UNIVERSITY OF SOUTHAMPTON

THE BIOSYNTHESIS, ASSEMBLY AND SECRETION OF VITELLOGENIN, AN OESTROGEN-INDUCED MULTICOMPONENT PROTEIN

by

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ABSTRACT

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The egg-yolk protein precursor, vitellogenin, is biosynthesized and secreted by Xenopus laevis (South African clawed toad) liver in response to a single dose of oestradiol-17-β . The protein has previously been characterized as a Ca^{2+}-binding glycolipophosphoprotein ; it is a dimer, mol. wt. 450,000-550,000, made up of two similar sized subunits. The biosynthesis of vitellogenin has been studied using an *in vitro* liver slice system, concentrating particularly on the post-translational phosphorylation and glycosylation of the vitellogenin polypeptide.

In pulse-chase experiments with (^{3}H) leucine, cellular fractionation followed by SDS polyacrylamide gel electrophoresis has allowed the identification of 200,000 mol. wt. polypeptide precursors of vitellogenin associated with the microsomes. However, further experiments with (^{32}P) phosphate and (^{3}H) sugars showed that the level of phosphorylation and glycosylation of the microsomal precursors is very low compared with secreted vitellogenin. A second distinct population of precursors has been identified in the post-microsomal fraction, but in contrast to the microsomal precursors, these are phosphorylated and glycosylated to a similar extent to secreted vitellogenin.

The possible involvement of glycolipid intermediates during the glycosylation of vitellogenin has also been studied. It has been shown that oestrogen-treatment results in an increase in the formation of glycolipid intermediates, both in isolated microsomal preparations and in liver slices, and that these have properties consistent with them being of the dolichol phosphate sugar-type.

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TO MY PARENTS,

REBECCA AND RACHEL

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CHAPTER 1

Chapter 1

INTRODUCTION

The primary translation products of secretory proteins undergo considerable structural changes before the secretion of the completed protein molecule. Table 1 shows a list of some common secreted proteins ; these examples illustrate that the post-translational modifications may involve the addition of non-protein groups, such as phosphorus, carbohydrate or lipid, and/or the cross-linking of polypeptide chains to form multichain proteins. In addition, many secreted proteins are produced as larger precursor molecules which must subsequently be cleaved to give the final product. In this introduction, some aspects of the post-translational modification of secreted proteins will be reviewed. First, however, the way in which the polypeptides of secretory proteins are biosynthesized and transported through the cell to the site of secretion will be described.

TABLE 1 THE COMPONENTS OF SOME COMMON SECRETORY PROTEINS

Protein	Mol. wt.	No. of chains (mol. wt.)	Carbohydrate	Lipid	Phosphate
Thyroglobulin	660,000	? (20-300,000)	◆		
Vitellogenin	450-550,000	2 (200,000)	◆	◆	◆
Collagen	345,000	3 (100,000)	◆		
Immunoglobulins	160,000	4 (23,000 + 50,000)	◆		
Serum Albumin	65,000	1			
Ovalbumin	45,000	1	◆		◆
Casein			◆		◆
Insulin	6,000	2			

1.1 The Basic Secretory Pathway

The classical model for the secretory pathway was proposed by Palade (1975), and this was based on an analysis of the intracellular transport of secretory proteins in the pancreatic exocrine cell. Palade's model suggests the following basic steps in the secretory process:-

(i) the synthesis of the polypeptides of the secretory proteins on polysomes attached to the membrane of the rough endoplasmic reticulum;
(ii) the segregation of the newly synthesized polypeptides within the cisternal space of the endoplasmic reticulum;
(iii) the intracellular transport of the polypeptide from the intercisternal space of the endoplasmic reticulum to the Golgi complex;
(iv) the concentration and possible intracellular storage of the secretory protein;
(v) the discharge of the completed protein from the cell.

Research into the biosynthesis, intracellular transport and secretion of other proteins has led to this model of the basic secretory pathway being generally accepted. Some of this work will now be considered.

1.1.1 The "Signal-Hypothesis"

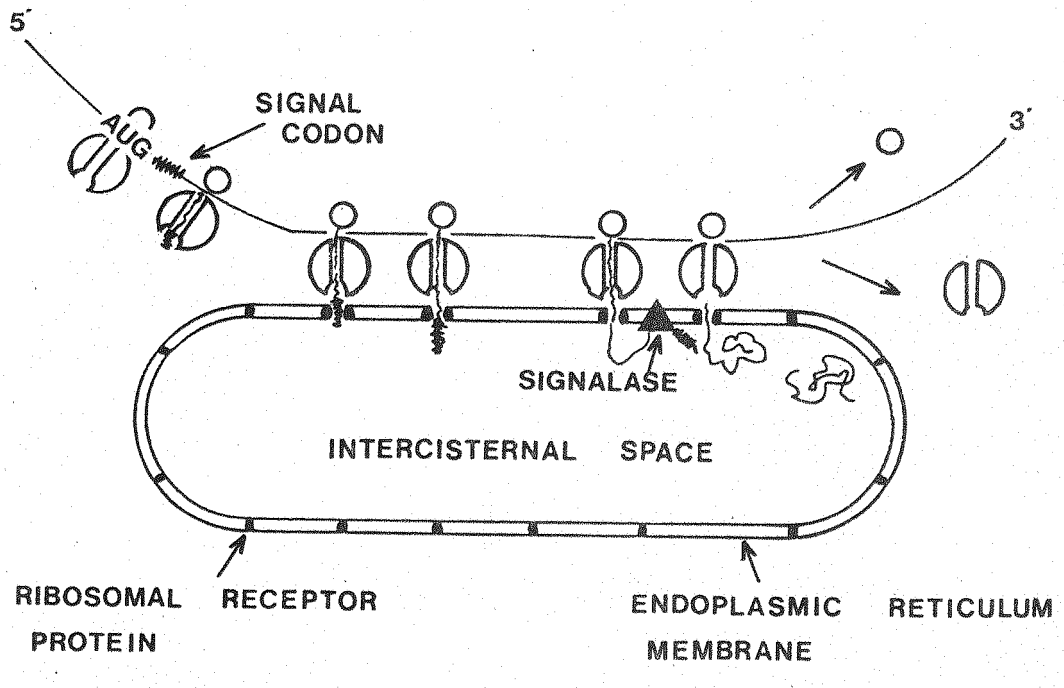
The "Signal-Hypothesis" was originally proposed by Blobel and Sabatini (1971) to explain the synthesis of the polypeptides of secretory proteins on the ribosomes of the rough endoplasmic reticulum. The hypothesis has since been elaborated, primarily due to the pioneering work of Milstein *et al* (1972), to provide a scheme for the transport of the translation products across the membrane of the endoplasmic reticulum, and their segregation within the intercisternal space.

The basis of the "Signal-Hypothesis" is that

secretory proteins are initially translated as polypeptides with an NH_2-terminal extension, termed the "signal-peptide"; the suggested events are outlined in Figure 1. According to the scheme, the newly translated signal-peptide of the nascent chain is channelled through the large ribosomal subunit, and, as it emerges, penetrates into the endoplasmic reticulum membrane resulting in the aggregation of specific receptor proteins in the membrane to form a proteinaceous tunnel. The large ribosomal subunit then binds to the membrane, linking the channel in the subunit with the newly formed tunnel in the membrane, and this allows the passage of the signal-peptide, followed by the rest of the nascent chain, through the membrane and into the cisternal space. Prior to the completion of the nascent chain, the signal-peptide is cleaved by a membrane-bound protease, referred to as "signalase", and, finally, the completed polypeptide is released into the intercisternal space, and the ribosome detached from the membrane. As a result of the folding of the completed polypeptide to give a bulky tertiary structure it is unable to pass back across the membrane, and transport becomes essentially unidirectional.

The evidence for the "Signal-Hypothesis" has come from experiments in which the mRNA coding for various secretory proteins has been translated in heterologous cell-free systems. The first report of a specific secretory protein being translated as a precursor with an NH_2-terminal extension was by Milstein et al (1972) ; they showed that when mRNA coding for immunoglobulin light chain was translated in a reticulocyte lysate system, the product had a molecular weight approximately 1,500 greater than authentic light chain. This observation was subsequently confirmed by Blobel and Dobberstein (1975, a b), and since then NH_2-terminal extensions have been identified in the primary translation products of many secretory proteins, including pancreatic secretory proteins (Devillers-Thiery et al, 1975), parathyroid hormone (Habener et al, 1976), insulin (Chan et al, 1976), albumin (Strauss et al, 1977) and egg-white ovomucoid (Thibodeau et al, 1978). Although

Figure 1



The "Signal Hypothesis"

amino-acid sequence data has revealed considerable variation in the length (15 - 30 amino-acids) and composition of the NH_2-terminal extensions, a common feature is their strongly hydrophobic character (Thibodeau et al, 1978). This marked hydrophobicity may represent an essential feature of their proposed function, that is, the establishment of a connection between the nascent chain on the ribosomes and the membrane of the endoplasmic reticulum.

However, secretory proteins are not the only class of proteins to be synthesized on membrane-bound ribosomes (see Shore and Tata, 1977 for a relevant review). In particular, proteins destined to become components of subcellular organelles are also biosynthesized on the ribosomes of the rough endoplasmic reticulum, for example, mitochondrial (Bingham and Campbell, 1972) and plasma-membrane (Bergeron et al, 1975) proteins, as well as proteins of the endoplasmic reticulum itself (Dallner et al, 1966, a b), and after translation these may also remain associated with the membrane. Indeed, further evidence (Shore and Harris, 1977) has indicated that the majority, if not all, of the proteins normally translated by the membrane-bound ribosomes are passed into or across the membrane, and that few, if any, are released into the cytosol. Therefore, although the evidence obtained from the study of specific secretory proteins is consistent with the "Signal-Hypothesis", it has not yet been established whether the primary translation products of other proteins biosynthesized on the membrane-bound ribosomes also include NH_2-terminal "signal-peptides", or whether this is a characteristic unique to secretory proteins. It is relevant that in E. Coli, outer membrane lipoprotein has been shown to be synthesized as a precursor with a hydrophobic NH_2-terminal extension (Di Rienzo et al, 1978), which is presumed to facilitate the insertion of the protein into the membrane. This observation suggests that such sequences may be a general feature of proteins destined to be associated with membranes, whether they are to become an integral part of the membrane structure, or simply to be transported across it. Lastly, it is interesting that ovalbumin, the major protein synthesized and secreted by the

hen oviduct, has been shown to be synthesized without a transient NH_2-terminal extension (Palmiter et al, 1978), which suggests that other factors must be involved in the transport of this protein across the endoplasmic reticulum membrane.

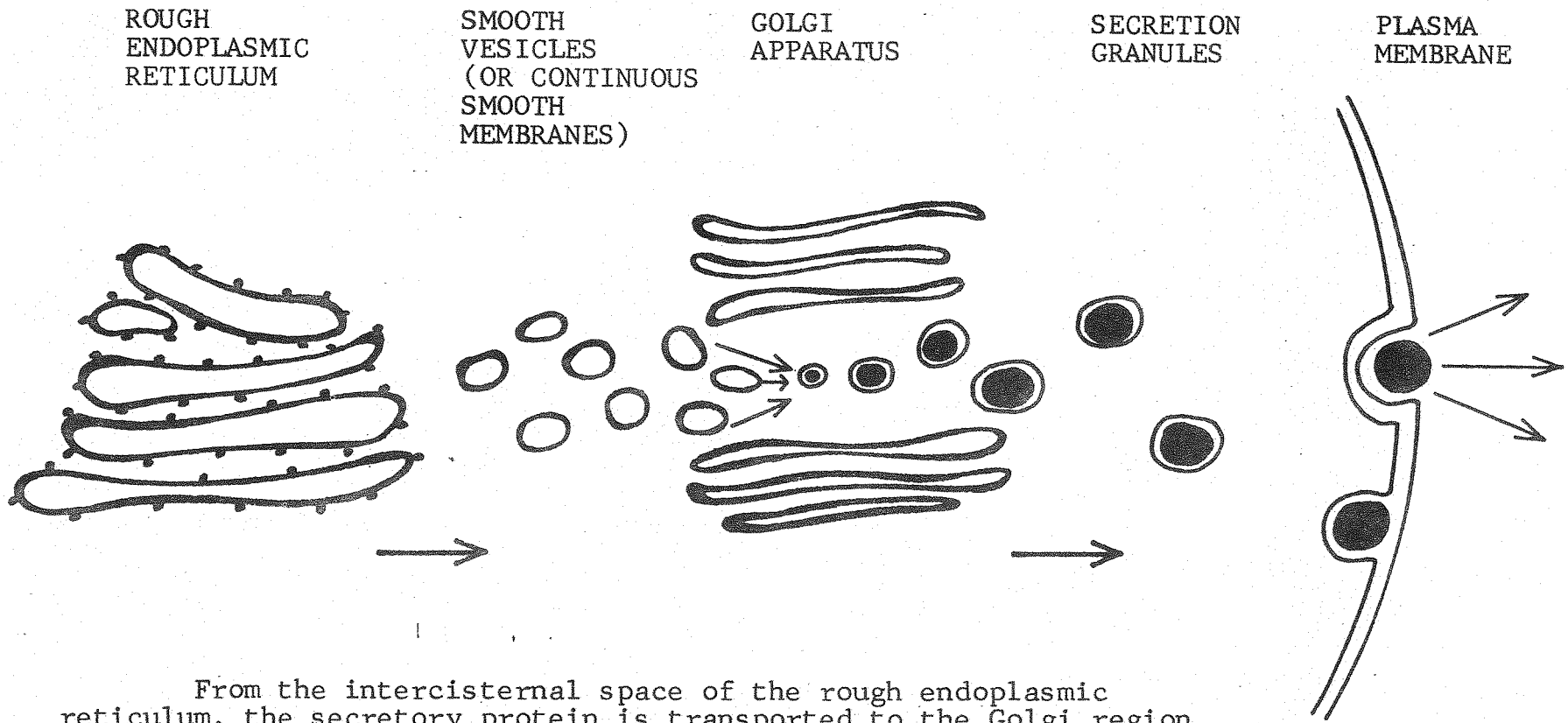
1.1.2 The Intracellular Transport of Secretory Proteins

After the release of the newly synthesized polypeptides of secretory proteins into the intercisternal space of the rough endoplasmic reticulum, they must be transported through the cell to the site of secretion, the plasma membrane.

The initial evidence for Palade's model of intracellular transport in the pancreatic exocrine cell came from autoradiographic studies after in vivo pulse-labelling (Caro and Palade, 1964). The results suggested that the polypeptides of the secretory proteins were transported from the endoplasmic reticulum to the Golgi complex, where they were packaged into secretion (zymogen) granules prior to secretion from the cell. Later, in vitro pulse labelling followed by subcellular fractionation indicated the transport of labelled protein from the rough microsomal fraction, derived from the membranes of the rough endoplasmic reticulum, to the smooth microsomal fraction, derived from small, smooth-surfaced vesicles and fragments of the Golgi complex, and then to zymogen granules (Jamieson and Palade, 1967, a b). A combination of the results obtained from autoradiography and subcellular fractionation suggested the transport process illustrated in Fig 2. It is proposed that after being released into the intercisternal space, the secretory protein moves out of the endoplasmic reticulum in small, smooth-surfaced vesicles formed as "buds" from the endoplasmic reticulum membrane. These vesicles then travel to the Golgi region of the cell, where they empty their contents into condensing vacuoles, which, when filled with protein, become mature zymogen granules and migrate to the cell wall and are secreted by exocytosis.

Peters et al (1971) studied the biosynthesis and

The Intracellular Transport of Secretory Proteins



From the intercisternal space of the rough endoplasmic reticulum, the secretory protein is transported to the Golgi region of the cell in smooth surfaced vesicles which are formed as 'buds' from the endoplasmic reticulum membrane (alternatively, there may be continuous smooth membrane connections between the rough endoplasmic reticulum and the Golgi region). In the Golgi region, the secretory protein is concentrated in vacuoles which when mature form secretion granules which are transported to the plasma membrane and discharged from the cell.

Figure 2

intracellular transport of serum albumin in rat liver. Animals were given an *in vivo* injection of (^{14}C) leucine and the incorporation of label into albumin immunoprecipitates derived from various subcellular fractions was followed. The results indicated the passage of the newly synthesized albumin from the rough to the smooth microsomes ie, from the rough to the smooth endoplasmic reticulum, and then to a Golgi-rich fraction prior to secretion. Similar intracellular transport pathways have been demonstrated for serum - α_1 acid glycoprotein (Jamieson and Ashton, 1973) and serum lipoproteins (Glaumann *et al*, 1975) biosynthesized in rat liver, and also for secretory proteins biosynthesized in other tissues, and these will be referred to later. It is relevant to point out that although the rough microsomal or membrane fraction is undoubtedly derived from the membranes of the rough endoplasmic reticulum, the nature of the smooth microsomal or membrane fraction is less well defined ; for example, whereas in the pancreas the smooth membrane fraction was derived mainly from the Golgi complex (Jamieson and Palade, 1967a), in the liver the smooth membrane fraction was derived mainly from the smooth endoplasmic reticulum (Peters *et al*, 1971). In general the smooth membrane fraction is heterogeneous, and may consist of elements of the smooth endoplasmic reticulum, the Golgi complex and also fragments of plasma membrane and other smooth membranes (see Dallner and Ernster, 1968, for a review), and this should be borne in mind during the following discussion.

A key point of the basic intracellular transport pathway is that once segregated within the intercisternal space, the newly synthesized protein is kept within specialized membrane bound compartments at all stages prior to secretion, and that it is not released free into the cytoplasm. This dogmatic view has been criticized by Rothman (1975) who argues that although the available evidence is consistent with this hypothesis, it does not offer conclusive proof of its existence, nor exclude alternatives. On the basis of independent experiments into the synthesis and transport of pancreatic enzymes, he proposes a model for

secretion in which the intracellular membrane compartments are in equilibrium, both with each other, and with the cytoplasm, and therefore that the secretory protein is not completely segregated from the cytoplasm.

However, in spite of such criticism, the idea that the newly biosynthesized secretory protein is compartmentalized throughout its intracellular life is widely accepted ; indeed, it is generally believed that such compartmentalization is an essential prerequisite for the post-translational modification processes which are necessary before the secretion of the completed protein (see Campbell and Blobel, 1976 for a review). Three aspects of the post-translational modification of secretory proteins will now be discussed ; the addition of carbohydrate residues to form glycoproteins, the addition of phosphate groups, and the way in which the primary translation products are converted into multi-chain proteins.

1.2 The Glycosylation of Secretory Proteins

With the exception of albumin, all of the main secretory proteins are glycoproteins, that is, they contain covalently bound carbohydrate groups. Glycoproteins are also important constituents of cell membranes and organelles, and a major class of structural proteins, the collagens, are also glycosylated, therefore the addition of carbohydrate groups is an important and widely occurring post-translational modification. A great deal of work has been carried out to investigate the structure and biosynthesis of glycoproteins, and some aspects of this work will be presented in this section.

1.2.1 The Structure of Glycoproteins

An appreciation of the biosynthesis of glycoproteins requires an understanding of some basic features of their structure. (For reviews on the structure of glycoproteins, see Spiro, 1969, 1970, 1973.)

Glycoproteins can be conveniently classified according to the nature of the covalent linkage between the

carbohydrate group and the polypeptide chain. Three distinct types of glycopeptide bond have been shown to occur in proteins from animal tissues, the linkage always involves the C-1 of the most internal sugar residue and a functional group of an amino-acid within the peptide chain. The three types of linkage are:-

(i) an N-glycosidic bond between the amide group of aspargine and N-acetyl glucosamine ;
(ii) an O-glycosidic bond between the hydroxyl group of serine or threonine and N-acetyl galactosamine, mannose, galactose or xylose, eg various mucins and glycoproteins with blood group activity ;
(iii) an O-glycosidic bond between the hydroxyl group of hydroxylysine and a galactose residue, eg basement membranes and collagens.

The following discussion will be concerned with glycoproteins involving the N-glycosidic type of linkage since this has been identified in a variety of glycoproteins from many sources, including most of the secretory proteins.

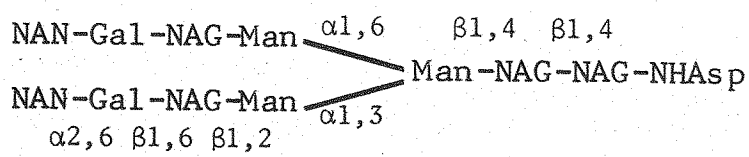
Table 2 shows the structures of the N-glycosidically linked carbohydrate groups of four secretory proteins. The oligosaccharides attached to the proteins are usually branched structures, and there are two distinct types of carbohydrate groups, the complex type, and the high-mannose type. Thyroglobulin contains both types of oligosaccharide chain ; each molecule of thyroglobulin has 7-8 high-mannose groups (termed Unit A chains) and 22 complex groups (termed Unit B chains) (Spiro and Spiro, 1973). Immunoglobulin G has 2 complex groups, one bound to each heavy chain (Kornfeld et al, 1971), in contrast, immunoglobulin M (a pentamer) contains a large amount of carbohydrate with 4-6 carbohydrate groups per heavy chain including both complex and high-mannose type (Hickman et al, 1972). Ovalbumin has a single high-mannose type group (Montgomery et al, 1965 ; Tai et al, 1975), and phosvitin has a single complex type group (Shainkin and Perlmann, 1971).

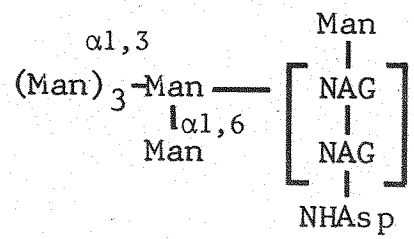
Two basic features of the structure of the carbo-

TABLE 2 THE STRUCTURE OF THE CARBOHYDRATE CHAINS ATTACHED TO FOUR SECRETORY GLYCOPROTEINS

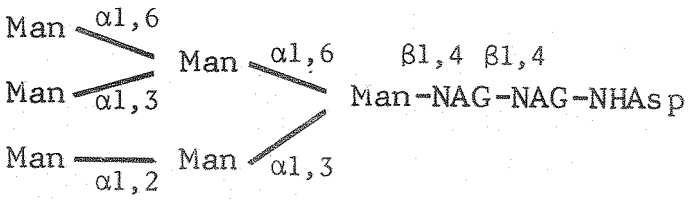
	COMPLEX TYPE	HIGH MANNOSE TYPE
THYROGLOBULIN	eg $(NAN\text{-}Gal\text{-}NAG)_3\text{-}(Man)_3\text{-}NAG\text{-}NAG\text{-}NHAsp$ ($\alpha 2,3$ $\beta 1,4$; $\beta 1,4$ $\beta 1,4$) (Unit B)	$(Man)_x\text{-}Man\text{-}Man\text{-}Man\text{-}NAG\text{-}NAG\text{-}NHAsp$ ($\alpha 1,2$; $\beta 1,4$ $\beta 1,4$) Man-Man ($\alpha 1,2$) Man-Man ($\alpha 1,2$) (Unit A)
IMMUNOGLOBULIN G	eg NAG-Man ($\beta 1,2$; $\alpha 1,6$) Gal-NAG-Man ($\beta 1,4$ $\beta 1,2$; $\alpha 1,3$) Man-NAG-NAG-NHAsp ($\beta 1,4$ $\beta 1,4$ β ; $\alpha 1,6$) Fuc	

IMMUNOGLOBULIN M

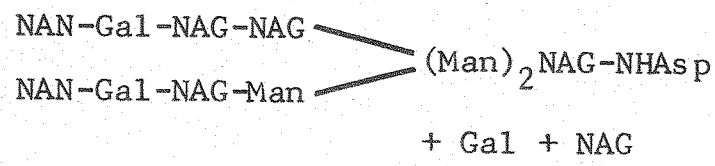




OVALBUMIN



PHOSVITIN

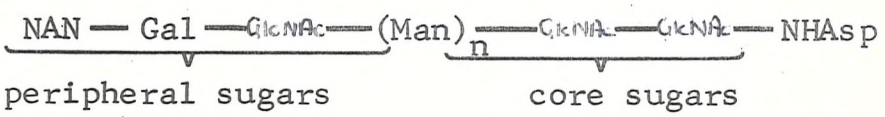


hydrate groups are important in the following discussion ;

(i) A sequence of mannose and N-acetyl glucosamine residues is usually attached to the aspargine of the polypeptide chain, ie:-

This sequence is referred to as the "core" of the oligosaccharide chain and it occurs in both complex and high-mannose type carbohydrate groups.

(ii) In the complex type oligosaccharide chains, the sequence of the peripheral sugars attached to the core is frequently sialic acid (N-acetylneuramic acid), galactose, N-acetyl glucosamine ie:-



(NAN - sialic acid, Gal - galactose, GlcNAc - N-acetyl glucosamine, Man - mannose, NHAsp - aspargine.)

In addition to the glycoproteins shown in Table 2, this sequence is also found in the complex oligosaccharide chains of fetuin, chorionic gonadotrophin, α- acid glycoprotein and other glycoproteins (Spiro, 1969).

