This isoenzyme has been also described in renal cell carcinomas (Hada, Higashino, Okochi, Yamamura, 1978), indicating that the expression of this enzyme is not hepatoma-specific.

The appearance of alkaline phosphatase with properties similar to those of PAP and other tissue APs has been demonstrated in a number of HeLa cell lines which were originally derived from a carcinoma of the cervix as well as several other cell lines derived from various human

tumours (Singh, Tsang and Blakemore, 1978; Singer and Fishman, 1974; Rutigan et al, 1974). Heterogeneity between HeLa cell-lines for alkaline phosphatase expression was observed in a study of eight cell lines of HeLa, in which six lines contained placental-like alkaline phosphatase, while the others were liver-like and a new form distinguishable from all other known isoenzymes (Benham, Povey and Harris, 1978). These PAP-like enzymes, although cross reactive with PAP when polyclonal antibodies are used, fail to show the clear electrophoretic polymorphism of PAP, and can be distinguished catalytically by their different pattern of inhibition with L-leu, L-Leu-Gly-Gly, Phe-Gly-Gly (Millan, Eriksson and Stigbrand, 1983). Thus there is question as to the extent of the similarity between these PAP-like enzymes and the placental enzyme.

The frequency of detectable concentration of PAP-like isoenzymes in screening studies of cancer patients falls in a range between 0% (Jacoby and Bagshawe, 1972) and almost 100% (Usategui-Gomez et al, 1974) with several reported values in the range 5-25% (Lehman, 1975a; Stolbach, Kraut and Fishman, 1969). These variations were mainly due to the lack of the sensitivity and specificity of the different assays employed and to some extent to the origin of the malignancy McLaughlin, Gee and Johnson (1983). Although the re-expression of different alkaline phosphatases has been documented both in cell lines in vitro and in human tumours in vivo, the application of serological assay for monitoring malignancy patients has not achieved widespread clinical usefulness (Winkleman, Nadler, Demitrian and Pileggi, 1972). Recently a highly sensitive and specific solid-phase enzyme immunoassay (EIA) for PAP based on the application of monoclonal antibodies (H317) has been

reported by Mclaughlin et al (1983), with a detection limit of approximately 0.07 µg/l The use of the H317 monoclonal antibody in solid-phase EIA detected measurable levels of circulating PAP-like enzymes in significant proportion of ovarian (34%) and cervical (25%) carcinoma patients, but did not show detectable levels in other malignancies, including breast carcinoma. Thus it may be a useful and sensitive serological parameter particularly in ovarian carcinoma. Another sensitive immunoassay (sandwich enzyme-linked) has been reported by Millan and Stigbrand (1982). Further studies will reveal more clearly whether such assays will increase the diagnostic value of alkaline phosphatase in cancer.

1:4 Membrane Proteins

Since PAP is associated with the syncytiotrophoblastic plasma membrane, it is essential to understand the nature of the intimate association between such proteins and the membranes. The key to our present concept of the structure of membranes was the discovery that membrane proteins retain their globular structure and could actually span the membrane in an asymmetric fashion. The idea was taken up in the "Fluid Mosaic Model", proposed by Singer and Nicolson (1972), which attempted to explain this structure in thermodynamic terms and drew two conclusions. Firstly, the lipid and protein molecules must be arranged in a tightly packed manner in which their hydrophobic regions can interact in the core of the membrane whilst the hydrophilic region of each amphipathic molecule is exposed to the aqueous environment. The second conclusion is that the individual components are free to move about in the plane of the membrane with respect to each other. Above the melting temperature of the bilayer lipid, the hydrocarbon chains in the core of the membrane are in a fluid-like state approaching that of a liquid hydrocarbon. This fluidity of biological membranes is a variable property which depends on the packing of the hydrocarbon chains of the phospholipids. The more tightly packed they are the less fluid is the membrane. Although this model is frequently presented as an accurate description of membrane structure this is not really the case. It is based on the concept that the lipid is the major structural entity in membranes, which might be true for myelin membranes which contain only 25 percent protein by weight, while the plasma membrane and the mitochondrial inner membrane contain 50 and 75 percent protein

repectively (Houslay and Stanley, 1982), and thus might have a quite different structure. A second tenet of the original fluid mosaic model is the absence of long range order in the protein molecules.

Different types of membrane proteins interact with the phospholipid bilayer very differently. On this basis membrane proteins can be divided into two classes:

Peripheral membrane proteins: proteins of this class are bound to the phospholipid bilayer only indirectly, via a primary interaction with another membrane protein. In contrast to integral membrane proteins, they can be removed from the membrane by treatments which do not destroy the lipid bilayer, such as extraction with salt solutions of low or high ionic strength. These proteins, then, are specifically localized to one side or another of a cellular membrane. Some such as fibronectin and beta-2-microglobulin, are localized to the outer surface of the plasma membrane. Others such as spectrin, actin, and other cytoskeletal proteins are believed to be synthesized as soluble, cytoplasmic proteins which bind to specific proteins (or other molecules) in the membrane only after their synthesis.

Integral membrane proteins : these proteins are firmly embedded in the lipid matrix where they exihibit hydrophobic and electrostatic interactions with the surrounding lipid. Two key types of experiments have established the transmembrane orientation of most of these proteins. First, proteolytic digestion of membrane vesicles which removes most of the polypeptide backbone, leaving the imbedded portion of the protein within the lipid bilayer. Secondly, detergent or solvents solubilization which release the intact polypeptide. Because of the large number and the varied functions of integral membrane proteins, it is unlikely that a single model of interaction with a lipid

bilayer will apply to all of them. Lodish, Braell, Schwartz, Strouts and Zilberstine (1981) proposed the following classification which reflects primarly the differences in interactions of the polypeptide chain with the phospholipid matrix.

A. Transmembrane glycoproteins which span the phospholipid bilayer once: this class includes proteins of quite diverse origin (influenza HA glycoprotein, erythrocyte glycophorin, the heavy chain of the HLA-A and H2 antigens and VSV G protein) and have in common a number of of key structural characteristics. All of these polypeptides span the lipid bilayer once and in all of them, a region of 10-30 amino acids at the C-terminus lies within the cytoplasm. Phosphorylated serine residues have been reported in the corresponding regions of H2 antigen (Houslay, 1981) and HLA antigens (Pober, et al, 1978). Adjacent to this region is a sequence of 20-25 very hydrophobic amino acids which are believed to form be the segment which spans the lipid bilayer. Close to either the amino or the carboxy end of this stretch is often a series of lysine and arginine residues; possibly this interacts with the phosphate residues of the phospholipid bilayer. The remainder of the polypeptide chain (which varies in size among these polypeptide species) and its attached carbohydrate chains is on the external surface of the membrane. These carbohydrate residues may be either N-linked (on asparagine) or O-linked (to serine or threonine). The role of these carbohydrates in membrane proteins is largely undefined.

B. Integral membrane proteins which pass through the phospholipid bilayer several times: some of these proteins, like rhodopsin and erythrocyte band III protein, constitute a major portion of the membrane protein in their respective cell types. The striking differences

between the configurations of these proteins and those in the previous class pose interesting questions as to their mechanism of biosynthesis, since in many respects the generally accepted models are inadequate.

C. Integral membrane proteins which do not span the phospholipid bilayer: most of these proteins are enzymes, such as glucose-6-phosphatase, cytochrome b_5, cytochrome b_5 reductase , cytochrome P_{450} as well as several glycosyl-transferases. These proteins are anchored to the plasma membranes via hydrophobic segments at either their carboxy or amino terminals.

Cells have evolved systems to restrict the mobility of membrane proteins in the lipid bilayer to a greater or lesser extent. Specific interactions between intrinsic membrane proteins and cytoskeletal elements are thought to exist, (Flanagan and Koch, 1978; Koch and Smith, 1978; Mescher, et al, 1981; Sheterline and Hopkins, 1981). Controlled attachment and detachment of the cytoplasmic portion of integral membrane proteins to internal peripheral membrane proteins and cytoskeletal elements may occur. The maintenance of such specific membrane protein-cytoskeletal interactions may mediate responses to intra- and extra cellular stimuli (Houslay, 1981). Such interactions could provide a mechanism by which certain types of extracellular signals can be transmitted to the interior of the cell.

There seems to be a general agreement that these transmembrane proteins, like most secretory proteins, are synthesized on membrane-bound polysomes in precursor forms containing, at their amino termini, signal peptides, which are sequences of predominantly hydrophobic amino acids not present in the mature proteins. The majority of data accumulated in this field can be fitted into the "signal hypothesis" (Blobel and Dobberstein, 1975) which proposes that a nascent polypeptide

destined for export binds to the membrane via the signal sequence. The associated ribosomes then forms a complex with membrane proteins, and the growing polypeptide chain is extruded through the membrane. The signal sequence is proteolytically removed during or immediately after the insertion of the nascent polypeptide to generate the mature protein. An alternative, the "membrane-trigger hypothesis" (Wickner, 1979), envisions a different role for the signal sequence. Here it is proposed that the extra amino acids confer a special structure on the precursor, which then associates with the membrane. This interaction triggers a conformational change resulting in the insertion of the protein into the lipid bilayer. This model invokes self assembly whereas the signal hypothesis requires the involvement of a protein complex that actively translocates the growing chain. A comparison of several signal sequences of secretory and membrane integrated proteins of the Vesicular Stomatitis virus (VSV) (Lingappa, Katz, Lodish and Blobel, 1978) revealed several common features, such as the presence of short amino terminal hydrophilic signal followed by a hydrophobic segment of amino acids. In 1975 Blobel and Dorbberstein assumed that the microsomal membrane could select sequences bearing nascent chains by means of a specific receptor. Evidence which confirmed the existance of such a receptor has come from studies of Lingappa et al (1978); Jackson, Walter and Blobel (1980); Prehn, Nurnberg and Rapaport (1980), in which synthetic signal sequences and other secretory and membrane proteins were shown to compete with proteins undergoing translation; and translocation could be prevented by inactivating the microsomal proteins. This evidence brought into doubt the theory that nascent chains alone possessed all the features needed to ensure their transfer

through a membrane (Wickner, 1979; Engelman and Steitz, 1981). Over the past few years the role played by the microsomes in the interpretation of the signal sequence has become better understood. The most recent view of the initial events occurring in vectorial transfer indicates the participation of two cytoplasmically-disposed proteins. The signal recognition protein (SRP) has a molecular weight of 250,000 (Walter and Blobel, 1980) and the docking protein (DP) with a molecular weight of 72,000 (Meyer, Louvard and Dobberstein, 1982). As a nascent secretory or membrane protein reveals itself through the exposure of its signal sequence, cytoplasmic SRP will recognize it and shut down its further translation. This block persists until the nascent chain-SRP-ribosomal complex contacts and binds to the endoplasmic reticulum-specific SRP receptor, the docking protein. As a result of this SRP-DP interaction, the block is removed, translation continues and transfer through the membrane commences (for review see Meyer, 1982).

Although such studies suggest similarities between the structural prerequisites for the early events in protein secretion and those of membrane assembly, questions regarding the specific mechanism by which nascent chains cross the lipid bilayer of the rough endoplasmic reticulum have not been answered.

CHAPTER 2

ALKALINE PHOSPHATASE AS A MARKER FOR HUMAN PLACENTAL MICROVILLOUS MEMBRANES

2:1 INTRODUCTION

Human placental alkaline phosphatase is a tissue-specific enzyme of the placenta. Histochemical staining indicated that alkaline phosphatase is enriched in the syncytiotrophoblastic microvilli (Jones and Fox, 1976). One of the common ways of preparing syncytiotrophoblastic microvilli is to gently extract the diced or minced placenta in saline by the method of Smith et al (1974). Using this method, Davies, Parry and Sutcliffe (1981) found that the microvilli could be separated by sucrose density gradient centrifugation into two populations, which showed similar polypeptide patterns on SDS polyacrylamide gels, but showed different bindng properties for labelled insulin, transferrin and IgG. Truman, Wakefield and Ford (1981) have also reported that two fractions of microvilli can be prepared in a similar way. In order to confirm the placental origin of these fractions of microvilli it was necessary to know if placental alkaline phosphatase was present in both.

2:2 Materials and Methods

2:2.1 Materials

All chemicals for the staining and assay of alkaline phosphatase were purchased from the Sigma Chemical Co. Nonidet NP40 was obtained from BRL, Rockville, Maryland, U.S.A. Rabbit antisera to human serum albumin and human transferrin were purchased from Behring Diagnostics. Rabbit antisera to placental microvilli which were rich in anti-placental alkaline phosphatase were a kind gift of Dr. P.M. Johnson, Liverpool University. The antisera were absorbed with adult human serum before use. Sepharose CL-6B and DEAE Sepharose CL-6B were obtained from Pharmacia (GB) Ltd. Ultrogel ACA 34 was obtained from LKB.

Placentae were obtained immediately after delivery and were stored at 4°C and used within 4h. Other tissues were obtained from autopsy material within ten hours of death.

2:2.1 Methods

1. Plasma Membrane Preparations

Placental microvilli were prepared by the method of Smith et al, (1974). Washed term placentaltissue was diced, placed in 0.16M NaCl, agitated for 30 min at 4°C, and the 1.0 G supernatant was used as the source of microvilli; the residual placental tissue was used as the source of "microvillus-depleted placenta". The two fractions of microvillous membranes were finally separated by sucrose gradient density centrifugation (Davies et al, 1981).

Plasma membranes were prepared from microvilus-depleted placental tissue using the method of Snary, Woods and Crumpton (1976). After extraction of microvilli, the microvillus-depleted tissue was passed through a specially designed press and the membrane fraction was removed by differential centrifugation (Snary et al, 1976).

2. Extraction of Alkaline Phosphatase

Alkaline phosphatase was prepared from placental and other tissues by extraction with butanol/H_2O using the method of Boyer (1963) except that the incubation time for butanol was increased to 1 h at 20°C in order to improve the separation between the aqueous and butanol phases. For microvilli, the material was first homogenised in 0.16 M NaCl, 10 mM phosphate buffer (pH 7.4) and then extracted against butanol. The aqueous phase of the microvillous extract was then aspirated and centrifuged (10,000 g for 15 min). These aqueous extractions were used for electrophoresis. For gel filtration, the aqueous extracts were filtered through Whatman No.4 paper.

To test the effect of other membrane disruption procedures, microvilli and homogenates of microvillus-depleted placentae were suspended in 0.16 M NaCl, 10 mM phosphate buffer pH 7.4. Aliquots (2 ml) of these starting materials, containing 1 mg protein/ml were treated as follows. For solvent extraction a 2 ml aliquot was added to 2 ml chloroform:methanol::2:1. For detergent extractions, separate aliquots were adjusted with nonidet NP40 (to a final concentration of 1%; v/v), Na deoxycholate to 0.1 M, or Triton X-100 (0.05-2.0%). In all cases the mixtures were vigorously stirred at room temperature for 30 min., before centrifugation at 3000 g for 15 min. The aqueous phases were aspirated and analysed on starch gels.

3. Column Chromatography

Columns of Ultrogel ACA 34 (74 x 5 cm^2) and Sepharose CL-6B (24 x 1.8 cm^2) were equilibrated in 4.6 mM tris, 2.5 mM succinic acid, pH 7.0 with or without the addition of Triton X-100 as stated in the results.

Alkaline phosphatase extracts from microvilli and microvillus-depleted placenta were applied at room temperature at 20-6 ml/h. The following proteins were used for estimation of molecular weight : alpha-2-macroglobulin (725,000), IgG (150,000), bovine serum albumin (67,000), pregnancy associated protein SP3 (mol. wt. 340,000; Horne and Nisbet, 1979).

4. Electrophoresis

Horizontal starch gel electrophoresis was performed according to the method of Robson and Harris (1965) as follows : starch gels (12%) were made using succinic acid/tris buffer pH 6 (0.0025M succinic acid, 0.0046M tris) and the bridge solution consisted of a citric acid/sodium hydroxide buffer (0.41M citric acid adjusted to pH 6 usning 10M NaOH). Electrophoresis was carried out at 8 V/Cm for 5h in the cold room. After electrophoresis, gels were sliced and stained for enzyme activity using β-naphthyl phosphate (25 mg) as substrate in a solution consisting of 50 ml borate buffer (0.06 M pH 9.7), 25 mg fast blue RR salt and 60 mg $MgSO_4$ (Boyer 1961).

For SDS/polyacrylamide gel electrophoresis (Laemmli, 1970), sample preparation and staining was carried out using tris-buffered slab gels as described by Sutcliffe et al (1980).

5. Assays

Alkaline phosphatase was assayed in a system of 62 mM. sodium carbonate buffer pH 10.75, 12.5 mM. magnesium chloride, 32.5 mM "Sigma 104" phosphatase substrate and 5-20 µl. of enzyme solution in a final volume of 50 µl. In all assays a control without enzyme and a control without substrate were included. The assay solutions were incubated for

30-60 min at room temperature. The reactions were then stopped by the addition of 0.4M glycine-sodium hydroxide buffer pH 10.3 to a final volume of 1 ml. The optical density of the solutions was measured spectrophotometrically at 400 nm. The enzyme specific activities were calculated by subtracting the sum of the controls from the full systems, and using the molar extinction coefficient of p-nitrophenol (1.775×10^4 under these conditions) to express the results in micromoles substrate hydrolysed per hour per ml of the enzyme solution (Sutcliffe, Brock, Robertson, Scrimgeour and Monaghan, 1972).

Double immunodiffusion in 1% agarose was carried out according to the method of Ouchterlony (1976).

2:3 RESULTS

2:3.1 Starch Gel Electrophoresis

When butanol extracts of either unfractionated placental tissue or of microvilli were subjected to starch gel electrophoresis in the absence of Triton X-100, the gels (Figure 1a) showed the A and B forms of placental alkaline phosphatase as described by Boyer (1963) and Robson and Harris (1965). Extracts of microvilli often gave considerable smearing of enzyme activity between the A and B bands (see Figure 3). However, when Triton X-100 was added to the starch gel at a final concentration of 0.5%, alkaline phosphatase from both the total placental extract and the placental microvilli showed three bands of enzyme activity (Figure 1b). The extra band appeared between the A and B zones and was particularly prominent in extracts of placental microvilli (Figure 1b, Tracks "m"). It was at a very low concentration relative to the A and B forms in extracts of placentae which had been depleted of microvilli (Figure 1b, Track "mdp"). Because the extra band of enzyme activity between the A and B forms was at relatively high activity in placental microvilli, it has been called placental alkaline phosphatase "M". Figure 1c shows that both of the fractions of microvillous membrane reported by Davies *et al* (1981) have a small amount of the A form, some B form and a preponderance of the M form.

The method of Smith *et al* (1974) is for the purification of microvilli from the syncytiotrophoblastic brush border. It is a gentle method which is not expected to extract the whole plasma membrane of the trophoblast. This raised the question of whether some alkaline phosphatase A or B might be preferentially located in plasma membrane which did not extract under the conditions used by Smith *et al* (1974).

Accordingly, microvillus-depleted placenta was subjected to disruption using the mechanical press method of Snary et al (1976). Plasma membranes were harvested in sucrose gradients (Davies et al, 1981), extracted with butanol and the enzyme was examined on starch gels. The results (Figure 2) show that the placental plasma membrane (ppm) prepared from microvillus-depleted placenta were as enriched in the M form as were the microvilli (see Figure 1b, c). In contrast, the A and B forms of the enzyme located preferentially in the supernatant fraction(s) which overlaid the ppm though, as with the microvilli, some was detectable in the membrane preparation.

Experiments were included to investigate whether the M form of placental alkaline phosphatase could be generated during extraction through artifactual aggregation of polypeptides at low ionic strength. Aliquots of placental microvillous membranes were resuspended in 0.1, 0.2, 0.3 M NaCl in 10 mM phosphate buffer pH 7.4 and then subjected to butanol extraction. The aqueous phases were analysed for alkaline phosphatase on starch gels in the presence of 0.5% (v/v) Triton X-100. The M form of alkaline phosphatase was prominent in all the aliquots and there was no discernable effect of NaCl (Figure 3a). In addition, the aqueous phases were analysed in starch gels in the absence of Triton X-100 (Figure 3b). As expected, no M form was visible in these gels. After electrophoresis, the filter paper sample inserts were stained for residual enzyme activity and considerable activity was detected (Figure 3d). No residual activity was detected in inserts from starch gels containing Triton X-100 (Fig. 3c). This suggests that a fraction of placental alkaline phosphatase requires the detergent to be soluble in the starch gel system. This fraction is likely to be the M form since

it is absent from starch gels without Triton X-100. Further, the finding (Fig. 3a and b) that the quantity of A form in the gel is unaffected by Triton X-100 argues that Triton X-100 is not artifactually converting A to M (also see results of gel filtration, below).

The appearance of the M band was unaffected when microvilli were extracted not in butanol, but in choloroform:methanol, nonidet NP40, sodium deoxycholate, or Triton X-100 (Figure 3e).

Since the A form of placental alkaline phosphatase is polymorphic, the mobility of the M form in extracts of placentae which exhibited the different phenotypes of the enzyme polymorphism and their respective microvilli were examined. Butanol extracts were made from homogenates of whole placental tissue and microvilli and subjected to electrophoresis in starch gels containing 0.5% v/v Triton X-100. Bands of the M form of placental alkaline phosphatase were observed between the A and B bands (Figure 4) and the A form shows the homozygote and heterozygote phenotypes 1-1, 1-2 and 2-2, according to the nomenclature of Donald and Robson (1974). For each track, the mobility of the M form correlated with the mobility of the A form.