Another feature of the structure of the carbohydrate groups is that although a given carbohydrate unit may have a basic structural pattern, it may appear in various stages of completion, or with minor modifications ; this phenomenon has been termed "microheterogeneity" (Spiro, 1969, 1970).

In view of the similarities between the carbohydrate sequences of different secretory glycoproteins, it is possible that any information concerning the synthesis of a particular glycoprotein may be generally applicable to the assembly of other glycoproteins. Research into the glycosylation process will now be discussed.

1.2.2 The Assembly of the Carbohydrate Units of Glycoproteins

It is now generally accepted that the assembly of the carbohydrate groups of glycoproteins occurs sequentially as the newly synthesized polypeptide is transported through

the cell prior to secretion. The classical theory involves the action of specific glycosyl transferases and the transfer of single sugar residues direct from nucleotide-sugars. However, evidence is accumulating to support the idea that the inner core region of the oligosaccharide is pre-assembled and added to the polypeptide chain "en bloc" from an oligosaccharide-lipid intermediate. Evidence for the sequential addition of the peripheral sugars will be described, followed by a discussion of the possible role of lipid-intermediates in glycosylation.

a) The Sequential Addition of Sugars to the Oligosaccharide Chain

Two main approaches have been adopted to study the process of carbohydrate addition to secretory proteins ; first, experiments in which intracellular intermediates have been identified, and second, investigations to localize the specific glycosyl transferases involved in sugar addition (for a review see Schachter, 1974). Examples of both of these approaches will be discussed briefly.

Immunoglobulin G contains 2 complex-type carbohydrate chains, one attached to the carboxyterminal half of each heavy chain. Light chains are usually free of carbohydrate, but some plasma-cell tumours synthesize and secrete kappa type light chains containing a single carbohydrate chain with a sequence closely resembling that of Ig G (Melchers, 1969). Melchers has studied the assembly of the complex type oligosaccharide chain of immunoglobulin using two plasma-cell tumours, MOPC 21 and MOPC 46, synthesizing and secreting Ig G and kappa-light chain respectively. A comparison of the kinetics of incorporation of (^{3}H) leucine and various radioactive sugars into intracellular and secreted immunoglobulin (detected by immunoprecipitation) suggested that mannose and glucosamine, the sugars of the core region, were added soon after the translation of the polypeptide chain, whereas galactose, a sugar present in the peripheral sequence, was added later (Melchers, 1970). Also, although labelled fucose was detectable in the secreted protein, it was not present in intracellular immunoglobulin, suggesting that fucose was added just prior to secretion.

To confirm these kinetic results, the carbohydrate content of immunoglobulin isolated from various subcellular fractions was analysed (Melchers, 1971), and a summary of the results obtained from the fractionation of plasma-cells synthesizing Ig G is shown in Table 3. Although only a crude sucrose-density gradient procedure was used for fractionation, there was a clear difference in the carbohydrate composition of the immunoglobulin associated with the two main subcellular fractions : the immunoglobulin associated with the rough membranes, presumably derived mainly from the rough endoplasmic reticulum, contained the core-sugars, glucosamine and mannose, but was deficient in galactose, whereas the smooth membranes, presumably derived from the smooth endoplasmic reticulum and the Golgi-complex, contained immunoglobulin with glucosamine, mannose, and galactose. The secreted form of immunoglobulin contained glucosamine, mannose and fucose. It was interesting that the cytoplasmic supernatant fraction also contained immunoglobulin ; however, since the amount recovered in this fraction varied between 3 and 30% depending upon the fractionation procedure adopted, it seemed likely that this was, at least in part, an artefact of this procedure. Similar results were obtained in experiments performed by Choi et al (1971). The results indicated that intracellular immunoglobulin which differed in carbohydrate could be found at different subcellular sites, and the results supported the idea that the addition of the peripheral sugars of the carbohydrate unit occurs in several steps as the immunoglobulin is transported through the cell prior to secretion.

These conclusions were further corroborated by the results of later experiments (Knopf et al, 1975). Labelled intracellular light chain was isolated by immunoprecipitation and digested with trypsin and Pronase. The resulting glycopeptides were resolved into four species ; the major glycopeptide species contained only the core sugars, N-acetyl glucosamine and mannose, another glycopeptide species contained the core sugars and galactose, and two glycopeptide species contained the core sugars, galactose, and either one or two residues of sialic acid. Again, these

TABLE 3 CHEMICAL ANALYSIS OF THE CARBOHYDRATE COMPONENT OF MOPC 21, 1g G MYELOMA PROTEIN PURIFIED FROM VARIOUS SUBCELLULAR FRACTIONS

1g G isolated from	Sugars Present in Carbohydrate Unit			
	$GlcNH_2$	Man	Gal	Fuc
Rough Membranes	◆	◆		
Smooth Membranes	◆	◆	◆	
Cytoplasmic Supernatant	◆	◆	◆	(◆)
Secreted	◆	◆	◆	◆

($GlcNH_2$ - glucosamine, Man - mannose, Gal - galactose, Fuc - fucose)

Simplified from Melchers (1971).

results supported the idea that the addition of the peripheral sugars occurs in several steps. The fact that all of the intermediates identified contained the full complement of core sugars favoured the idea that the core is added to the polypeptide "en bloc", rather than by the addition of single sugar residues - this will be discussed in the next section.

The enzymatic assembly of the complex Unit B carbohydrate moiety of thyroglobulin has been investigated by Spiro and Spiro (Spiro and Spiro, 1973 ; Spiro et al, 1974). They isolated from thyroid particles sialic acid and galactosyl transferases with properties consistent with a role in the addition of the peripheral sugars residues to the Unit B oligosaccharide chain (Spiro and Spiro, 1968). The specificity of the transferases was tested using exogenous glycopeptide acceptors, prepared by Pronase digestion of thyroglobulin ; the glycopeptides produced were used either intact, having the structure:-

Peptide - Core Sugars - Man-GlcNAc-Gal-NAN

or after the stepwise removal of the peripheral sugars from the oligosaccharide chain by selective acid hydrolysis and glycosidase digestion.

The preferred substrates of the two enzymes were as shown below:-

Galactosyl Transferase

Peptide - Core Sugars - Man - GlcNAc

Sialyl Transferase

Peptide - Core Sugars - Man - GlcNAc - Gal

The product of the galactosyl transferase reaction was the preferred substrate for the sialyl transferase, so the results suggested that the two transferases act in sequence in the addition of the two terminal sugars of the Unit B oligosaccharide. It was also shown (Spiro and Spiro, 1973) that the sialyl and galactosyl transferases, together with an N-acetyl glucosaminyl transferase, could act in concert in the addition of the sialic acid - galactose - N-acetyl glucosamine chain to the core sugars of the Unit B oligosaccharide. The sialyl and galactosyl transferases were found to be localized in a light membrane fraction, presumably representing the membranes of the smooth endoplasmic reticulum and the Golgi apparatus, and this was consistent with the peripheral sugar residues being added at a later stage during intracellular transport than the core sugars. However, these studies gave no clue as to the enzymes involved in the assembly and addition of the core sugar residues. The evidence to suggest the possible role of lipid-intermediates in the addition of the core sugar residues will now be reviewed.

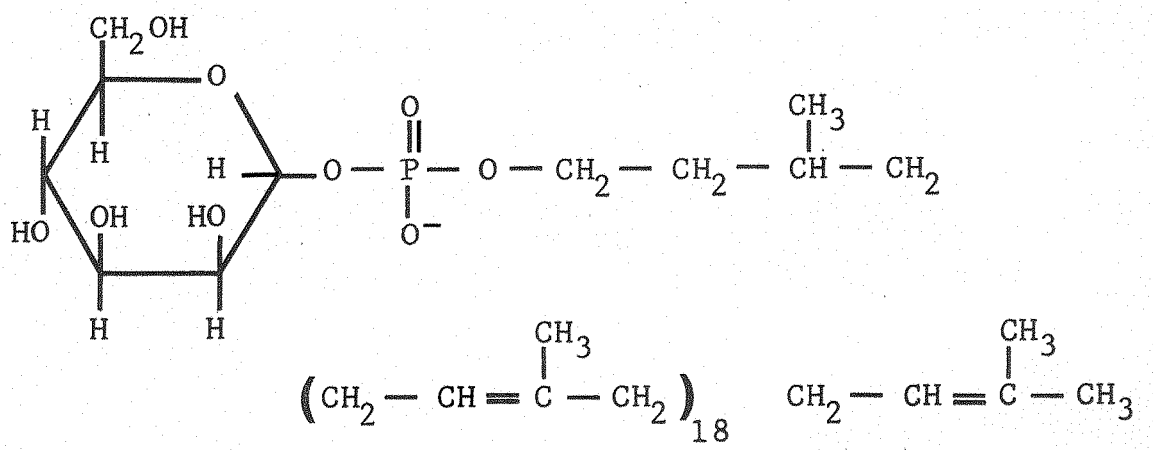
b) The Role of Lipid-Linked Sugars in Glycoprotein Biosynthesis

Over the past 15 years there has been extensive research into the role played by lipid-intermediates in the glycosylation of proteins. In mammallian systems, the major class of lipids found to be involved are the dolichols. The dolichols are a family of isoprenols with a chain length ranging from C80 - C100. They were first isolated from mammallian tissue in 1963 by Hemming's group (Burgos et al, 1963), and the structure of dolichol was suggested to be:-

$$H\,(CH_2 - \overset{CH_3}{C} = CH - CH_2)_{19} CH_2 - \overset{CH_3}{CH} - CH_2 - CH_2 - OH$$

Dolichols are widely distributed in mammallian systems and are mainly esterified to long-chain fatty acids, although the free alcohol is also present (Butterworth and Hemming, 1968).

The original discovery by Strominger's group (Anderson et al, 1965) that a lipid-carrier was involved in the transfer of glycopeptide groups during bacterial cell wall synthesis, and the subsequent identification of the lipid-carriers as isoprenol derivatives (Hiyashi et al, 1967 ; Scher and Lennarz, 1969) prompted numerous investigations into the possible role of similar lipid-carriers in eukaryotic glycoprotein biosynthesis. In early studies in mammallian systems, Caccam et al (1969) showed that membrane preparations from liver, oviduct, and myeloma tumours catalysed the transfer of (^{14}C) mannose from GDP (^{14}C) mannose to an endogenous acceptor lipid, and they suggested that the glycolipid formed was an isoprenol derivative. The first clear indication that the lipid-intermediates involved were derivatives of dolichol came from Behrens and Leloir (1970) ; they found that rat liver microsomes catalysed the transfer of glucose from UDP-glucose to a lipid acceptor, to form a product which had properties consistent with it being dolichol monophosphate glucose. Unambiguous identification of dolichol intermediates came from Hemming and Evans (1973) ; they showed that pig liver microsomes catalysed the transfer of (^{14}C) mannose from GDP (^{14}C) mannose to a lipid acceptor, forming a glycolipid which was identical to synthetic dolichol monophosphate mannose prepared by Warren and Jeanloz (1973).



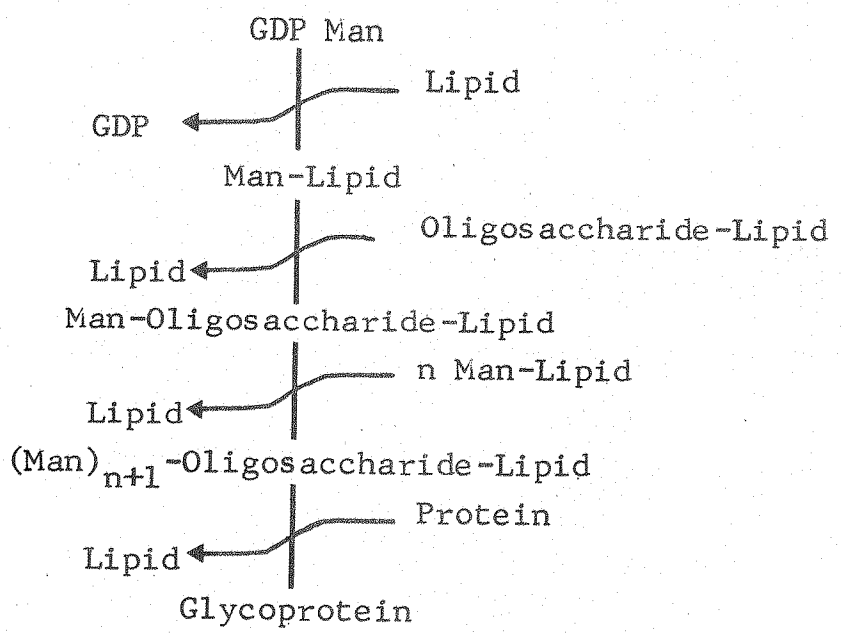
Dolichol phosphate mannose

Tetas et al (1970) showed that rat and rabbit liver microsomal preparations catalysed the transfer of N-acetyl glucosamine from UDP-N-acetyl glucosamine to a lipid acceptor, and the product was subsequently identified as dolichol diphosphate N-acetyl glucosamine (Behrens et al, 1971a; Palamarczyk and Hemming, 1974). During the same period, microsomal preparations from a variety of other tissues were found to catalyse the transfer of monosaccharides from nucleotide-sugars to dolichol-type acceptor lipids. Also, several of these early investigations (Behrens and Leloir, 1970 ; Richards and Hemming, 1972 ; Baynes et al, 1973) demonstrated the transfer of labelled sugar from the isolated lipid-sugar intermediate to endogenous microsomal protein, suggesting a role for the intermediates in glycoprotein synthesis.

In further studies Behrens et al (1971b showed that in addition to monosaccharide-lipid derivatives, a second type of lipid-sugar intermediate was formed. This compound had the unusual property of being insoluble in chloroform/methanol mixtures but soluble in chloroform/methanol/water (10:10:3), and it was tentatively identified as an oligosaccharide containing approximately 20 monosaccharide residues linked to a lipid, presumably dolichol, by a pyrophosphate bridge. The isolated oligosaccharide-lipid was shown to serve as a donor of the oligosaccharide chain to endogenous microsomal protein (Behrens et al, 1973). Since these early investigation, there has been extensive research to identify the role of lipid-sugar intermediates in glycoprotein synthesis (for reviews see Hemming, 1974 ; Lennarz, 1975 ; Waechter and Lennarz, 1976). The evidence to suggest the participation of lipid-sugar intermediates, presumably derivatives of dolichol, during glycoprotein synthesis in the hen oviduct will now be reviewed as a specific example. This system is of particular relevance to a discussion on the post-translational modification of secretory proteins since a major product of oviduct protein synthesis is the secretory glycoprotein, ovalbumin.

Waechter et al (1973) showed that particulate membrane fractions of hen oviduct catalyzed the transfer

of (^{14}C) mannose from GDP (^{14}C) mannose to an acceptor lipid, to form a mannolipid which had the characteristics of dolichol phosphate mannose. However, two other labelled components were also identified. The first, which was termed soluble mannosylated endogenous acceptor (mannosyl-s-acceptor), was insoluble in chloroform-methanol (2:1), but soluble in chloroform-methanol-water (10:10:3), this property suggested that it was an oligosaccharide-lipid intermediate similar to that described by Behrens et al (1971b). The second, which was termed residual mannosylated endogenous receptor (mannosyl-r-acceptor), was insoluble in both solvent mixtures, and the evidence suggested that it was a glycoprotein. The kinetics of the transfer of labelled mannose from GDP-mannose to the three types of endogenous acceptors suggested that the mannolipid formed was an intermediate in the synthesis of the oligosaccharide-lipid (mannosyl-s-acceptor) and the glycoprotein (mannosyl-r-acceptor). More direct evidence for this relationship was the finding that the isolated and purified mannolipid could serve as a donor of mannose in the synthesis of the oligosaccharide-lipid and the glycoprotein (Waechter et al, 1973). In subsequent experiments it was shown that the isolated oligosaccharide-lipid served as a donor for the introduction of oligosaccharide chains into the glycoprotein (Lucas et al, 1975). The results were consistent with the scheme shown below:-



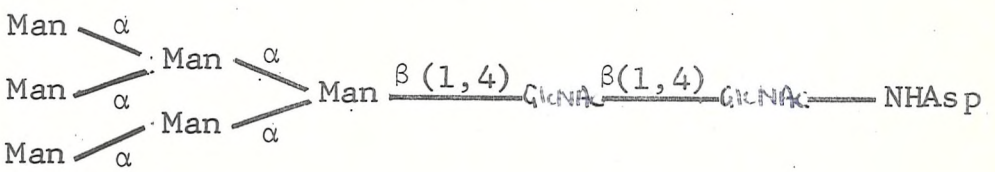
Double-label experiments (Lucas et al, 1975) indicated that the oligosaccharide chain of the oligosaccharide-lipid contained both N-acetyl glucosamine and mannose, and also provided evidence that the oligosaccharide chain was transferred "en bloc" during glycoprotein synthesis.

To further investigate the relationship between the oligosaccharide-lipid and the glycoprotein, the oligosaccharide chains derived from both the oligosaccharide-lipid and the glycoprotein were characterized and compared by a variety of techniques, including the use of highly purified glycosidases (Chen et al, 1975). All of the evidence was consistent with the conclusion that the oligosaccharide chain present in the oligosaccharide-lipid intermediate was identical to the oligosaccharide chain of the glycoprotein. The structure of the oligosaccharide was proposed to be:-

$$(Man)_n \text{——} Man \overset{\beta(1,4)}{\text{——}} GlcNAc \overset{\beta(1,4)}{\text{——}} GlcNAc$$

This evidence strongly supported the idea of the "en bloc" transfer of the oligosaccharide chain from the lipid intermediate to the protein.

A most important point which must be emphasized is that the core structure of the oligosaccharide chain from the lipid intermediate ie $(Man)_n$ - $(GlcNA)_2$ is identical to the core structure of many soluble, secretory glycoproteins. A comparison of the structure of the oligosaccharide chain with the structure of the carbohydrate moiety of ovalbumin (Tai et al, 1975):-

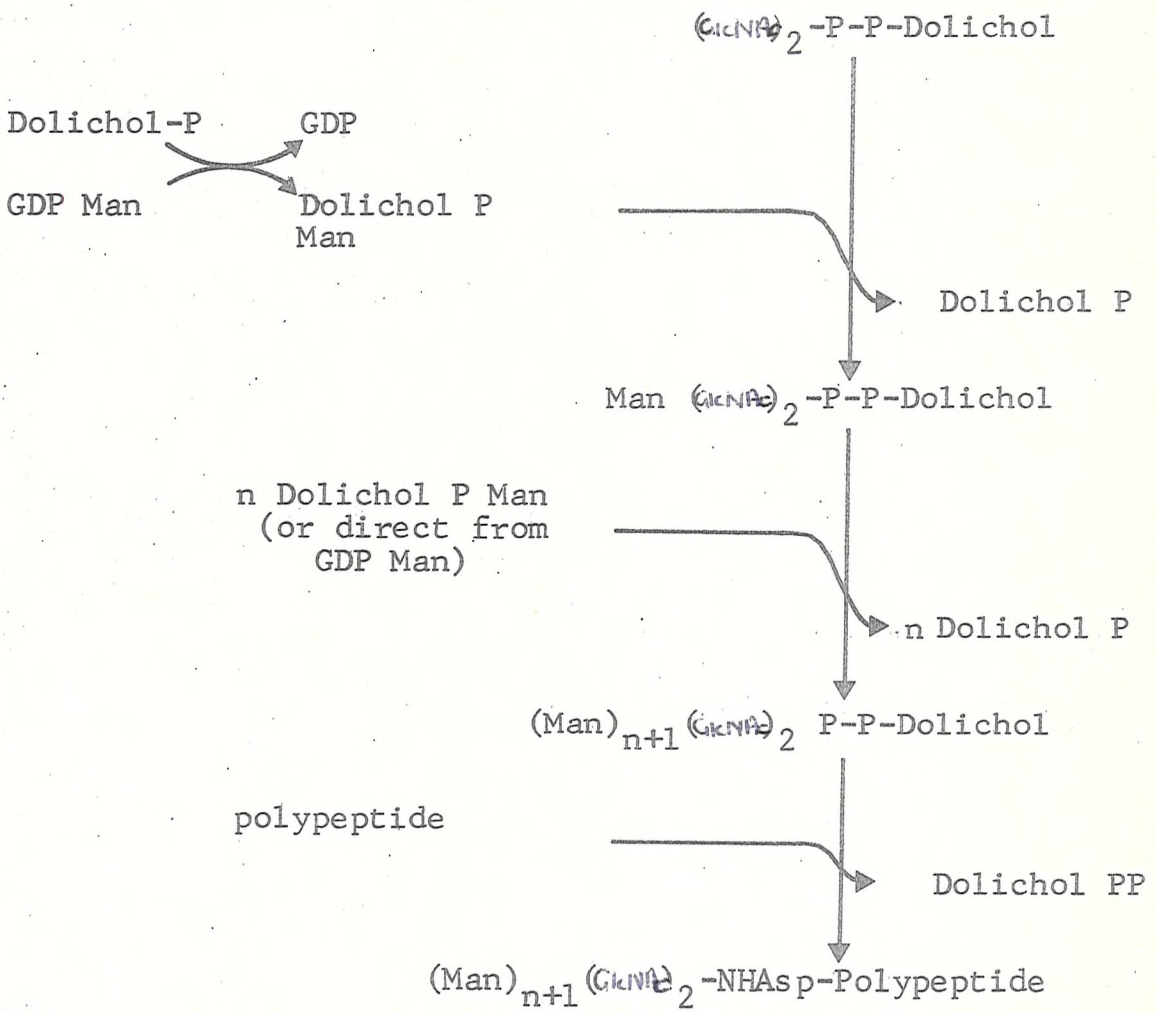


indicates that it would serve as an appropriate donor of the core sugars of this protein. Hsu et al (1974) working on mouse myeloma preparations, identified an oligosaccharide-lipid intermediate with a similar structure to the one found in the hen oviduct, and the evidence again suggested

that the oligosaccharide chain was transferred "en bloc" during glycoprotein synthesis.

The next step was to investigate the nature of the proteins glycosylated by the oligosaccharide-lipid intermediate, and to establish whether the lipid-linked pathway is indeed involved in the glycosylation of secretory glycoproteins. Pless and Lennarz (1975) analysed the proteins labelled by (^{14}C) mannose-oligosaccharide-lipid using sodium dodecyl sulphate polyacrylamide gel electrophoresis. Under the incubation conditions used the principal acceptor of the oligosaccharide chain was a polypeptide of molecular weight approximately 25,000, however, no significant label was incorporated into a protein which could be identified as ovalbumin. Similarly, Hsu et al (1974) reported that in mouse myeloma preparations, which synthesize large amounts of immunoglobulin light chain, most of the protein labelled by (^{14}C) mannose-oligosaccharide-lipid had a molecular weight of 20-50,000, but that only 10-20% of the labelled protein was light chain. In view of the large quantities of secretory proteins synthesized by both of these tissues, these results were disappointing since they suggested that the labelled oligosaccharide was incorporated primarily into membrane-bound glycoproteins, but they did not completely rule out the possibility of lipid intermediates participating in the addition of the core sugars to ovalbumin and immunoglobulin. However, based on these and other studies (see Waechter and Lennarz, 1976) it was possible to propose a scheme to explain the role of lipid intermediates (generally assumed to be derivatives of dolichol) in the biosynthesis of glycoproteins, and this is outlined in Figure 3. It must be stressed that this is only a tentative pathway and is still not fully understood ; for example, the precise mechanism of assembly of the initial dolichol diphosphate disaccharide is not clear, and it also seems likely that some of the mannose residues in the oligosaccharide are added directly from GDP-mannose and not via a dolichol phosphate mannose intermediate (Kang et al, 1978).

Figure 3 The Role of Glycolipid Intermediates in the Glycosylation of Proteins



Although it was not possible to demonstrate the glycosylation of endogenous ovalbumin by oligosaccharide-lipid, subsequent experiments have been successful in showing the transfer of oligosaccharide from oligosaccharide-lipid to a denatured and chemically modified form of ovalbumin (Pless and Lennarz, 1977). Sulfitolysis was employed for the denaturation and disulphide cleavage of various proteins and they were then incubated with an oviduct membrane preparation and isolated, labelled, oligosaccharide-lipid. Sodium dodecyl sulphate polyacrylamide gel analysis indicated the incorporation of oligosaccharide from the oligosaccharide-lipid to the denatured forms of the three secretory proteins, ovalbumin, α-lactalbumin, and ribonuclease A. Since α-lactalbumin and ribonuclease A are unrelated to ovalbumin in amino-acid sequence, and completely foreign to the oviduct, it would appear that the enzyme specificity for this glycosylation involves at most a limited region of the polypeptide chain. It is suggested that the tri-peptide sequence Asn-X-Threonine (or Serine) is necessary for glycosylation ; however since other denatured proteins that were tested also included this sequence but were not glycosylated, other factors must also be involved (see also Struck et al, 1978). The results of the investigation were interpreted as suggesting that the unfolding of the native polypeptide structure is necessary for the exposure of the site of carbohydrate attachment. If extended to the physiological situation, this would imply that the transfer of the oligosaccharide to the polypeptide occurs before the newly synthesized protein adopts its folded structure, and therefore either while it is still attached to the ribosome or very soon after being released. This conclusion is consistent with the observation that the core sugars, N-acetyl glucosamine and mannose, are added to ovalbumin nascent chains (Kiely et al, 1976).

Further evidence for the participation of oligosaccharide-lipid in ovalbumin glycosylation has come from a convincing series of experiments using the antibiotic

tunicamycin (Struck and Lennarz, 1977). Tunicamycin specifically inhibits the lipid-linked pathway for glycoprotein synthesis by blocking the first step in the assembly of the oligosaccharide-lipid, the synthesis of N-acetyl glucosamine diphosphate dolichol, as shown in Figure 3 (Tkacz and Lampen, 1975). It was found that tissue slices incubated in the presence of tunicamycin synthesized the polypeptide chain of ovalbumin at almost normal rates, but that the newly synthesized protein did not contain labelled N-acetyl glucosamine or mannose, and had the properties of unglycosylated ovalbumin. Tunicamycin has also been shown to inhibit the glycosylation of immunoglobulins (Hickman and Kornfeld, 1978). These results provide strong evidence in favour of a lipid-linked pathway being involved in the assembly of the core oligosaccharide of ovalbumin and the immunoglobulins.

Thus, the biosynthesis of an oligosaccharide-lipid containing N-acetyl glucosamine and mannose has been established, and its role as a donor of its oligosaccharide chain to form both membrane and secretory glycoproteins has been demonstrated. However, the results of more recent studies extending the previous work on the biosynthesis and structure of the oligosaccharide-lipid have indicated that the situation is considerably more complex than previously suggested. Oviduct membrane preparations have been shown to synthesize a large oligosaccharide-lipid containing glucose in addition to the N-acetyl glucosamine and mannose present in the intermediate described previously (Chen and Lennarz, 1978a). It has been demonstrated that this oligosaccharide is transferred "en bloc" to an endogenous membrane protein, molecular weight 25,000, and also to an exogenous soluble protein, S-carboxymethylated α-lactalbumin. However, it has also been shown that after transfer to the protein, the oligosaccharide is processed enzymically and the glucose residues removed resulting in a smaller oligosaccharide with a molecular weight similar to $(Man)_2(GlcNAc)_2$ (Chen and Lennarz, 1978b).

Spiro et al (1976) had previously demonstrated the

biosynthesis of a large oligosaccharide-lipid by thyroid slices and other tissues, including the oviduct. The oligosaccharide-lipid from the thyroid contained 14-15 monosaccharide residues, including glucose, and its transfer to an endogenous protein acceptor was shown. They pointed out that if the oligosaccharide-lipid acts as the donor of oligosaccharide to thyroglobulin, not only would the glucose residues have to be removed, but the oligosaccharide would also have to undergo considerable processing to remove excess mannose residues in order to achieve carbohydrate-units of the complex-type, which contain only 3 mannose residues in the core structure. The processing of high-mannose containing oligosaccharides to complex-type oligosaccharide-units has been demonstrated by Tabas et al (1978).

Therefore in summary, there is evidence for the participation of oligosaccharide-lipid intermediates in the glycosylation of secretory proteins. However, it now seems that this is unlikely to involve the simple "en bloc" transfer of a pre-formed core oligosaccharide unit to the protein, but the transfer of a large glucose-containing oligosaccharide which must subsequently be processed by specific glycosidases to give the appropriate core structure. The evidence suggests that the core sugars are added to the protein during or soon after translation, and that the peripheral sugars are then added in stages during intracellular transport.

1.3 The Phosphorylation of Secretory Proteins

The first report to discuss the phosphorylation of a secretory protein came from Sanger and Hocquard (1962) ; they identified dephospho-ovalbumin as an intermediate in the biosynthesis of ovalbumin in hen oviduct. The presence of intracellular dephospho-ovalbumin suggested that the phosphorylation of ovalbumin takes place after the synthesis of the polypeptide chain. More recently there has been extensive research into the role of phosphorylation and dephosphorylation in regulatory processes (for a review,

see Rubin and Rosen, 1975), but less attention has been paid to the post-translational phosphorylation of individual secretory proteins, with the exception of the milk protein, casein. Some of the research into the mechanism of casein phosphorylation will be described here.

1.3.1 The Phosphorylation of Casein

The major components of borine casein are α_{S1}-casein (45%), β-casein (33%) and κ-casein (15%) (Bingham and Farrell, 1977). The determination of the amino-acid sequences of the caseins has defined accurately the location of the phosphate groups (Mercier et al, 1971 ; Mercier et al, 1973 ; Ribadeau-Dumas et al, 1972) ; the α_{S1}-, β- and κ-caseins contain 8, 5 and 1 phosphoserine residues respectively. A prominent feature of the primary structure of the α_{S1}- and β-caseins is a region of homology which includes a cluster of 4 phosphoseryl residues, the sequence of this region in α_{S1}-casein is as shown below:-

$$\overset{63}{\text{-Glu}} - \underset{\underset{P}{|}}{\text{Ser}} - \text{Ile} - \underset{\underset{P}{|}}{\text{Ser}} - \underset{\underset{P}{|}}{\text{Ser}} - \underset{\underset{P}{|}}{\text{Ser}} - \text{Glu} - \overset{70}{\text{Glu}} -$$

in α_{S1}-casein this sequence extends over residues 63-70, and it is repeated in residues 14-21 of β-casein, with the substitution of leucine for isoleucine.

Early kinetic studies (Singh et al, 1967) indicated that phosphorylation of casein occurs after the completion of the polypeptide chain, and efforts were directed towards locating enzymes responsible for the phosphorylation. Bingham et al (1972) identified a protein kinase in the Golgi fraction of bovine mammary gland that incorporated phosphate into dephosphorylated α_{S1}-casein at more than ten times the rate of protein kinases from other subcellular fractions. The Golgi casein kinase was a cyclic-AMP independent protein kinase and used ATP as the phosphate donor. Dephosphorylated α_{S1}-, β- and κ-caseins were the best substrates for the enzyme and were phosphorylated

at much higher rates than native caseins ; histones, phosvitin, β-lactoglobulin and α-lactalbumin were not appreciably phosphorylated. The presence of Ca^{2+} or Mg^{2+} was essential for catalysis.

The observation that dephosphorylated caseins were preferred substrates suggested that the enzyme was incorporating phosphate into sites which had originally been phosphorylated, however, there was no direct evidence that this was the case. More recently, a Golgi protein kinase has been identified in bovine mammary gland which appears to have the ability to rephosphorylate dephosphorylated casein specifically (Mackinlay et al, 1977). When dephosphorylated α_{S1}-casein was rephosphorylated with (γ^{32}-P) ATP, the labelled phosphate incorporated was confined to two lengths of the polypeptide chain which between them included all of the phosphate groups of the native casein, this indicated a specificity of action of the casein kinase *in vitro* similar to that occurring *in vivo*. To confirm this specificity, two peptides which included all of the phosphoserine residues of α_{S1}-casein were prepared from native α_{S1}-casein, dephosphorylated and then rephosphorylated with (γ-^{32}P) ATP. The labelled phosphate was found to be incorporated only at the sites occupied by phosphoserine residues in the native protein. These results strongly supported the idea that this protein kinase is responsible for the *in vivo* phosphorylation of casein.

α_{S1}-casein contains 16 serine residues, only 8 of which are phosphorylated (Mercier et al, 1971). Mercier proposed a theory for the specificity of casein kinase based on an examination of the amino-acid sequences of the three bovine caseins. In α_{S1}-casein, each phosphorylated serine has a glutamic acid or a phosphoserine residue two residues to the right in the sequence, but this sequence does not occur when the serines are phosphate-free. Thus it is proposed that casein kinase recognizes a potential phosphorylation site corresponding to the tripeptide sequence Ser - X - Ser P (or Glu), where X is any amino-

acid, this hypothesis is also supported by the β-casein sequence. The ability of the specific casein kinase isolated from bovine mammary gland to specifically rephosphorylated peptides derived from casein as well as the native protein (Mackinlay et al, 1977) favours the idea that the enzyme recognizes a particular amino-acid sequence and does not require a particular conformation in the native protein. If the phosphorylation does indeed require recognition of Ser - X - Ser P or Ser - X - Glu sites, it must be assumed that phosphorylation is a sequential process, with a Ser - X - Glu site being phosphorylated first, in order to generate a Ser- X - Ser P site. However, other milk proteins contain potential sites for phosphorylation, but do not conform to the proposed theory (Bingham and Farrell, 1977) ; for example, lactalbumin has three potential sites but contains no phosphate, and κ-casein has four potential sites, none of which are phosphorylated. The lack of phosphorylation at these sites may be the result of steric hinderance due to the conformation of the protein or the proximity of carbohydrate residues.

In contrast to the 8 phosphate groups of α_{s1}-casein, the region of the vitellogenin molecule destined to become the yolk-protein, phosvitin, contains 56% serine residues, most of which are phosphorylated (Follett and Redshaw, 1971). A protein kinase has been identified in the liver of Xenopus laevis (Tenner and Wallace, 1972), but there is no evidence that it is the specific kinase involved during the biosynthesis of vitellogenin. It would be interesting to discover whether oestrogen-treatment results in the induction of a specific vitellogenin kinase in order to process such a highly phosphorylated molecule, and to determine the characteristics involved in its specificity.

1.4 The Formation of Multichain Proteins

The previous sections have described the biosynthesis and intracellular transport of the polypeptides of secretory proteins, and the addition of carbohydrate and phosphate groups to the polypeptides. Since several secretory proteins

are made up of two or more covalently-linked polypeptide chains (see Table 1) another type of post-translational modifications involves the formation of multichain proteins from the primary translation products. The formation of a multichain protein depends on the correct assembly of its component polypeptide chains, and the processes involved in the assembly of insulin, collagen and the immunoglobulins will be described here.

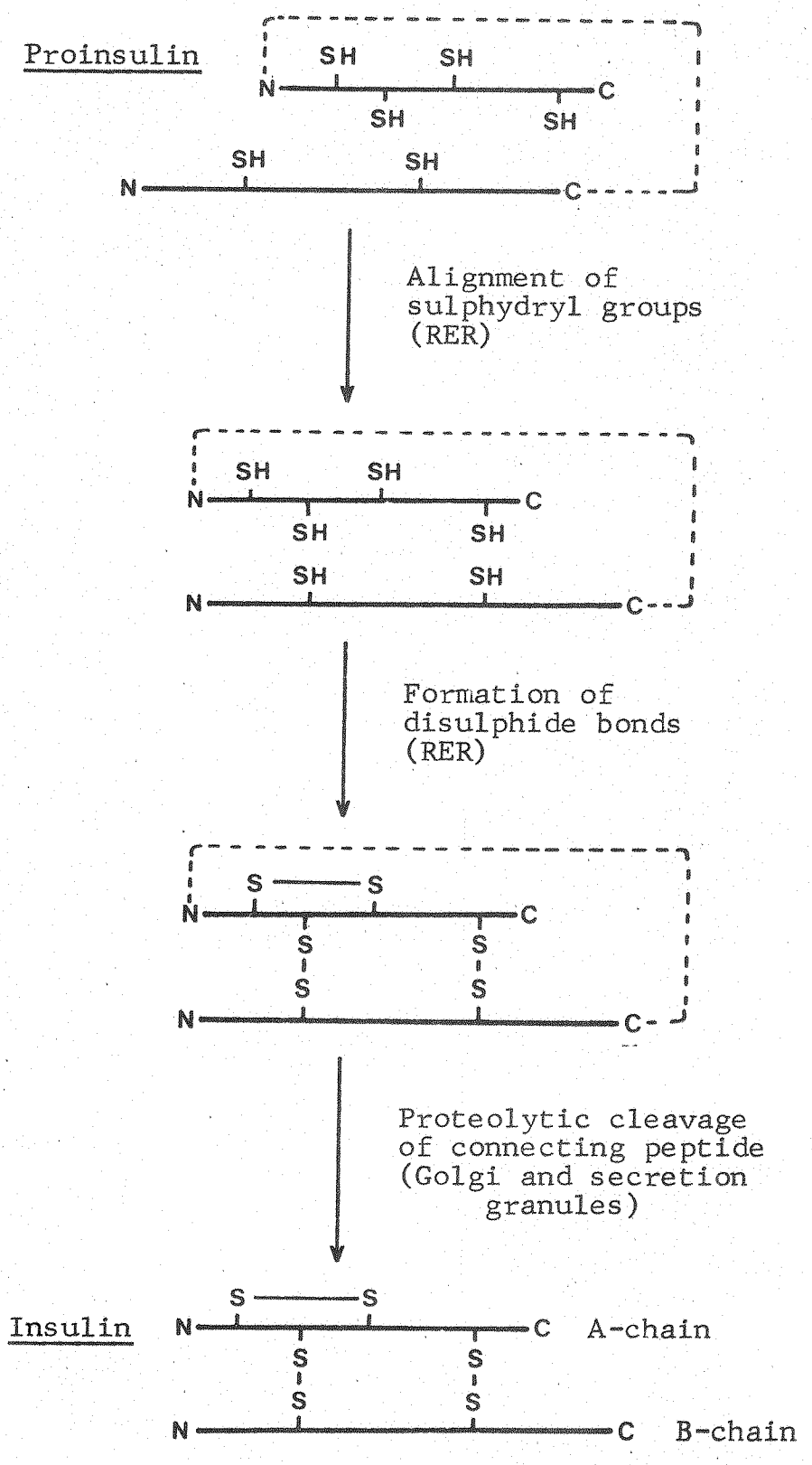
1.4.1 The Assembly of Insulin

The polypeptide hormone insulin (mol. wt. 6,000) is made of of 2 different polypeptide chains, the A and B chains, which are linked by 2 disulphide bridges (Sanger, 1945) (see Figure 4). Therefore during the assembly of insulin the two polypeptide chains must be brought together by some mechanism so that the interchain linkages are formed in the correct places. Both polypeptide chains of insulin are biosynthesized as part of a single precursor molecule, proinsulin (mol. wt. 9,000) (Steiner and Oyer, 1967 ; Chance et al, 1968). The proinsulin molecule has a B-chain amino-acid sequence at the NH_2-terminus with a 22 amino-acid sequence linking the COOH-terminus of the B-chain with the NH_2-terminus of the A-chain, and terminates with the normal A-chain amino-acid sequence (Chance et al, 1968). The native conformation adopted by the folding of the proinsulin molecule results in the A- and B-chain regions being brought together in the correct position for the formation of the interchain linkages (Steiner and Clark, 1968) and this is followed by the cleavage of the connecting peptide to form the final insulin molecule (see Figure 4).

The formation of the disulphide bridges is believed to take place in the rough endoplasmic reticulum soon after the release of the proinsulin molecule from the ribosomes, but the proteolytic cleavage of the connecting peptide does not appear to begin until the polypeptide reaches the Golgi apparatus and then continues for several hours after the formation of secretion granules (Steiner et al, 1974). However, it should be pointed out that the primary

Figure 4

The Assembly of Insulin from Proinsulin



translation product is not proinsulin, but pre-proinsulin, which includes the NH_2-terminal extension, the signal-peptide, but this is assumed to be cleaved prior to the processing of proinsulin (Chan et al, 1976).

1.4.2 The Assembly of Collagen

The basic unit of the collagen fibril is the tropocollagen molecule (or collagen fibril monomer), this is insoluble at pH 7.4 and 37°C and aggregates in a highly specific manner to form insoluble fibrils. The formation of the collagen monomer poses a more difficult assembly problem than insulin since it is made up of three polypeptide chains, termed α-chains, each with a mol. wt. of approximately 100,000, and these are wrapped around each other in a right-handed, triple-helical formation. The three α-chains have their ends approximately aligned, and although the helical structure extends throughout most of the collagen molecule, there are regions at the NH_2- and COOH-termini of approximately 15-20 amino-acids which are non-helical. The amino-acid sequences of the individual α-chains making up the helical structure need not be the same, for example, the collagen molecules of bone, tendon and skin, Type I collagens, consist of two distinct types of α-chain, α1(I) and α2, in the ratio of 2:1 ; in contrast cartilage collagen, Type II, consists of three identical α-chains, designated α1(II). Type III and Type IV collagens are found in other tissues and again these are made up of α-chains with distinct primary structures.

The α-chains contain about 1050 amino-acids, of which glycine accounts for one-third of the total and this is evenly distributed so that every third residue is glycine and one of the other two is usually proline ; the α-chains contain no cysteine residues. Before the assembly of the collagen molecule, proline and lysine residues in the α-chains must be hydroxylated in order to allow the formation of the two types of interchain bond which are responsible for the stability and rigidity of the helical

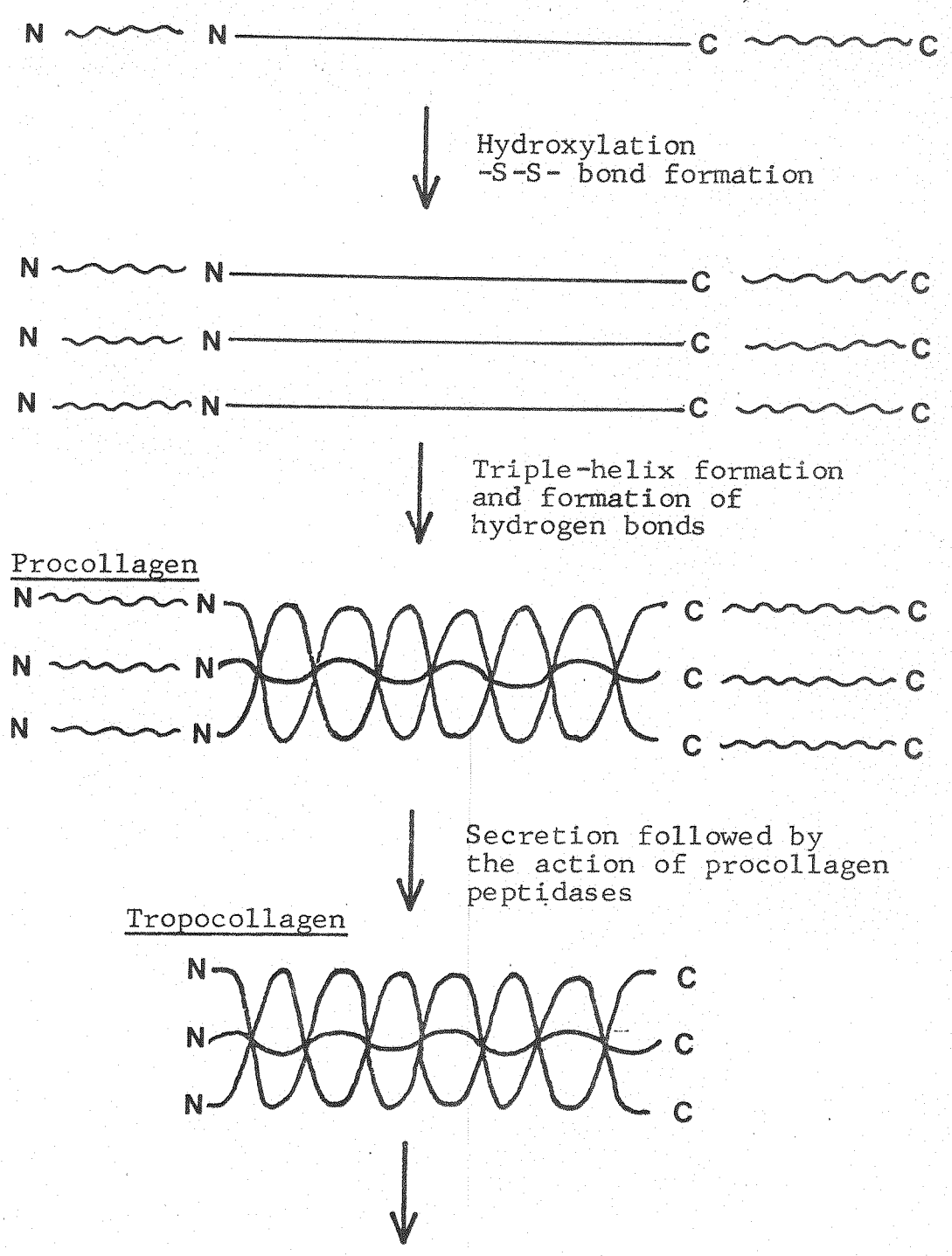
structure - these linkages are hydrogen-bonds between hydroxyproline residues and water molecules and covalent bonds between hydroxylysine residues. (For reviews see Kuhn, 1969 ; Bornstein, 1974 ; Grant and Jackson, 1976 ; Fessler and Fessler, 1978).

The problem of how the three polypeptide chains are assembled to form the collagen molecule has been solved to some extent by the discovery that collagen is synthesized as a large, soluble precursor molecule called procollagen (see Grant and Jackson, 1976 ; Fessler and Fessler, 1978). Procollagen consists of three polypeptide chains, designated pro- α -chains, which are significantly larger (mol. wt. approximately 145,000) than the α-chains of the final molecule. The pro- α -chains have an NH_2-terminal extension (mol. wt. approximately 18,000) and a COOH-terminal extension (mol. wt. 22-35,000) (Byers et al, 1974). The cysteine residues of the COOH-terminal extension are able to form interchain disulphide bonds (Byers et al, 1974) and it has been shown that disulphide bond formation is closely correlated with helix formation (Schofield et al, 1974 ; Harwood et al, 1977). On the basis of these results it is proposed that the formation of disulphide bonds between the COOH-terminal extensions brings about the correct alignment of the three pro- α -chains and promotes the assembly of the triple-helix (see Figure 5). The helical structure is then stabilized by the formation of hydrogen-bonds between hydroxyproline residues and water moleucles (Berg and Prockop, 1973 ; Sakakibara et al, 1973).

The assembly of the pro- α -chains into procollagen occurs during the passage of the molecules from the rough to the smooth endoplasmic reticulum (Harwood et al, 1973). However, the final conversion of the procollagen molecule to collagen does not occur until after secretion, when the NH_2- and COOH-terminal extensions are digested in a step-wide fashion by procollagen peptidases (Davidson et al, 1975). The removal of the extension peptides markedly influences the solubility properties of the molecule and the cleavage results in tropocollagen molecules which

Figure 5

The Assembly of Collagen Pro α-chains

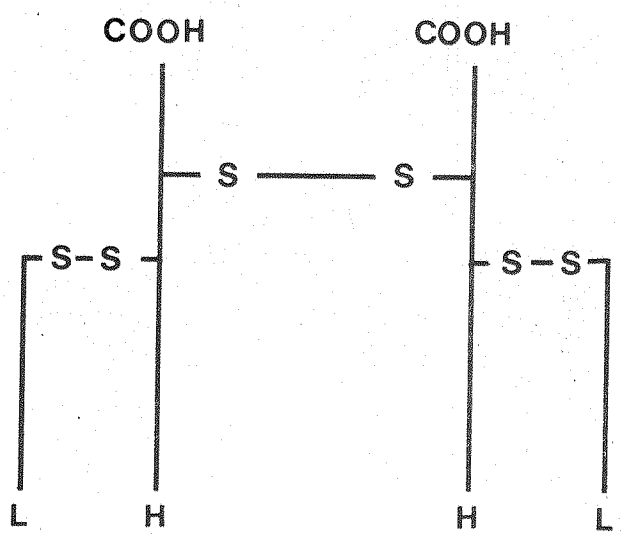


aggregate to form insoluble fibrils. The formation of interchain covalent linkages, either Schiff's bases or α- β-unsaturated aldols, between hydroxylysine residues of adjacent polypeptide chains stabilizes the structure of the fibrils and confers on them the high tensile-strength necessary for their structural function (Tanzer, 1973).

Thus the successful assembly of collagen differs from the formation of insulin since it involves the association of three individual polypeptide chains, and not the biosynthesis of a single large precursor molecule.

1.4.3 The Assembly of the Immunoglobulins

The immunoglobulin represents an example of a multi-chain protein with four polypeptide chains. All of the immunoglobulin classes have a basic 4-chain monomer structure consisting of two heavy chains (mol. wt. 50,000) and two light chains (mol. wt 25,000) (Porter, 1967) ; each heavy chain (H) is disulphide linked to a light chain (L) to form a half-molecule (HL), and two half-molecules are disulphide linked via their heavy chains to form the complete monomer. This basic monomer structure is shown below:-



Thus the biosynthesis and assembly of the immunoglobulins requires the correct alignment and covalent linkage of the four polypeptide chains (see Bevan et al, 1972, for a review on immunoglobulin biosynthesis).

The heavy and light chains of immunoglobulin are synthesized on separate polysomes (Williamson and Askonas, 1967), the heavy chains on polysomes consisting of 11-18 ribosomes and the light chains on polysomes with 4-5 ribosomes. However, as mentioned earlier in this introduction, the primary translation products are slightly larger than the authentic heavy and light chains since they include the NH_2-terminal extension, the signal-peptide ; this peptide is cleaved prior to the assembly of the immunoglobulin monomer.

The immunoglobulin monomer is assembled from its component heavy and light chains by a sequence of disulphide-bridge formation and studies have been directed towards determining the order of disulphide linkage of the polypeptide chains (see Bevan et al, 1972). Pulse-chase experiments have resulted in two basic pathways for immunoglobulin assembly being identified:-

1)	2)
$H+H \longrightarrow H_2$	$H+L \longrightarrow HL$
$H_2+L \longrightarrow H_2L$	$HL+HL \longrightarrow H_2L_2$
$H_2L+L \longrightarrow H_2L_2$	
Intermediates - H_2 and H_2L	Intermediates - HL

A study of the intermediates involved in the biosynthesis of immunoglobulins in a number of species has shown that each case falls into one or other of the two categories, with the exception of IgG_2b, where a mixed pattern of intermediates was observed (see Bevan et al, 1972).