To investigate the immunological cross-reactivity of the different forms of placental alkaline phosphatase, pure fractions of A and M and butanol extracts of other human organ phosphatases were applied to the sample wells of Ouchterlony immunodiffusion plates and stained for enzyme activity (Figure 5). The results show lines of complete identity between A and M-PAP. No reactions were observed with alkaline phosphatases from other organs, nor did extracts of other organs interfere with the precipitation lines of A and M placental alkaline phosphatase.

2:3.2 Chromatography

Molecular weight determinations were performed by molecular sieving using Sepharose CL-6B equilibrated in tris-succinate buffer (Figure 6). All enzyme activity was eluted in a single peak. Aliquots of the filtration fractions labelled 1-12 (Figure 6, upper panel) were subjected to electrophoresis in starch gels and the results are shown in the lower panel of Figure 6, where the M form of the enzyme can be seen to elute before the A form. The results (Figure 6) also show that M form of the enzyme was co-eluted with alpha-2-macroglobulin (MW 725,000).

It was necessary to find out whether this high molecular weight represents the real molecular weight of the enzyme in microvilli, or whether it was an artifact due to the way in which the enzyme had been extracted from microvilli and prepared for gel filtration. Gel filtration was therefore carried out using AcA 34 in order to find out whether the enzyme of lower molecular weight could be generated by changing the conditions of enzyme extraction and elution. Ultrogel AcA 34 was selected as a mixture for gel filtration. It has an exclusion limit of 750,000 and a linear range of 20,000-350,000. This choice was made in order to detect additional species of sizes between the A molecule (127,000) and the species of 725,000. The first experiments were designed to determine whether the molecular weight could be affected by the presence of Triton X-100. Figure 7 shows the elution profile of alkaline phosphatase from a column equilibrated in 0.5% Triton X-100, tris-succinate buffer. Aliquots of the filtration fractions labelled 1-10 (Figure 7, upper panel) were subjected to electrophoresis in starch gels and the results are shown in the lower panel of Figure 7. In further experiments where Triton X-100 was absent

from the gel filtration system, the respective elution patterns of the A and the M were the same as in Figure 7. It was also found that the M form of the enzyme co-eluted with alpha-2-macroglobulin confirming the results of Sepharose CL-6B medium. A further experiment was conducted where the gel filtration medium was equilibrated in 0.16 M NaCl, tris-succinate without Triton X-100. Here microvilli in 0.16 M NaCl, 10mM K phosphate pH 7.5 were extracted with a half volume of butanol, dialysed against the equilibration buffer and subjected to gel filtration on AcA 34. The results showed (Figure 8) that the M form of the enzyme was detected co-eluting with alpha-2-macroglobulin, and that there was no substantial conversion of the enzyme to smaller species. Experiments were also carried out to exclude the possibility that the M form of the enzyme is an artifactual hydrophobic aggregate of the A form molecules. Enzyme was extracted from microvilli with Triton X-100 instead of butanol, before being analysed on AcA 34 equilibrated in 0.16 M NaCl, tris-succinate buffer in the presence of 0.5% Triton X-100. No conversion of the enzyme to smaller species could be detected. Together these gel filtration results show that the presence of Triton X-100 is not essential for the detection of a high molecular weight form of alkaline phosphatase in butanol extracts of microvillous membrane.

Placental alkaline phosphatase is more stable at 55-65°C than are alkaline phosphatases from other tissues (Neale et al, 1965). The heat stability of the A and the M forms of PAP were compared by taking fraction numbers 12 and 19, respectively, from the AcA 34 gel filtration experiment (Figure 8). Figure 9 shows that the enzyme in both fractions remained stable and active for at least for 60 min. at 60°C. For comparison, the alkaline phosphatase activity of human male serum was almost inactivated after 20 min. at 60°C.

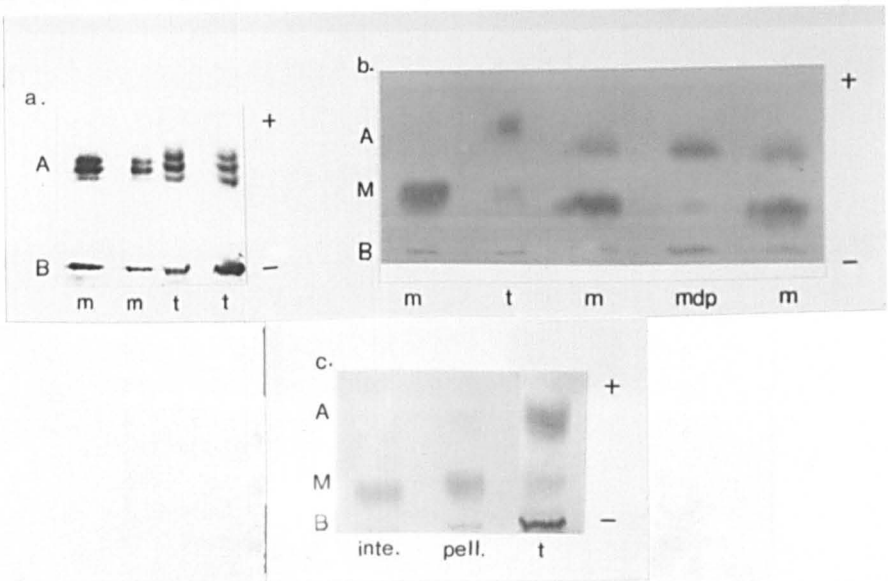

Figure 1. The effect of Triton X-100 on the mobility of placental alkaline phosphatase in starch gels.

Starch gel electrophoresis of various extracts of placenta, stained for alkaline phosphatase. In (a) the gels did not contain Triton X-100. The gels in (b) and (c) contained 0.5% (v/v) Triton X-100. The samples applied to the origin (o) were butanol extracts of : total placental homogenate (t); microvilli (m); microvillus-depleted placenta (mdp). Fig. 1 (c) shows the interface and pellet fractions from the sucrose gradient purification of microvilli by the method of Davies et al (1981).

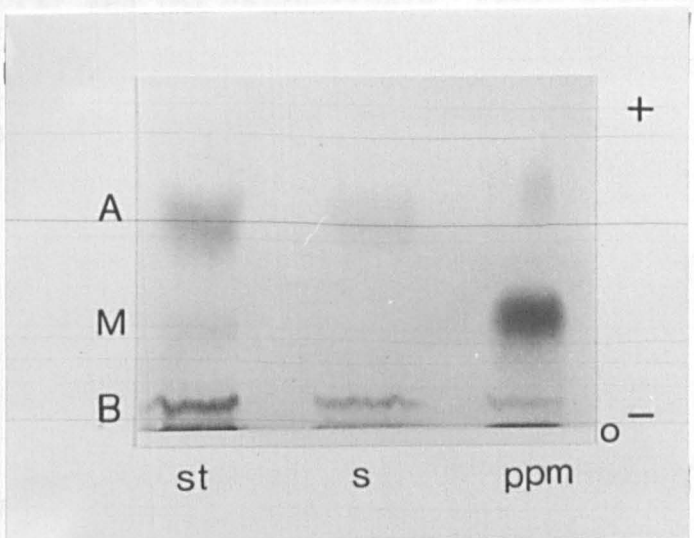

Figure 2. The electrophoretic mobility of placental alkaline phosphatase after sub-cellular fractionation of microvillous-depleted placenta.

The samples analysed were butanol extracts of the plasma membrane fraction (ppm) and the supernatent (s) fraction from a discontinuous sucrose gradient. Sample (st) was a butanol extract of microvillus-depleted placenta which had not been separated on a sucrose gradient.

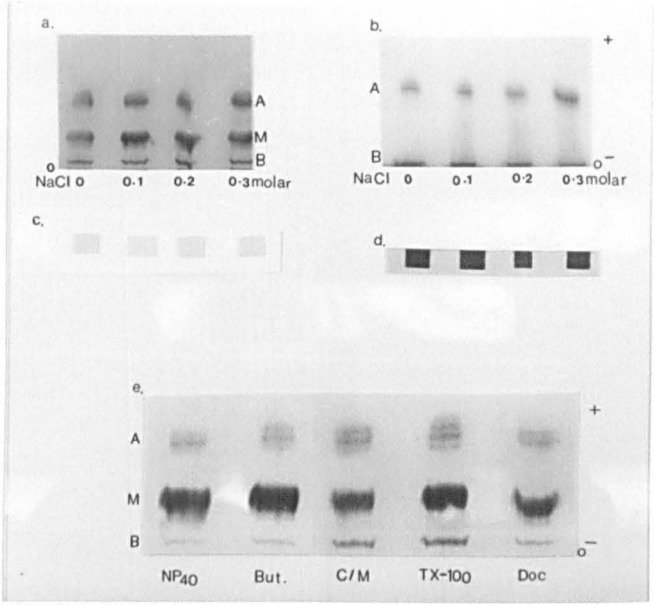

Figure 3. Experiments to test the effect of various extraction systems on the electrophoretic mobility of microvillous alkaline phosphatase.

In figure (a and b) the samples were extracted (see text) into NaCl solutions of 0-0.3 M as shown. In Figure (a) the gel contained 0.5% (v/v) Triton X-100; in (b) the gel did not contain Triton X-100. Figure (c) and (d) show the enzyme activity in sample inserts removed from the gel in Figure (a) and (b) respectively, after electrophoresis had finished. In Figure (e) microvilli were extracted with 0.1 M Na deoxycholate (DOC); 2:1::chloroform:methanol (C/M); 1% (v/v) nonedit (NP40); volume of butanol (B); 2% v/v Triton X-100 (TX-100). The extracts were loaded onto a starch gel containing 0.5% Triton X-100.

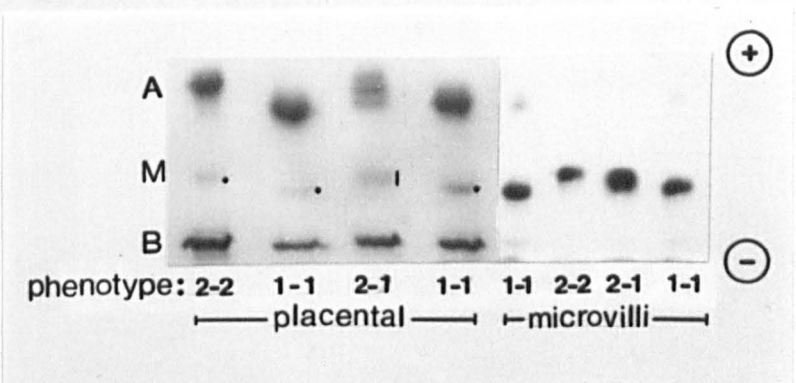

Figure 4. The mobility of the M form in different phenotypes of placental alkaline phosphatase.

Butanol extracts of total placental tissue and placental microvilli were applied to starch gels in the presence of 0.5% v/v Triton X-100. The phenotypes are noted below each sample.

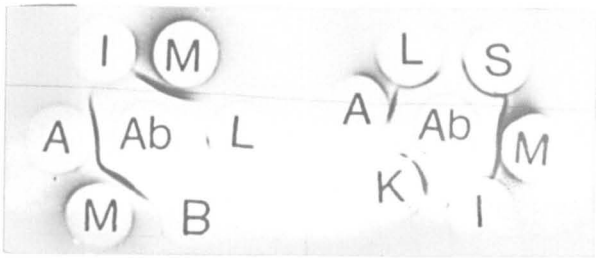

Figure 5. Immunodiffusion plate of the A and M forms of alkaline phosphatase.

Double diffusion of pure samples of PAP forms A and M (A,M) and tissue extracts of intestinal (I) liver (L),bone (B), spleen (S), and kidney (K) alkaline phosphatase. The antiserum (Ab) is rabbit anti-microvillous antiserum. The precipitation lines were stained for alkaline phosphatase activity. Equal activities of enzyme were loaded in each well.

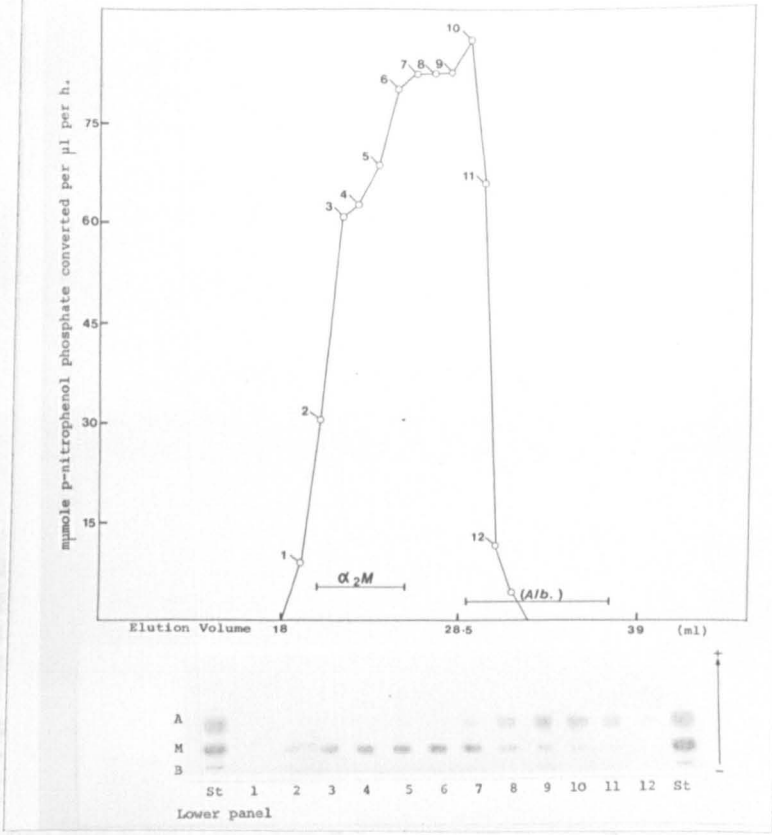

Figure 6. Gel filtration of placental microvillous alkaline phosphatase.

Upper panel: Sepharose CL-6B gel filtration of microvillous alkaline phosphatase extracted against butanol. Enzyme activity is plotted against elution volume in ml. Aliquots of the fractions marked 1-12 on this graph were taken for analysis by starch gel electrophoresis. Lower panel: Electrophoresis of aliquots 1-12 in starch gels containing 0.5% v/v Triton X-100. The aliquots were loaded according to the numbers below the tracks. Sample (st) is an aliquot of the extract originally applied to the Sepharose CL-6B column. The gels were stained for alkaline phosphatase activity and the origin (o) and the mobilities of the A, B and M forms are shown.

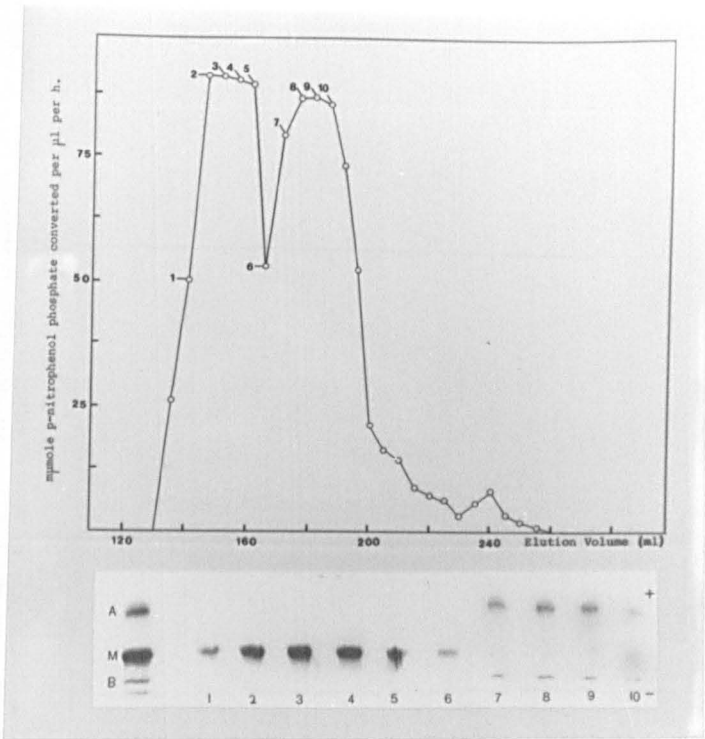

Figure 7. Gel filtration of placental microvillous alkaline phosphatase in the presence of Triton X-100.

Upper panel: AcA34 gel filtration of microvillous alkaline phosphatase extracted against butanol. Enzyme activity is plotted against elution volume in ml. Aliquots of the fractions marked 1-10 in this graph were taken for analysis by starch gel electrophoresis.

Lower panel: Electrophoresis of aliquots 1-10 in starch gels containing 0.5% v/v Triton X-100. The aliquots were loaded according to the numbers below the tracks. Sample (st) is an aliquot of the extract originally applied to the AcA 34 column. The gels were stained for alkaline phosphatase and the origin (o) and the mobilities of the A, B and M forms are shown.

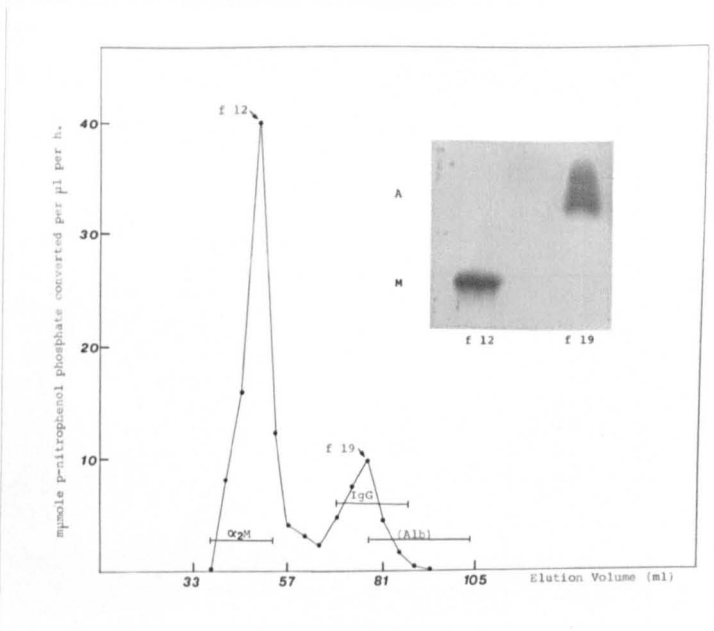

Figure 8. Gel filtration of placental microvillous alkaline phosphatase in the absence of Triton X-100.

A butanol extract of microvilli, dialysed against tris-succinate buffer (see text), was applied to an AcA 34 column in the absence of Triton X-100. The enzyme activity was assayed (—●—●—) and the elution ranges of alpha-2-macroglobulin (α_2M), IgG and human serum albumin (Alb.) were determined. The enzyme activity peaks in fraction numbers f12 and f19 were further analysed on starch gels containing 0.5% v/v Triton X-100. and the results are shown in the insert.

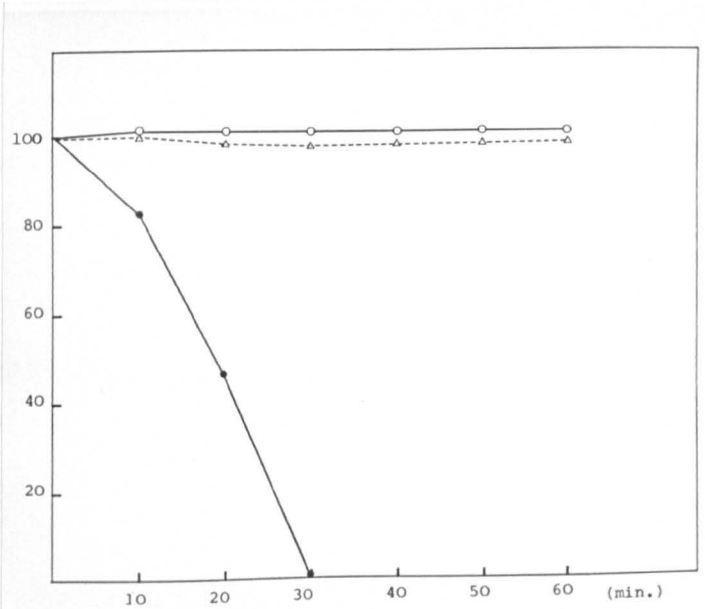

Figure 9. Heat stability of the A and M forms and of the alkaline phosphatase in normal male serum.
Aliquots of gel filtration fractions number f12 (—o—o—o—) and f19 (- -▽- -▽-) were taken to represent the M and the A forms, respectively. Together with a control of serum from an adult male (—●—●—●—), the aliquots were incubated at 60°C for between 10 and 60 min. Samples were taken at the various times and assayed for alkaline phosphatase activity. The activities were expressed as percentages of the activity in unheated reference samples.

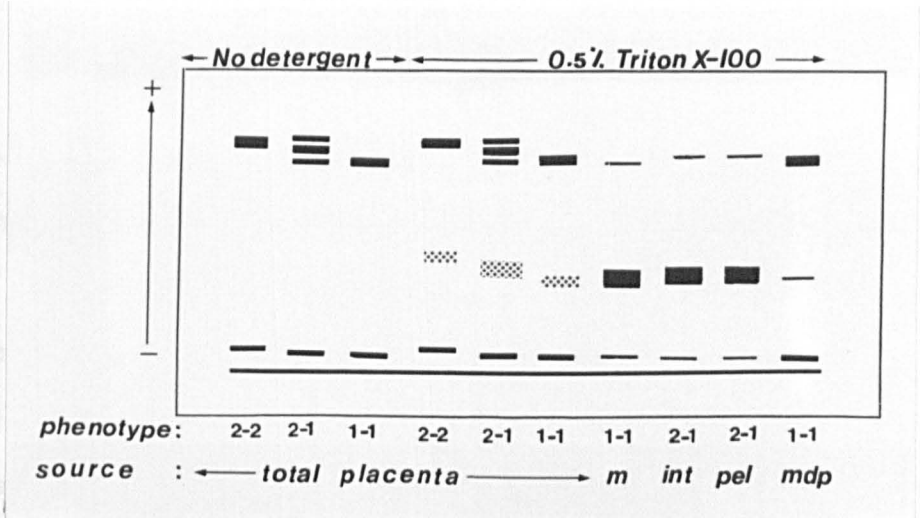

Figure 10. Summary of observed genetic and non-genetic variation in placental alkaline phosphatase.
The diagram summarises results both by Robson and Harris (1965) and in this study, both using starch gel electrophoresis, but differing in that the present study employed 0.5% Triton X-100 in the gel. The sources of the samples and the phenotypes of the placentae from which they came are noted below the diagram :
m = microvilli, mdp = microvillus-depleted placenta, int = interface and pel = pellet of sucrose gradient fractionated microvilli.