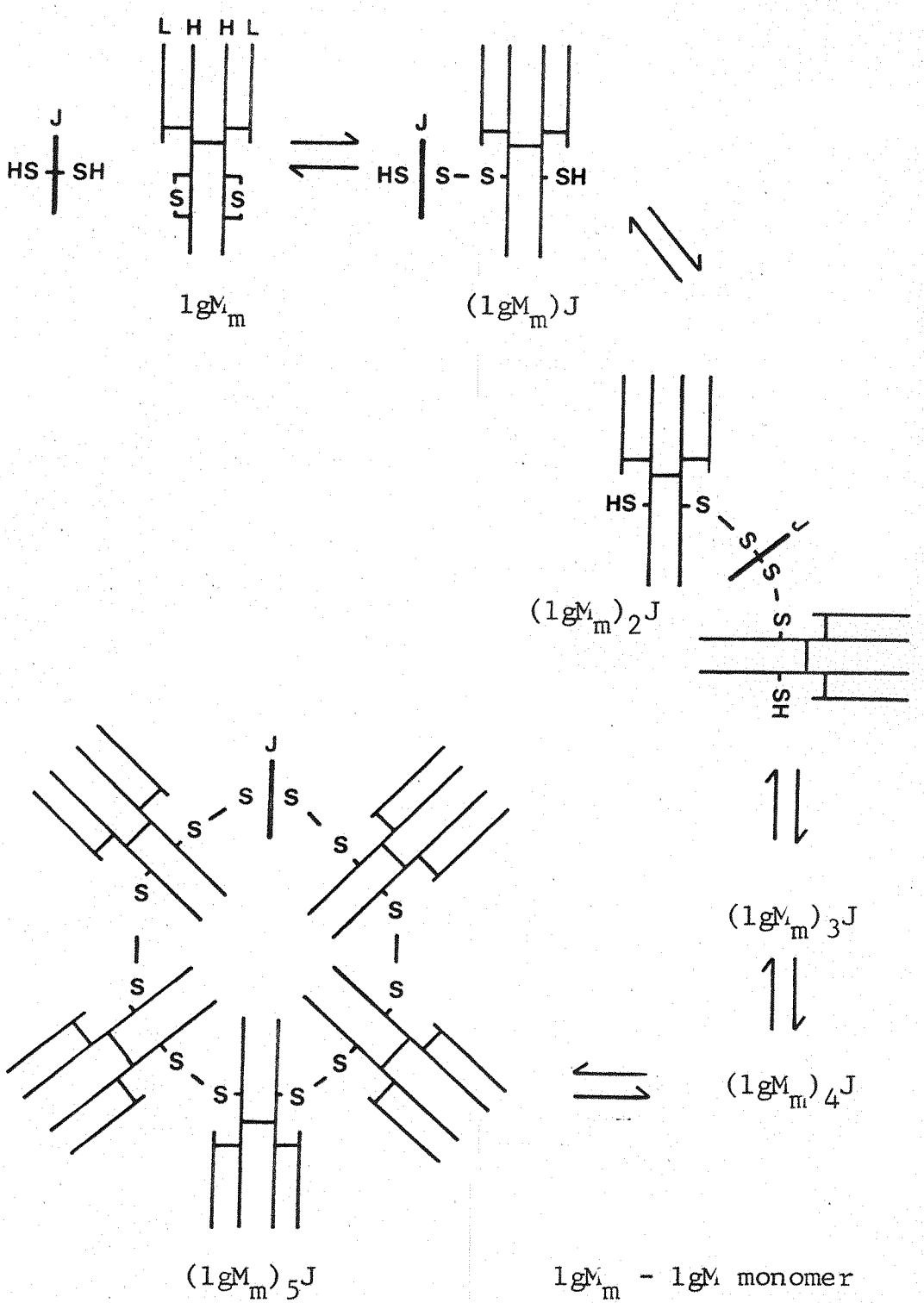
However, although these experiments explain the order of the covalent linkage of the heavy and light chains, they give no information as to how the chains are initially aligned to allow the correct disulphide bridges to be

formed. It is thought that whereas covalent linkage does not occur until after the release of the chains into the intercisternal space of the endoplasmic reticulum (Laskov et al, 1971), non-covalent linkages may be formed between free light chain molecules and heavy chain molecules which are still attached to the polysomes (Shapiro et al, 1966), and that the formation of covalent bonds follows the non-covalent assembly of the chains into a stable structure (see Bevan et al, 1972). There is no evidence for the involvement of large precursor molecules in the assembly of the chains.

The assembly of immunoglobulin M (IgM) and immunoglobulin A (IgA) poses even more problems, since they are secreted in polymeric forms, IgM as a pentamer, consisting of 5 IgM monomers, and IgA as a dimer, consisting of 2 IgA monomers. In both cases the monomers are linked together by inter-monomer H-chain disulphide bonds. Since no polymeric forms of either IgM or IgA can be detected intracellularly (Parkhouse and Askonas, 1969 ; Parkhouse, 1971), polymerization probably occurs just before or at the time of secretion ; it is suggested that the sulphydryl groups responsible for the covalent linkages between the monomers are blocked within the cell so that no polymeric form is produced until the time of secretion (Askonas and Parkhouse, 1971).

Once the polymeric forms of immunoglobulin are assembled they contain a third type of polypeptide chain, called the J-chain, which has a molecular weight of approximately 23,000 (Halpern and Koshland, 1970 ; Mestecky et al, 1971). The J-chain is also biosynthesized in the secretory cell and is distinguishable from light chain since it is immunologically distinct and has a unique peptide map (Kaji and Parkhouse, 1975), it is also relevant that the J-chain has a high cysteine content. It is proposed that the J-chain is essential for the polymerization of IgM and IgA (Della Corte and Parkhouse, 1973) and that the introduction of the J-chain results in the assembly of the polymeric forms by a series of disulphide exchanges, beginning with

Figure 6



The Polymerization of lgM
(from Chapuis and Koshland, 1974)

the formation of a J-chain dimer ; a possible mechanism for the polymerization of 1 g M is shown in Figure 6 (Chapuis and Koshland, 1974). The proposed mechanism requires an oxidation step for the formation of the final disulphide bond. A problem with this mechanism is how polymerization is limited to the formation of a pentamer? A possible explanation is that the oxidation step required for the closing of the pentamer is coupled with secretion, and that the pentameric form is the only acceptable substrate for this reaction ; alternatively, steric constraints may prevent the closing of a non-pentameric form of the chain (Chapuis and Koshland, 1974).

From the three examples described it is clear that there is no common mechanism involved in the assembly of multichain proteins. The process may involve the synthesis of a single large precursor molecule, as in the case of insulin, or the association of individual polypeptide chains, as with collagen and the immunoglobulins.

1.5 Vitellogenin - a Glycolipophosphoprotein

In summary it may be said that progress has been made in elucidating the mechanisms involed in the biosynthesis and modification of specific secretory proteins. However, since it is clear that there is wide variation in both the nature and the extent of the post-translational processing steps which the primary translation products must undergo, there is room for considerable diversity in the precise mechanisms involved during the biosynthesis of individual proteins ; therefore it is valuable to investigate the biosynthesis of as many different secretory proteins as possible. Vitellogenin is a glycolipophosphoprotein (see Table 4) and as yet there is little information concerning the process involved in the addition of the non-protein components. The work presented in this thesis looked at the addition of the phosphate and carbohydrate groups to the vitellogenin polypeptide.

TABLE 4 STRUCTURAL PROPERTIES OF XENOPUS VITELLOGENIN, LIPOVITELLIN AND PHOSVITIN

PROTEIN	VITELLOGENIN (serum)	LIPOVITELLIN (yolk platelet)	PHOSVITIN (yolk platelet)
MOL. WT. ($\times 10^{-3}$)	500 (200,000)	290 (120,31)	55 (35, 17?)
PHOSPHORUS (%)	1.3	0.7	10
SERINE (% residues)	12	4	56
(% residues phosphorylated)			72
METHIONINE	+	+	–
CALCIUM (% protein-bound)	1.6		
LIPID (%)	12.0	20.0	0.5
CARBOHYDRATE (%)	1.6	0.3	10.0
BILIVERDIN (molar ratio)	2	1	0

Figures in parenthesis give the molecular weights of individual subunits : the vitellogenin molecule contains 2 sub-units, each consisting of a lipovitellin dimer and 1 molecule of phosvitin.

(Data combined from:- Bergink and Wallace, 1974a ; Ansari et al, 1971 ; Redshaw and Follett, 1971 ; Clemens, 1974 ; Tata, 1976 ; Ohlendorf et al, 1977).

CHAPTER 2

Chapter 2

MATERIALS AND METHODS

2.1 Materials

Special chemicals were obtained from the sources given below. All other chemicals were ex-stock from BDH or Koch-Light.

2.1.1 Chemicals

Acrylamide (pure)	Koch-Light Laboratories, Bucks
Ammonium persulphate (Analar)	BDH Chemicals, Poole, Dorset
Bromophenol Blue	BDH Chemicals, Poole, Dorse
Coomassie Brilliant Blue	E.T. Gurr, through Baird & Tatlock, Romford, Essex
Cycloheximide	Sigma Chemicals, London
DEAE-Cellulose Acetate (DE 52)	Whatman, Maidstone, Kent
Dimethyl formamide	BDH Chemicals, Poole, Dorset
L-Leucine	BDH Chemicals, Poole, Dorset
N^1 N^1-Methylene bis acrylamide	BDH Chemicals, Poole, Dorset
17-β oestradiol	BDH Chemcials, Poole, Dorset
Silica gel GF254	Merck Chemicals, Germany
Sodium dodecyl sulphate	BDH Chemicals, Poole, Dorset
N, N, N^1, Tetramethyl-ethylenediamine	BDH Chemicals, Poole, Dorset
Tris (hydroxymethyl) methylamine	Sigma Chemicals, London

2.1.2 Chemicals for Liquid Scintillation Counting

Butyl PBD	Koch-Light Laboratories, Bucks
Dimethyl POPOP (scintillation grade)	Cambrian Chemicals Ltd., U.K.

Dimethyldially-tartardiimide	Aldrich Chemicals, Gillingham, Dorset
Hyamine hydroxide (1 M sol^{n} in methanol)	Nuclear Enterprises, Edinburgh
NCS Tissue Solubliser	Hopkins & Williams, Essex
PPO (Scintillation grade)	International Enzymes, Berks
Synperonic NXP	I.C.I., U.K.
Toluene (Sulphur-free)	Koch-Light Laboratories, Bucks
Xylene (puriss. A.R.)	Koch-Light Laboratories, Bucks

2.1.3 Liquid Scintillation Fluids

Toluene-Butyl PBD for non-aqueous samples

Butyl PBD	20 g
Toluene	2.5 l

Tritoscint II for aqueous samples

Dimethyl POPOP	0.5 g
PPO	4.0 g
Synperonic NXP	333 ml
Xylene	667 ml

2.1.4 Radiochemicals

All radiochemicals were obtained from the Radiochemical Centre, Amersham, Bucks. The specific activities of the radiochemicals used were as follows:-

	Specific Radioactivity
Acetate	
(U-^{14}C) Acetic acid, sodium salt	59 mCi/m mol
(^{3}H) Acetic acid, sodium salt	250 mCi/m mol
Amino Acids	
L-(4, 5-^{3}H) Leucine	40-60 Ci/m mol
L-(methyl-^{3}H) Methionine	70 Ci/m mol
L-(3-^{3}H) Serine	17 Ci/m mol
L-(2, 5-^{3}H) Histidine	50 Ci/m mol
Phosphate	
(^{32}P) Phosphate	Carrier-free

	Specific Radioactivity
Sugars	
D-(1-^{3}H) Galactose	22 Ci/m mol
D-(6-^{3}H) Glucosamine	38 Ci/m mol
D-(1-^{3}H) Mannose	5 Ci/m mol
Uridine diphospho-D-(6-^{3}H) galactose	16.3 Ci/m mol
Guanosine diphospho-(U-^{14}C) mannose	173 mCi/m mol
Uridine diphospho-N-acetyl-d-(U-^{14}C) glucosamine	323 mCi/m mol

2.1.5 Liquid Scintillation Counting

All samples were counted at 12^{o}C in either a Phillips Liquid Scintillation Counter or an Intertechnique ABAC SL 40. Automatic correction was made for quenching from quench curves constructed using chloroform/methanol (2:1) as a quenching agent. The accuracy of the quench correction was $\pm$ 5% (S.D.).

In dual label (^{3}H)/(^{32}P) experiments, corrections were made for cross-over between the two counting channels.

2.1.6 Abbreviations

ATP	Adenosine-5'-Triphosphate
Bis	N, N^1 methylenebisacrylamide
Butyl PBD	5-(4-biphenyl)-2-(4-tert-butylphenol)-1-oxa-3, 4-diazole
DATD	Diethydiallyltartardiimide
DEAE	Diethylaminoethyl
DMFA	Dimethylformamide
EDTA	Ethylenediamine tetra-acetic acid
HCG	Human chorionic gonadotrophin
Hyamine	Benzyldimethyl (2-(2-(4-(1, 1, 3, 3, -tetramethylbutyl)-tolyloxy)ethoxy) ethyl ammonium hydroxide)
PCA	Perchloric acid
PPO	2, 5, Diphenyloxazole
Dimethyl POPOP	1, 4, Di(2-(4-me)5-phenyloxazolyl)-benzene
SDS	Sodium dodecyl sulphate (sodium lauryl sulphate)
TEMED	N, N, N^1, N^1-tetramethylethylenediamine
TCA	Trichloroacetic acid
Tris	Tris (hydroxymethyl) methylamine

2.2 Methods

2.2.1 Animals

Female Xenopus laevis weighing approximately 100 g were used in all experiments. The animals were kept in fresh water at 22°C and were fed weekly on diced ox liver.

2.2.2 Injections

In experimental animals a single dose of 17-β oestradiol was injected into the dorsal lymph sac. In control animals an injection of 0.2 mls of olive oil was given in the same location.

Injections of radioisotopes in 0.2 mls of distilled water were given intra-muscularly into the upper leg muscle.

2.2.3 Preparation of Plasma Vitellogenin

Vitellogenin was prepared from the plasma according to the method described by Munday et al (1968). Oestrogen treated Xenopus were pithed and their blood collected by cardiac puncture. 0.2 mls of citrate (15 mg/ml) was added to the blood to prevent clotting and the plasma was isolated from the red blood cells by centrifugation. The plasma was then cooled to 0°C, treated with 3.5 M dimethylformamide (0.37 mls DMFA/1 ml plasma), and the pH of the resulting solution adjusted to 7.0 with 0.2 M acetic acid. The mixture was allowed to stand at 0°C for 1 hour and the resulting thick green precipitate was collected by centrifugation at 3,000 rpm for 5 minutes at 5°C. The precipitate was then washed once with 2.5 mls of 3.5 M DMFA in 0.15 M NaCl and reharvested by a second centrifugation step. The resulting vitellogenin was then dissolved in 0.9% NaCl (Approximately 3-5 mls), dialysed against distilled water for 3 hours, and freeze-dried for storage. Vitellogenin prepared by this procedure was stable, and could be stored deep-frozen for long periods (at least 12 months).

2.2.4 In vitro Incubation of Liver Slices

a) Incubation Medium

In preliminary experiments, the medium used was identical to the phosphate saline buffer described by Dulbecco and Vogt (1954), except for the addition of 20 mM sodium pyruvate as an energy source. To avoid the precipitation of calcium phosphate, three separate solutions were prepared and then mixed and brought to pH 7.4 with 1 M HCl. They were as follows:-

Solution 1		Solution 2		Solution 3	
NaCl	4.0 g	$CaCl_2 6H_2O$	0.1 g	$MgCl_2 6H_2O$	0.1 g
KCl	0.2 g				
Na_2HPO_4 (anhyd)	1.5 g				
KH_2PO_4 (anhyd)	0.2 g				
Sodium pyruvate	2.2 g				
/800 ml H_2O		/100 ml H_2O		/100 ml H_2O	

However, in subsequent experiments the incubation medium was based on a 50% dilution of Eagle's Minimal Essential Medium with Earle's Salts, containing a final concentration of 20 mM N-2-hydroxyethylpiperazine-N^1-2-ethanesulphonic acid (HEPES), 200 units/ml of penicillin, 200 µg/ml of streptomycin, 70 µg/ml of gentomycin and 20 units/ml of mycostatin (see Table 5).

b) Liver Slice Preparation

Animals were pithed and their livers removed into ice-cold incubation medium. Liver slices of approximately 5 x 3 mm were cut using fine scissors and washed 3 times with 10 mls of incubation medium. The slices were blotted dry on filter paper and weighed on foil.

c) Incubation Conditions

Incubations were carried out in 25 ml glass conical flasks with a tissue to medium ratio of 0.5 g of liver slices to 4 mls of medium or 1 g of liver slices to 10 mls

TABLE 5 COMPOSITION OF THE INCUBATION MEDIUM

A stock solution was prepared as follows:-

	g/litre		mg/litre
NaCl	6.8	Choline Cl	1.0
KCl	0.4	Folic Acid	1.0
*NaH_2PO_4	0.15	Inositol	2.0
$MgSO_4.7H_2O$	0.2	Nicotinamide	1.0
$CaCl_2$	0.2	Ca-Pantothenate	1.0
Glucose	1.0	Pyridoxal HCl	1.0
$NaHCO_3$	2.2	Riboflavin	0.1
		Thiamine HCl	1.0
		Phenol red	10.0

*This was omitted in (^{32}P) phosphate incorporation experiments.

A 50% dilution of the stock solution with the following additions was used for the incubations:-

	Final conc^n
Penicillin	200 units/ml
Streptomycin	200 µg/ml
Gentomycin	70 µg/ml
Mycostatin	20 units/ml
HEPES	20 mM

The pH was adjusted to pH 7.4 with NaOH.

of incubation medium.

In time-course experiments in which samples of both incubation medium and tissue were required, one flask was set up for analysis at each sampling time.

Samples were incubated at 22°C with constant shaking at 45 strokes/min, which allowed the slices to move gently from side to side within the flask. The incubations were allowed to equilibrate for 10 minutes before the addition of isotope.

d) Pulse-Chase Incubations

Flasks of liver slices were set up as described above. The slices were incubated with the appropriate labelled precursor and at the end of the required pulse-period further protein synthesis was prevented by the addition of cycloheximide (100 µg/ml medium) ; the incubation was then continued in the same medium. Half way through the subsequent chase period a second dose of cycloheximide was given as a precautionary measure to ensure that there was no recovery from the effect. Any variations from this basic procedure will be described as appropriate.

2.2.5 Isolation of Vitellogenin from the Incubation Medium

After the required incubation time the incubation medium was removed and the vitellogenin precipitated from it by the dimethylformamide/acetic acid technique described in section 2.2.3, and then dialysed and freeze-dried for storage. The specific activity of the isolated vitellogenin was determined as described in section 2.2.7 d).

2.2.6 Cellular Fractionation Studies

a) Isolation of the Microsomal Fraction and the Post-Microsomal Supernatant

At the end of the required incubation time the incubation medium was removed and the liver slices were washed twice in 5 mls of TKMS buffer (0.25 M sucrose, 0.05 M Tris-HCl, 0.025 M KCl and 0.005 M $MgCl_2$, pH 7.5). The

slices were then transferred to a glass homogenizing tube with 2.5 mls of ice-cold TKMS buffer containing 50 µl/ml of the protease inhibitor phenylmethylsulphonylfluoride, PMSF (from a stock solution of 6 mg/ml in 90% ethanol). The tissue was homogenized with a tight-fitting pestle using a Tri-R-Stir R tissue homogenizer at speed setting 3.5-4 ; 12 passes of the pestle were made. If incorporation of radioactivity into total tissue protein was to be determined a sample of the homogenate was kept at this stage.

Cell debris, nuclei and mitochondria were sedimented from the total tissue homogenate by centrifugation at 4,000 g (6,000 rpm) for 10 minutes at 4°C, in the 16 x 15 fixed-angle rotor of an MSE High-Speed 18 centrifuge. The resulting post-mitochondrial supernatant was red with a layer of yellow lipid floating on the surface, and the 4,000 g pellet was black. The lipid associated with the post-mitochondrial supernatant was removed by filtering the supernatant through absorbant cotton wool. The filtrate was then decanted into 10 ml polycarbonate tubes and the microsomal fraction sedimented by centrifugation at 105,000 g for 60 minutes at 4°C, in the 10 x 10 ml titanium fixed-angle rotor of an MSE 50 centrifuge. After centrifugation a brown, jelly-like microsomal pellet was obtained and a pale red post-microsomal supernatant. Both fractions were retained.

The microsomal pellet obtained from 0.5 g of liver slices was resuspended in 2 ml of ice-cold TKMS buffer by gentle homogenization, either within the centrifuge tube or after transfer to a test-tube, with a loose-fitting pestle at speed setting 2.5 until the suspension was of uniform appearance.

b) Isolation of the Post-Microsomal Pellet

A sample (0.5 ml) of the post-microsomal supernatant fraction was retained for gel analysis and the remainder was transferred to a 10 ml polycarbonate tube. The post-microsomal pellet was sedimented by centrifugation at 105,000 g for 3 hours. The small red pellet which resulted was resuspended in ice-cold TKMS buffer by gentle

homogenization ; the volume of TKMS used was the same as the volume of post-microsomal supernatant from which the pellet was sedimented. The final supernatant was retained (Scheme 1 summarizes the cellular fractionation procedure.)

c) Solublization of the Microsomal and Post-Microsomal Pellet Fractions

The microsomal pellet from 0.5 g of liver slices was resuspended in 2 ml of TKMS buffer as described above. The suspension was incubated at room temperature for 30 minutes in the presence of the protease inhibitor PMSF, this was added at a concentration of 50 µl/ml (from a stock solution of 6 mg/ml in 95% ethanol). At the end of the incubation period, sodium dodecyl sulphate (SDS), 2-mercaptoethanol, and Na_2CO_3 were added to give final concentrations of 2%, 10% and 50 mM respectively (from a stock solution containing 50% 2-mercaptoethanol, 10% SDS and 250 mM Na_2CO_3), and the suspension solublized by boiling at 100°C for 5 minutes.

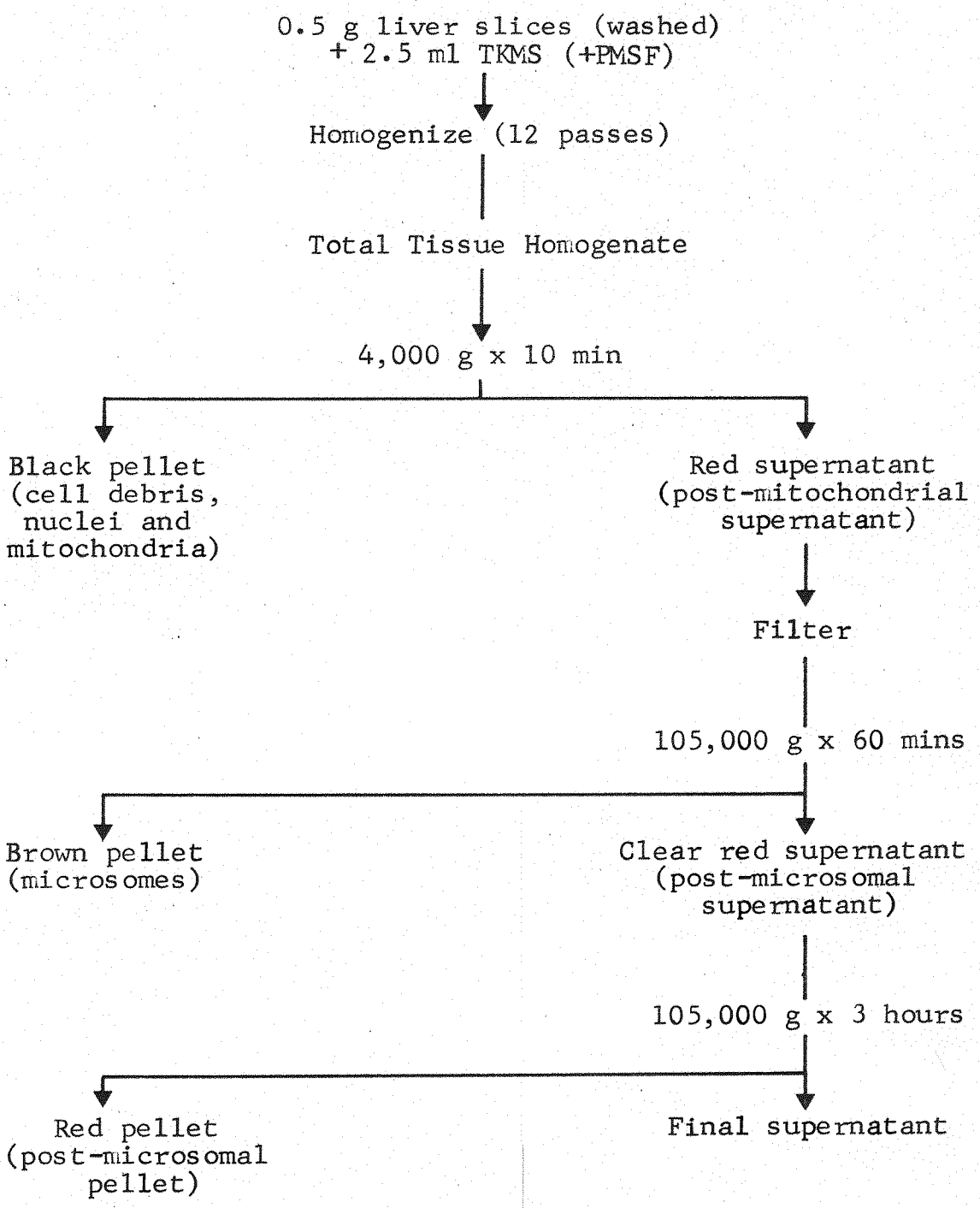
The post-microsomal pellet was resuspended in TKMS buffer, the volume used being the same as the volume of post-microsomal supernatant from which it was sedimented. PMSF was added to the suspension at a concentration of 50 µl/ml (from the stock solution) and SDS, 2-mercaptoethanol and Na_2CO_3 were added to give final concentrations of 2%, 10% and 50 mM respectively. The suspension was then solublized by boiling at 100°C for 5 minutes. (N.B. - the post-microsomal pellet suspension was not incubated for 30 minutes with PMSF prior to solublization.)

After solublization by this procedure, these fractions were ready to be applied to SDS polyacrylamide gels.

2.2.7 Measurement of Protein-Bound Radioactivity

The term 'protein-bound radioactivity' refers to the covalent incorporation of radioactive precursors into protein. Although the methodology used to determine the level of radioactive incorporation into particular

SCHEME 1 CELLULAR FRACTIONATION OF XENOPUS LIVER



fractions varied, in all cases it was necessary to remove all non-covalently bound label before determining the incorporation into protein. To obtain a low background level of radioactivity the following precautions were taken:-

(i) samples labelled with radioactive precursor were diluted with excess unlabelled precursor ;
(ii) adequate time was allowed for the precipitation of the protein by TCA ;
(iii) the precipitates were washed, using TCA containing unlabelled precursor, by repeated rotomixing and centrifugation. Care needed to be taken during the centrifugation steps since if the suspension was centrifuged for longer than 1 minute at top speed the precipitate formed a hard pellet which was difficult to break-up and could trap non-covalently bound label.

a) Analysis of the Incubation Medium Protein for the Incorporation of Radioactive Precursors

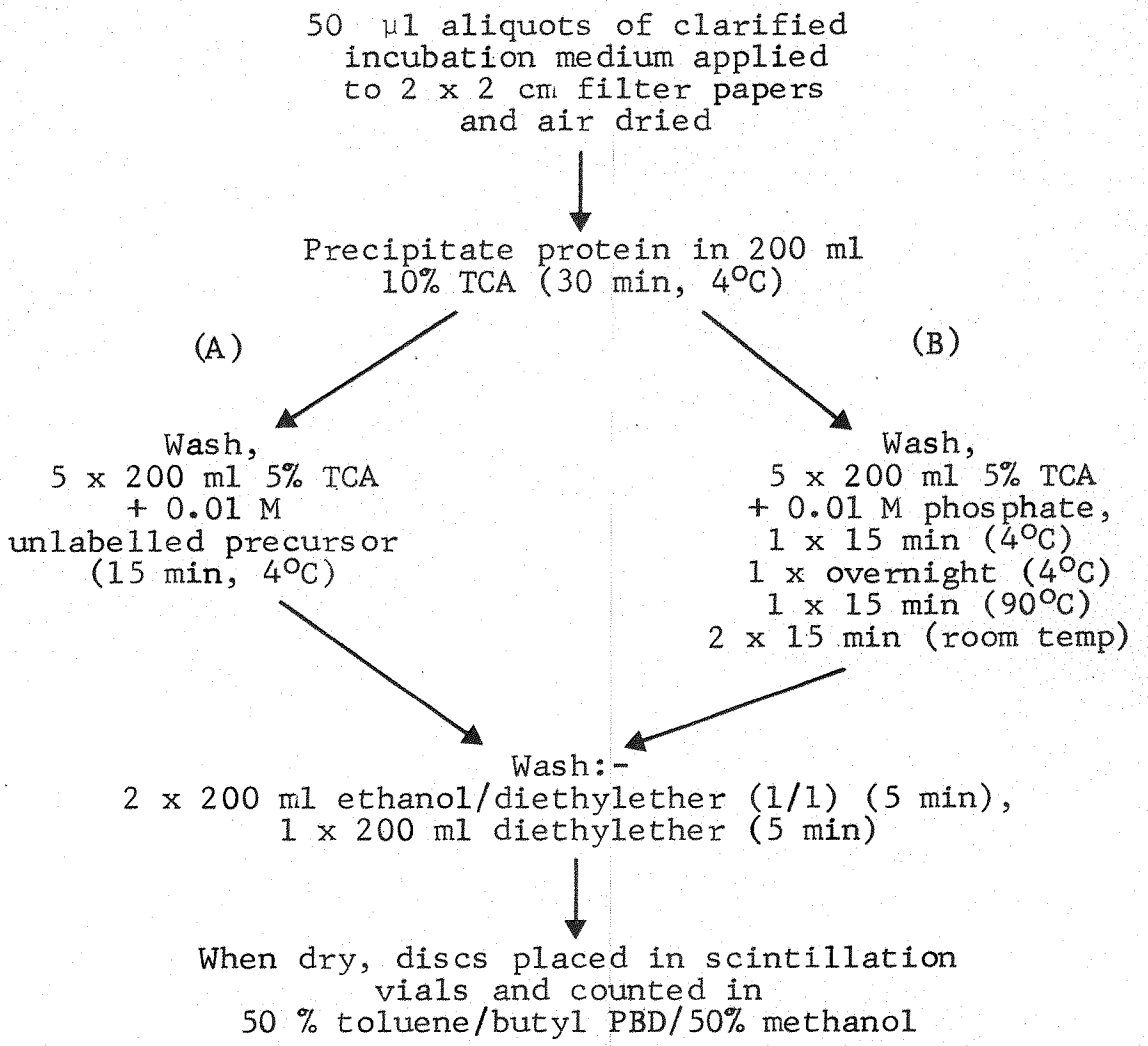
The filter paper disc technique was used to determine the incorporation of radioactive precursors into incubation medium protein.

0.3 ml samples of incubation medium were centrifuged to remove red blood cells and other debris, and then 50 µl aliquots of the clarified medium were applied to individual pieces of Whatman 3 mm filter paper measuring approximately 2 cm x 2 cm. The incorporation of radioactivity into the aliquots was then analysed as shown in Scheme 2. For most of the isotopes used the washing procedure (A) was sufficient to reduce the background radioactivity to 150-300 dpm. However, for incubations in which 200 µCi of (^{32}P) phosphate was used, the washing procedure (B) was necessary in order to reduce the background radioactivity to an acceptable level (approximately 4,000 dpm).

b) Analysis of the Total Tissue Protein for the Incorporation of Radioactive Precursors

1 ml aliquots of liver slice homogenate were used to determine the incorporation of radioactivity into total tissue protein as shown in Scheme 3.

SCHEME 2 FILTER PAPER DISC METHOD FOR THE ANALYSIS OF INCORPORATION OF RADIOACTIVITY INTO INCUBATION MEDIUM PROTEIN



SCHEME 3 ANALYSIS OF TOTAL TISSUE PROTEIN FOR THE INCORPORATION OF RADIOACTIVE PRECURSORS

1 ml aliquot of total tissue homogenate

↓

Add 3 ml, 0.1 M unlabelled precursor

↓

Precipitate protein with 30% TCA to a final concentration of 10%

↓ 2 hours at 4°C

Wash 4 x 5 ml, 5% TCA containing 0.01 M unlabelled precursor,
1 x 90°C (30 min)
3 x 4°C

↓

Add 5 ml, 30% H_2O_2 to pellet to bleach protein (60°C, 3-4 hrs)

↓

Re-precipitate protein at final concentration 10% TCA

↓

Wash, 2 x 5 ml 5% TCA (4°C)

↓

Wash, 2 x 3 ml acetone (15 min)
1 x 5 ml ether (15 min)

↓

Dissolve precipitate in 0.9 ml hyamine hydroxide at 50°C. To 0.3 ml aliquot add 15 mls toluene/butyl PBD + 2 drops acetic acid and measure radioactivity

SCHEME 4 ANALYSIS OF MICROSOMAL PROTEIN FOR THE INCORPORATION OF RADIOACTIVE PRECURSORS

1 ml aliquot of microsomal suspension

↓

Add 3 ml, 0.1 M unlabelled precursor

↓

Precipitate protein with 30% TCA to a final conc^{n} of 10%

↓ 1 hour at 4°C

Wash 6 x 3 ml, 5% TCA containing 0.01 M unlabelled precursor,
5 x 4°C
1 x 90°C (15 min)

↓

Wash, 2 x 3 ml acetone (15 min)
1 x 3 ml ether (15 min)

↓

Dissolve precipitate in 0.3 ml hyamine hydroxide at 50°C, add 15 ml toluene/butyl PBD + 1 drop glacial acetic acid and measure radioactivity

Since the total tissue protein fraction is black, due to the presence of melanin pigment, it was necessary to bleach the protein with hydrogen peroxide prior to counting as the dark colour would result in a high level of quench and make counting unreliable. After bleaching, the sample had to be washed throughly to remove all traces of hydrogen peroxide.

c) Analysis of the Microsomal Protein for the Incorporation of Radioactive Precursors

Resuspended microsomal protein was analysed for the incorporation of radioactivity as shown in Scheme 4.

d) Determination of the Specific Activity of Vitellogenin Isolated from the Incubation Medium

A small sample (approximately 0.25 mg) of the labelled vitellogenin isolated from the incubation medium was dissolved in 0.5 mls of 0.15 M NaCl. The protein concentration of 25-100 µl samples of this solution was determined by the method of Lowry et al (1951). Similar aliquots of the solution were applied to filter papers and the protein-bound radioactivity was determined as shown in Scheme 3. Alternatively, the protein-bound radioactivity was determined by adding aliquots of the labelled vitellogenin solution to 1 ml of a solution containing approximately 5 mg of cold, carrier vitellogenin. The protein was then TCA precipitated and washed as shown in Scheme 4. Knowing the protein concentration and protein-bound radioactivity the specific activity of the vitellogenin was calculated.

2.2.8 Polyacrylamide Gel Electrophoresis

Two methods of polyacrylamide gel electrophoresis were used in this work:-

a) Non-SDS polyacrylamide gel electrophoresis

b) SDS polyacrylamide gel electrophoresis.

Both of these sytems involve the use of double-stack polyacrylamide gels, with a large-pore upper gel acting as a

stacking gel and concentrating the protein into a fine band before separation in the small pore lower gel.

a) Non-SDS Polyacrylamide Gel Electrophoresis

Preparation of Gels

Stock acrylamide and buffer solutions were prepared as shown in Table 6a. The gels were cast in 8 cm perspex tubes which were sealed at one end with a double-layer of Parafilm.

Stock solutions for the lower, small pore gel were mixed in the proportions shown in Table 6b to give the desired gel concentration. (In this work only 5% gels were used, but details of 4-10% gels are included for reference.) The mixture was then de-aerated on a water pump. The gels were poured to a height of approximately 6 cms and a thin layer of distilled water was pipetted over the top in order to achieve a flat surface after polymerization. The gel solution took about 30 minutes to polymerize.

When polymerization of the lower gel was complete, the solutions for the upper, large-pore gel were mixed in the proportions shown in Table 6b and de-aerated. The layer of water was removed from the top of the lower gel and the upper solution added to a depth of 0.75 cms ; water was again overlaid. The gels were exposed to light from a 200 watt bulb placed directly above them so that the photo-polymerization of the acrylamide in the upper gel could be initiated by the action of riboflavin. Polymerization took about 15 minutes, after which the upper gel appeared white and opaque. The gel was left for a further 30 minutes and a small hole was made in the Parafilm before electrophoresis.

Preparation of Samples for Electrophoresis

50-100 µl samples of incubation medium were applied directly to the gels. When precipitated vitellogenin was to be analysed, it was dissolved in Tris/glycine reservoir buffer, and a sample volume of 10-100 µl containing up to 50 µg of protein was applied to the gels.

TABLE 6a SOLUTIONS FOR NON-SDS POLYACRYLAMIDE GEL ELECTROPHORESIS

Lower Gel
(Small-Pore)

Gel Buffer (1a)

Tris	36.3 g
N.HCl	48.0 ml
TEMED	0.92 ml
H_2O to	100 ml

Acrylamide Solution (2a)

Acrylamide	30.0 g
Bis	0.8 g
$K_3Fe(CN)_6$	15 mg
H_2O to	100 ml

Initiator (3a)

Ammonium persulphate	0.28 g
H_2O to	100 ml

(freshly prepared)

Upper Gel
(Large-Pore)

Gel Buffer (1b)

Tris	5.8 g
$N.H_2PO_4$	25.6 ml
H_2O to	100 ml

Acrylamide Solution (2b)

Acrylamide	10.0 g
Bis	2.5 g
H_2O to	100 ml

Initiator (3b)

Riboflavin	4 mg
H_2O to	100 ml

Reservoir Buffer - Tris/glycine

Tris	6 g
Glycine	28.8 g
H_2O to	1 litre
pH	8.6

(Solutions had a shelf-life of 3 months when kept refridgerated in amber glass bottles)

TABLE 6b PREPARATION OF NON-SDS GELS

Solutions were mixed in the following proportions:-

Lower Gel

Solution	% Acrylamide						
	4	5	6	7	8	9	10
1a	1.0	1.0	1.0	1.0	1.0	1.0	1.0
2a	1.3	1.6	2.0	2.3	2.7	3.0	3.3
3a	4.0	4.0	4.0	4.0	4.0	4.0	4.0
H_2O	3.7	3.4	3.0	2.7	2.3	2.0	1.7

Upper Gel

1b	1.0
2b	2.0
3b	1.0
H_2O	4.0

Electrophoresis

The electrophoresis was carried out in a Shandon disc-gel electrophoresis apparatus using the same buffer in the upper and lower chambers. A drop of saturated sucrose solution containing bromophenol blue was added to the samples - since the dye runs with the buffer front, this allowed the distance of migration to be followed. A sample volume of 10-100 µl, containing up to 50 µg of protein was then layered over each gel, the sucrose in the indicator solution giving the sample sufficient density to displace the upper reservoir buffer.

A Shandon power pack was used for the electrophoresis and was set to deliver a constant current, with the electrode in the lower buffer connected to the anode. A current of 2 mA/gel was passed for approximately 5 minutes to allow the protein to migrate into the large-pore gel and form a fine band. The current was then increased to 5 mA/gel. The electrophoresis was continued until the dye front had reached 0.5-1 cm from the end of the gel tube ; this took about 1 hour.

The tubes were removed from the apparatus and the gels squeezed out into 7% v/v acetic acid. After fixing theprotein bands for 1 hour, the gels were stained for protein with 0.2% Coomassie Brilliant Blue solution in 10% v/v acetic acid, 50% v/v methanol for 1½-2 hours. The gels were then destained by allowing the stain to diffuse out into 10% v/v acetic acid, 20% v/v methanol, with frequent changes of the destainer.

Destained gels could then be scanned for protein in a Joyce-Loebl gel scanner at 550 nm.

b) SDS Polyacrylamide Gel Electrophoresis

Preparation of Gels

The preparation of the gels and the electrophoresis procedure was based on the method of Neville (1971). However, in the lower gel, the normal cross-linker, bis, was replaced by diethyldiallyltartardiimide (DATD), this

meant that the gels could be solublized in 2% periodate solution instead of NCS solublizer (see later). Stock acrylamide and buffer solutions were prepared as shown in Table 7a.

The stock solutions for the lower, small-pore gel were mixed in the proportions shown in Table 7b to give the desired concentration. (In this work only 7.5% gels were used, but details of 5% and 11% gels are included for reference.) After de-aeration, TEMED was added to initiate polymerization. The gels were cast to a height of 5-6 cms in Parafilm sealed Perspex tubes and distilled water was layered over the surface. When polymerization was complete (20-30 minutes), the water layer was removed and the upper gel solution mixed as shown in Table 7b, de-aerated, and applied to a depth of 0.75-1 cm. The upper gel was left to chemically polymerize on top of the lower gel, this took approximately 10 minutes, and the gels were left for a further 30 minutes prior to electrophoresis. The gels could be prepared up to 24 hours before use and kept overnight in a refridgerator.

Preparation of Samples

Samples were prepared for electrophoresis as shown in Table 8.

Electrophoresis

The gels were placed in the electrophoresis apparatus and the upper and lower reservoirs were filled with the appropriate buffers. Up to 250 µl of sample could be applied to each gel, containing as much as 150 µg of protein. A drop of saturated sucrose solution containing bromophenol blue was added to the samples before they were applied to the gels. The samples were electrophoresed towards the anode at 1 mA/gel for about 10 minutes to allow the protein to form a fine band in the upper gel. The current was then increased to 2.5 mA/gel, and the dye front took 2-2½ hours to run the length of the tube. The gels were removed from the tubes and the dye front marked with a piece of Ni-chrome wire.

TABLE 7a SOLUTIONS FOR SDS POLYACRYLAMIDE GEL ELECTROPHORESIS

Lower Gel
(Small-pore)

Acrylamide Solution (1a)

Acrylamide	27.5 g
DATD	0.369 g
H_2O to	100 ml

Gel Buffer (2a)

Tris	12.85 g
H_2O to	100 ml

pH to 9.18 with HCl

Initiator (3a)

Ammonium persulphate	0.3 g
H_2O to	100 ml

(freshly prepared)

Upper Gel
(Large-pore)

Acrylamide Solution (1c)

Acrylamide	7.5 g
Bis	0.6 g
H_2O to	100 ml

Gel Buffer (2c)

Tris	1.64 g
H_2O to	100 ml

pH to 6.1 with H_2SO_4

Initiator - as (3a)

Reservoir Buffers

Upper Buffer

Tris/SDS/Borate

Tris	4.965 g
SDS	1.0 g
H_2O to	1 litre

pH to 8.64 with solid boric acid

Lower Buffer

Tris/HCl

Tris	51.39 g
H_2O to	1 litre

pH to 9.5 with HCl

TABLE 7b PREPARATION OF SDS GELS

Solutions were mixed in the following proportions:-

Lower Gel

Solution	% Acrylamide		
	11	7.5	5
1a	6.0	4.0	3.0
2a	6.0	6.0	6.0
3a	3.0	2.5	2.0
H_2O	-	2.0	5.5
TEMED	22 µl	22 µl	22 µl

Upper Gel

1c	2.0
2c	2.0
3a	1.0
TEMED	7 µl

TABLE 8 PREPARATION OF SAMPLES FOR SDS POLYACRYLAMIDE GEL ELECTROPHORESIS

Sample	Treatment
Precipitated Vitellogenin	Dissolved in 2% SDS, 10% 2-mercaptoethanol, 50 mM Na_2CO_3
Incubation Medium	Incubated at a final concentration of 4% SDS, 10% 2-mercaptoethanol, 50 mM Na_2CO_3 at 100°C for 5 min
Microsomal and Post-Microsomal Pellets	Resuspended in TKMS and solublized at a final concentration of 2% SDS, 10% 2-mercaptoethanol, 50 mM Na_2CO_3 at 100°C for 5 min (in presence of PMSF)
Post-Microsomal Supernatant* and Final Supernatant	Incubate at a final concentration of 2% SDS, 10% 2-mercaptoethanol, 50 mM Na_2CO_3 at 100°C for 5 min (in presence of PMSF)

(*The post-microsomal supernatant fraction was always analysed immediately on isolation.)

After fixing for 1 hour in 7% v/v acetic acid, the gels were stained in Coomassie Brilliant Blue solution in 10%v/v acetic acid, 50% v/v methanol, at a concentration of 0.1% (stained overnight) or 0.25% (stained for approximately 2 hours). The gels were then destained in 10% v/v acetic acid, 25% v/v methanol with frequent changes of destainer. Destained gels could be scanned for protein at 550 nm using a Joyce Loebl gel scanner.

c) The Determination of Radioactivity on Polyacrylamide Gels

In order to determine the distribution of radioactivity on polyacrylamide gels they were sliced, and the slices solublized and counted. In order to do this the gel to be counted was first frozen in an enamel dish containing crushed solid CO_2 and then sliced, either using a Mickel automatic gel slicer, or by placing the frozen gel on graph paper and using a razor blade to cut slices of the required size.

Solublization of Non-SDS Gels

Non-SDS gels, in which bis was used a cross-linker, were solublized using NCS solublizer. 2 mm slices were cut and each slice was placed in a scintillation vial containing 0.5 mls of NCS. The vials were tightly capped and left at 50°C overnight. After this treatment the samples were allowed to cool and 15 mls of toluene/butyl PBD was added to each sample. It should be emphasized that during this procedure although the protein was digested, the actual gel slice was left intact. The vials were left to cool in the dark for at least one hour before counting.

Solublization of SDS Gels

During the preparation of SDS gels, in most cases the normal cross-linker, bis, was replaced by diethyldiallyltartardiimide (DATD) in equimolar quantities (Baumann and Chrambach, 1976), and this meant that the gel slices could be solublized in 2% periodate instead of NCS. Also, because of the number of gels to be analyzed during some of the experiments, the size of the slices cut from the lower half

of the gels was often increased from 2 mm to 5 mm, and thus this reduced the number of samples to be counted. Each 2 mm slice was placed in a scintillation vial containing 0.4 mls of 2% sodium periodate (for 5 mm slices this was increased to 0.8 mls) and the slices left to dissolve at room temperature, this took 30-60 minutes. In this method the slices were completely dissolved. 1 ml of absolute alcohol, 10 mls of Tritoscint and a drop of glacial acetic acid were then added to each vial and the vials left to cool in the dark for at least one hour before counting.

2.2.9 The Incorporation of Labelled Nucleotide-Sugars into Glycolipid Intermediates by Microsomal Preparations

a) Preparation of Microsomes

Control or oestrogen-treated Xenopus were pithed and their livers removed into ice-cold TKMS buffer. 2 g of liver was weighed out and the tissue finely minced using scissors and then transferred to 10 mls of fresh TKMS buffer in an homogenizing tube. The mince was homogenized with just 3 full strokes of a loose-fitting pestle (approximately 1 mm clearance) using a Tri-R-Stir-R homogenizer at speed setting 3.5. The microsomes were then prepared from the tissue homogenate as described in section 2.2.6 a).

b) Resuspension of Microsomes

The microsomes prepared from 2 g of liver were resuspended by gentle homogenization in 2.5 mls of 0.05 M Tris/maleate, pH 7.5, at 0°C (to give a protein concentration of approximately 4-8 mg/ml). The protein concentration was determined accurately by the method of Lowry et al (1951).

c) Incubation Conditions

The incubation conditions were similar to those described by Palamarczyk and Hemming (1971). 0.4 or 0.8 ml aliquots of fresh microsomal suspension were incubated with the following additions ; 8 mM $MnCl_2$, 1 mM EDTA, 8 mM 2-mercaptoethanol (these were added from a 10x concentration stock solution) and either UDP-D (6-^{3}H) galactose

(0.5 - 2 μCi), UDP-N-acetyl-D (U-^{14}C) glucosamine (0.05 - 0.1 μCi) or GDP (U-^{14}C) mannose (0.05 - 0.1μCi). The incubations were normally for one hour at 22°C, with constant shaking, at 60-70 strokes/minute. At the end of the incubation period the incubations were terminated by the addition of 4 volumes of 2:1 (v/v) chloroform/methanol and rotomixing. In some experiments 0.3 ml aliquots of the incubation mixture were retained for polyacrylamide gel analysis before the addition of chloroform/methanol.

d) Extraction of Lipid from the Microsomal Incubation Mixture

Lipid was extracted from the microsomal incubation mixture as shown in Scheme 5.

2.2.10 The Incorporation of Labelled Sugars into Glycolipid Intermediates by Liver Slices

a) Preparation of Liver Slices and Incubation Conditions

Liver slices were prepared and incubated with either 50 μCi of (^{3}H) galactose or 50 μCi of (^{3}H) glucosamine as described in section 2.2.4. At the end of the incubation time the incubation medium was removed and the slices washed twice with 5 mls of ice-cold TKMS buffer. The slices were then transferred to an homogenizing tube with 2 mls of TKMS buffer and the slices homogenized with a tight-fitting pestle using a Tri-R-Stir-R homogenizer at a speed setting 3.5 ; twelve passes of the pestle were made.

b) Extraction of Lipid from the Liver Slice Homogenate

Lipid was extracted from the liver slice homogenate as shown in Scheme 6. The procedure was similar to the extraction of lipid from the microsomal incubation mixture but after extraction with 2:1 chloroform/methanol, the residue was further extracted with 10:10:3 chloroform/methanol/water. The washed 2:1 chloroform/methanol extract and the 10:10:3 chloroform/methanol/water extract were pooled for chromatographic analysis.