2:4 D I S C U S S I O N

Though previous biochemical studies have been made on placental alkaline phosphatase, this is the first electrophoretic study on enzyme extracted from microvilli and it reports a new "M" form of the enzyme. Figure 10 summarises the various isotypic and allotypic forms of heterogeneity now known for PAP. Although the M form was detected in extracts of whole placental tissue which were made according to the techniques of previous authors, previous studies did not involve the addition of Triton X-100 to starch gels and this step is essential to allow the M form to enter the gel and be stained as a descrete band. It may be that the failure of Doellgast et al (1977) to detect the M form in polyacrylamide gels which contained Triton X-100 was due to the strength of their gels being too high to permit the M and B forms of the enzyme to be resolved.

2:4.1 Nature Of The M Form

Various experiments argue that the M form of the enzyme is not an artifact. Although Triton X-100 is needed for the detection of the M form in starch gels, it cannot be argued that Triton X-100 is creating the M form from the A form, since inclusion of Triton X-100 in starch gels had no quantitative or qualitative effect on the detection of the A form, neither did it generate the M form from purified A form. Instead, the effect of Triton X-100 in the starch gel system was found to be that it enabled a fraction of PAP to enter the gel instead of remaining at the origin, and that this occurred without affecting the detection of the A form of the enzyme (Figure 3). The electrophoretic mobility of the M form was unaffected when the concentration of NaCl was increased

during and after the butanol extraction step (Figure 3a). The detection of the M form in chloroform-methanol, nonidet, Triton X-100 and sodium deoxycholate extractions argues against it being an artifact of butanol extraction (Figure 3e). The molecular weight of the M form (approximately 725,000) is unaffected by the inclusion of Triton X-100 or NaCl in the chromatography buffer. This argues against the M form being generated artifactually or as a Triton-enzyme micelle as in the case of bacterial membrane carboxypeptidase (Umbreit and Strominger, 1973). Finally, the possibility that the M form of the enzyme is an artifactual aggregate, created by hydrophobic protein interactions after the membrane lipids had been extracted with butanol was considered. Such aggregation phenomena have been found for cytochrome b_5 and cytochrome b_5 reductase, and involve hydrophobic amino acid domains whose marked tendency to aggregate in aqueous media can be prevented by the addition of detergents (Spatz and Strittmatter, 1971; 1973). It was found that extraction of microvilli with Triton X-100 did not alter the molecular weight of the M form nor did it alter its characteristic mobility in starch gels.

It is clear that the "M" form of the enzyme is a band of placental alkaline phosphatase activity, since the M and A forms cross-react serologically (Figure 5) and the M form is heat stable at 60°C (Figure 9). The M band also showed reproducible variation in mobility which correlated with the heterozygous and homozygous phenotypes of the A form of the enzyme (Figure 4). Taken together, these results indicate that the A and the M form share at least part of the same enzymatic polypeptide. The results in chapter 3 will also show that purified samples of that A and M forms showed a polypeptide of the same molecular

weight (69,000), in agreement with the findings from previous work on the A form by Doellgast et al (1977), Ghosh and Fishman (1968) and Gottlieb and Sussman (1968).

Gel filtration gave estimates of the molecular weight of the A form of PAP of 127,000, a values in good agreement with previously published data (references quoted above). However, the estimate of the molecular weight of the M form by gel filtration gave a much greater MW value of 725,000. These estimates from gel filtration were unaffected by the presence of 0.16 M NaCl or 0.5% Triton X-100 in the membrane extraction or in the filtration system. The results in this chapter show that this discrepancy between the gel filtration and the electrophoresis is unlikely to be due to an experimental artifact. It may be that the M form is composed of either an aggregate of 5 or 6 A form molecules, or a lesser number of molecules with other material. Both these types of possibilities have been raised by Doellgast et al (1977) in an attempt to examine the structure of the B form of alkaline phosphatase, which in their hands had a molecular weight of over 200,000. A fuller analysis of the biochemical composition of the A, and M forms of PAP will be discussed in the following chapters.

2:4.2 Location

Carlson et al (1976) were among the first to show biochemically that placental plasma membrane fractions are enriched in alkaline phosphatase. The present results indicate that the placental alkaline phosphatase associated with the microvillous plasma membrane is enriched in an "M" form of the enzyme. Further, the M form is equally represented in both the sub-fractions of placental microvilli which were described by Davies et al (1981) and by Truman et al (1981). The A form

of the enzyme is found predominantly in other parts of the placenta, though still presumably in the syncytiotrophoblastic cells, since alkaline phosphatase has been detected histochemically in the syncytiotrophoblast alone (Jones and Fox, 1976). The B form of the enzyme was found in both microvillous and non-microvillous material, but its concentration was not high enough to show if it had a preferential location. Evidence was sought that some of the A and B forms of the enzyme might be located in a fraction of trophoblast plasma which was not extractable by the method of Smith et al (1974). However, when a more severe form of extraction (due to Snary et al, 1976) was used after the removal of microvilli, the resultant plasma membranes were rich in M and showed as little of A and B as did the microvillous fraction (Figure 2). Thus no evidence could be found for a sub-fraction of trophoblast membrane which was enriched in enzyme other than the M form. It is worthnoting that the amounts of the A and B forms which can be seen in the microvillous preparations vary (Fig. 3) and can be washed off these membranes by phosphate buffered saline (Fig. 4).

2:4.3 Some Implications

In the absence of knowledge about the biological function of placental alkaline phosphatase, the functional implications of the presence of the M form of the enzyme in microvilli is quite unclear. However, the finding of the M form has some potential clinical significance. The concentration of PAP in maternal serum increases as pregnancy proceeds, suggesting that the enzyme could be of clinical use in monitoring or predicting complications of pragnancy which involve alterations in placental function, such as pre-eclampsia and placental insufficiency. Although the results of investigations into this have

proven sufficiently discouraging that the enzyme assay is now hardly ever used in clinical obstetrics, these studies did not take account of the molecular heterogeneity of the enzyme described in this study.

Clearly, many factors affect the concentration of the enzyme in maternal blood. These include maternal blood volume, maternal enzyme clearance rates and the size and functional status of the placenta. Further, the enzyme may enter the serum from the syncytiotrophoblast in any of at least three general ways : (a) secretion, (b) rupture of the syncytiotrophoblast and loss of cytoplasmic contents and (c) shedding of microvilli. It is not known whether alkaline phosphatase is actively secreted into the maternal blood. The findings of syncytiotrophoblastic fragments and fetal blood cells in the maternal circulation suggests that route (b) may contribute to maternal serum alkaline phoshatase. The knowledge that microvilli can readily be washed from the term placenta suggests that membrane loss can also occur during pregnancy. It is possible that the rate of such a loss could affect the transport capacity of the placenta, and that the extent of this loss might be estimated by measuring the serum level of the M form of PAP.

CHAPTER 3

PURIFICATION AND CHARACTERIZATION OF MICROVILLOUS ALKALINE PHOSPHATASE

3:1 INTRODUCTION

Human placental alkaline phosphatase (PAP) is a marker of syncytiotrophoblastic microvillous membranes (Carlson, Wada and Sussman 1976). Genetic and structural studies on the A isotype of PAP (A-PAP) have shown that the enzyme is a homodimer composed of two identical polypeptides of molecular weight 70,000 (Robson and Harris 1967, Ghosh and Fishman 1968). The B-PAP has a molecular weight of over 200,000 and was considered by Ghosh and Fishman (1968) to be a molecular aggregate of A-PAP. Later studies by Doellgast *et al* (1977) suggested that B-PAP results from the polymerization of A-PAP with other material. The results in chapter 2 (Abu-Hasan *et al*, 1984) have shown that the major form of PAP in syncytiotrophoblastic microvillous membranes has a native molecular weight of 725,000, which is much higher than that reported for A-PAP and B-PAP. This microvillous form of PAP was termed M-PAP and has a mobility between the A and B forms in starch/Triton X-100 gels. Antigenic, polymorphic and heat stability studies showed that all three forms of PAP share a common polypeptide, though it is not known whether M-PAP also contains other materials. Since Triton X-100 was necessary for M-PAP to enter starch gels, Abu-Hasan *et al* (1984) suggested that M-PAP was substantially more hydrophobic than either A-PAP or B-PAP. The present data reports the purification of M-PAP and subsequent structural and kinetic comparisons with A-PAP.

3:2 MATERIALS AND METHODS

3:2.1 Materials

Iodoacetic acid, iodoacetamide/dithiothreitol, Cyanogen bromide (CNBr) and all chemicals for the staining and assay of alkaline phosphatase were purchased from the Sigma Co. Isoelectric point markers, Amberlite Monobed Resin and iodoaceamide were purchased from BDH Co. Hydrophobic chromatography (Agarose C n series) were obtained from Miles Co. Ampholines were obtained from LKB. DEAE Sepharose CL-6B was obtained from Pharmacia (GB) Ltd. Placentae were obtained immediately after delivery and were stored at 4°C and used within 4 hours.

3:2.2 Methods

1. Preparation and Extraction of Microvilli

Placental microvilli were prepared as a 1 G supernatant by the method of Smith, Brush and Luckett (1974). Alkaline phosphatase was prepared from placental tissue by extraction with butanol/H_2O using the method of Boyer (1963) except that the incubation time for butanol was increased to 1 h at 20°C in order to improve the separation between the aqueous and butanol phases. Extraction of microvilli, was carried out as described in chapter 2.

2. Column Chromatography

A column of DEAE Sepharose CL-6B (2.5 x 25 cm) was equilibrated with tris-succinate in the presence of 0.5% Triton X-100, pH 7.0 (equilibration buffer). Butanol extracts of alkaline phosphatase prepared from microvilli of 7 heterozygous placentae were made up with

glycerol to 5.0% (v/v) and Triton X-100 to 0.5% (v/v). The material was passed through the column at 150 ml/h in equilibration buffer and was eluted at 20 ml/h with a linear gradient of 400 ml of equilibration buffer containing a linear gradient of 0-0.5M NaCl at room temperature.

3. Electrophoresis

Horizontal starch gel electrophoresis at pH 6.0 (Robson and Harris 1965) was performed as described in chapter 2 (Abu-Hasan et al, 1984). Non-dissociating polyacrylamide gel electrophoresis in both tubes and slabs was carried out using 7% (w/v) polyacrylamide gels made by the method of Ryrie (1975) with the modification that Triton X-100 was added to the gel to a final concentration of 0.5% (v/v). Gels were stained for alkaline phosphatase activity and also for protein using Coomassie blue.

SDS/polyacrylamide gel electrophoresis (Laemmli, 1970), sample preparation and staining was carried out using tris-buffered slab gels as described by Sutcliffe et al (1980).

4. Reduction and Alkylation

Acetone precipitates of pure fractions of the A and M forms of the enzyme were dissolved at 1mg/ml in 10M urea. Reduction was carried out by adding 1/5 volume of a solution of 36 mM dithiothreitol/0.8M urea/0.24M Tris-HCl, pH 8.4. This was incubated at 37°C for 30 min and then made up to 0.1M with respect to iodoacetamide/iodoacetic acid. Varying the proportions of these two reagents can be used to titrate cysteine residues (Mossman and Williamson, 1980). Incubation was continued for 30 min and terminated by the addition of 5 vol. of acetone. The reduced and alkylated samples were recovered as a

precipitate after centrifugation at 5000 g and were resuspended in freshly deionised 10 M urea.

5. Isoelectric Focusing

Thin layer plates of polyacrylamide gel, 5 x 10 x 0.1cm, were prepared by the polymerization of degassed 5% (v/v) acrylamide deionized with Amberlite Monobed Resin (BDH) containing 2% (w/v) Ampholine carrier ampholites (pH 3.5-10.0) and freshly deionized 8M urea. The gel was polymerised with 10% ammonium persulphate, stood at 4°C for 15 min. before one glass plate was carefully removed from the gel. Protein samples were loaded on Whatman 3mm filter paper. The cathodal and anodal buffers were 1 M solutions of NaOH and H_3 PO4 respectively, and isoelectric focusing was carried out over a water-cooled platter at an initial current of 30 mA for a period of 3-4 h. After focusing, the gels were washed in several changes of 12.5% (w/v) trichloracetic acid, 3.5% sulphosalicylic acid for 5-8 h. This was followed by a 30 min wash in destain (methanol/water/acetic acid in the ratio of 5:4:1) before staining in 0.25% coomassie blue desolved in destain. The pH gradient was determined using LDH protein markers, range pH 4.7-10.6.

6. Cyanogen Bromide (CNBr) Fragmentation of Proteins

CNBr degradation at low pH was carried out as using a modification of the acidic acetonitrile method of Nikodem and Frisco (1979) as follows. Cyanogen bromide (225-250mg) was dissolved in acetonitrile (100 µl) to a final volume of 150 µl, an aliquot (20 µl) of this reagent was aded to each sample of protein (25-30 µg in 50 µl), followed by 200 µl of 0.6 N HCl. The mixture of cyanogen bromide and protein was left at

room temperature for 90 min. The reaction was then terminated by drying the sample under low pressure vacume. As a control, the cyanogen bromide was omitted and a solution of acetonitrile and HCl was aded to the protein.

7. Hydrophobic Chromatography

A small column of 1.0 ml of alkyl agarose (Miles) was equilibrated with tris-succinate buffer pH 7.4. An aliquot of dialysed butanol extract of microvillous membranes was passed through the column at 6ml/h and 0.4 ml fractions were collected. The column was then washed in the equilibration buffer and the bound protein was eluted with 10% butanol in tris-succinate buffer, pH 7.4 (see above). The fractions were assayed for enzyme activity and analysed on starch gels containing 0.5% (v/v) Triton X-100.

8. A s s a y s

Protein was estimated by the method of Lowry et al (1951) using bovine serum albumin as standard. For those fractions containing Triton X-100, the modification of Bonsal and Hunt (1971) was used, in which SDS was added to prevent the formation of water-insoluble complexes between phosphomolybdate and Triton X-100. Alkaline phosphatase was assayed in a system of 62 mM. sodium carbonate buffer pH 10.75, 12.5 mM. magnesium chloride, 32.5 mM. p-nitrophenol as substrate and 5-20 µl of enzyme in a final volume of 50 µl (Sutcliffe, Brock, Robertson, Scrimgeour and Monaghan, 1972). Albumin and transferrin were assayed by antibody-antigen crossed electrophoresis (Laurell) as described by Sutcliffe et al (1980).

3:3 RESULTS

3:3.1 Ion-exchange Chromatography

To study the polypeptide composition of the enzymes, alkaline phosphatase was extracted with butanol from the microvilli of seven placentae and subjected to ion-exchange chromatography in the presence of Triton X-100. The results (Figure 1) show that some protein did not bind to the column and that four discrete peaks of protein were eluted with the salt gradient between 0.04M and 0.15M NaCl. Alkaline phosphatase activity was found to elute from the column in two peaks, at 0.04M and 0.1M NaCl, which correspond to the first and third eluted peaks of protein. When transferrin and serum albumin were assayed in the fractions by antibody-antigen crossed electrophoresis, the proteins were detected at positions corresponding, respectively, to the second and fourth eluted peaks of protein (Figure 1). Aliquots of the fractions labelled 48-61 were taken and studied by electrophoresis in starch gels containing 0.5% (v/v) Triton X-100. The results in the lower panel of Figure 1, show that M-PAP eluted at 0.04M NaCl and that A-PAP eluted at 0.1M NaCl. The B form of the enzyme eluted close to M-PAP but was present at a much lower activity.

3:3.2 Polyacrylamide Gel Electrophoresis

The purity of the alkaline phosphatase in the two eluted peaks of enzyme activity (cf. Figure 1) was assayed in non-denaturing polyacrylamide gels (Ryrie, 1975) stained either for enzyme activity or with Coomassie blue. Figure 2 shows the results obtained with gels, some of which contained 0.5% (v/v) Triton X-100. With 0.5% (v/v) Triton

X-100 in the gel, two zones of enzyme activity were observed in fractions 49-51 (Tracks 1-3; Figure 2) whilst fractions 59-61 showed a single band of alkaline phosphatase of faster mobility (Figure 2). When stained with Coomassie blue (Tracks 4 - 6 and 8), bands of protein staining were observed which correspond to the bands of enzyme activity. The main conclusion from this experiment was that a substantial purification of alkaline phosphatase had been affected. However, the finding of three bands of enzyme activity in the polyacrylamide gels (with Triton X-100) prompted the question of whether there was a simple correspondance between these bands and the A, B and M forms of PAP. Since fractions 59-61 showed mainly the A form of the enzyme in starch gels (Figure 1, lower panel), the most anodal band of enzyme activity in polyacrylamide/Triton X-100 gels (Figure 2, Track 7) corresponds to the A form of the enzyme. A very strong band of intermediate mobility was observed when a butanol extract of microvilli was run in polyacrylamide in the presence of 0.5% Triton X-100 (Figure 2, Track 16). Since the bands of slower and faster mobility stain more weakly, the fast band was deduced to be A-PAP and the strong intermediate band is M-PAP. The identity of the slowest band is not clear. Tracks 1-3 show approximately equally intense staining for both cathodal bands and, since starch gels show an excess of M-PAP over B-PAP in fractions 49-52, we cannot at present consider that the slowest mobility form is B-PAP.

The extent of the separation of the cathodal two bands of alkaline phosphatase depended on the conditions of electrophoresis. Omission of Triton X-100 from the gel led to the enzymes being found at or around the origin (Figure 2, Tracks 9 -15), with only the A form entering the gel. In the presence of the detergent, the two bands could be resolved

in gels up to 7% acrylamide. In 10% acrylamide they had virtually the same mobility and were difficult to distinguish.

The ion-exchange fractions containing alkaline phosphatase were analysed on SDS/polyacrylamide gels (figure 3). In the presence of reducing agents, a single protein band was observed which had the same mobility in the fractions containing the M (tracks 1-6 and 14) and A (tracks 11-13) forms of the enzyme. Fractions 54-57 of the ion-exchange elution contained transferrin (Figure 1, upper panel; Tf) and a protein band of the mobility of transferrin was observed in the SDS gel (Figure 3, Tracks 7-10).

Aliquots of pure fractions of A-PAP and M-PAP and butanol extracts of microvillous membranes were lyophylysed and incubated without reduction in different concentrations of urea at room temperature, before SDS/polyacrylamide gel electrophoresis. The results (Figure 4, tracks 4,5,6, and 7) show aliquots of M-PAP incubated for 2 h in 10% SDS, 3M, 5M and 8M urea respectively. A doublet band occurred at approximately 116K and two minor bands of mobilities corresponding to 180K and 69K could also be seen. Further incubation of these samples for up to 48 h under the same conditions showed no detectable differences either in the mobility or in the intensity. Tracks 8 and 9 show aliquots of A-PAP incubated for 2 h in 8M and 5M urea respectively. They show a major band with a mobility corresponding to 130K and a minor band at 69K. Track 2 shows the behaviour of the butanol extracts incubated in 8M urea for 2 h, in which bands of mobilities corresponding to 180K, 130K, 116K and 69K can be seen. Tracks 1, 3 and 10 show the reduced butanol extracts, M-PAP and A-PAP respectively. All these samples show a band at 69K.

3:3.3 Reduction, Alkylation and Isoelectric Focusing

Reduced and alkylated A and M-PAP were compared in 8M urea in thin layer polyacrylamide isoelectric focusing gels of pH range 3.5-10. Figure 5 shows the patterns obtained. Multiple isoelectric bands were present in both forms under different alkylating conditions, but there were some bands of relativly high pI which were observed in M-PAP, but not in A-PAP. Preparations of M-PAP were also found to cause distortion to the surface of the gel, mainly at the high pI region. Regions of distortion are indicated by arrows. The considerable similarity between the banding patterns of the A and M forms remained whether alkylation was carried out with iodoacetamide, iodoacetic acid or a mixture of both. In those preparations alkylated with iodoacetamide, several isoelectric bands were observed between pH 5.65-5.95 (tracks 4,5). With iodoacetic acid, a lesser number of bands of pI 5.15-5.3 were observed (tracks 2,3). With a mixture of iodoacetic acid and iodoacetamide (5:1) both forms produced similar band numbers to those samples alkylated with iodoacetamide with pI values ranging between 5.15 and 5.65 (tracks 6,7). There were more clearly defined bands in preparations of A-PAP (tracks 2,4,6) than the M-PAP (tracks 3,5,7), though incorporation of 4% Nonedit NP40 into the isoelectric focusing system somewhat improved the resolution of M-PAP.

3:3.4 Cyanogen Bromide (CNBr) Fragmentation of PAP

Pure fractions of M and A-PAP were compared by CNBr fragmentation. The results (Fig. 6 tracks 1,2,3 and 4) show a very similar methionine cleavage patterns in both polypeptides. The controls for this

experiment included samples of A-PAP, M-PAP and BSA without treatment with CNBr (tracks 6,7 and 8 respectively). Also included was a sample of BSA cleaved in the presence of CNBr (track 5).

3:3.5 Hydrophobic Chromatography

A comparison of the hydrophobicity of the different forms of placental alkaline phosphatase was made by investigating their affinities for hydrocarbon-coated agaroses that varied in the length of their alkyl side chains up to a length of C_{10}. The columns differed in their ability to bind the enzyme. Un-alkylated agarose (Miles type "c=o") did not bind either form of the enzyme. C_6 alkyl chains (Miles type "C=6") bound the A, B and M-PAP. Figure 7 shows the isozymes of PAP in the excluded and eluted fractions from a C_2 column loaded to its exclusion point with butanol extracts of microvillus membranes. Figure 7 (a) shows the starting material (st) and the electrophoretic pattern of the enzyme which failed to bind to the column. In the early fractions (1-5) the M band is absent, though some M-PAP can be seen in later fractions, perhaps due to saturation of the column. When the bound enzyme was eluted with 10% butanol solution, the eluted fractions showed only M-PAP (Figure 7 b).

3:3.6 Enzyme Kinetics

Michaelis constants (Figure 8) and pH optima (Figure 9) were determined for purified M-PAP and A-PAP, obtained from the gel filtration of butanol extracts of microvilli. As shown in table 1, A-PAP and M-PAP had very similar pH optima, namely mean values of 10.8 and 10.9, respectively. The Km values were also comparable, having means of 3.89 (mM) and 3.66 (mM) for the A and M forms, respectively.

TABLE 1 Km values and pH optima for A-PAP and M-PAP, showing means* and ranges.

Enzyme	Km (mM)	pH optimum
A-PAP	3.89 (3.2-4.4)	10.8 (10.7-11.1)
M-PAP	3.66 (3.2-4.0)	10.9 (10.7-11.2)

*calculated from 5 determinations

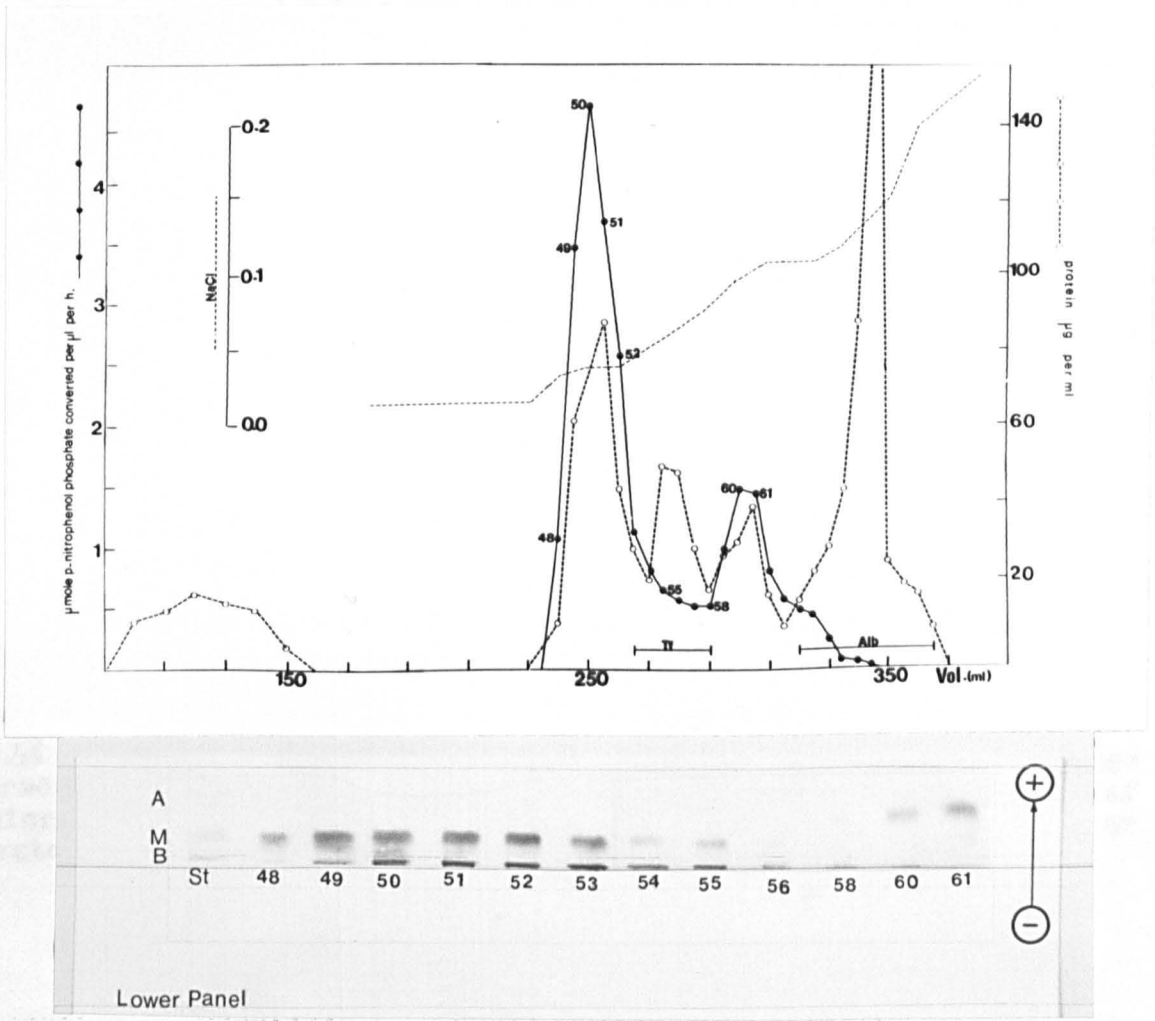

Figure 1. Ion-exchange chromatography of placental microvillous alkaline phosphatase in the presence of 0.5% Triton X-100.

Upper panel: DEAE Sepharose CL-6B chromatography of alkaline phosphatase extracted with butanol from placental microvilli. Enzyme activity, protein concentration and the concentration of NaCl are plotted against elution volume in ml. The horizontal bars represent the elution volume of transferrin (Tf) and albumin (Alb), detected by antibody-antigen crossed electrophoresis. Aliquots of the fractions marked 48-61 were taken for analysis by starch gel electrophoresis.

Lower panel: Electrophoresis of aliquots 48-61 in starch gels containing 0.5% (v/v) Triton X-100. The aliquots were loaded according to the numbers below the tracks. Sample "St" is an aliquot of the butanol extract originally applied to the ion-exchange column.

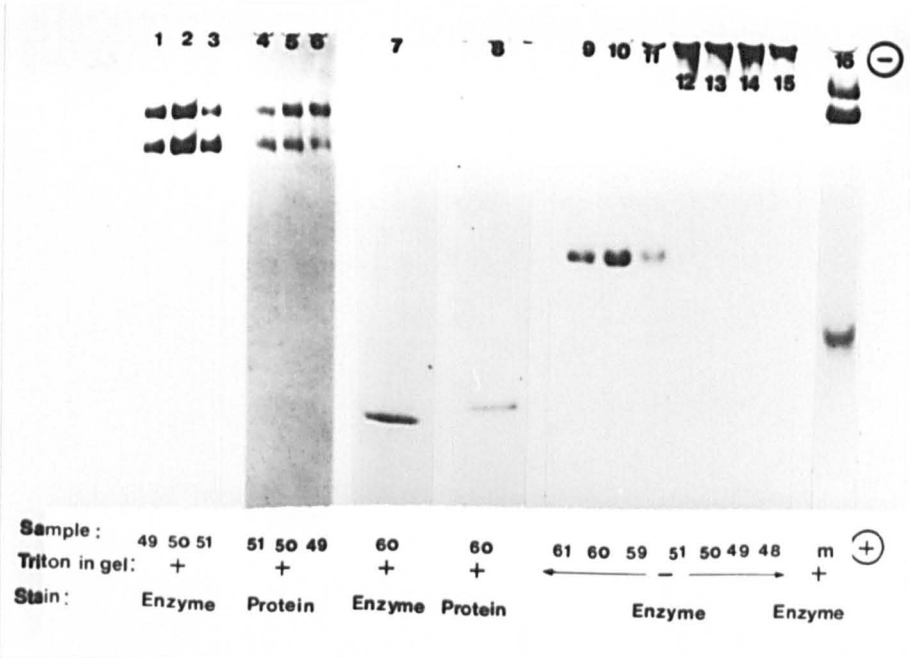

Figure 2. Analysis of fractions from ion-exchange chromatography in non-denaturing polyacrylamide gels.
Polyacrylamide gels (7%), some containing (+) and others lacking (-) 0.5% Triton X-100, were loaded with samples from the ion-exchange fractions (identified below each track) and with butanol extracts of microvilli (m). The gels were stained for either enzyme activity or protein as indicated below the tracks. The origin is at the cathode.

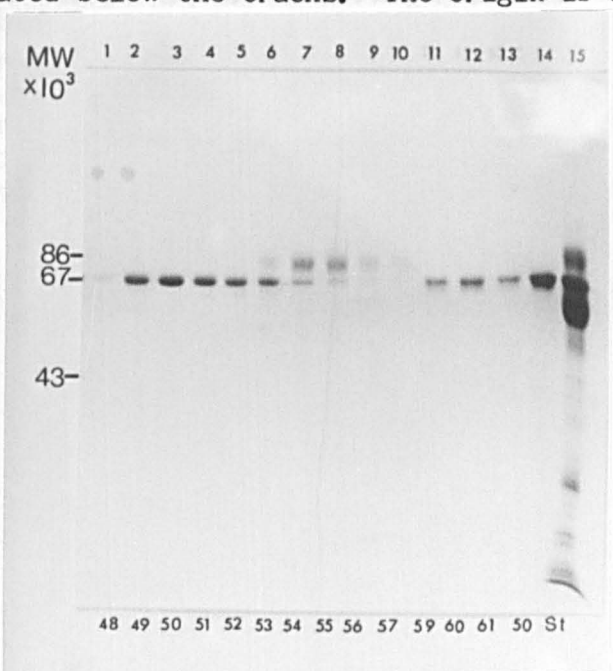

Figure 3. Analysis of fractions from ion-exchange Chromatography in SDS/polyacrylamide gels.
SDS/polyacrylamide gels (4.5-12%) were used to analyse the protein in the butanol/H_2O extract of microvilli which was applied to the ion-exchange column (sample "St", Track 15 and. Tracks 1-14, show various fractions (identified below the tracks) after treatment with β-mercaptoethanol. The origin is at the cathode. The molecular weight calibration is provided from standard proteins run in adjacent tracks.

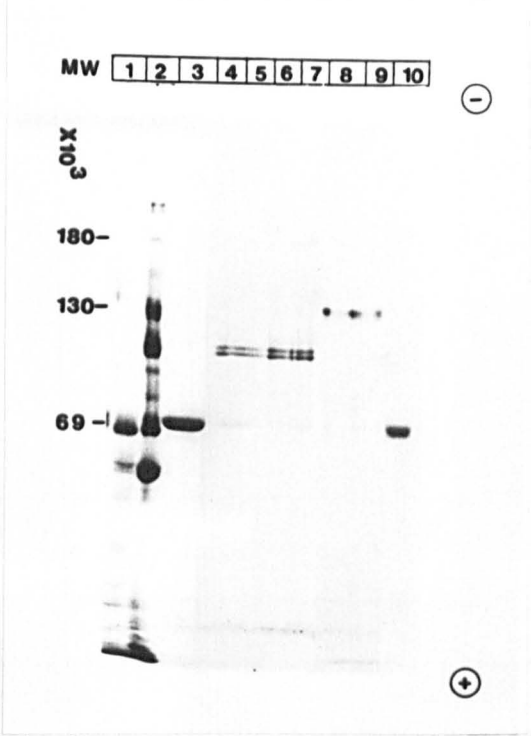

Figure 4. Analysis of A-PAP and M-PAP in SDS gels without reduction. Before electrophoresis, aliquots of A-PAP, M-PAP and butanol extracts of microvillus membranes were incubated for 2h either in SDS or in different concentrations of urea. In tracks 4,5,6,7 M-PAP was incubated in 10% SDS, or in 3M, 5M, and 8M urea, respectively. Aliquots of A-PAP (tracks 8,9) were incubated in 5M and 8M urea, respectively. Track 2 shows an aliquot of butanol extract incubated in 8M urea. Tracks 1,3,10 show reduced samples of butanol extract, M-PAP and A-PAP, respectively.

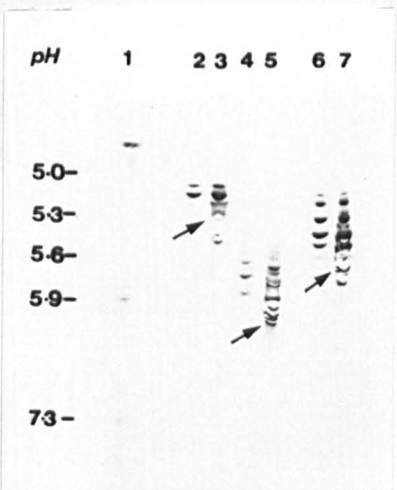

Figure 5. Isoelectric focussing of reduced and alkylated PAP. Reduced and alkylated A-PAP and M-PAP were resolved in a pH gradient of 3.5-10.0 in 8M urea/polyacrylamide gels. Aliquots of acetone-precipitated preparations of purified A-PAP (tracks 2,4,6) and M-PAP (tracks 3,5,7) were reduced with dithiotreitol and alkylated with iodoacetate (tracks 2,3), iodoacetamide (tracks 4,5), or a 5:1 molar ratio of iodoacetate : iodoacetamide (tracks 6,7). Track 1 contains pH markers.

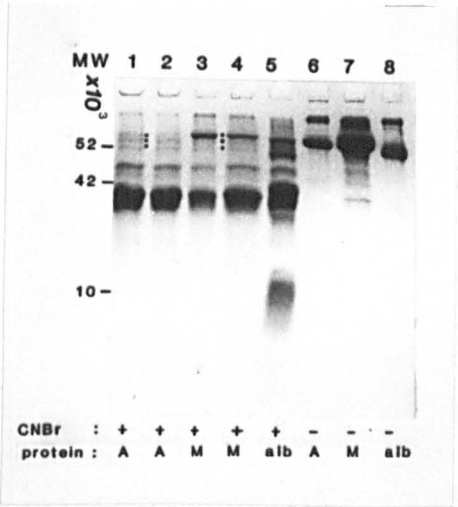

Figure 6. SDS gel analysis of CNBr peptides from purified preparations of M and A-PAP.

Aliquots of purified M and A-PAP were cleaved in acidic CNBr (denoted as '-' below tracks). Tracks 1 and 2 contain cleaved A-PAP, tracks 3 and 4 contain cleaved M-PAP. Track 5 contains cleaved BSA. Cleavage controls of A-PAP, M-PAP and bovine serum albumin (alb.) are shown in tracks 6,7 and 8 respectively ('-' shows were CNBr was omitted from the reaction).

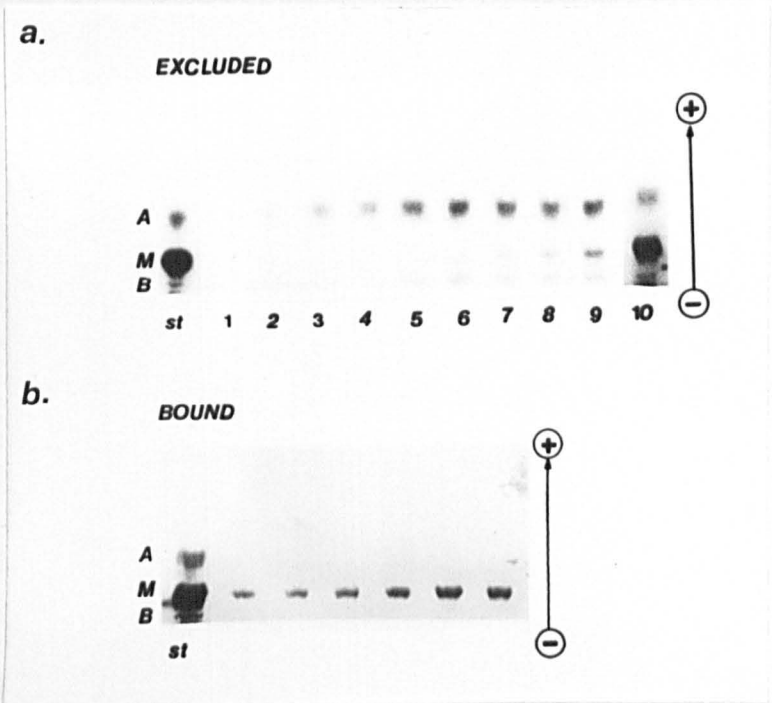

Figure 7. Hydrophobic affinity chromatography of PAP on a C_2 agarose column.

A C_2 agarose column was loaded with a butanol extract of microvillus membranes (samples "st"). The excluded enzyme fractions (gel a) and the bound enzyme (gel b) were analysed in starch gel electrophoresis.

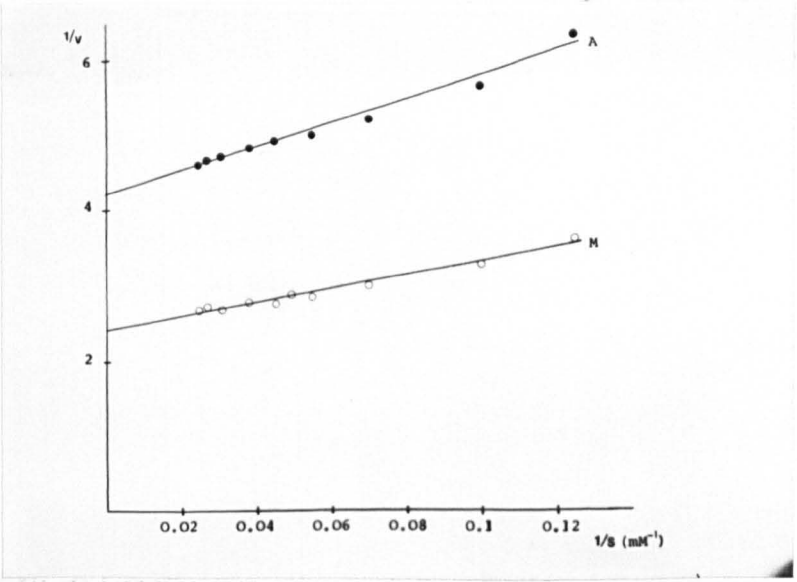

Figure 8. The effect of substrate concentration on the activities of the A and M forms of PAP.

Lineweaver-Bruk plots for the A and M forms of placental alkaline phosphatase. The velocity (v) is expressed as mμ mole of p-nitrophenol phosphate converted / μl in 30 min at 37°C.

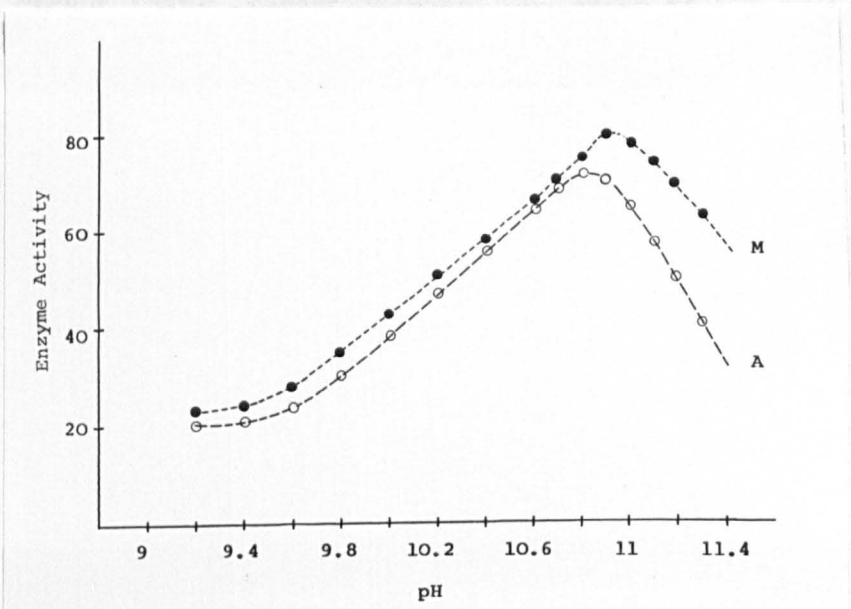

Figure 9. The effect of pH on the activities of the A and M forms of placental alkaline phosphatase.

Aliquots of purified M and A-PAP were assayed at different pH values. The pH-activity curves for the A and M forms of PAP with 32.5 mM p-nitrophenol phosphate as substrate were plotted. Enzyme activity is expressed as mμ moles of substrate converted / μl in 30 min at 37°C.