SCHEME 5 EXTRACTION OF LIPID FROM MICROSOMAL INCUBATIONS

Incubation terminated by
addition of 4 vols. $CHCl_3$/MeOH, 2:1 (v/v)

↓

Rotomix and centrifuge

↓

Upper aqueous layer
Lower organic layer
Residue at interface

↓

Remove upper aqueous layer and discard.
Carefully collect the lower organic layer
without disturbing the interface, and
retain the organic extract

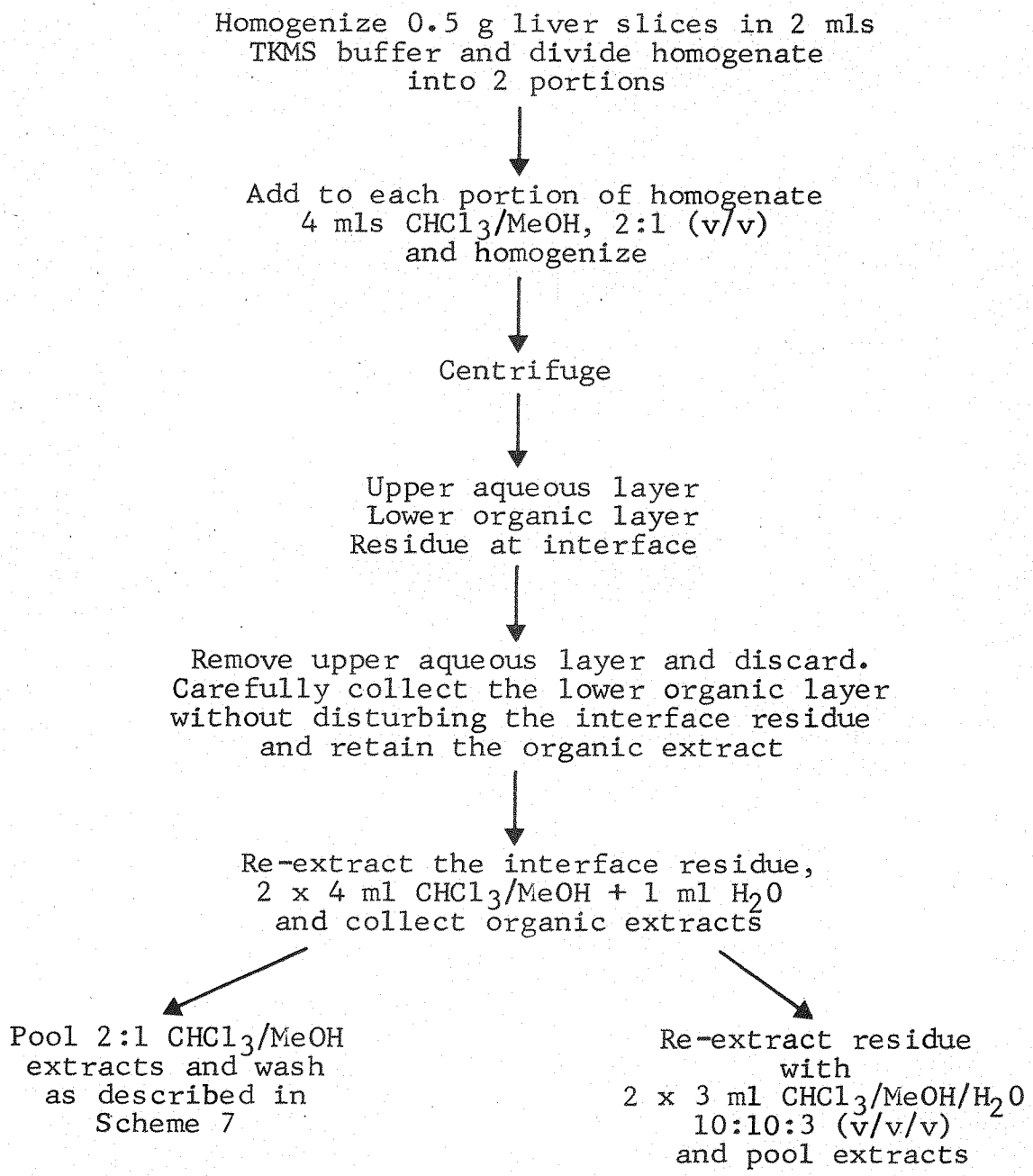
↓

Re-extract the interface residue,
1 x 2 ml $CHCl_3$/MeOH + 0.5 mls Tris/maleate
1 x 2 ml $CHCl_3$/MeOH + 0.5 mls H_2O
and collect organic extracts

↓

Pool 2:1 $CHCl_3$/MeOH extracts and wash
as described in Scheme 7

SCHEME 6 EXTRACTION OF LIPID FROM LIVER SLICE HOMOGENATE



SCHEME 7 WASHING OF THE 2:1 CHLOROFORM/METHANOL EXTRACT

To the 2:1 $CHCl_3$/MeOH extract
add 0.25 vols. of Theoretical Upper Phase*
and wash by gentle mixing

↓

Centrifuge, and discard upper
aqueous layer. Wash organic
layer once more as described above

↓

Centrifuge, and discard upper
aqueous layer. Wash organic
layer with 0.25 vols. of Salt-free
Theoretical Upper Phase*

↓

Centrifuge and discard aqueous layer

↓

Evaporate sample of washed extract
to dryness and dissolve in
50% toluene/butyl PBD/50% methanol
for counting

*Theoretical Upper Phase -
$CHCl_3$/MeOH/H_2O/1 M $MgCl_2$ (3:48:47:0.168)

Salt-free Theoretical Upper Phase -
$CHCl_3$/MeOH/H_2O (3:48:47)

(Folch et al, 1957)

2.2.11 DEAE-Cellulose Acetate Chromatography of the Labelled Lipid Extract

a) Preparation of Columns

The chromatographic procedure adopted was based on the method described by Barr and Hemming (1972). The DEAE-cellulose acetate was prepared by leaving DE 52 cellulose in contact with glacial acetic acid overnight. The excess acetic acid was washed out with methanol on a Buchner funnel until no smell of acetic acid remained. The DEAE-cellulose acetate was stored suspended in methanol. The columns were packed (dimensions 12 cm x 0.8 cm diameter) and washed with 50 mls of chloroform/methanol, 2:1 (v/v) before the application of the samples.

b) Preparation of Samples of Lipid Extract for Chromatography

For analysis of the lipid extract obtained from microsomal incubations:- the sample of washed 2:1 chloroform/methanol extract to be chromatographed was evaporated to dryness in a round-bottomed flask, and then re-dissolved in 2-3 ml of chloroform/methanol, 2:1 (v/v). The sample was then ready for application to the column.

For analysis of the lipid extract obtained from liver slice homogenates:- the washed 2:1 chloroform/methanol extract and the 10:10:3 chloroform/methanol/water extract were pooled and evaporated to dryness in a round-bottomed flask. The sample was then re-dissolved by first washing around the sides of the flask with 0.5 ml of 10:10:3 chloroform/methanol/water (v/v/v), and then adding 2.5 ml of chloroform/methanol, 2:1 (v/v) to this. The sample was then ready for application to the column.

c) Elution of Columns

The samples prepared as described above were applied to the columns and eluted successively with chloroform/methanol, 2:1 (v/v) ; methanol ; 0.01 M ammonium acetate in 2:1 (v/v) chloroform/methanol and 0.05 M ammonium acetate in 2:1 (v/v) chloroform/methanol. The ammonium acetate solutions were prepared by adding to chloroform/methanol, 2:1 (v/v), the appropriate volume of a stock solution of

2.5 M ammonium acetate in methanol, containing 3% v/v acetic acid.

For the analysis of samples obtained from microsomal incubations, the columns were eluted with:-

30 ml chloroform/methanol, 2:1 (v/v)
20 ml methanol
50 ml 0.01 M ammonium acetate
50 ml 0.05 M ammonium acetate

and for the analysis of samples obtained from total tissue homogenates, the columns were eluted with:-

50 ml chloroform/methanol, 2:1 (v/v)
30 ml methanol
50 ml 0.01 M ammonium acetate
50 ml 0.05 M ammonium acetate.

The elution was carried out at room-temperature and the flow-rate was approximately 60 ml/hour. 5 or 10 ml fractions were collected, and samples evaporated to dryness and counted for radioactivity by the addition of 10 mls of 50% toluene/butyl PBD/50% methanol.

2.2.12 Further Analysis of the Labelled Fractions Eluted from the DEAE-Cellulose Acetate Columns

a) Thin-Layer Chromatography

Thin layer plates were prepared manually using a slurry of non-fluorescent silica gel in water. The samples to be chromatographed (fractions eluted from the DEAE-cellulose acetate columns by ammonium acetate) were washed twice with 0.25 vols of water to remove the ammonium acetate salt which could affect the chromatography. They were then evaporated to dryness and the residue taken up in approximately 0.2 mls of chloroform/methanol, 2:1 (v/v). The samples were then spotted onto the plates. The solvent systems used were chloroform/methanol/water, 65:25:4 (v/v/v) and isopropanol/ammonia/water, 6:3:1(v/v/v). After running, the solvent front was marked and 1 cm bands were scraped from the plates for the determination of radioactivity ; the radioactivity was eluted for 30 minutes with 2 ml of methanol in a scintillation vial. 15 ml of

toluene/butyl PBD was then added to each vial and the samples counted.

b) Mild Acid Hydrolysis Procedure

The acid hydrolysis procedure was as described by Richards and Hemming (1972). The sample to be hydrolysed was evaporated to dryness and mixed with 1 ml of methanol/water, 1:1(v/v), containing 0.01 M HCl, and heated at 100°C for 20 minutes. The mixture was then neutralized by adding 1 ml of 0.1 M NaOH in methanol. 2 ml of chloroform and 2 ml of water were added and, after mixing, the mixture was separated into two layers by centrifugation. Samples of the aqueous layer and the chloroform layer (evaporated to dryness) were counted for radioactivity in 10 mls of Tritoscint or 10 ml of 50 % toluene/butyl PBD/50% methanol respectively.

c) Paper Chromatography of the Labelled Sugars Released by Acid Hydrolysis

The labelled sugars released from the lipid fraction into the aqueous phase during the acid hydrolysis procedure were analysed by descending paper chromatography using the upper layer formed from a mixture of butanol/ethanol/water 4:1:5 (v/v/v) as the solvent system. The aqueous sample containing the labelled sugar was first rotomixed with a small quantity of "Amberlite" resin in order to remove the salt resulting from the neutralization of the acid ; the resin was then removed by filtering the solution through glass wool. The sample was then evaporated to dryness, redissolved in 0.2 mls of distilled water and spotted on Whatman No. 1 chromatography paper. 20µg-40µg samples of glucose, raffinose, lactose and N-acetyl-glucosamine were spotted as standards. The chromatogram was run for 36-48 hours. After the run, the radioactivity was located by cutting the lane containing the radioactive sample into 1 cm strips and placing each strip into a scintillation vial. The radioactivity was eluted with 5 mls of methanol for 30 minutes ; 10 ml of toluene/butyl PBD was then added and the samples counted.

The positions of the standard sugars were determined by staining with silver nitrate. 0.2 ml of saturated

silver nitrate was diluted to 20 ml with acetone, and distilled water was added until the precipitate just dissolved. The paper was dipped into this solution and when dry was taken through 10% ethanolic NaOH and then through sodium thiosulphate to clear the background, and, finally, washed in cold, running water. The standard sugars stained as brown or black spots.

2.2.13 Assay of Glucose-6-Phosphatase and 5'-Nucleotidase Activity

a) Preparations of Fractions for Assay

2 control and 2 oestrogen-treated Xenopus were used to determine the glucose-6-phosphatase and 5'-nucleotidase activity of liver subfractions. 2 g samples of liver from each animal were sliced and homogenized in 8 mls of TKMS buffer. A sample of the total tissue homogenate was retained and the remainder was fractionated as shown in Scheme 1 to obtain the microsomal pellet, the post-microsomal pellet and the final supernatant. The microsomal pellet was resuspended in 5 ml of TKMS buffer and the post-microsomal pellet in 3 ml of TKMS buffer. 100µ and 200 µ l samples of total tissue homogenate, microsomal suspension, post-microsomal pellet suspension and final supernatant were assayed for glucose-6-phosphatase and 5'-nucleotidase activity as described below.

b) Glucose-6-Phosphatase Assay

100 µl and 200 µl aliquots of the fractions were incubated with 0.3 mls, 0.2 M dimethylglutarate (pH 6.5) ; 0.1 ml, 0.1 M EDTA and 0.1 ml, 0.1 M glucose-6-phosphate. Incubations were for 1 hour at 22°C with constant shaking at 70 strokes/minute. The reaction was stopped by the addition of 1.5 mls, 6% TCA and the protein was precipitated for 1 hour at 0°C ; the tubes were then centrifuged and the supernatant removed and assayed for phosphate as described below. The protein precipitate was resuspended in 2 mls H_2O and the protein content of 100µ and 200 µl samples determined by the method of Lowry et al (1951).

c) 5'-Nucleotidase Assay

100 µl and 200 µl aliquots of the fractions were incubated with 0.35 ml, 0.25 M Tris (pH 8.0) ; 0.05 ml, 0.05 M $MgCl_2$ and 0.1 ml, 0.025 M AMP. The tubes were incubated and the reaction terminated as in the glucose-6-phosphatase assay described above. Samples of the supernatant were assayed for phosphate as described below.

d) Inorganic Phosphorus Determination

0.4 ml samples of supernatant were used for an assay of the inorganic phosphate released during the incubations. 1.5 ml of 0.6% ammonium molybdate in 5% perchloric acid was added to 0.4 ml samples of supernatant. The phospho-molybdate complex was reduced with 0.5 ml (2 mg/ml) ascorbic acid and the blue colour was allowed to develop for 30 minutes and then read at 750 nm. A standard curve was set up using 0.05 - 1 µmole of phosphate. The enzyme activities were expressed as µ moles phosphate released/mg protein/hour after tissue and substrate blanks has been subtracted from experimental readings.

2.2.14 Electron Microscopy

a) The Microsomal Pellet

The microsomal pellet, prepared as described in section 2.2.6 a), was fixed in 1% osmium tetroxide, buffered with 0.05 M cacodylate (pH 7.4) and containing 0.25 M sucrose, for 90 minutes at room temperature, and then washed in cacodylate wash for 3 hours. The sample was dehydrated through graded ethanol, and then embedded in Spurr resin overnight at 70°C. Ultra-thin sections were cut on a Cambridge/Huxley Ultra-Microtome using glass knives and counter-stained with urenyl-acetate and lead citrate. The sections were then examined in an Hitachi HU-12 electron microscope.

b) The Post-Microsomal Pellet

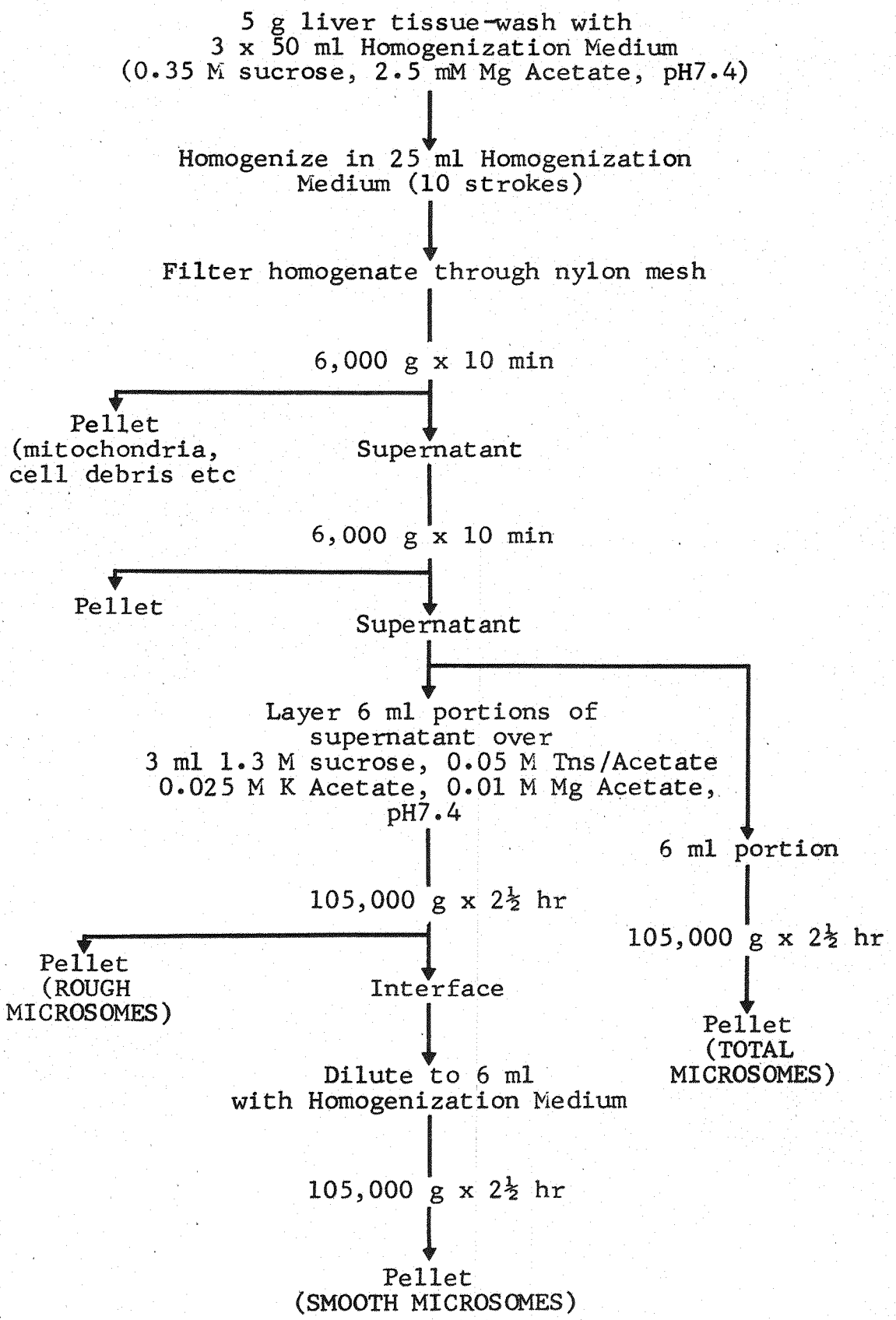
In order to improve the fixation of the post-microsomal pellet, equal volumes of the post-microsomal

supernatant and 6% glutaraldehyde, in 0.25 M glucose, 0.05 M cacodylate, were mixed and then centrifuged at 105,000 g for 3 hours to obtain the post-microsomal pellet. Fixation was terminated by a cacodylate wash, and then the tissue was sliced into smaller pieces, and the washing continued for a further 30 minutes. The sample was then post-fixed in 1% osmium tetroxide and processed as described for the microsomal pellet.

Addendum Preparation of Rough and Smooth Microsomal Fractions from Rat and Xenopus Liver

Rough and Smooth Microsomal Fractions were prepared as described by Lewis and Tata (1973). The method is outlined in Scheme 8. The pellets were resuspended in 0.35 M sucrose, 0.05 M Tris/Acetate, pH 7.4, 0.025 M K Acetate, 0.01 M Mg Acetate and the glucose-6-phosphatase activity of the fractions determined as described in 2 2 13

SCHEME 8 PREPARATION OF ROUGH AND SMOOTH MICROSOMAL FRACTIONS FROM RAT AND XENOPUS LIVER



CHAPTER 3

Chapter 3

THE INCORPORATION OF RADIOACTIVE PRECURSORS INTO SECRETED VITELLOGENIN BY LIVER SLICES

The livers of oestrogen-treated Xenopus laevis (South African clawed toad) biosynthesize and secrete into the blood large quantities of the serum protein vitellogenin (Munday et al, 1968 ; Follett and Redshaw, 1968 ; Wallace and Dumont, 1968). Vitellogenin is a Ca^{2+}-binding, glycolipophosphoprotein with a molecular weight of 450,000-550,000 (Ansari et al, 1971 ; Redshaw and Follett, 1971), and is a dimer, made up of two similar sized subunits, molecular weight 200,000-220,000 (Bergink and Wallace, 1974a; Clemens et al, 1975). Studies by Wallace have shown that, under the influence of gonadotrophins, vitellogenin is selectively removed from the serum by the ovaries, and is cleaved within the oocytes to give its two component proteins, lipovitellin and phosvitin (Wallace and Dumont, 1968 ; Wallace and Jared, 1969). These are the egg-yolk proteins and have subunit molecular weights of approximately 0.17×10^5 for phosvitin (Ohlendorf et al, 1971) and 1.1 and 0.3×10^5 for lipovitellin (Bergink and Wallace, 1974a).

The amino-acid and non-protein components of vitellogenin are extremely unevenly distributed between the regions of the parent molecule destined to become the two yolk-proteins ; thus phosvitin is unique in that 56% of its amino-acids are serine residues, and, since 70% of the serine residues are phosphorylated, the region of the parent molecule destined to become phosvitin contains most of the phosphorus. Almost all of the lipid associated with vitellogenin is accounted for by that in lipovitellin, so the lipovitellin region of the parent molecule contains most of the lipid (see Table 4 for the structural features

of Xenopus vitellogenin, lipovitellin and phosvitin).

During the period when this part of the work was being undertaken, experiments were being carried out in this laboratory to investigate the uptake, cleavage and processing of vitellogenin by human chorionic gonadotrophin (HCG)-primed, isolated oocytes. These studies required labelled vitellogenin of a very high specific activity so that the processing events inside the oocytes could be followed. The most successful way of obtaining very highly labelled vitellogenin for use in this work was by chemically iodinating vitellogenin purified from Xenopus serum with ^{125}I, using the lactoperoxidase method. Vitellogenin with a specific activity of 40 - 100 x 10^6 dpm/mg was obtained using this procedure.

However, it was felt that if possible it would be preferable to use non-chemically modified labelled vitellogenin for some of the uptake experiments. Previous work in this laboratory (Dolphin et al, 1971 ; Merry et al, 1973) has shown that during the in vitro incubation of liver slices from oestrogen treated toads, impressive amounts of labelled amino-acids are incorporated into vitellogenin secreted into the incubation medium. We planned to improve this system and to incorporate various labelled precursors into vitellogenin with a high specific activity, and then to isolate the pure protein for possible use in oocyte incorporation studies. With this system there was also the possibility of incorporating label into the regions of the parent molecule destined to become either phosvitin or lipovitellin, and this could be useful for following the cleavage of vitellogenin, and the appearance of the individual yolk-proteins.

3.1 The Incorporation of (^3H) leucine into Vitellogenin by Liver Slices

The first step was to establish that the labelled vitellogenin secreted into the incubation medium by liver slices could be isolated from the medium in an intact, undegraded form.

3.1.1 The Time-Course of Incorporation

Figure 8 shows the time-course of incorporation of (^{3}H) leucine into protein secreted into the incubation medium during the *in vitro* incubation of liver slices from oestrogen-treated toads. The lag phase of 90-120 mins associated with the secretion confirmed previous work in this laboratory (Dolphin *et al*, 1971 ; Merry *et al*, 1973) and the significance of this lag phase will be discussed in Chapter 4.

3.1.2 Polyacrylamide Gel Analysis of the Incubation Medium

Samples of the incubation medium taken after a 6 hour incubation period were analyzed by both non-SDS and SDS polyacrylamide gel electrophoresis. During non-SDS polyacrylamide gel electrophoresis, ie under non-denaturing conditions, native vitellogenin runs in its dimeric form, but any dissociation into the monomer form can be identified. During SDS polyacrylamide gel electrophoresis, vitellogenin is dissociated into its two component 200,000 mol. wt. sub-units.

The results of non-SDS polyacrylamide gel analysis (Figure 9) showed that nearly all of the protein-bound radioactivity released into the incubation medium was associated with a single protein peak (A), which corresponded to the position of the authentic vitellogenin dimer ; this confirmed the work of Dolphin *et al* (1971) and Merry *et al* (1973). The slight shoulder of protein and label (B), migrating just ahead of the main peak, represents a small proportion of vitellogenin monomer present in the sample ; the small peak (C) represents a small amount of polymerized vitellogenin.

Figure 10 shows the results of SDS polyacrylamide gel analysis ; all of the protein-bound radioactivity was associated with a single protein peak corresponding to the position of the 200,000 mol. wt. subunit of vitellogenin.