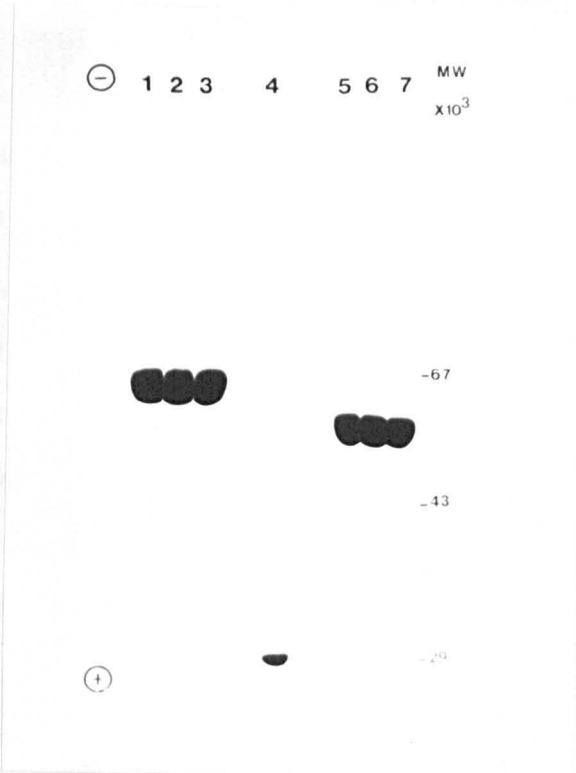

Figure 10. The effect of reduction on the mobility of bovine serum albumin in SDS/polyacrylamide electrophoresis

Bovine serum albumin (20 µg per track) was prepared for SDS polyacrylamide gel electrophoresis according to the materials and methods, except that β-mercaptoethanol was added to the protein applied to tracks 1-3. The protein applied to tracks 5-7 had not been treated β-mercaptoethanol. Track no. 4 contains molecular weight markers.

3:4 DISCUSSION

The finding that A-PAP and M-PAP have similar pH optima and Km values (Table 1) is consistent with the previous findings in chapter 2 (Abu-Hasan et al, 1984) that both forms are heat-stable, that they share antigenic determinants and exhibit the same genetic polymorphism. Studies on the polypeptide composition of A-PAP and M-PAP under reducing conditions in SDS/polyacrylamide gels showed that both forms contained a similar polypeptide subunit of 69,000 molecular weight. The molecular weights of the A-PAP subunit and of its dimer 130,000 are in good agreement with the previous results of Ghosh and Fishman (1968), Gottlieb and Sussman (1968), Sussman et al (1968), and Doellgast et al (1977). M-PAP also contains a disulphide-bonded dimer, though the mobility of the protein in SDS/polyacrylamide gels in the absence of reduction (116,000) is less than for the dimer of A-PAP. Since both forms have the same molecular weight of polypeptide under reducing conditions, the difference between the 116,000 and 130,000 bands cannot be due to proteolysis. An alternative explanation is that the dimers of A and M-PAP differ in their conformation and hence in their rates of migration in the SDS gel. Since the disparity in the molecular weight of A and M-PAP is only seen in the absence of reduction, it may be due to differences in the pattern of inter- or intra-domain disulphide bonding in the two forms. That this is a feasible possibility is illustrated in Figure 10, which shows that the mobility of serum albumin is increased under non-reducing conditions, presumably because the unreduced protein is in a more compact state. It was also observed that in the absence of reduction the M-PAP dimer showed two polypeptide

species within the 116,000 band (Figure 4). These two species appear to be components of M-PAP, since the samples analysed were substantially free of B-PAP and A-PAP.

Isoelectric focusing of reduced and alkylated PAP polypeptides revealed bands in common between the two isoenzymes. The charge heterogeneity in these bands could be due to in vivo modifications such as phosphorylation or addition of charged carbohydrate groups, or it could be due to artifactual modifications induced during the reduction and alkylation procedures. The most likely targets for such modifications are cysteine, asparagine, and glutamine. Cysteine can be readily oxidized to form cysteic acid while asparagine and glutamine are known to undergo spontaneous deamidation (O' Farrell, 1975). The considerable similarity between the banding patterns in both forms remained whether alkylation was carried out with iodoacetamide, iodoacetic acid or a mixture of both. In addition, there were some bands of relatively high pI which were observed only in M-PAP. These bands were found in association with some smearing and distortion in the surface of the gel. The cause of this distortion is not known and it was not observed in A-PAP. Incorporation of nonidet NP40 in the system reduced the smearing of these bands but, it did not affect either the presence of these extra bands or the distortions in the surface of the gel. O' Farrell (1975) has reported that the presence of multiple bands in association with smearing in IEF gels can be due to relative insolubility of certain proteins and that these effects can be improved by the presence of nonidet NP40. The hydrophobic properties of M-PAP described in this chapter and the previous observations in chapter 2 (Abu-Hasan et al, 1984) may account for the smearing and the additional bands which are observed when the polypeptide is resolved in IEF gels.

The molecular weight of A-PAP was estimated by gel filtration to be 127,000 (Abu-Hasan et al 1984); this is consistent with the value of 130,000 MW from non-reducing SDS polyacrylamide gels (Figure 4). The evidence from the SDS gels (Figure 4) shows that M-PAP is a homomer of 69,000 MW subunits and that it contains no other unrelated species of polypeptide. In the absence of reduction, M-PAP showed additional protein bands of mobilities corresponding to 116,000 and 180,000 MW. Whether the 180,000 band represents a trimer or a tetramer is unclear, since direct estimates of molecular weight cannot be obtained for disulphide bonded structures (cf Figure 4). However, this value is far lower than that of 725,000 mol. wt. which was observed for native M-PAP by gel filtration (chapter 2). The discrepancy between the gel filtration and electrophoresis data could be explained by the M-PAP being composed of either an aggregate of 5 to 6 dimers (10-12 polypeptides), or a lesser number of dimers together with some non-proteinaceous material which can be separated by SDS from the polypeptide component. Both these types of possibility have been raised by Doellgast et al (1977) in an attempt to examine the structure of B-PAP, which in their hands had a molecular weight of over 200,000.

The effect of Triton X-100 on the electrophoretic properties of M-PAP in starch gels and in non-denaturing polyacrylamide gels is consistent with a hydrophobic property which is expected of an integral membrane protein. The observation (Figure 6) of the hydrophobic affinity of M-PAP for alkyl agarose strongly supports the hydrophobic nature of the M-PAP and provides a useful purification technique for this form of the enzyme. The hydrophobicity of M-PAP is probably reflected in the chromatographic studies with DEAE Sepharose CL-6B, in

which Triton X-100 appears to change the elution point of M-PAP. The enzyme elutes at 0.04M and 0.18M NaCl in the presence and absence of Triton X-100 respectively, whereas the elution point of A-PAP did not change from 0.1M NaCl under these conditions. A similar effect of Triton X-100 on the binding of PAP to DEAE Sephadex was reported by Doellgast et al, (1974). Since their studies did not involve a detailed analysis of the different forms of the enzyme, their observation could be partially due to the hydrophobic character of the M-PAP. These observations suggest that the binding of M-PAP to DEAE Sephaarose CL-6B involves hydrophobic interactions which are reduced in the presence of Triton X-100. This is in close agreement with the finding that lipophilic proteins have a high tendency to bind detergents such as Triton X-100 and deoxycholate (Helenus et al, 1972). It has been proposed that non-ionic detergents bind primarily by hydrophobic interactions to hydrophobic regions on the proteins which are occupied by lipid in the native membrane (Utermann and Simons, 1974).

The evidence presented in chapter 2 (Abu-Hasan et al, 1984) and this chapter (Abu-Hasan and Sutcliffe, 1984) indicates that placental microvillus plasma membranes contain a hydrophobic form of PAP which is distinct from A-PAP. In certain other systems the fate of a polypeptide either to be retained in the cytoplasm, integrated into the membrane, or to be secreted from the cell, is determined by the location of hydrophobic signal sequences at the ends of the polypeptides. The presence of amino-terminal signal sequences on polypeptides determine whether they are to be secreted or to be retained in the cytoplasm (see Meyer, 1982). The presence of similar sequences at the carboxy-terminal ends of immunoglobulin heavy polypeptides determine whether they are to

be secreted from the cell, or integrated into the cell membrane as surface antibody (Early et al, 1980; Singer et al 1980). These models can cautiously be applied to PAP. Since A-PAP is heavily glycosylated, it is probable that it enters the cellular membrane system and that it therefore, has an amino-terminal signal sequence. This must also apply to M-PAP, since it is found in the microvillus plasma membrane. Either of these amino-terminal sequences could be proteolytically cleaved from the polypeptides after synthesis. The models also suggest that the M polypeptide may differ from A by the presence of another hydrophobic sequence at another site. If, as in the case of antibodies, the presence or absence of this sequence is controlled not by proteolysis, but by a genetic splicing system, then it is not possible to predict whether such a sequence would cause a difference in the molecular weights of the reduced A and M polypeptides. The difference between the mobilities of the dimers of A and M in SDS gels is probably due to a conformational difference. This may be due to differences in their primary structures. Another possibility is that A-PAP and M-PAP derive from exactly the same polypeptide sequence, but that there is more than one conformationally stable state in which the enzymic polypeptide can exist, possibly due to heterogeneity in post-translational processing. These differing conformational states could substantially effect the hydrophobicity and aggregation state of the PAP polypeptide, leading to the formation of the distinct and apparently highly stable A and M forms of the enzyme. Further discussion of these matters can be seen in chapter four.

CHAPTER 4

INTEGRATION OF PLACENTAL ALKALINE PHOSPHATASE INTO THE MICROVILLUS MEMBRANES

4.1 INTRODUCTION

The results reported in the previous chapters have shown that M-PAP exhibits the same genetic polymorphism as the A and B forms. It contains 69K polypeptides and cross-reacts serologically with A-PAP. However, compared with A-PAP and B-PAP, M-PAP is more hydrophobic, both in its affinity for alkyl agaroses and in needing Triton X-100 for it to be resolved on starch gels (Abu-Hasan and Sutcliffe, 1984). Such hydrophobic properties may be expected of an integral membrane protein.

Since M-PAP is associated with the microvillous membrane, it is necessary to ask whether the observed differences between M-PAP and A and B-PAP could be due to the presence in M-PAP of an intra-membranous, hydrophobic amino acid domain which might be specific to M-PAP. The results in this chapter establish the orientation of M-PAP in the microvillous membrane, and report some structural comparisons between the A and M forms of the enzyme.

4:2 MATERIALS AND METHODS

4:2.1 Materials

Subtilisin (EC 3.4.21.3) type VIII, trypsin (EC 3.4.21.4), phenyl methyl sulfonyl-fluoride (PMSF), p-toluenesulfonyl-L-arginine methyl ester (TAME) and all chemicals for the staining and assay of alkaline phosphatase were purchased from the Sigma Co. DEAE Sepharose CL-6B and Sephadex G150 were obtained from Pharmacia. Ultrogel AcA 34 was obtained from LKB. A-PAP, M-PAP and placental microvilli were purified as described in the previous chapters.

4:2.2 Methods

1. Proteinase Treatment of Microvillous Membranes and Proteins

The optimal conditions for the release of PAP by subtilisin from intact membrane vesicles were established in preliminary experiments using different enzyme concentrations and conditions. The following standard procedure was then adopted. Microvilli at a protein concentration of approximatly 10mg/ml in PBS buffer, pH 7.4 were treated with a concentration of subtilisin equal to 1/20 of the concentration of microvillous proteins. The reaction was carried out in a final volume of 3ml for 3hr at 37°C with frequent shaking. Proteolysis was stopped by the addition of PMSF and TAME to final concentrations of 2mM and 1mg/ml, respectively. The residual membranes were then sedimented at 100,000g for 30 min . Both the supernatant and the residual membranes were stored at -20° C. Trypsin cleavage was carried out under the same conditions, with the pH adjusted to 7.8. Purified M and A-PAP were cleaved in the same way as for microvilli.

2. Identification of the Amino-terminal Amino Acids

Edman degradation was carried out on a Beckman 890C automatic sequencer fitted with cold trap, using 0.25M Quadrol buffer and the delivery programme modified by Beckman from (Brauer, Margolies and Hober, 1975). Further details have been described by Brett and Findlay (1983). Proteins were solubilised and added to the cup in 90% formic acid and mixed thoroughly with 3 mg of polyprene (Tarr, Beecher, Bell and McKean, 1978). The resulting thiazolinones were converted to the corresponding phenylthiohydantions (PTH) with 200µl of 20% trifluoroacetic acid at 70°C for 15 min. PTH-derivatives were identified by reverse phase HPLC using DuPont 8800 pump system and quantitated using a Spectra Physics SP4100 computing integrator. The gradient was of acetonitrile (20-40%) in 12 mM-sodium acetate as modified from Zimmerman, Appella and Pisano (1977).

3. Polyacrylamide-gel Electrophoresis

SDS/Tris/polyacrylamide-gel electrophoresis was carried out as previously descibed by Laemmli (1970). Samples for electrophoresis were made up as described by Sutcliffe *et al* (1980), with the modification that the unreduced samples were made up in 0.8% SDS/23% v/v glycerol.

4:3 RESULTS

4:3.1 Released Enzyme Activity

Preparations of PAP were phenotyped before the enzyme was purified or analysed. Proteinase treatment of microvilli, with subtilisin or trypsin did not affect the activity of PAP (Table 1). The PAP released from microvilli treated with trypsin did not exceed that released in the absence of enzyme treatment. Thus trypsin did not release PAP activity from microvilli. In contrast, subtilisin solubilization released upto 80% of the enzyme activity into the supernatant, leaving about 20% of the total activity associated with the membranes.

4:3.2 Electrophoresis and Chromatography of Subtilisin Released PAP

The treatment of microvilli or purified M-PAP with subtilisin yielded PAP which, on starch gels, showed a single zone of enzyme activity of slightly faster mobility than that of native A-PAP (Figure1). This form of M-PAP did not require the presence of Triton X-100 to enter the gel system. On Ultrogel AcA 34 and Sephadex G-150 gel filtration chromatography, the subtilisin-cleaved M-PAP co-eluted with A-PAP at 130K (Figure 2). Ion-exchange chromatography in DEAE sepharose CL-6B revealed a significantly modified elution behaviour of cleaved M-PAP compared to that solubilized by butanol, with the cleaved product eluting between 0.08-0.115M NaCl in the absence of Triton X-100. This elution point is similar to that of A-PAP (0.10 M) and is substantially different from that of intact M-PAP (0.18 M under these conditions).

On SDS gels (Figure 3 tracks 1 and 3) aliquots of the subtilisin supernatants were found to contain a major polypeptide with a molecular weight of 67K which was slightly smaller than the molecular weight of

Table 1 The yield of PAP from microvilli treated with protease.

Microvillus protein (mg) (a)	Protease (mg) (b)	Ratio (a:b)	Enzyme activity expressed as % of initial activity in untreated microvilli	
			supernatant	microvillus pellet
	subtilisin			
23.75	1.19	20:1	83.0	22
23.75	0.59	40:1	71.0	25
23.75	0.12	200:1	66.0	38
23.75	0.00		9.7	96
	trypsin			
23.75	1.19	20:1	9.7	96

native PAP (69K). The bands at 27.6K in tracks 1 and 3 represent subtilisin, and the same band is visible in tracks 7 and 8, which have been run in a separate gel. Evidence for the 67K peptide being a cleaved form of PAP was obtained when it was found that purified preparations of M and A-PAP also yield a 67K polypeptide when treated with subtilisin (tracks 7 and 8). When membranes previously treated with subtilisin (Figure 3, tracks 2 and 4) were compared with that of the controls (track 6) there was a decrease in the intensity of the 69K band and an additional band at 67K, yielding a characteristic pair of bands in the gel. It is evident that subtilisin cleavage of purified M and A-PAP produced identical SDS profiles, in which a recoverable portion of MW 67K was indistinguishable from that cleaved from intact microvilli. This suggests that M-PAP is equally susceptible to limited proteolysis before or after its removal from the membrane. It also shows the presence of a similar cleavage site in the A-PAP polypeptide.

4:3.3 Amino-terminal sequence

The differences in the subunit molecular weights between the intact (69K) and the subtilisin-cleaved enzyme (67K) was attributed to the removal of a small peptide of about 2K believed to be situated in the plasma membrane. In order to locate which end of the PAP was inserted into the membrane, amino terminal sequences were determined for M-PAP before and after subtilisin cleavage. Table 2 shows the sequences of the N-terminal 13 amino acids for M-PAP extracted from microvilli with butanol. That sequence is compared with M-PAP cleaved from microvilli with subtilisin, and also with the sequence for A-PAP. All three sequences show identity upto position 13.

Table 2 N-terminal amino acid sequence of the butanol extracted M-PAP, subtilisin-cleaved M-PAP and A-PAP.*

		1										7			13
Butanol extracted M-PAP	+ :	Ile-	Ile-	Pro-	Val-	Glu-	Glu-	Glu-	Asn-	Pro-	Asp-	Phe-	Trp-	Asn-	
Subtilisin cleaved M-PAP	++:	Ile-	Ile-	Pro-	Val-	Glu-	Glu-	Glu-	Asn-	Pro-	Asp-	Phe-	Trp-	Asn-	
Butanol extracted A-PAP	+:	Ile-	Ile-	Pro-	Val-	Glu-	Glu-	Glu-	Asn-	Pro-	Asp-	Phe-	Trp-	Asn-	

* The phenotypes used for this study were SS for M-PAP and pooled phenotypes for A-PAP.

\+ Purified as described (Abu-Hasan and Sutcliffe, 1984).

++ Purified from the supernatant of subtilisin treated microvilli by ion-exchange chromatography and gel filtration (see text).

4:3.4 The Behaviour of Subtilisin-cleaved PAP on Non-redusing SDS Gels

In the previous chapters SDS gels showed mobility differences between the dimers of M and A PAP, corresponding to approximatly 116K and 130K, respectively. These differences were believed to be due to conformational differences. Thus, after cleavage with subtilisin, M and A-PAP were analysed on SDS gels in the absence of reduction. The results (Figure 4 track 3) show cleaved M-PAP with an apparent molecular size of 130K , with a mobility clearly slower than that of uncleaved M-PAP (118K ; track 4). This was not the case for A-PAP which, when cleaved, resulted in a slight decrease in its apparent molecular size by about 2K (track 2). Variations in the mobilities of the different allelic forms of PAP were also observed when subtilisin-cleaved M-PAP from placental micovilli of SS, FF, and FS phenotypes (Figure 4 tracks 6,7 and 8, respectively) were analysed under these conditions. Molecular size estimates for the SS and FF phenotypes in the absence of reduction were 129K and 124K, respectively.

4:3.5 The Effect of Trypsin Solubilization on PAP

Trypsin treatment failed to release microvillous PAP activity above control levels (Table 1), and no 69K band was detected in the membrane supernatants (Figure 5 tracks 2 and 3). However, the intensity of the M-PAP band in the membrane pellet was decreased by the addition of trypsin (tracks 5-7), with the concomitant appearance of a band of about 60K (tracks 5-7) which was absent from the control (track 4). Butanol extracts of both the control and the trypsin-treated membrane pellets (tracks 8 and 9 respectively) showed several bands including

prominent ones at 69K and 60K which were found only in trypsin-treated membranes. Similar results were obtained when purified M and A-PAP were digested with trypsin (Figure 5 track 11). Although trypsin was unable to release M-PAP from the membrane, it appears to cleave a portion of about 9K from one end of the subunit, leaving an enzymatically active portion of PAP associated with the membranes.

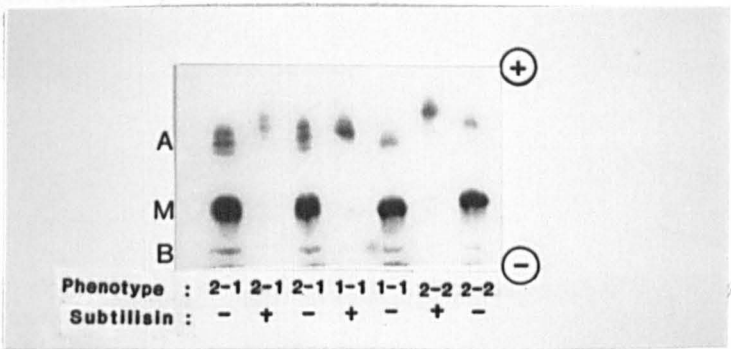

Figure 1. Starch gel electrophoresis of subtilisin-cleaved M-PAP. Aliquots of placental microvillous membranes of SS, FS, and FF phenotypes were incubated with subtilisin for 3 h at 37°C at a ratio of 10:1 (w/w PAP:protease). After incubation, the supernatants were separated and analysed on starch gels containing 0.5% Triton X-100; together with butanol extracts of untreated microvilli of corresponding phenotype.

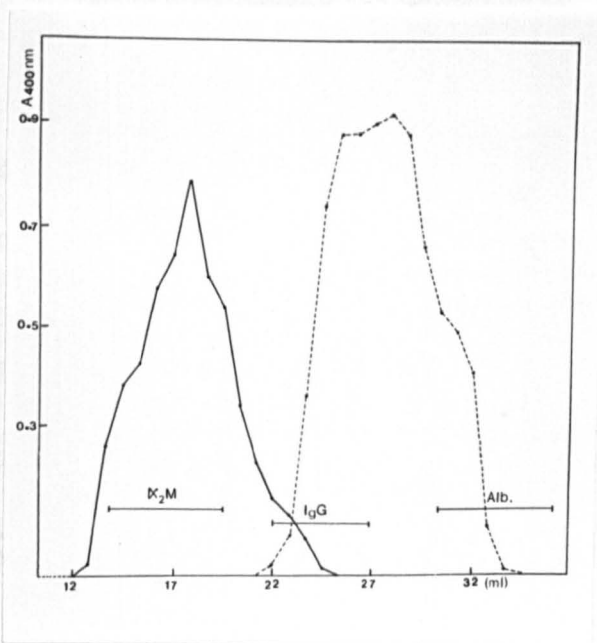

Figure 2. Gel filtration of placental alkaline phosphatase after proteolytic cleavage from microvilli.
After subtilisin cleavage of microvilli, the released PAP was applied to an AcA34 column (25x1cm^2) and eluted in Tris-succinate buffer pH 7.4. The enzyme activity was plotted against the elution volume (-----) and compared with the elution of a butanol extract of PAP (———) prepared without the addition of subtilisin. The elution ranges of alpha-2-macroglobulin (α_2M), IgG and human serum albumin (Alb.) were determined.