Figure 8

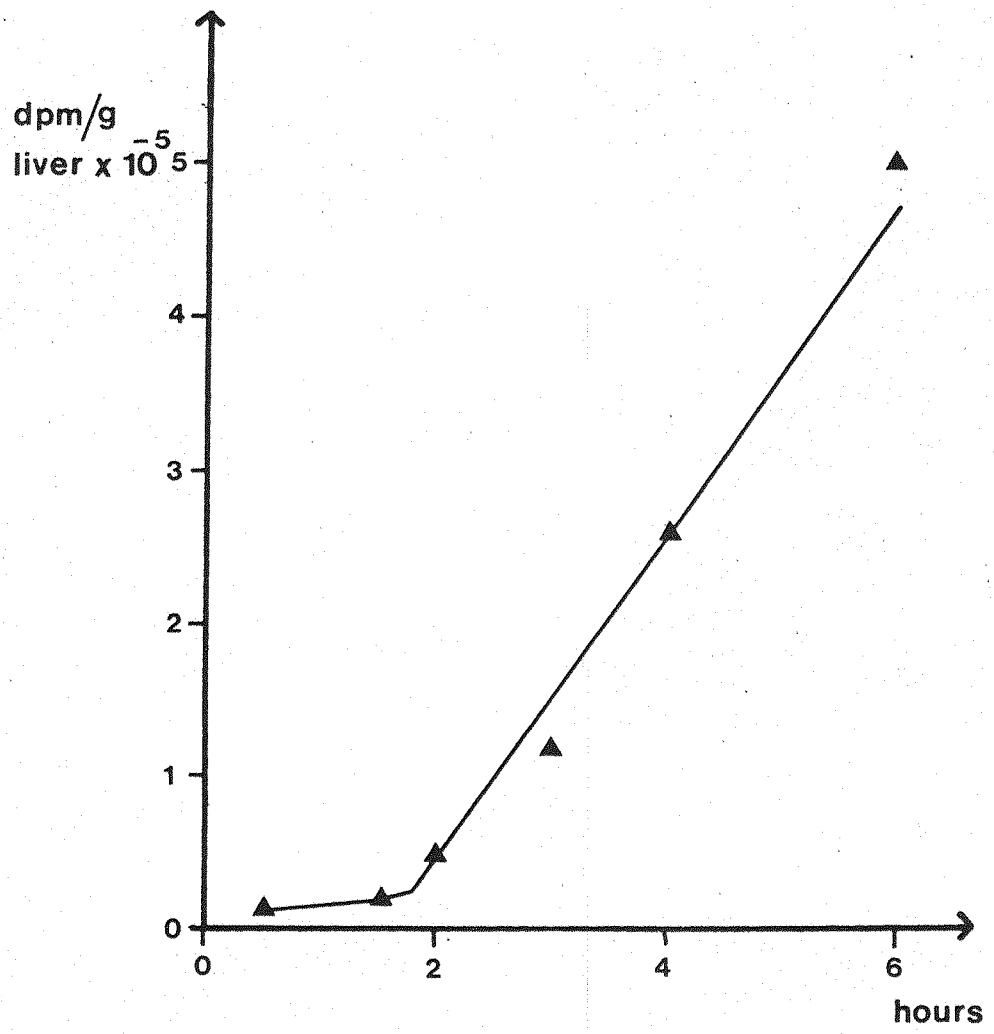


Figure 8 Incorporation of (^{3}H) Leucine into Secreted Protein

Liver slices (1 g) were incubated for 6 hours in 10 ml of phosphate-saline buffer with 10 μCi (^{3}H) leucine. At various times, 0.3 ml samples of the incubation medium were removed and analysed for the incorporation of radioactivity into secreted protein by the filter paper disc method as described in Chapter 2.

Figure 9

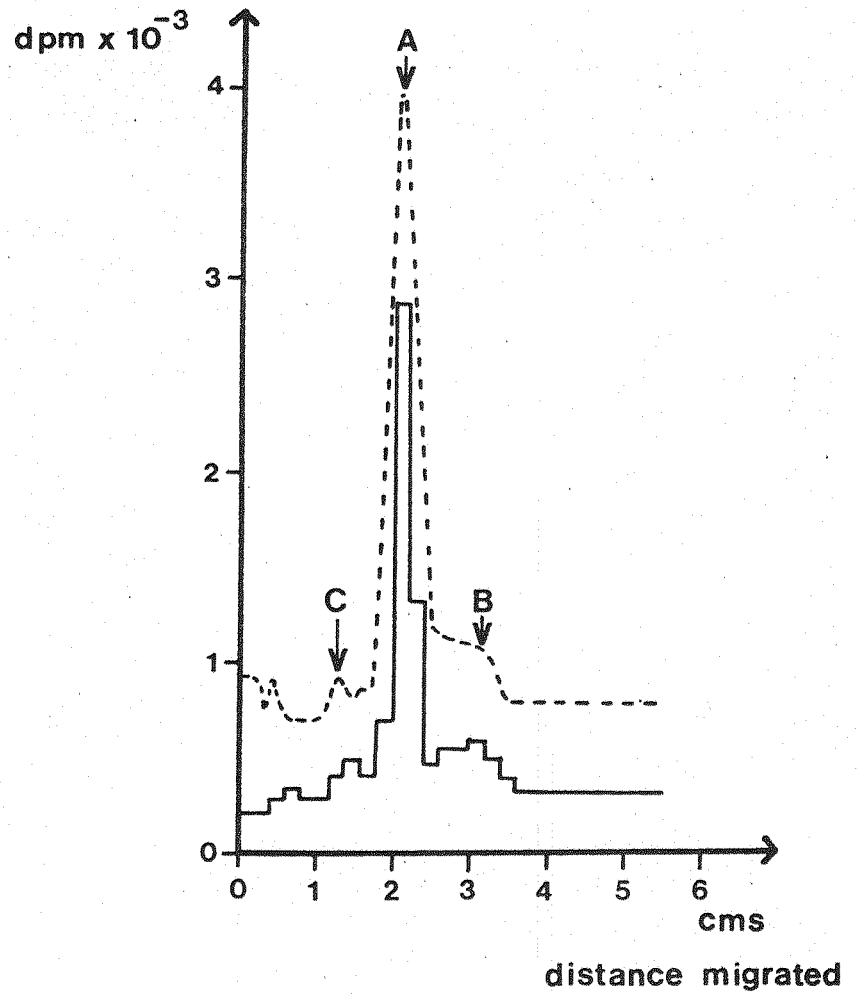


Figure 9 Non-SDS Polyacrylamide Gel Analysis of (^{3}H) Leucine-Labelled Incubation Medium

A 100 µl sample of the incubation medium obtained from the experiment described in Figure 8 was analysed on a 5% non-SDS polyacrylamide gel. The gel was stained for protein, scanned at 550 nm (dashed line) and the radioactivity in 2 mm slices determined (histogram).

Figure 10

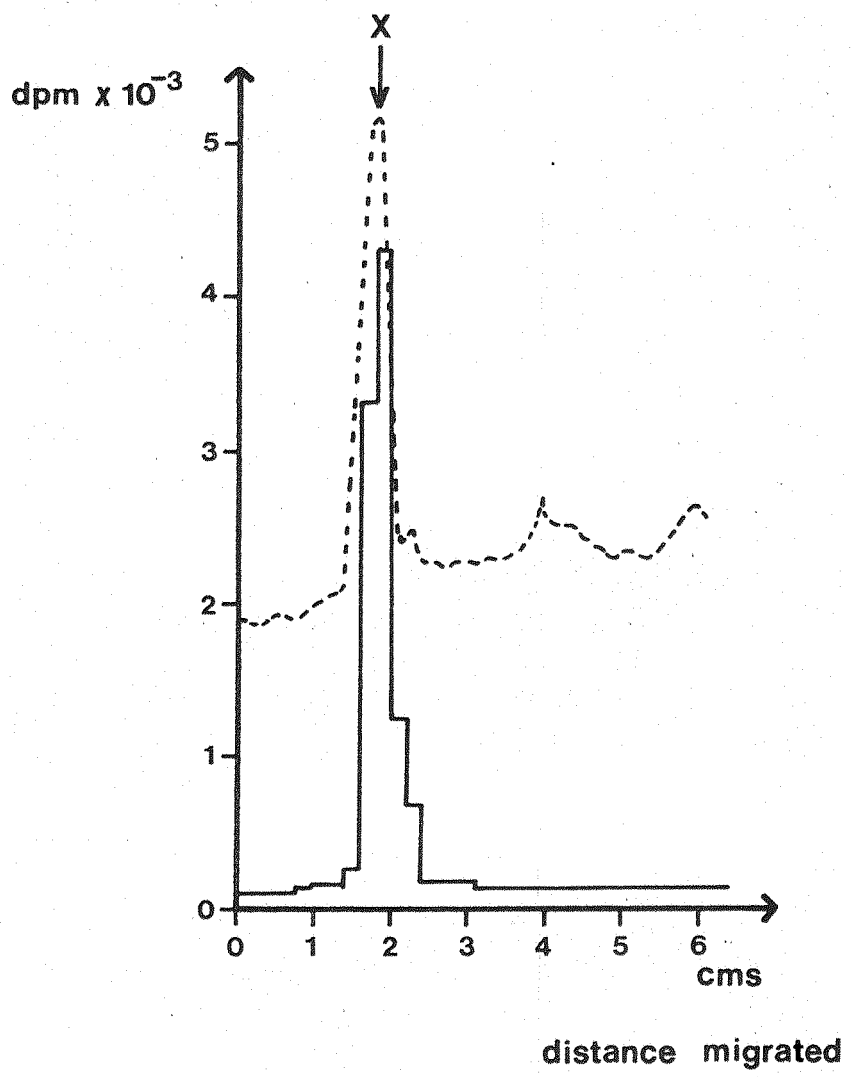


Figure 10 SDS Polyacrylamide Gel Analysis of (^{3}H) Leucine-Labelled Incubation Medium

A 100 µl sample of incubation medium obtained from the experiment described in Figure 8 was analysed on a 7.5% SDS polyacrylamide gel. The gel was stained for protein, scanned at 550 nm (dashed line) and the radioactivity in 2 mm slices determined (histogram). 'X' marks the position of polypeptide of mol. wt. approx. 200,000.

3.1.3 The Isolation of Vitellogenin from the Incubation Medium

Labelled vitellogenin was isolated from the incubation medium using the specific dimethylformamide/ acetic acid technique described by Munday et al (1968). After precipitation, the vitellogenin was dissolved in 0.9% NaCl, dialysed and freeze-dried for storage as described in Chapter 2. 3-5 mg of vitellogenin was isolated from each incubation. Non-SDS and SDS poly cryla-mide gel analysis confirmed that during this isolation procedure the vitellogenin maintained both its dimer structure and its undegraded 200,000 mol. wt. subunits. However, care was required during the acidification stage of the procedure, since over-acidification could lead to the dissociation of the dimer into its monomer subunits. In this section of the work it was necessary to analyse the vitellogenin obtained using both types of gels, since for oocyte incorporation studies it was required in its native dimer form with undegraded subunits ; previous work in this laboratory had shown that it was possible for a sample to appear in an authentic dimer form on non-SDS gels, but for SDS gel analysis to reveal that the component subunits were actually extensively degraded.

Therefore it was possible to use the in vitro liver slice technique to prepare labelled vitellogenin which could be isolated from the incubation medium in its native form.

3.1.4 The Specific Activity of the Isolated Vitellogenin

When 1 g of liver slices, taken from animals at 8-14 days after a single injection of oestrogen, were incubated with 10 μCi of (^{3}H) leucine for 6 hours in phosphate-saline buffer, they routinely incorporated 300,000-600,000 dpm/g liver into incubation medium vitellogenin, although there was considerable variation between animals. The estimated specific activity of the vitellogenin isolated from the incubation medium was 80-200,000 dpm/mg protein. For labelled vitellogenin to be considered suitable for isolated oocyte incorporation

studies, a specific activity of at least 1-2 x 10^6 dpm/mg was required to enable the uptake of the vitellogenin to be followed. The two possible ways of increasing the specific activity were either to increase the amount of labelled precursor added, or, to prolong the incubation period - a combination of the two would give the most economical use of the available precursor.

3.2 Modifications to the Incubation Medium for 24 hour Incubations

In previous work, the in vitro incubation of liver slices was performed in phosphate-saline buffer. However, to enable vitellogenin with a higher specific activity to be produced it was decided to extend the incubation period to 24 hours ; for this, an incubation medium similar to those used for long-term tissue culture experiments was more appropriate. A 50% dilution of Eagle's minimal essential medium with Earle's salts (minus leucine) with a final concentration of 20mM HEPES, as used by Wangh and Knowland (1975) was chosen. The deletion of leucine from the medium meant that the specific activity of the added (^{3}H) leucine would not be reduced. It was found that when liver slices were incubated in this medium for 24 hours with (^{3}H) leucine, the incorporation of radioactivity into secreted vitellogenin was approximately double that obtained in a parallel incubation with phosphate-saline buffer (Table 9). The effect of the modifications described below on the level of incorporation were tested, and the results are shown in Table 9.

(i) Amino-acids. Completely omitting all of the amino-acids from the incubation medium had no effect on the incorporation of (^{3}H) leucine into secreted vitellogenin during a 24 hour incubation.

(ii) Insulin. In some tissue culture procedures, insulin is added to facilitate the uptake and incorporation of amino-acids. However, insulin had no effect on this system.

(iii) Antibiotics. When vitellogenin was precipitated from 6 hour incubation medium, and then

TABLE 9

Expt 1 1 g of liver slices were incubated with 10 μCi of (^{3}H) leucine in either a) 10 mls of phosphate-saline buffer or b) 10 mls of Eagle's minimal essential medium + Earle's salts (-Leucine). At 6 hours and 24 hours, the incorporation of radioactivity into incubation medium protein was determined as described in Chapter 2.

Expts 2-4 1 g samples of liver slices were incubated with 10 μCi (^{3}H) leucine in 10 mls of Eagle's minimal essential medium + Earle's salts. The effect of omitting amino-acids from the incubation medium (Expt 2), the addition of insulin (Expt 3) and the addition of antibiotics (Expt 4) on the incorporation of radioactivity into incubation medium protein was determined in parallel incubations.

TABLE 9 THE INCORPORATION OF (^{3}H) LEUCINE INTO SECRETED PROTEIN WITH VARIOUS MODIFICATIONS TO THE INCUBATION MEDIUM

Medium	Modification	Incorporation into Secreted Protein (dpm/g liver x 10^{-5})	
		6 hours	24 hours
Expt 1			
a) Phosphate-Saline buffer		1.9	12.9
b) MEM + salts (-Leucine)		3.8	22.2
Expt 2			
MEM + salts	a) -Leucine	5.5	13.0
	b) -Amino-acids	5.0	13.1
Expt 3			
MEM + salts (-Leucine)	a) -Insulin	2.7	-
	b) +Insulin	2.8	-
Expt 4			
MEM + salts (-amino-acids)	a) -Antibiotics	8.5	14.5
	b) +Antibiotics	6.6	13.6

analysed by SDS polyacrylamide gel electrophoresis and the gel stained for protein with Coomassie Blue, there was only one major protein band, corresponding to the 200,000 mol. wt. subunit of vitellogenin (Figure 11a). However, when the vitellogenin precipitated from 24 hour incubation medium was analysed (Figure 11b), the 200,000 mol. wt. subunit was found to be extensively degraded, with less than 50% remaining as the intact vitellogenin subunit. The addition of the antibiotics penicillin, streptomycin, gentomycin and mycostatin, at final concentrations of 200 units/ml, 200 µg/ml, 70 µg/ml and 200 units/ml respectively prevented this degradation without reducing the level of incorporation, thus allowing intact vitellogenin to be isolated after a 24 hour incubation. The degradation of the vitellogenin was probably the result of its proteolytic digestion by enzymes released from the lysozomes after the breakdown of the liver slice tissue by bacterial action. The effect of the antibiotics may be to inhibit the breakdown of the liver slice tissue and thus prevent the release of the proteolytic enzymes.

Therefore, the incubation medium used for all subsequent <u>in vitro</u> liver slice incubations described in this thesis was a 50% dilution of Eagle's minimal essential medium with Earle's salts, minus amino-acids, with a final concentration of 20mM HEPES containing the antibiotics penicillin, streptomycin, gentomycin and mycostatin. The full composition is given in Table 5 in Chapter 2.

3.3 The Specific Activity of Vitellogenin Preparations isolated after 24 hour Incubations

Vitellogenin labelled with several precursors was isolated from the incubation medium after 24 hour incubations. Table 10 shows the specific activities of the vitellogenin preparations obtained. All samples were analysed by both SDS and non-SDS polyacrylamide gel electro-

Figure 11 SDS Polyacrylamide Gel Analysis of Vitellogenin Precipitated from a) 6 hour and b) 24 hour Incubation Medium

Vitellogenin was precipitated from samples of incubation medium taken at 6 hours, a), and at the end of a 24 hour incubation, b). Samples (approx. 40 µg) of the vitellogenin were analysed on 7.5% SDS polyacrylamide gels. The gels were stained for protein and scanned at 550 nm.

Figure 11

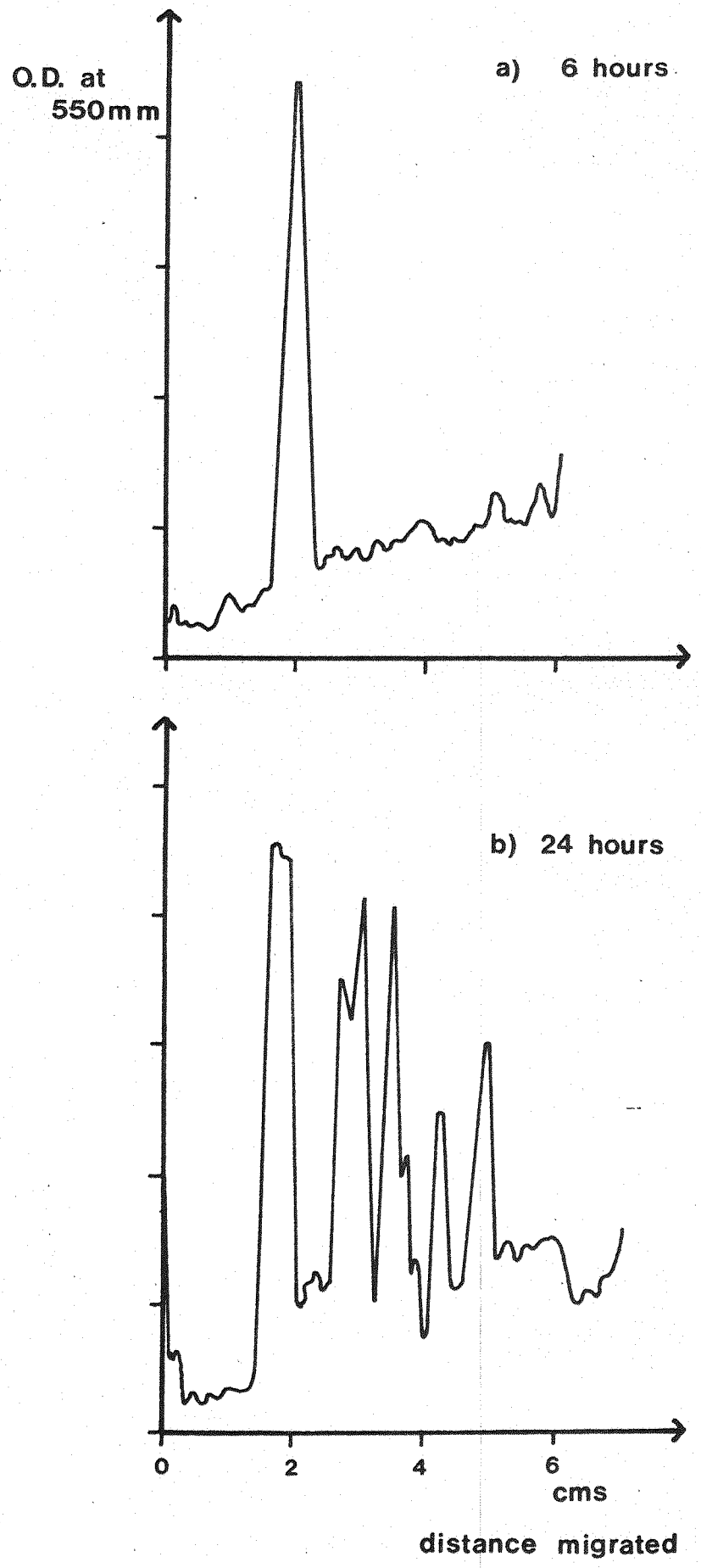


TABLE 10

1 g samples of liver slices were incubated in 7.5 mls of incubation medium with various isotopes for 24 hours. At the end of the incubations, vitellogenin was precipitated from the incubation medium and the specific activity determined as described in Chapter 2.

TABLE 11

At 10-12 days after oestrogen treatment, animals were injected with the isotopes shown. 48 hours later the animals were sacrificed, the blood collected, the vitellogenin prepared from the serum, and the specific activity determined as described in Chapter 2.

TABLE 10 THE SPECIFIC ACTIVITY OF THE LABELLED VITELLOGENIN ISOLATED FROM IN VITRO LIVER SLICE INCUBATIONS

Isotope	µCi	Spec. Act. of Vg (dpm/mg)	% isotope incorporated
(^{3}H) Leu	100	2-5 x 10^{6}	15-20
(^{3}H) Met	15	0.7 x 10^{6}	15 (L)
(^{3}H) Ser	50	0.5 x 10^{6}	2.6 (P)
(^{32}P) phosphate	500	4-8 x 10^{6}	1-2 (P)
(^{14}C) Acetate	100	0.8 x 10^{6}	0.4
(^{3}H) Acetate	500	1.5 x 10^{6}	0.15

TABLE 11 THE SPECIFIC ACTIVITY OF VITELLOGENIN ISOLATED FROM XENOPUS SERUM AFTER IN VIVO INJECTION OF ISOTOPE

Isotope	µCi	Spec. Act. of Vg (dpm/mg)
(^{3}H) Leu	100	0.18 x 10^{6}
(^{32}P) phosphate	500	0.02-0.06 x 10^{6}
(^{3}H) Leu	150	0.16-0.4 x 10^{6}

phoresis, typical results are shown in Figure 12 and 13. Non-SDS analysis (Figure 12) showed that although most of the vitellogenin was in the dimer form (A), some was dissociated into the monomer form (B), the proportion of monomer varied between preparations. SDS analysis (Figure 13) confirmed that the subunits were undegraded.

3.4 A comparison of in vitro Incorporation with the in vivo Incorporation of Injected Isotope

The in vivo incorporation of radioactive precursor into serum vitellogenin by oestrogen-treated Xenopus provided an alternative method for obtaining the labelled protein required for oocyte incorporation studies. 10-12 days after oestrogen treatment, toads were injected with 100 µCi of (^{3}H) leucine, 250 µCi of (^{32}P) phosphate or 50 µCi each of (^{3}H) leucine, (^{3}H) serine and (^{3}H) histidine (the isotopes were injected in 0.2 ml of distilled water). 48 hours later, the animals were sacrificed, the serum collected and the vitellogenin isolated as described in Chapter 2. The specific activities of the labelled vitellogenin preparations obtained by this method are shown in Table 11.