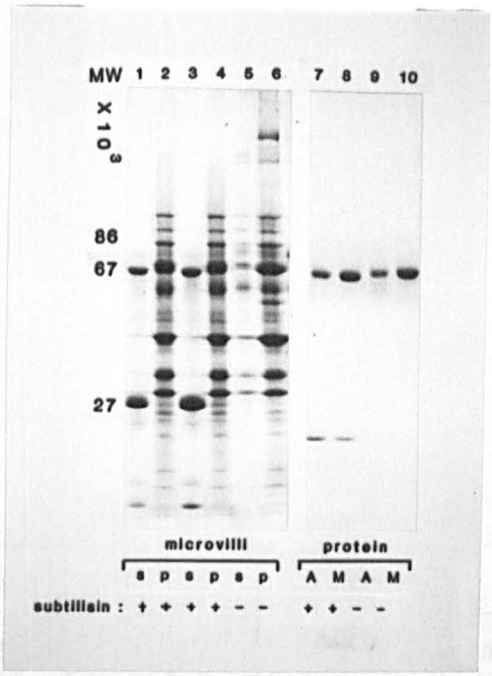

Figure 3. SDS-polyacrylamide gels of subtilisin treated placental microvillous membranes and purified PAP.

A suspension of microvillous membranes was incubated for 3 h at 37°C with subtilisin at a ratio (w/w PAP:protease) of 10:1 or 20:1 or without subtilisin. After incubation, the samples were centrifuged at 100,000g for 30 min. The supernatants were removed and the pellets were washed once and resuspended in PBS. Aliquots of the supernatants (tracks 1,3 and 5) and the pelleted membranes (tracks 2,4 and 6) were analysed on SDS-PAGE. Microvilli were treated with a ratio 10:1 subtilisin in tracks 1 and 2, with 20:1 subtilisin in tracks 3 and 4, and without subtilisin in tracks 5 and 6. Tracks 7-10 contain purified samples of A-PAP and M-PAP either treated with subtilisin or not, as indicated in the figure (A=A-PAP; M=M-PAP; -=control; +=after treatment with subtilisin).

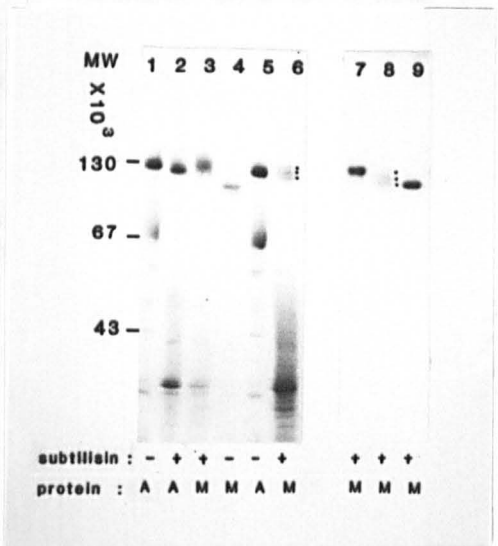

Figure 4. Subtilisin cleavage of M-PAP and A-PAP resolved on SDS polyacrylamide gel in the absence of reduction.

Purified preparations of M and A-PAP were incubated with subtilisin for 3 h at 37°C at a ratio of 10:1 (w/w). The cleaved proteins were then analysed on SDS gels in the absence of reducing agents. Tracks 1 and 5 contain A-PAP. Tracks 2 and 3 contain subtilisin treated A and M-PAP, respectively. Track no. 4 contains M-PAP. Tracks 6,7,8 and 9 show the mobility of subtilisin cleaved M-PAP obtained from separate samples of microvilli with PAP phenotypes of FS, SS, FS and FF respectively. The three bands in the FS phenotype are marked by black dots.

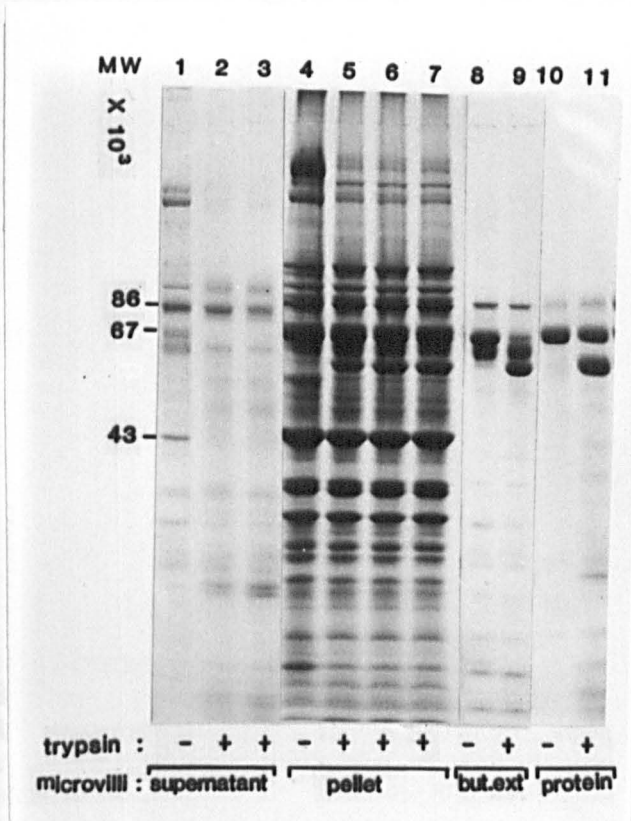

Figure 5. SDS polyacrylamide gel of microvilli and purified PAP after treatment with trypsin.

Aliquots of microvillous membrane suspension were incubated for 3h at 37 °C with trypsin at ratios (w/w PAP:protease) of 10:1, 20:1 and 40:1. The results are shown in tracks 1-9. After incubation and centrifugation, the membrane supernatants were resolved in SDS gels : track numbers : 1 (no trypsin), 2 (20:1 trypsin), 3 (10:1 trypsin). The pellets were analysed in SDS gel track numbers : 4 (no trypsin), 5 (40:1 trypsin), 6 (20:1 trypsin) and 7 (10:1 trypsin).

Aliquots of untreated membranes and those treated with 10:1 trypsin were also extracted with 50% butanol; the aqueous phases of these extractions were resolved in tracks 8 and 9, respectively. Track no. 10 contains M-PAP and no 11 contains M-PAP treated with trypsin.

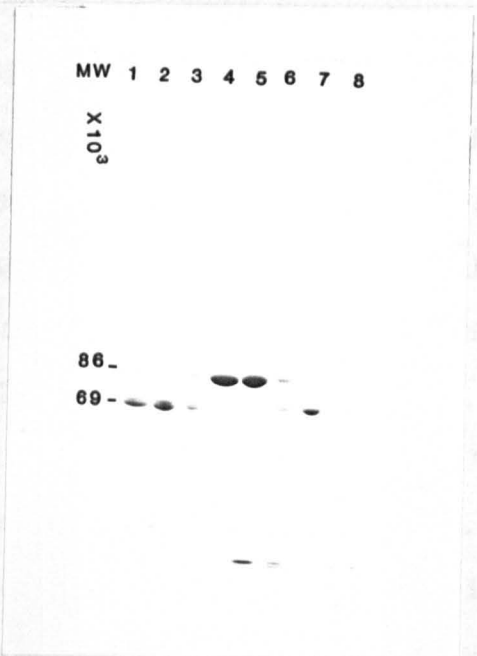

Figure 6. SDS polyacrylamide gel of ion-exchange fractions from a microvillus preparation containing degraded M-PAP.
A butanol extract of placental microvillus membranes were applied to DEAE sepharose CL-6B. The elution was carried out with a gradient of increasing sodium chloride concentration. The fractions were assayed on starch gels and then aliquots of the fractions containing M and A-PAP were analysed on SDS-PAGE. Tracks 1,2 and 3 show M-PAP with a doublet band at 67K and 69K. Tracks 6,7 and 8 contain A-PAP. The band at 86K in tracks 4 and 5 is transferrin.

4:4 DISCUSSION

4:4.1 Location of the Membrane Domain of M-PAP

Detergents and proteinases are commonly used as methods for solubilizing integral membrane proteins; they release microvillar membrane hydrolases of kidney and intestine without loss of hydrolase activity. Detergent solubilization yields a protein of intact (native) primary structure, whereas proteases cleave off the external (hydrophilic) domains of proteins from the hydrophobic peptides integral to the plasma membrane (for review, see Kenny and Maroux, 1982). The available evidence indicates that M-PAP behaves somewhat similarily to kidney and intestinal hydrolases. The majority of M-PAP can only be released from microvilli by delipidation or proteolysis. Treatment of placental microvillous membranes with subtilisin releases PAP with a polypeptide molecular weight of about 2K smaller (67K) than the molecular weight of native M-PAP obtained by butanol extraction of microvilli (69K). It is probable that this 2K difference in molecular weight is due to the presence of a membrane binding domain in the butanol form (M-PAP) which is absent in the subtilisin released form. A small hydrophobic domain which may function in this way has been identified in intestinal alkaline phosphatase (Komoda et al, 1981). Integral proteins can interact with the phospholipid bilayer in various ways, particularly with respect to the number and the location of the amino acids which are embedded in the membrane. M-PAP seems to fit in the same category as cytochrome b_5, NADH cytochrome b_5 reductase and most of the kidney and intestinal microvillar hydrolases in which most of the protein mass is located on one side of the membrane and only a

small peptide segment lies within the membrane. This differs from other integral membrane proteins, such as rhodopsin and erythrocyte band III, which have most of their mass inserted in the lipid bilayer (for reviews, see Kenny and Maroux, 1982; Lodish, Braell, Schwartz, Strous and Zilberstein, 1981).

N-terminal amino acid sequencing was carried out in order to determine whether M-PAP is integrated into the plasma membrane at its carboxy-terminus or elsewhere. Previous dansyl-Edman sequencing studies on PAP, purified from butanol extracts of total placental homogenate, yielded the amino-terminal sequence of Ile-Ile-Pro-Val. (Green and Sussman, 1973). The sequencing data (Table 2) agree and extend those findings . It also shows that native and subtilisin cleaved M-PAP show common N-termini, indicating that the 2K membrane binding domain must be located at the C-terminus of the PAP polypeptide. Limited sequence studies on detergent and proteolysed forms of human and pig intestinal alkaline phosphatases (Komoda et al, 1981; Colbeau and Maroux, 1978) have shown that intact and proteolysed forms had the same N-terminal peptides (in the human Phe-Ile-Pro and in the pig Phe-Ile), suggesting that both enzymes are integrated at their carboxy-termini. Alkaline phosphatases therefore contrast with some of the other microvillar hydrolases of kidney and intestine which are integrated at the N-terminal (Kenny and Maroux, 1982). The incidence of hydrophobic amino acids in the first four positions in PAP (Table 2) is not surprising. Many other secreted proteins have 2-4 such residues which are probably the carboxyl end of the hydrophobic leader sequence the majority of which has been cleaved off during polypeptide maturation (Harwood, 1980).

Since M-PAP consists of two identical 69K subunits it is probable that it is integrated into the membrane by both anchoring domains. However, it is worth noting that a few samples of M-PAP prepared by butanol extraction showed a doublet band at 67K and 69K (Figure 6 tracks 1-3), most probably due to endogenous proteases acting at the junction between the hydrophilic and the binding domain toward the carboxyl end of the molecule. These samples were probably exceptional, care over the duration and temperature of the microvillous extraction usually prevents the formation of the 67K band. It is not clear why such degradation was not found in the A-PAP fractions prepared from those samples (Figure 6 tracks 6-8). Perhaps the residual A-PAP associated with the microvillous preparations is entrapped within the membrane vesicles, and hence protected from proteolysis.

Since M-PAP has an affinity for hydrophobic alkyl agarose which allows it to be separated from A-PAP, we may consider the working hypothesis that M is an integral membrane protein because it differs from A-PAP in having a hydrophobic carboxy-terminal domain anchoring it in the membrane. However, A-PAP and M-PAP have similar CNBr maps and both express the same genetic polymorphism, located in the 67K hydrophilic fragment of the polypeptide. If this polymorphism defines a unique, allelic exon then the presence of different carboxy-termini could imply a process of exon shuffling, or altered transcription or altered maturation of the primary transcript. Such a phenomenon was found in the lymphocyte IgM molecule, which exists as a secretory water-soluble molecule as well as an integral membrane protein. The two forms of the molecule are so similar that for a long time no difference between them could be detected. More recently, it has been shown that the difference between the two forms reside at the C-terminus of the μ

chains; membrane-bound μ chains have a 26 residue long stretch of hydrophobic amino acids, absent from secretory μ chains (Singer and Singer, 1980; Rogers, Early, Carter, Bond, Hood and Wall, 1980; Early, Rogers, Davis, Calame, Bond, Wall and Hood, 1980). It has been shown that the two forms of the molecule are coded for by separate messenger RNAs, which originate by different splicing of a common precursor RNA (Rogers et al, 1980; Early et al, 1980). However, we must also consider that the differences could be due to post-translational events, though it is difficult to be clear about mechanism. Glycosylation could render a hydrophobic ("M") domain relatively hydrophilic (making it perhaps into "A"), but if glycosylation occurs within the microsomal membrane system of the cell, it is difficult to understand how glycosylation could occur to a domain which, being up to that point hydrophobic, would presumably be located within the plasma membrane. Other modifications (e.g. phosphorylation) meet with similar difficulties, unless it is possible to modify the nascent polypeptide chain before it enters the endoplasmic reticulum. Since the reduced 69K polypeptides of M and A are indistinguishable on SDS gels (69K), as also are the 67K subtilisin polypeptides, it seems improbable that A is a proteolytic product of M.

4:4.2 Conformational Heterogeneities

Under non-reducing conditions subtilisin cleaved and uncleaved M-PAP had molecular sizes of 130K and 118K respectively; the cleaved form showing a bigger apparent molecular size (Figure 4). This unexpected finding might have been due to the hydrophobic segment in uncleaved M-PAP binding more SDS and hence moving faster in the gel due to the extra negative charge. However, this is unlikly as such an effect did not

occur under reducing conditions. The difference in the effect of subtilisin on the two forms of the enzyme argues that the dimers of A and M probably have different conformations. These conformations might be caused by structural differences inherent in the 67K domains. Alternatively, they could be due to a more complex interaction in the intact molecules, whereby the 2K fragments of A and M induce different conformational folding (and perhaps disulphide bonding) in the 67K domains. Further sequence data is awaited to help answer these questions.

Molecular size variations of the different allelic forms were observed in starch gels, and in SDS gels in the absence of reduction, both for the cleaved and uncleaved forms of PAP (Figure 4). Since this effect is not lost by the removal of the 2K domain, this molecular heterogeneity must reside within the 67K domain of the molecule. On SDS gels, the fast and slow phenotypes of intact A-PAP were reported to have apparent dimeric molecular weights of 119K and 122K, respectively (Holmgren and Stigbrand, 1976). Although our estimates are higher than these molecular sizes, we confirm the existence of this heterogeneity. It is unusual to be able to distinguish polymorphic forms of enzymes in SDS gels, even in the absence of reduction. Since the allelic forms of PAP are indistinguishable in SDS gels <u>with</u> reduction, the differences in the <u>absence</u> of reduction suggest that the allelic forms of PAP differ substantially in conformational structure. We cannot deduce the number of amino acid changes needed to produce such conformational differences. Simple amino acid substitutions are generally thought to account for enzyme polymorphisms, and this is the case in some common variants of haemoglobin as well as in the recently reported analysis of <u>Drosophila</u>

alcohol dehydrogenase (Kreitman, 1983). Such substitutions might cause conformational changes big enough either to be detectable in non-reducing SDS gels, or to result in notable allele-specificity amongst monoclonal antibodies to PAP (Slaughter et al, 1981, 1983; Millan et al, 1982). However, multiple amino acid substitutions can sometimes underly allelic differences, as has been found from sequencing analyses of histocompatibility antigens (Weiss, Golden, Kakut, Millor, Fahren/Kvist and Flavell, 1983). The matter must remain open so far as PAP is concerned until more sequence data is available.

4:4.3 Native Molecular Weight Estimates

Molecular weight estimates from gel filtration of M-PAP (725,000) suggest that the enzyme is in an aggregated form (chapter 2). After subtilisin treatment M-PAP was much reduced in size and co-eluted with A-PAP (130K). This indicates that M-PAP aggregates through non-covalent associations between hydrophobic C-termini. It remains to be determined whether this aggregation exists in intact microvillous membranes. High molecular weight forms of PAP and PAP-like enzymes have been previously reported. B-PAP has a molecular weight of about 200,000 (Doellgast et al, 1977). A large hydrophobic form of heat stable alkaline phosphatase has been described in JAR choriocarcinoma cells by Neuwald and Brooks (1981). In that case, the enzyme had a molecular weight of at least 225,000, though large forms of the enzyme were also evident in non-denaturing gels. The enzyme could be converted from a slow mobility hydrophobic form to a faster mobility, hydrophilic form through trypsin proteolysis. Though there are parallels between JAR alkaline phosphatase and placental microvillous AP, the JAR enzyme is more sensitive to

inhibition by phenylalanylglycylglycine than is PAP, and M-PAP cannot be cleaved into hydrophobic and hydrophilic domains by trypsin treatment, since that protease primarily cleaves the PAP polypeptide at a position close to its NH_2-terminus. This difference may be indicative of fundamental structural differences between PAP and the PAP-like enzyme of JAR cells.

CHAPTER 5

GENERAL DISCUSSION

General Discussion

This chapter focuses attention on the possible relationship between the microvillous alkaline phosphatase (M-PAP) and A-PAP and examines some areas of interest for future experimental research into the nature, genetics and function of placental alkaline phosphatase.

The results presented in the previous chapters demonstrate the existence of a membrane bound form of human placental alkaline phosphatase (M-PAP) in association with the microvillous plasma membranes. The physical characteristics displayed by M-PAP on gel filtration (chapter 2 fig 6-8), ion-exchange chromatography (chapter 3 fig 1) and on hydrophobic chromatography (chapter 3 fig 7) were typical of integral membrane proteins, and distinct from those of the previously described A and B forms of PAP prepared from total placental extracts. Such behavioural differences appear to support the view that M-PAP could represent a product of a distinct gene from that of A-PAP. However, the expression of the same genetic polymorphism (chapter 2 fig 4) and the identical N-terminal 13 amino acid sequences (chapter 4 table 2) in both forms suggests that A and M-PAP are very closely related and probably, at least in part, share the same enzymatic polypeptide. Evidence of antigenic cross-reactivity (chapter 2 fig 5), and the data on heat stability (chapter 2 fig 9), CNBr peptide maps (chapter 3 fig 6), cleavage sites for subtilisin (chapter 4 fig 3) and trypsin (chapter 4 fig 5), subunit molecular weight (chapter 3 fig 3) and kinetic studies (chapter 3 table 1) also strongly argue against the involvement of two distinct genes for the expression of the A and M forms of the enzyme. If so, then one would be led to believe that both forms are produced

essentially by a single gene and therefore the different properties of A and M-PAP could be due to post-translational processing or differential splicing. Post-translational processing such as glycosylation, phosphorylation, acylation and proteolysis may give rise to different functional and structural properties displayed by these molecules. The finding of a similar subtilisin cleavage in A-PAP, as judged on SDS polyacrylamide gels (chapter 4 fig 3), argues against this form being a subtilisin cleavage product of M-PAP in vitro. However, this possibility is only weakly supported by SDS-electrophoretic patterns, since small differences in molecular weight (69K versus 67K) cannot be definitely shown. Whether a similar in vivo proteolytic cleavage accounts for A-PAP is not clear. It may be that covalently bound lipid is associated with the carboxy-terminal of M-PAP and accounts for its behavioural characteristics. Such bound lipid has been found in association with the membrane-spanning fragments of certain viral glycoproteins (Schmidt, Bracha, and Schlesinger, 1979; Schmidt and Schlesinger, 1979; Schmidt, 1982) and with the variant surface glycoproteins of Trypanosoma brucei (Cardoso de Almeida and Turner, 1983). In the latter case both lipids and carbohydrates were found to bind to the carboxy-terminal domain. These fatty acid residues were assumed to provide a type of anchor that could bind these glycoproteins to the membranes. Furthermore the process of acylation and deacylation reported by Bishr Omary and Trowbridge (1981) for the transferrin receptors in human lymphoma cells, would modulate the affinity of a glycoprotein for the membrane. The exact mechanism of the attachment of these glycoproteins is poorly understood (Schmidt, 1982).

Subtilisin digestion has been used as an alternative to butanol solubilization for the release of M-PAP. The mechanism of release

appears to involve proteolytic removal of the major portion of the molecule (67K) from a short anchoring peptide, approximately 15-20 amino acids at the carboxy-terminal (chapter 4 fig 3). The removal of the binding domain of M-PAP has not been shown to affect the catalytic activity of the enzyme (chapter 4 table 1). Nevertheless this domain exerts a major influence on the structural properties of M-PAP. Following digestion with subtilisin, M-PAP showed behavioural characteristics similar to that of A-PAP under different electrophoretic and chromatographic systems. On starch gels cleaved M-PAP did not require the presence of Triton X-100 and showed a slightly faster mobility than that of A-PAP (chapter 4 fig 1). On SDS polyacrylamide gels both forms showed very similar mobilities (chapter 4 fig 3). Cleaved M-PAP was also found to elute with A-PAP in both ion-exchange and gel filtration columns (chapter 4 fig 2). This change in the behavioural properties in M-PAP appears to correlate with the removal of approximately 15-20 amino acid residues from the carboxy-terminal. These findings together with the behaviour of the subtilisin cleaved and un-cleaved dimeric polypeptides of both forms on SDS polyacrylamide gels in the absence of reduction (chapter 4 fig 4), where the removal of the binding domain of M-PAP seems to have a significant influence on the compaction of the molecule, provide some indirect evidence for the presence of some structural differences between the carboxy termini of these two molecules. Since M-PAP displays physical characteristics typical of integral membrane proteins, a more likely hypothesis is that M differs from A-PAP in having a hydrophobic carboxy-terminal domain anchoring it in the membrane. However, both A and M-PAP express the same genetic polymorphism (chapter 2 fig 4), located in the 67K

hydrophilic fragment of the polypeptide and have identical N-terminal amino acid sequences up to position 13 (chapter 4 table 2). If this polymorphism defines a unique shared exon between A and M-PAP, then the presence of different carboxy-termini would imply that their C-termini are encoded on different exons. This will also require the presence of different RNA splicing sites. Such a phenomenon exists for the membranous and secreted forms of immunoglobulins and is brought about by separate species of mRNA differing only in their 3' coding regions (Rogers, et al, 1980; Early, et al, 1980). A possible role for alternative acceptor splice sites in the mouse H-2K gene has been described by Kress, Glaros, Khoury and Jay, (1983) in which two alternative transcripts were found to encode H-2K antigens differing in their C-termini . Different models can be proposed to achieve the two forms of PAP differing in their C-termini. RNA polymerase could read through the whole gene and generate nuclear RNAs with common 3' ends (containing all five exons), or transcription could be terminated at different sites producing RNAs with different 3' ends. Subsequent differential splicing of the exons 4 and 5 in both models could give rise to the A and M forms of mRNA which would encode the two forms of the enzyme (see Fig. 1).

5:1 Further Characterization of the C-termini

At this stage of knowledge one can only speculate as to the relationship between the A and M forms of the enzyme. Whatever the physical basis for the difference between the two forms, it seems probable that its elucidation, perhaps involving direct amino acid sequencing of the carboxy-termini of the A and M forms, will contribute to the knowledge of the biosynthesis and expression of membrane

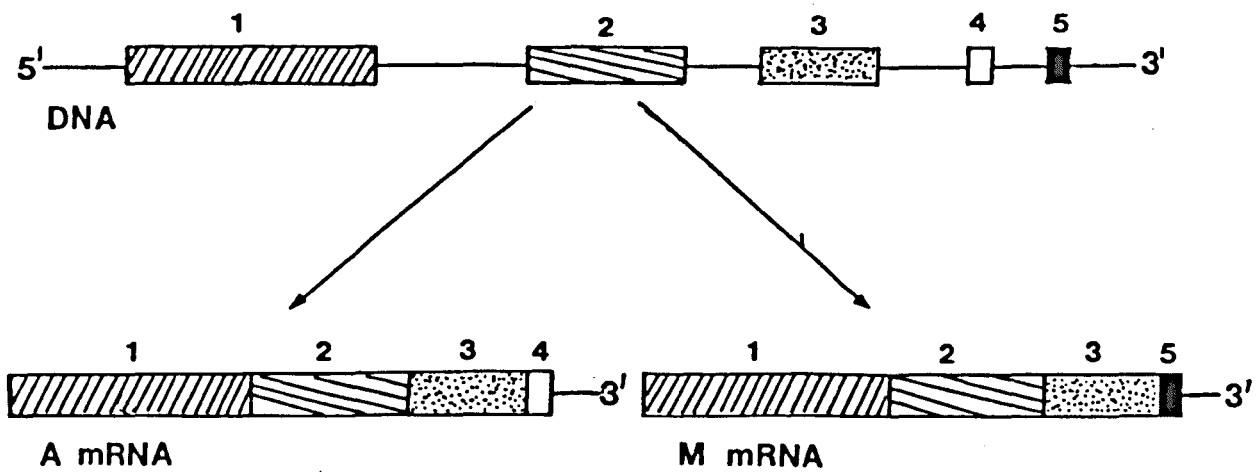

Figure 1. Proposed RNA processing patterns that could generate two different mRNAs.

proteins. Since subtilisin treatment of the butanol form of M-PAP effectively cleaves the hydrophilic domain (67K) from the binding domain (2K), it is possible to isolate and characterize these latter fragments. However, there are inherent difficulties in the purification, handling and detection of such peptides. The use of LH 20 columns with different solvent systems seems to isolate small hydrophobic peptides (Brett and Findlay, 1983). The detection of these peptides can be facilitated by labelling with ^{125}I as the probability that such hydrophobic segments containing tyrosine residues is high. It is also possible to improve the resolution of SDS polyacrylamide gels and to detect small fragments down to 10 amino acids by increasing the bis acrylamide and the incorporation of Urea in these gels (Swank and Munkres, 1971).

5:2 The Need For Molecular Biological Studies

There is no doubt that molecular studies of the PAP gene at the DNA level would give some insight into the problem of the expression of membranous and non-membranous forms of PAP. It would also serve to determine the genetic basis of the allelism, the nature and location of AP loci in placental and other DNA, as well as to enquire into the nature of PAP-like isoenzymes in tumours. cDNA clones from mRNA of PAP, can be made by priming reverse transcription either with oligo dT, or with synthetic oligonucleotides. The amino acid sequence found in this study can provide the bases for 4 alternative 11-nucleotide oligomers (with two mixed sites), one of these oligomers would be complementary to PAP mRNA. Although PAP is expressed in large quantities at term, D. A. Nickson finds that the message is not present at high levels and therefore is difficult to isolate. This could be due to the stability

of the enzyme and therefore low levels of the mRNA, or to the inactivation of the gene prior to parturition (Nickson, personal communication). Selected tumour cell lines such as HeLa subline TCRC-1, (Singer and Fishman, 1974; Beckman et al, 1970), or Osteosarcoma cells (Singh, Tsang and Blakmore, 1978) which have been found to produce PAP, on the basis of electrophoretic and inhibition studies, might be a rich source for PAP message. However, the only problem in dealing with such cell lines is the lack of structural studies on the enzyme produced in comparison with PAP.

An alternative strategy is to probe a cloned genomic library using synthetic oligomers. This technique has been used for multiple copy-genes where there is no requirement for screening large number of clones whereas looking for a single-copy gene, which might be the case for PAP, would involve analysing a large number of clones some of which might be hybridizing due to the presence of similar sequences in other parts of the genome, and not PAP gene itself. The 11-oligonucleotide oligomers could be extended by using them as a primer for mRNA reverse transcription. Such a possibility will greatly reduce the number of genomic clones that would hybridize with the probe. However, the advantages of this strategy are that it would circumvent the potential problem of cDNA cloning of mRNA which might be unstable or present at low concentrations. It would also detect the presence of other sequences involved in the control and expression of PAP, should they exist.

5:3 Nature and Origin of Serum PAP

Placental alkaline phosphatase becomes detectable in the serum of pregnant women between the 16th and 20th weeks of pregnancy, increasing

progressively up to the onset of labour and disappearing within three to six days of the delivery of the placenta (Moss, 1982). Previous studies on serum PAP did not involve any structural studies neither in comparison with the A and B forms of the enzyme nor with other alkaline phosphatases. The methods employed by various investigators were restricted to heat inactivation, the use of inhibitors, immunoassays and starch gel electrophoresis. The allotypic variation in electrophoretic mobility that is characteristic of the isoenzyme prepared from placentae is reflected also in serum (Boyer, 1961; Beckman, Bjorling and Christodoulou, 1966). This sera seems to contain mainly the A form of PAP and some of the B form which can be seen late in pregnancy (Ghosh and Fishman, 1974). It is not yet known whether PAP is actively secreted into the maternal blood or to what extent microvillous PAP from syncytiotrophoblast microvilli that have broken away into maternal circulation account for PAP levels during pregnancy. The finding that microvillous M-PAP can be proteolytically cleaved from the membranes to yield a form with a mobility slightly faster than that of A-PAP on starch gels raised the question whether M-PAP contributes to serum PAP in a similar way. This should now be taken into consideration when starch gel zones of activity of pregnancy sera are being interpreted. It is also of great importance to ask whether M-PAP has any clinical value in monitoring the rate of loss of microvilli in normal and complicated pregnancies.

5:4 Candidate Substrates for PAP

Despite the quantity of PAP in microvillous membranes and in pregnancy sera, its precise biological function is not clear. This uncertainty applies to other alkaline phosphatases, though a variety of

tentative hypotheses have been advanced (see, McComb, 1979). Alkaline phosphatases from three different preparations (calf intestine, bovine liver, and Escherichia coli), have been found in vitro to dephosphorylate phospho-tyrosine-histones as well as some other membrane proteins (Swarup, Cohen, and Garbers, 1982). It was also found that some human tumour cells contain microsomal alkaline phosphatases which can dephosphorylate receptors of epidermal growth factor (EGF) after the receptor had been phosphorylated by EGF (Horlein, Gallis, Brautigan, and Bornstein, 1982). EGF receptors exist on membranes prepared from whole placentae (O'Keefe, Hollinbergand, and Cuatrecasas, 1974) and were also reported to exist on placental microvillous plasma membranes (Richards, Beardmore, Brown, Molloy and Johnson, 1983). It is interesting to speculate that the expression of PAP in syncytiotrophoblast may control the binding of EGF and thus reduce the ability of the terminally differentiated syncytium to respond to EGF.

BIBLIOGRAPHY

Abu-Hasan, N. S., Davies, M. and Sutcliffe, R. G. (1984) A high molecular weight species of placental alkaline phosphatase in human syncytiotrophoblast microvilli. Placenta (in press).

Abu-Hasan, N. S. and Sutcliffe, R. G. (1984) Purification and analysis of the microvillous (M) and A forms of placental alkaline phosphatase. Placenta (in press).

Aleem, F. A. (1972) Total and heat stable alkaline phosphatase in normal and abnormal pregnancies. Obstet. Gynecol. 40, 163-172.

Badger, K. S. and Sussman, H. H. (1976) Structural evidence that human liver and placental alkaline phosphatase isoenzymes are coded by different genes. Proc. Natl. Acad. Sci. USA 73, 2201-2205.

Beckman, G. and Beckman, L. (1969) The placental alkaline phosphatase polymorphism; variations in Hawaiian subpopulations. Hum. Hered. 19, 524-529.

Beckman, G., Beckman, L. and Lundgren, E. (1970) Isoenzyme variations in hunan cells grown in vitro : identity between alkaline phosphatases from HeLa cells and placenta . Hum. Hered. 20, 182-186.

Beckman, G. and Beckman, L. (1975) The placental alkaline phosphatase variant D and natural selection. Relation to complications of pregnancy and to "the Regan isoenzyme". Heriditase 81, 85-88.

Beckman, L. and Beckman, G. (1968) A genetic variant of placental alkaline phosphatase with unusual electrophoretic properties. Acta. Genet. (Basel) 18, 543-552.

Beckman, L., Beckman, G., Christodoulou, C. and Ifekwunigwe, A. (1967) Variations in human placental alkaline phosphatase. Acta. Genet. (Basel) 17, 406-412.

Beckman, L., Bjorling, G. and Christodoulou, C. (1966) Pregnancy enzymes and placental polymorphism. 1. alkaline phosphatase. Acta. Genet. 16, 59-64.

Beer, A. E., Billingham, R.E. and Yang, S. L. (1972) Further evidence concerning the autoantigenic status of the trophoblast. J. Exp. Med. 135, 1177-1184.

Behrman, S. J., Yoshida, T., Amano, Y. and Paine, P. (1974) Rhesus and squirrel monky placental specific antigen(s). Am. J. Obstet. Gynecol. 118, 616-625.

Bishr Omary, M. and Trowbridge, I. S. (1981) Biosynthess of the human transferrin receptor i cultured cels. J. Biol. Chem. 256, 12888-12892.

Benham, F. J., Povey, M. S. and Harris, H. (1978) Heterogeneity of alkaline phosphatase in different HeLa lines. Somatic cell genet. 4, 13-25.

Bennett, M. J., Grudzinskas, J. G., Gordon, Y. B. and Turnbull, A. C. (1978) Circulating levels of alpha-fetoprotein and prgnancy specific beta-glycoprotein in pregnancies without an embryo. Br. J. Obstet. Gynecol. 85, 348-349.

Billington, W. D. (1979) The immunology of trophoblast, In: Immunology of human reproduction. (Ed.) Scott, J. S. and Jones, W. R. pp 81-102 London. Academic press.

Blobel, G. and Dobberstine, B. (1975) Transfer of proteins across membranes: I. Presence of proteolytically processed and un processed nascent immunoglobulin light chains on membrane-bound ribosomes of murine myeloma. J. Cell Biol. 67, 835-851.

Bonsall, R. W. and Hunt, S. (1971) Characteristics of interactions

between surfactants and the human erythrocyte membrane. Biochim. Biophys. Acta 249, 266-280.

Boyer, S. H. (1961) Alkaline phosphatase in human sera and placentae. Science 134, 1002-1004.

Boyer, S. H. (1963) Human organ alkaline phosphatases. Discrimination by several means including starch gel electrophoresis of antienzyme-enzyme supernatant fluids. Ann. N. Y. Acad. Sci. 103, 938-951.

Brauer, A. W., Margolies, M. N. and Haber, E. (1975) The application of 0.1M Quadrol to the microsequence of proteins and sequence of tryptic peptides. Biochem. 14, 3029-3035.

Brett, M. and Findlay, J. B. C (1983) Isolation and characterization of the CNBr peptides from the proteolytically derived N-terminal fragment of ovine opsin. Biochem. J. 211, 661-670.

Burgos, M. H., Rodriguez, E. M. (1966) Specialized zones in the trophoblast of the human term placenta. Am. J. Obstet. Gynecol. 96, 342-356.

Butterworth, P. J. and Moss, D. W. (1966) Action of neuraminidase on human kidney alkaline phosphatase. Nature (Lond.) 209, 805-806.

Carlson, R. W., Wada, H. C. and Sussman, H. H. (1976) The plasma membrane of human placenta, isolation of microvillus membrane and characterization of proteins and glycoproteins subunits. J. Biol. Chem. 251, 4139-4146.

Chang, C. H., Angellis, D. and Fishman, W. H. (1980) Presence of the rare D-variant heat stable, placental type alkaline phosphatase in normal human testis. Cancer Res. 40, 1506-1510.

Chen, S. and Andersson, W. (1972) Regan isoenzyme of alkaline phosphatase in cancer patients. Gastroenterology 62, 732-738.

See page 120

Doellgast, G. J. and Benirschke, K. (1979) Placental alkaline phosphatase in hominidae. Natuer 280, 601-602.

Colbeau, A. and Maroux, S. (1978) Integration of alkaline phosphatase in the intestinal brush border membrane. Biochim. Biophs. Acta 511, 39-51.

Currie, A. R., Beck, J. S., Ellis, S. and Read, C. H. (1966) Immunofluorescent localization of a growth-hormone-like factor in normal and abnormal syncytiotrophoblast. J. Pathol. Bacteriol. 92, 395-399.

Davis, B. D. and Tai, P-C. (1980) The mechanism of protein secretion across membranes (a review). Nature (lond.) 283, 433-438.

Davies, M., Parry, J. E. and Sutcliffe, R. G. (1981) Examination of different preparations of human plasma membrane for the binding of insulin , transferrin and immunoglobulins. J. Reprod. Fert. 63, 315-324.

DeBroe, M. E., Borgers, M. and Wieme, R. J. (1975) The separation and characterization of liver plasma membrane fragments circulating in the blood of patients with cholestasis. Clin. Chim. Acta 59, 369-372.

Doellgast, G. J. and Fishman, W. H. (1976) L-leucine, a specific inhibitor of a rare human placental alkaline phosphatase phenotype. Nature (lond.) 259, 49-51.

Doellgast, G. J., Spiegel, J., Guenther, R. A. and Fishman, W. H. (1977) Studies on human placental alkaline phosphatase: Purification by immunoabsorbtion and comparison of the A and B forms. Biochim. Biophys. Acta 484, 59-78.

Doellgast, G. J. and Fishman, W. H. (1974) Purification of human placental alkaline phosphatase. Biochem. J. 141, 103-112.

Donald, L. J. and Robson, E. B. (1973) Rare variants of placental

See page 120

alkaline phosphatase. Ann. Hum. Gent. (Lond.) 37, 303-313.

Early, P., Rogers, J., Davis, M., Calame, K., Bond, M., Wall, R. and Hood, L. (1980) Two mRNAs can be produced from a single immunoglobulin μ gene by alternative RNA processing pathways. Cell 20, 313-319.

Edward, R. G. (1972) Early human development : From oocyte to implantation. In Scientific foundations of obstetric and gynecology, 2nd. ed. Philip, E. A., Barnes, J. and Newton, M. (eds.), pp 175-252. London: Heinemann.

Engelman, D. M. and Steitz, T. A. (1981) The spontaneous insertion of proteins into and across membranes. Cell, 23, 411-422.

Faulk, P. and Johnson, P. M. (1977) Immunological studies of human placentae : identification and distribution of proteins in mature chorionic villi. Clin. Exp. Immunol. 27, 365-375.

Fishman, L., Miyayama, H., Driscoll, S. G. and Fishman, W. H. (1976) Developmental-phase specific alkaline phosphatase isoenzymes of human placenta and their occurrence in human cancer. Cancer Res. 36, 2268-2273.

Fishman, W. H., Inglis, N. R., Stolbach, L. L. and Kraut, M. J. A. (1968) Serum alkaline phosphatase isoenzyme of human neoplastic origin. Cancer Res. 28, 150-154.

Fox, H. (1967) The incidence and significance of vasculo-syncytial membranes in the human placenta. J. Obstet. Gynaecol. Brit. Commonwealth 74, 28-34.

Ghosh, N. K. and Fishman, W. H. (1968) Purification and properties of molecular weight variants of placental alkaline phosphatase. Biochem. J. 103, 779-792.

Goldstine, D. J. and Harris, H. (1979) Human placental alkaline

See page 120

phosphatase differes from that of other species. Nature 280, 602-605.

Goldstine, D. J., Rogers, C. E. and Harris, H. (1980) Expression of alkaline phosphatase loci in mammalian tissues. Proc. Natl. Acad. Sci. USA 77, 2857-2860.

Goldstine, D.J., Blasco, L. and Harris, H. (1980a) Placental alkaline phosphatase in non-malignant human cervix. Proc. Natl. Acad. Sci. USA 77, 4226-4228.

Goodfellow, P. N., Banstable, C. J., Bodmer, W. F., Snary, O. and Crumpton, M. J. (1976) Expression of HLA system antigens in placenta. Transplantation 22, 595-603.

Gottlieb, A. J. and Sussman, H. H. (1968) Human placental alkaline phosphatase: molecular weight and subunit structure. Biochim. Biophys. Acta 160, 167-171.

Green, P. J. and Sussman, H. H. (1973) Structural comparison of ectopic and normal placental alkaline phosphatase. Proc. Natl. Acad. Sci USA 70, 2936-2940.

Hada, T., Higashino, K., Okochi, T., and Yamamura, Y. (1978) Kasahara-variant alkaline phosphatase in a renal cell carcinoma. Clin. chim. Acta 89, 311-316.

Hagashino, K., Kudo, S. and Yamamura, Y. (1974) Investigation of a variant of the placental alkaline phosphatase in hepatic carcinoma. Cancer Res. 34, 3347-3351.

Hamilton, W. J. and Hamilton, D. V. (1977) Development of the human placenta. In : Scientific foundations of obstetrics and gynaecology, 2nd.ed. Philip, E. A., Barnes, J. and Newton, M. (eds.), pp 292-358. London: Heinemann.

Harris, H., Hopkinson, D. A. and Robson, E. B. (1974) The incidence

of rare alleles determining electrophoretic variants : data on 43 enzyme loci in man. Ann. Hum. Genet. 37, 237-253.

Harris, H. (1982) The Harvey lectures, series 76, pp. 95-124. Acadimic press, London.

Harwood, R. (1980) in The enzymology of post translational modification of proteins. vol. 1 (Freedman, R.B. and Hawkins, H.c., eds) pp. 3-53, Academic press. New York.

Higashino, K., Hashinotsume, M., Yang, K. Y., Takahashi, Y., Yamamura, Y. (1972) Studies on a variant of alkaline phosphatase in sera of patient with hepatocellular carcinoma. Clin. Chim. Acta 40, 76-81.

Higashino, K., Otani, R., Kudo, S. and Yamamura, Y. (1977) A fetal intestinal type alkaline phosphatase in hepatocellular carcinoma tissue. Clin. Chem. 23, 1615-1623.

Holmgren, P. A, Stigbrand, T. and Damber, M. and Schoultz, B. V. (1978) Serum levels of placental alkaline phosphatase in high-risk pregnancies. J. Obstet. Gynecol. 54, 631-634.

Holmgren, P. A. and Stigbrand, T. (1976) Purification and partial characterization of two genetic variants of placental alkaline phosphatase. Biochem. Genet. 14, 777-789.

Horlein, D., Gallis, B., Brautigan, D. L. and Bornstein, P. (1982) Partial purification and characterization of phosphotyrosyl-protein phosphatase from Ehrlich Ascites tumourcells. Biochemistry 21, 5577-5584.

Horne, C. H. W., Towler, C. M., Pugh-Humphreys, R. G. P., Thomson, A. W. and Bohn, H. (1976) pregnancy specific beta-1-glycoprotein - a product of the syncytiotrophoblast. Experiantia 32, 1197,1199.

See page 120

Horne, C. H. W. and Nisbet, A. D. (1979) Pregnancy proteins : A review. Invest. Cell Pathol. 2, 217-231.

Hunter, R. J., Pinkerton, J. H. M., Johnston, H. (1970) Serum placental alkaline phosphatase in normal pregnancy and preeclampsia. Obstetric Gynecology 36, 536-546.

Inglis, N. R., Kirley, S., Stolbach, L. L. and Fishman , W. H. (1973) Phenotypes of the Regan isoenzyme and identity between the placental D-variant and the Nagao isoenzyme. Cancer Res. 33, 1657-1661.

Jackon, R. C., Walter, B. and Blobel, G. (1980) Secretion requires a cytoplasmically disposed sulphydryl of the RER membrane. Nature (Lond.) 286, 174-176.

Jacoby, B. and Bagshawe, K. D. (1972) A radioimmunoassay for placental-type alkaline phosphatase. Cancer Res. 32, 2413-2420.

Jones. C. J. P. and Fox, H. (1976) An ultrahistochemical study of the distribution of acid and alkaline phosphatase in placentae from normal and complicated pregnancies. J. Path. 118, 143-151.

Johnson, P. M. and Faulk, W. P. (1978) Immunological studies of human placentae: identification and distribution of proteins in immature chorionic villi. Immunol. 34, 1027-1035.

Kelley, L. K., King, B. F., Johnson, L. W. and Smith, C. H. (1979) Composition and structure of human placental microvillous membrane. Exp. Cell Res. 123, 167-176.

Kenny, J. A. and Maroux, S. (1982) Topology of microvillar membrane hydrolases of kidney and intestine. Physiol. Rev. 62, 91-128.

Komoda, T., Sakagishi, Y. and Sekine, T. (1981) Multiple forms of human intestinal alkaline phosphatase: chemical and enzymatic properties, and circulating clearances of the fast- and slow-

See page 120

moving enzymes. Clin. Chim. Acta 117, 167-187.

Komoda, T. and Sakagishi, Y. (1978) The function of carbohydrate moiety and alteration of carbohydrate composition in human alkaline phosphatase isoenzymes. Biochim. Biophys. Acta 523, 395-406.

Kreitman, M. (1983) Nucleotide polymorphism at the alcohol dehydrogenase locu of Drosophila melanogaster. Nature (London) 304, 412-417.

Kress, M., Glaros, D., Khoury, G. and Jay, G. (1983) Alternative RNA splicing in expression of the H-2K gene. Nature 306, 602-604.

Krystal, M., Elliot, R. M., Benz, E. W., Young, J. F. and Palese, P. (1982) Evolution of influeza A and B viruses : conservation of structural features in the hemaglutinin genes. Proc. Natl. Acad. Sci. USA 97, 4800-4804.

Laemmli, U. K. (1970) Cleavage of structural proteins during the assembly of the head of bacteriophage T4. Nature (lond.) 227, 680-685.

Lehman, F. G. (1975) Immunological methods for human placental alkaline phosphatase (Regan isoenzyme). Clin. Chim. Acta 65, 271-282.

Lehman, F. G. (1975) Immuonlogical relationship between human placental and intestinal alkaline phosphatase. Clin. Chim. Acta 65, 257-269.

Lin, T. M. and Halbert, S. P. (1976) Placental localization of human pregnancy associated plasma proteins. Science 193, 1249-1252.

Lingappa, V. R., Katz, F. N., Lodish, H. F. and Blobel, G. (1978) A signal sequence for the insertion of a transmembrane

glycoprotein. Journal of Biological Chemistry 253, 8667-8670.

Lodish, H. F., Braell, W. A., Schwartz, A. L., Strous, G. J. A. M. and Zilberstein, A. (1981) Synthesis and assemply of membrane and organelle proteins. Inter. Review of Cytology, Suplement 12, 247-307.

McComb, R. B., Bowers, G. N. and Posen, S. (1979) Alkaline phosphatases. New York Plenum.

McIntyre, J. A. and Faulk, W. P. (1979) Trophoblast modulation of maternal allogeneic recognition. Proc. Natl. Acad. Sci. USA 76, 4029-4032.

McLaughlin, P. J., Gee, H. and Johnson, P. M. (1983) Placental type alkaline phosphatase in pregnancy and malignancy plasma : specific estimation using monoclonal antibody in solid phase enzyme immunoassay. Clin. Chem. Acta 130, 199-209.

Meyer, D. I. (1982) The signal hypothesis-a working model. Trends. Biol. Sci. 7, 320-321.

Meyer, D.I., Louvard, D. and Dobberstein, B. (1982) Characterization of molecules involved in protein translocation using a specific antibody. J. Cell. Biol. 92, 579-583.

Millan, J. L. Eriksson, A. and Stigbrand, T. (1982) A possible new locus of alkaline phosphatase expressed in human testis. Hum. Genet. 62, 293-295.

Millan, J. L., Stigbrand, T., Ruoslahti, E. and Fishman, W. H. (1982) Characterization and use of an allotype-specific monoclonal antibody to placental alkaline phosphatase in the study of cancer-related phosphatase polymorphism. Cancer Res. 42, 2444-2449.

See page 121

Millan, J. L., Beckman, G., Jeppsson, A. and Stigbrand, T. (1982) Genetics variants of placental alkaline phosphatase as detected by monoclonal antibodies. Hum. Genet. 60, 145-149.

Moss, D. W., Eaton, R. H., Smith, J. K. and Whitby, L. G. (1966) Alteration in electrophoretic mobility of alkaline phosphatase after treatment with neuraminidase. Biochem. J. 98, 32c-33c.

Moss, D. W. (1982) In "Isoenzymes" pp 17-30, Chapman and Hall Ltd, London.

Mossman, T. R. and Williamson, A. R. (1980) Structural mutations in a mouse immunoglobulin light chain resulting in a failure to be secreted. Cell 20, 283-292.

Mulivor, R. A., Hannig, V. L. and Harris, H. (1978) Developmental changes in human intestinal alkaline phosphatase. Proc. Natl. Acad. Sci. USA 75, 3909-3912.

Mulivor, R. A., Ploktin, L. I. and Harris, H. (1978) Differential inhibition of the products of the human alkaline loci. Ann. Hum. Genet. (Lond.) 42, 1-13.

Nakayama, T., Yoshida, M. and Kitamura, M. (1970) L-leucine sensitive heat-stable alkaline phosophatase isoenzyme detected in a patient with pleuritis carcinomatosa. Clin. Chim. Acta 30, 546-548.

Neale, F. C., Clubb, J. S., Hotchkis, D. and Posen, S. (1965) Heat stability in human placental alkaline phosphatase. J. Clin. Path. 18, 359-363.

Neuwald, P. D. and Brooks, M. (1981) Altered form of placental alkaline phosphatase produced by JAR choriocarcinoma cells. Cancer Res. 41, 1682-1689.

Nikodem, V. and Fresco, J. R. (1979) Protein fingerprinting by SDS-gel electrophoresis after partial fragmentation with CNBr.

Analyt. Biochem. 97, 382-386.

O' Farrell, P.H. (1975) High resolution two-dimentional electrophoresis of proteins. J. Biol. Chem. 250, 4007-4021.

Ogbimi, A. O., Johnson, P. M., Brown, P. J. and Fox, H. (1979) Characterisation of the soluble fraction of human syncytiotrophoblast microvillous plasma membrane associated proteins. J. Reprod. Immmunol. 1, 127-140.

Ogbimi, A. O. and Johnson, P. M. (1980) Further immuonological and chemical characterisation of human syncytiotrophoblast microvillous plasma membrane associated components. J. Reprod. Immunol. 2, 99-108.

Okamura, K., Powell, J. E., Lee, A. C. and Stevens, V. C. (1981) Characterization of solubilized microvillous membrane proteins and glycoproteins from human placental syncytiotrophoblast. Placenta 2, 117-128.

O'Keefe, E., Hollenberg, M. D. and Cuatrecasas, P. (1983) Epidermal growth factor. Characteristics of specific binding in membranes from liver, placenta and other target tissues. Arch. Biochem. Biophys. 164, 518-526.

Ouchterlony, O. (1976) In handbook of experimental immunology (e.d, Weir, D. M.) pp. 655-706, Blackwell Scientific Publications, Oxford.

Palade, B. E, (1975) Intracellular aspects of the process of protein secretion. Science 189, 347-358.

Prehn, S., Nurnberg, P. and Rapoport, T. A. (1980) A receptor for signal segments of secretory proteins in rough endoplasmic reticulum membranes. FEPS Lett. 123, 79-84.

Robinson, J. C. and Pierce, J. E. (1964) Differential action of neuraminidase on human serum alkaline phosphatases. Nature 204, 472-473.

Redman, C. W. G. (1983) HLA-DR antigen on human trophoblast: A review. Am. J. Reprod. Immunol. 3, 175-177.

Richards, R. C., Beardmore, J. M., Brown, P. J., Molly, C.M. and Johnson, P. M. (1983) Epidermal growth factor receptors on isolated human placental syncytiotrophoblast plasma membrane. Placenta 4, 133-138.

Robson, E. B. and Harris, H. (1965) Genetics of the alkaline phosphatase polymorphiosm of the human placenta. Nature 18, 1257-1259.

Robson, E. B. and Harris, H. (1967) Further studies on the genetics of placental alkaline phosphatase. Ann.Hum. Genet. 30, 219-232.

Rogers, J., Early, P., Carter, C., Bond, M., Hood, L., and Wall, R. (1980) Two mRNAs with different 3' ends encode membrane-bound and secreted forms of immunoglobulin μ chain. Cell 20, 303-312.

Ruoslahti, E. and Seppala, M. (1972) Studies of carcino-fetal proteins. III. Development of a radioimmunoassay for alpha-fetoprotein in serum of healthy human adults. Int. J. Cancer 8, 374-383.

Rutigan, R., Kelly, J. P. W., Klark, L. A., Inglis, N. and Fishman, W. H. (1974) Regan type alkaline phosphatase in a human heteroploid cell line. Cancer Res. 34, 1908-1915.

Ryrie, I. J. (1975) Purification and partial characterisation of the oligomycin-sensitive ATPase from yeast mitochondria. Arch. Biochem. Biophys. 168, 712-719.

Swank, R. T. and Munkers, K.D. (1971) Molecular weight analysis of oligopeptides by electrophoresis in polyacrylamide gels with sodium dodecyle sulfate. Anal. Biochem. 39, 462-477.

Seargeant, L. E. and Stinson, R. A. (1979) Evidence that three

See page 121

Sheterline, P. and Hopkins, C. R. (1981) Transmembrane linkage between surface glycoproteins and components in neutrophil leukocytes. J. Cell Biol. 90, 743-754.

structural genes code for human alkaline phosphatases. Nature (London) 281,152-154.

Schmidt, M.F.G. (1982) Acylation of proteins-a new type of modification of membrane glycoproteins. Trin. Biol. Sci. 322-324.

Schmidt, M. F. G., Bracha, M. and Schlesiger, M. J. (1979) evidence for covalent attachment of fatty acids to S indbis virus glycoproteins. Proc. Natl. Acad. Sci. USA 76, 1687-1691.

Schmidt, M. F. G. and Schlesinger, M. J. (1979) Fatty acid binding to vesicular stomatitis virus glycoprotein : a new type of posttranslational modification of the viral glycoprotein. Cell 17, 813-819.

Singer, R. M. ad Fishman, W. H. (1974) Characterization of two Hela sublines-TCRC-1 produces Regan isoenzyme and TCRC-2 produces non-Regan isoenzyme. J. cell Biol. 60, 777-780.

Singer, P. A., Singer, H. H. and Williamson, A. R. (1980) Different species of mRNA encode receptor and secretory IgM μ chains differing at carboxy termini. Nature (Lond.) 285, 294-300.

Singh, I., Tsang, K. Y. and Blakemore, W. S. (1978) Placental like-alkaline phosphatase from human osteosarcoma cells. Cancer Res. 38, 193-198.

Slaughter, C. A., Coseo, M. C., Cancro, M. P. and Harris, H. (1981) Detection of enzyme polymorphism by using monoclonal antibodies. Proc. Natl. Acad. Sci. USA 78, 1124-1128.

Slaughter, C.A., Gogoli, K.J., Coseo, M.C., Meyer, L.J., Lesko, J. and Harris, H. (1983) Discrimination of human placental alkaline phosphatase allelic variants by monoclonal antibodies. Amer. J. Hum. Genet. 35, 1-20.

See page 121

Smith, C. H., Nelson, D. N., King, B. F., Donohue, T. M., Ruzycki, S. M. and Kelley, L. K. (1977) Characterisation of microvillus membrane preparation from human placental syncytiotrophoblast : A morphologic, biochemical, and physiological study. Am. J. Obstet. Gynecol. 128, 190-196.

Smith, N. C., Brush, M. G. and Luckett, S. (1974) Preparation of human placental villous surface membrane.Nature (Lond.)252, 302-303.

Snary, D., Woods, F. R. and Crumpton, M. J. (1976) Disruption of solid tissue for plasma membrane preparation. Analyt. Biochem. 74, 457-465.

Spatz, L. ans Strittmatter, P. (1971) A form of cytochrome b_5 that contains an additional hydrophobic sequence of 40 amino acid residues. Proc. Natl. Acad. Sci. USA 68, 1042-1046.

Spatz, L. and Strittmatter, P. (1973) A form of reduced nicotinamide adenine dinucleotide cytochrome b_5 reductase containing both the catalytic site and additional hydrophobic membrane binding segment. J. Biol. Chem. 248, 793-799.

Stinson, R. A. and Seargeant, L. E. (1981) Comparative studies of pure alkaline phosphatase from five human tissues. Clin. Chim. Acta 110, 261-272.

Stolbach, L. L., Kraut, M. J. and Fishman, W. H. (1969) Ectopic production of an alkaline phosphatase isoenzyme in patients with cancer. New Engl. J. Med. 281, 757-762.

Sunderland, C. A., Redman, C. W. G. and Stirrat, G. M. (1981) HLA A, B, C antigens are expressed on non-villous trophoblast of the early human placentae. J. Immunol. 127, 2614-2615.

Sutcliffe, R. G., Brock, D. J. H., Robertson, J. G., Scrimgeour, J. B. and Monaghan, J. M. (1972) Enzymes in amniotic fluid: a study of

specific activity patterns during pregnancy. J. Obstet. Gynaecol. Brit. Commonwealth 79, 799-809.

Sutcliffe, R. G., Kukulska-Langland, B. M., Coggins, J. R., Hunter, J. B. and Gore, C. H. (1980) Studies on human pregnancy associated plasma protein A. Biochem. J. 191, 799-809.

Swarup, G., Cohen, S. and Gabers, D. L. (1981) Selective dephosphorylation of proteins containing phosphotyrosine by alkaline phosphatase. J. Biol. Chem. 265, 8197-8201.

Tarr, G. E., Beecher, J. F., Bell, M. and McKean, D. J. (1978) Polyquarternary amines prevent peptide loss from sequenators. Analyt. Biochem. 84, 622-627.

Truman, J. P., Wakefield, J. and Ford, C. H. (1981) Microvilli of human term placenta. Isolation and subfractionation by centrifugation in sucrose density gradients. Biochem. J. 196, 121-132.

Umbreit, N. J. and Strominger, L. J. (1973) D-alanine carboxypeptidase from Bacillus Subtilis membranes. J. Biol. Chem. 248, 6759-6766.

Usategui-Gomez, M., Yeager, F. M. and Fernandez de Castro, A. (1973) A sensitive immunochemical method for the detrmination of the Regan isoenzyme in serum. Cancer Res. 33, 1574-1577.

Usategui-Gomez, M., Yeager, F. M. and Fernandez de Castro, A. (1974) Regan isoenzyme in normal human sera. Cancer Res. 34, 2544-2545.

Walter, P. and Blobel, G.(1980) Purification of a membrane associated protein complex required for protein translocation across the endoplasmic reticulum. Proc.Natl.Acad. Sci. USA 77, 7112-7116.

Warnock, M. L. and Reisman, R. (1969) Variant alkaline phosphatase in

See page 121

human hepatocellular cancers. Clin. Chim. Acta 24, 5-11.

Wegmann, T. G., Singh, B. and Carlson, G. A. (1979) Allogeneic placenta is a paternal strain antigen immuno-absorbent. J. Immunol. 22, 270-275.

Wei, S. C. and Doellgast, G. J. (1980) Specificity of allozyme-absorbed antisera to human placental alkaline phosphatase for individual phenotypes. Biochem. Genet. 18, 1097-1107.

Wei, S. C. and Doellgast, G.J. (1981) Immunochemical studies of human placental-type variants of alkaline phosphatase. Structural differences between the Nagao isoenzyme and the placental D-variant. Eur. J. Biochem. 118, 39-45.

Weiss, E., Golden, L., Kakut, R., Mellor, A., Fahrner, K., Kvist, S. and Flavell, R. A. (1983) The DNA sequence of the $H2K^b$ gene: evidence for gene conversion as a mechanism for the generation of polymorphism in histocompatability antigens. Europ. Molec. Biol. Org. 2, 453-462.

Whyte, A. and Loke, Y. N. (1979) Antigenes of the human trophoblast plasma membrane. Clin. Exp. Immunol. 37, 359-366.

Wickner, W. (1979) The assembly of proteins into biological membranes :the membrane trigger hypothesis. Ann. Rev. Biochem. 48, 23-45.

Winkleman, J., Nadler, S., Demeterian, J. and Pileggi, V. J. (1972) The clinical usefulness of alkaline phosphatase isoenzyme determinations. Am. J. Clin. Pathol. 57, 625-634.

Zimmerman, C. L., Appella, E. and Pisano, J. J. (1977) Rapid analysis of amino acid phenylthiohadantions by high-performance liquid chromatography. Analyt. Biochm. 77, 569-573.

APPENDIX : PUBLISHED PAPERS

Abu-Hasan, N. S., Davies, M. and Sutcliffe, R. G. (1984) A high molecular weight species of placental alkaline phosphatase in human syncytiotrophoblast microvill. Placenta (in press).

Abu-Hasan, N. S. and Sutcliffe, R. G. (1984) Purification and analysis of the microvillous (M) and A forms of placental alkaline phosphatase. Placenta (in press).

SUPPLEMENT

Cardoso de Almida and Turner, M, J. (1983) The membrane form of variant surface glycoproteins of Trypanosoma brucei. Nature 302, 349-352.

Coligan, J. E., Kidt, T. J., Vehara, H., Martinko, J. and Nathenson, S. G. (1981) Primary structure of a murine transplantation antigen. Nature 291, 35-39.

Faulk, W. P. and Temple, E. (1976) Distribution of β-microglobulin and HLA in chorionic villi of human placentae. Nature 262, 799-802.

Fishman, W. H. and Ghosh, N. K. (1967) Isoenzymes of human alkaline phosphatase. In Advances in clinical chemistry (e.d., Bodansky, O and Stewart, C. P.) pp 255-370. Academic Press, New York.

Flanagan, J. and Koch, G. L. E. (1978) Cross-linked surface Ig attaches to actin. Nature 273, 278-281.

Fox, F. and Khakonger, H. N. (1969) Enzyme histochemistry of the Hofbauer cells of the human placenta. J. Obstet. Gynocol. Brit. Comm. 76, 918-921.

Getzowa, S. and Sadowsky, A. (1950) The structure of the human placenta with full term and immature foetus, living or dead. J. Obstet. Gynocol. Brit. Empire. 57, 388-396.

Helenius, A. and Kai, S. (1972) The binding of detergent to lipophilic and hydrophilic proteins. J. Biol. Chem. 247, 3656-3661.

Higashino, K., Kudo, S. and Yamamura, Y. (1974) Further invistigation ofa variant of placental alkaline phosphatase in human hepatic carcinoma. Cancer Res. 34, 3347-3351.

Houslay, M. D. (1981) Membrane phosphorylation : a crucial role in the action of insulin, EGF, and pp60 Src. Bioscience reports 1, 19-34.

Houslay, M. D. and Stanley, K. K. (1982) Dynamics of biological

membranes. John Willey and Sons. New York.

Ito, F. and Chou, J. Y. (1983) Biosynthesis and processing of placental alkaline phosphatase. Biochem. Biophys. Res. Comm. 111, 611-618.

Koch, G. L. E. and Smith, M. J. (1978) An association between acine and major histocompatibility antigen H-2. Nature 273, 274-277.

Lowry, O. H., Rosebrough, N. J., Farr, A. L. and Randal, R. J. (1951) Protein measurment with the folin phenol reagent. J. Biol. Chem 193, 265-275.

Mescher, M. F., Jose, J. L. J. and Blak, S. P. (1981) Actin-containing matrix associated with the plasma membrane of murine tumour and lymphoid cells. Nature 289, 139-144.

Poper, J. S. Guild, B. C. and Strominger, J. L. (1978) Phosphorylation in vivo and in vitro of human histocompatibility antigens, (HLA-A and HLA-B) in the carboxy terminal intracellular domain. Proc. Natl. Acad. Sci. USA 75, 60002-6006.

Seargeant, L. E. and Stinson, R. A. (1979) Evidence that three structural genes code for human alkaline phosphatases. Nature, 281, 152-154.

Timperley, W. R., Turner, P. and Davis, S. (1971) Alkaline phosphatase in carcinopharyngiomas. J. Pathol. 103, 257-262.

Utermann, G. and Simons, K. (1974) Studies on the amphipathic nature of the membrane proteins in Simliki Forest Virus. J. Mol. Biol. 85, 569-587.

Wahren, B., Holmgren, P. A. and Stigbrand, T. (1979) Placental alkaline phosphatase, alphaphetoprotein and carcinoembryonic antigin in testicular tumors. Tissue typing by means of cytologic smears. Int. J. Cancer 24, 749-753.