In vivo incorporation resulted in vitellogenin of a much lower specific activity than was obtained using the same amount of labelled precursor in an in vitro liver slice incubation ; thus when (^{3}H) leucine was injected, the specific activity of the labelled vitellogenin obtained was 0.18×10^{6} dpm/mg, compared with $2\text{-}5 \times 10^{6}$ dpm/mg from an in vitro incubation, and when (^{32}P) phosphate was injected, the specific activity of the vitellogenin was only 0.02-0.06×10^{6} dpm/mg compared with $4\text{-}8 \times 10^{6}$ dpm/mg from an in vitro incubation. However, at 10-12 days after oestrogen-treatment, the serum contains up to 50 mg of vitellogenin per ml, and therefore the in vivo method has the advantage of enabling up to 300 mg of labelled vitellogenin to be isolated from each animal, compared with only about 5 mg obtained from the medium after the incubation of 1 g of liver slices. But, since the uptake of vitellogenin by isolated oocytes was only of the order of 0.1 µg/oocyte/

Figure 12 Non-SDS Polyacrylamide Gel Analysis of Vitellogenin Precipitated from the Incubation Medium

Liver slices (1 g) were incubated for 24 hours in 10 ml of incubation medium with either a) 100 µCi (^{3}H) leucine or b) 500 µCi (^{32}P) phosphate. At the end of the incubation the vitellogenin was precipitated from the medium and samples (40 - 80 µg) were analysed on 5% non-SDS polyacrylamide gels. The gels were stained for protein, scanned at 550 nm (dashed line) and the radioactivity in 2 mm slices determined (histogram).

a) (^{3}H) leucine-labelled vitellogenin

b) (^{32}P) phosphate-labelled vitellogenin

Figure 13 (overleaf) SDS Polyacrylamide Gel Analysis of Vitellogenin Precipitated from the Incubation Medium

Samples (approx. 50 µg) of the vitellogenin obtained from the experiments described in Figure 12 were analysed on 7.5% SDS polyacrylamide gels. The gels were stained for protein, scanned at 550 nm (dashed line) and the radioactivity in 2 mm slices was determined (histogram) X-position of polypeptide of mol. wt. approx. 200,000.

a) (^{3}H) leucine-labelled vitellogenin

b) (^{32}P) phosphate-labelled vitellogenin

Figure 12

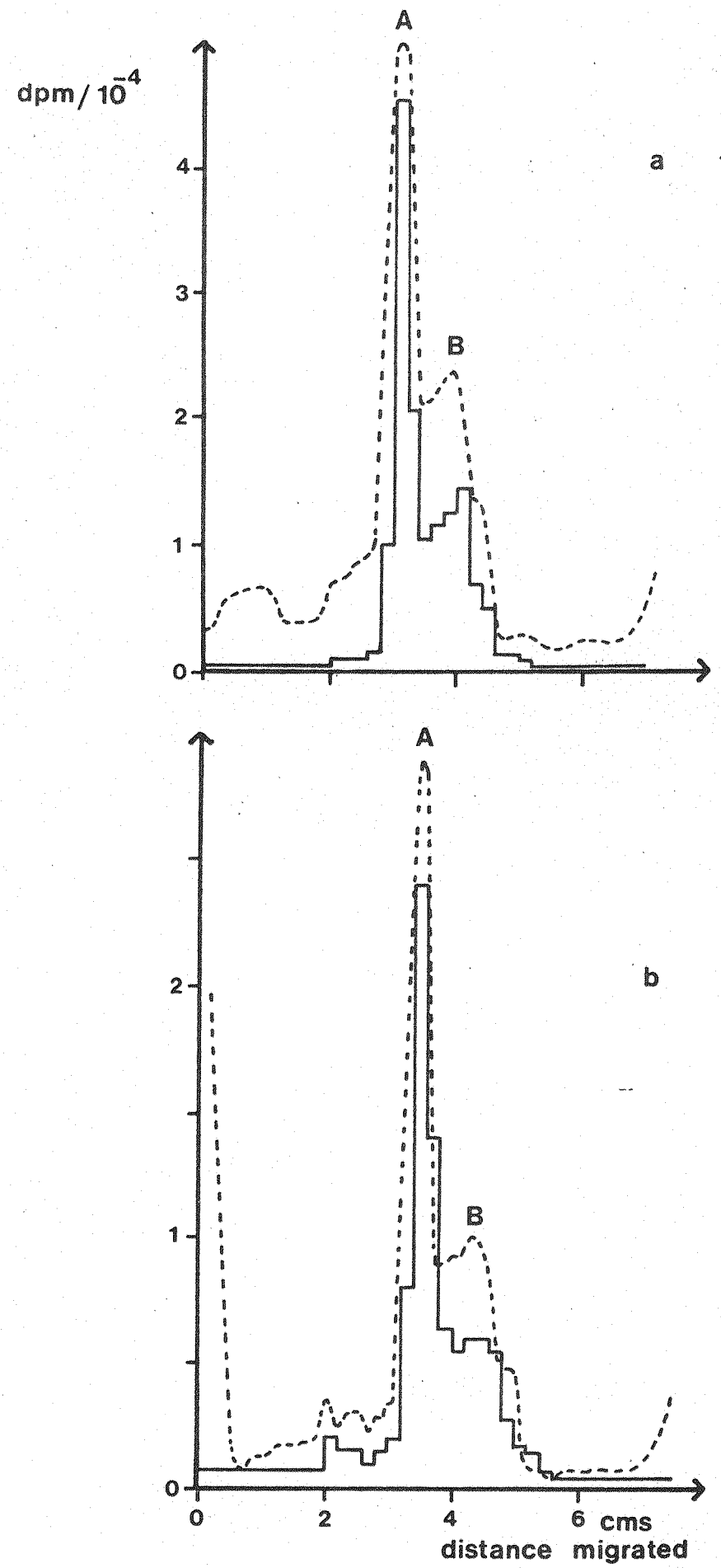
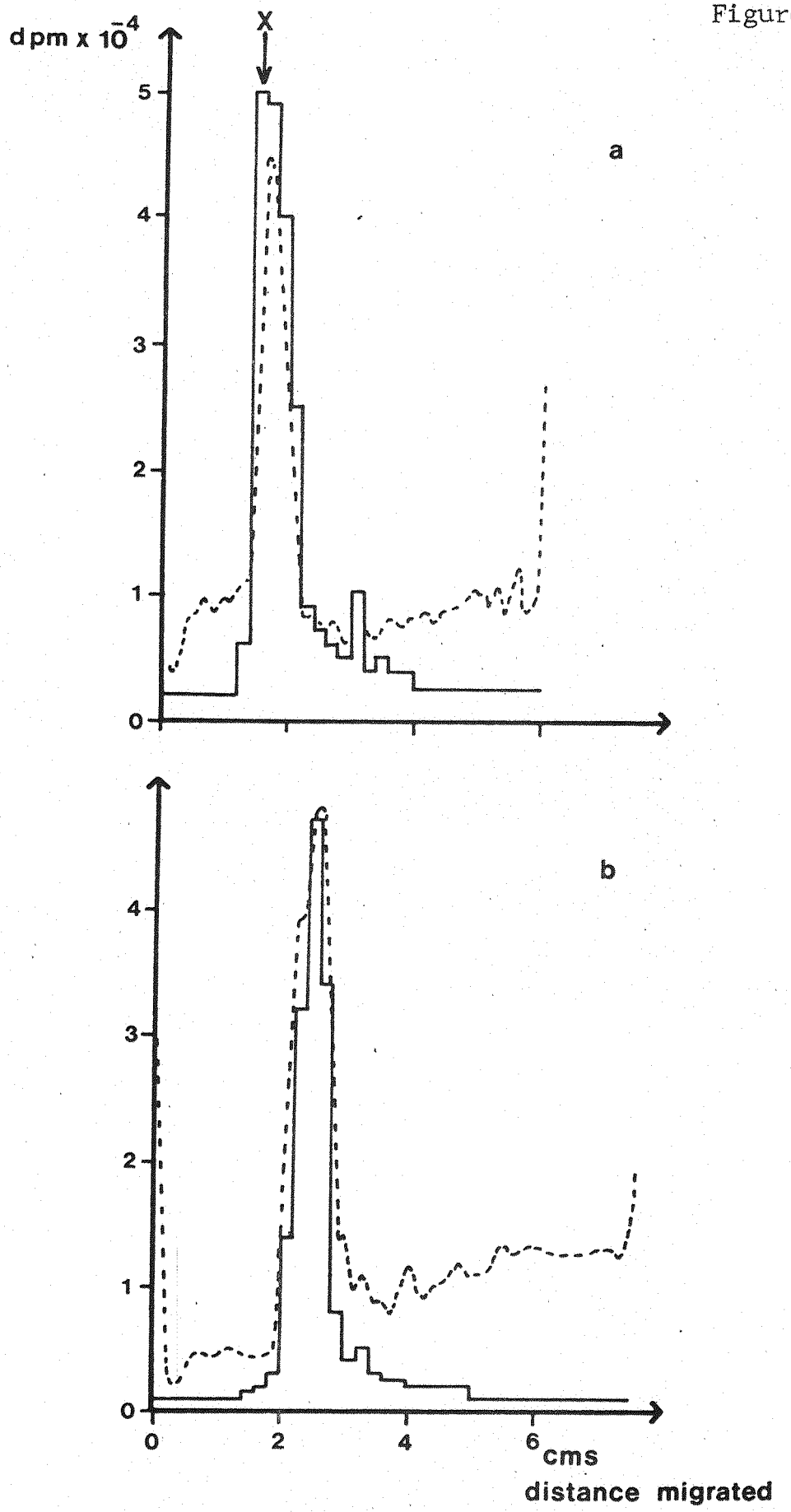


Figure 13



24 hours, the specific activity of the vitellogenin obtained by the in vivo procedure was not high enough to be useful for these experiments.

3.5 The Uptake of Labelled Vitellogenin (prepared from in vitro incubations) by Isolated Oocytes

These results were obtained in this laboratory by J.M. Pratt (unpublished observations).

Figure 14 shows the results of an experiment in which isolated oocytes from HCG-treated toads were incubated with either (^{3}H) leucine- or (^{32}P) phosphate-labelled vigellogenin prepared as described in the previous section ; the specific activity of both samples was 2×10^{6} dpm/mg. Both (^{3}H) leucine- and (^{32}P) phosphate-labelled vitellogeenin was incorporated to approximately the same extent, 0.1 µg/ oocyte/24 hours, however insufficient counts were incorporated to allow the distribution of the radioactivity inside the oocyte to be analysed by polyacrylamide gel electrophoresis.

Table 12 shows the results of a second experiment in which (^{3}H) leucine- and (^{3}H) methionine-labelled vitellogenin (specific activities 0.5×10^{6} and 3×10^{6} dpm/mg respectively) and (^{125}I) labelled vitellogenin (prepared by chemical iodination of serum vitellogenin) were incubated with isolated oocytes.

TABLE 12

Vitellogenin labelled with	Incorporation (µgVg/10 oocytes/ 24 hours)	Ratio dimer : monomer
(^{3}H) met	0.43	2:1
(^{3}H) leu	0.11	1:4
(^{125}I)	0.73	3:1

Although each incubation contained the same concentration of vitellogenin, there was considerable variation in the level of incorporation. Non-SDS polyacrylamide gel analysis of the vitellogenin samples revealed that the

Figure 14

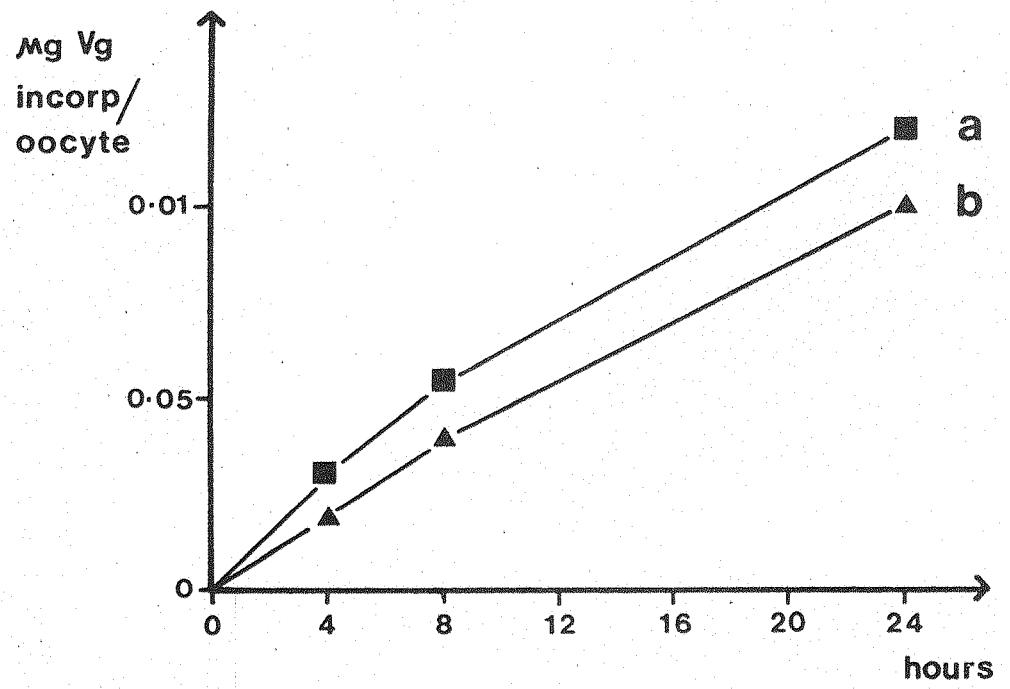


Figure 14 The Uptake of a) (^{3}H) Leucine- and b) (^{32}P) Phosphate-Labelled Vitellogenin by Isolated Oocytes

Isolated oocytes obtained from HCG-treated Xenopus were incubated with either a) (^{3}H) leucine- or b) (^{32}P) phosphate-labelled vitellogenin for 24 hours. The incorporation of radioactivity into the oocytes was determined at intervals.

observed uptake was related to the ratio of vitellogenin dimer to vitellogenin monomer present. Thus in the (^{3}H) methionine-labelled vitellogenin, the ratio of dimer to monomer was 2:1 and the incorporation was 0.43 µg/10 oocytes/24 hours, whereas in the (^{3}H) leucine-labelled vitellogenin the ratio of dimer to monomer was 1:4 ie most of the vitellogenin was dissociated into its monomer subunits, and the incorporation was only 0.11 µg/10 oocytes/24 hours ; the (^{125}I) labelled vitellogenin had the highest ratio of dimer to monomer, 3:1, and also gave the highest incorporation, 0.73 µg/10 oocytes/24 hours. These results suggest that the oocytes are able to distinguish between the dimer and monomer forms of vitellogenin and that the uptake of the native dimer form is favoured.

CHAPTER 4

Chapter 4

THE PHOSPHORYLATION AND GLYCOSYLATION OF THE VITELLOGENIN POLYPEPTIDE

Workers in this laboratory have shown that when liver-slices from oestrogen-treated Xenopus laevis are incubated with radioactive amino-acids there is a 90-120 minute lag-period before the secretion of labelled vitellogenin into the medium (Dolphin et al, 1971 ; Merry et al, 1973). This lag-period is the time taken for the biosynthesis, assembly and secretion of the final vitellogenin molecule (Merry et al, 1973). Further studies have identified a single microsomal precursor of secreted vitellogenin with a mol. wt. of approximately 200,000, very similar to the mol. wt. of the vitellogenin subunit (Penning et al, 1977). This precursor is present on the microsomes from an early stage in the lag-period, and the results suggest that the phosvitinyl and lipovitellinyl moieties of the parent vitellogenin molecule are biosynthesized as part of a single large polypeptide. These findings are consistent with results showing that vitellogenin mRNA codes for a protein of mol. wt. approximately 200,000 (Shapiro et al, 1976).

Since vitellogenin is a Ca^{2+}-binding, glycolipophosphoprotein, the primary translation product must undergo several post-translational modifications before the secretion of the completed molecule. As yet there is little direct evidence to suggest when the phosphorus, carbohydrate and lipid moieties are added to the newly translated vitellogenin polypeptide. The aim of the experiments presented in this chapter was to investigate at what stage prior to secretion the intracellular precursors of vitellogenin are phosphorylated and glycosylated.

4.1 The in vitro Incorporation of (^{3}H) Leucine into Vitellogenin by Liver Slices and the Identification of Microsomal Precursors

Several preliminary experiments were performed to demonstrate the characteristics of (^{3}H) leucine incorporation into vitellogenin by liver slices. It was also necessary to confirm that (^{3}H) leucine labelled microsomal precursors of vitellogenin could be reliably identified before proceeding with more complex experiments.

Figure 15a shows the time-course of secretion of labelled vitellogenin into the medium during the incubation of liver slices from oestrogen-treated toads with (^{3}H) leucine. The characteristic lag-period of 90-120 minutes - representing the time taken for the biosynthesis, assembly and secretion of the final vitellogenin molecule - was a consistent feature of these experiments. If after 1 hour further peptide-bond formation (and consequently translation) was inhibited by the addition of cycloheximide, authentic labelled vitellogenin was secreted into the medium for about 90 minutes after the end of the lag-period (Figure 15b). This result indicated that prior to cycloheximide addition at 1 hour, completed (^{3}H) leucine-labelled polypeptide precursors of vitellogenin must already have been present intracellularly ; it was also evident that any modifications taking place during the second hour of the lag-period did not require the formation of any new peptide-bonds.

To identify the microsomal precursors of vitellogenin, liver slices were pulsed for 1 hour with (^{3}H) leucine. At the end of the pulse period, cycloheximide was added and the incubations continued for a subsequent 4 hour chase period. Samples of liver slices were taken at 1 hour, 2 hours and 5 hours and the microsomal fraction harvested, solublized in the presence of the protease inhibitor PMSF, and the solublized microsomal protein analysed by SDS polyacrylamide gel electrophoresis as described in Chapter 2. The results are shown in Figure 16. After 1 hour only one major labelled polypeptide was associated with the microsomes, and this had a mol. wt. of about 200,000, similar to the subunit

Figure 15 The Incorporation of (^{3}H) Leucine into Secreted Protein, a) in the Absence and b) in the Presence of Cycloheximide

a) Liver slices (0.5 g) were incubated for 6 hours in 5 ml of incubation medium with 10 μCi (^{3}H) leucine. At various times, 0.3 ml samples of the medium were removed and the incorporation of radioactivity into secreted protein was determined by the filter paper disc method as described in Chapter 2.

b) Liver slices were incubated with (^{3}H) leucine as described in a). At 1 hour, cycloheximide was added to the incubation to give a final concentration of 100 μg/ml, and the same amount was added at 3 hours and 5 hours. The incorporation of radioactivity into secreted protein was determined at various times.

Figure 15

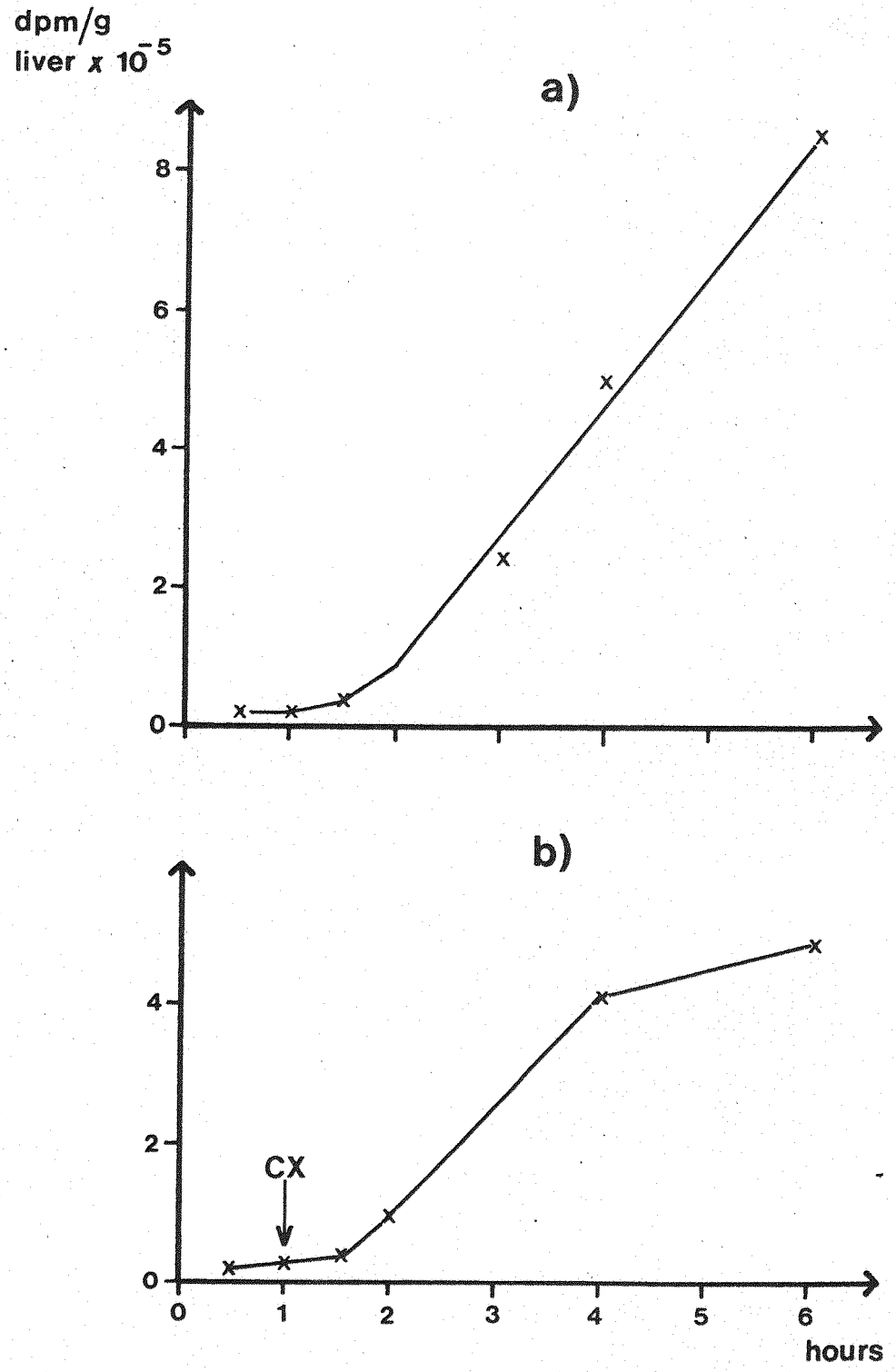
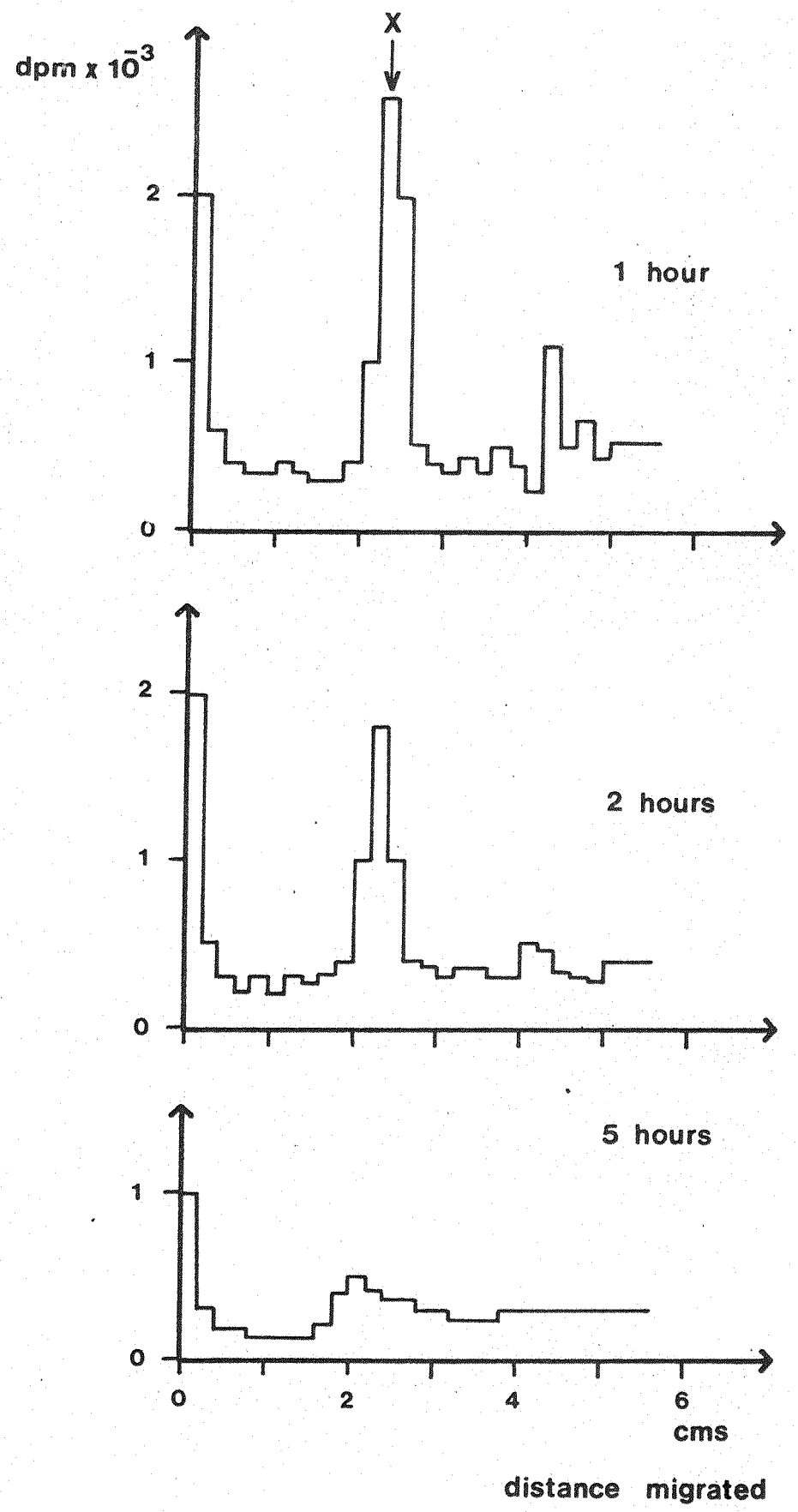


Figure 16 SDS Polyacrylamide Gel Analysis of (^{3}H) Leucine-Labelled Microsomal Protein

Liver slices (3 x 0.5 g) were incubated in 5 mls of incubation medium with 20 µCi (^{3}H) leucine. Cycloheximide (100 µg/ml) was added at 1 hour (and again at 3 hours and 5 hours). Samples of liver slices were taken at 1, 2 and 5 hours and the microsomal fraction harvested and solublized with SDS and 2-mercaptoethanol as described in Chapter 2. 150 µl samples of the solublized microsomal protein were analysed on 7.5% SDS polyacrylamide gels and the radioactivity in 2 mm slices was determined. 'X' - position of a polypeptide of mol. wt. approx. 200,000.

Figure 16


dpm x 10^{-3}
X
2
1
0
1 hour
2
1
0
2 hours
1
0
5 hours
0
2
4
6
cms
distance migrated

mol. wt. of secreted vitellogenin. This species was still present on the microsomes at 2 hours ie 1 hour after the addition of cycloheximide, but was almost completely lost at 5 hours, the loss corresponding to the appearance of labelled vitellogenin in the incubation medium ; similar results were obtained when the slices were incubated with (^{3}H) leucine for 2 and 2½ hours before cycloheximide addition. These results confirmed the earlier work of Penning et al (1977) and indicated that during the lag-period, completed 200,000 mol. wt. polypeptide precursors of vitellogenin accumulate on the microsomes, and that at the end of the lag-period these precursors are released into the incubation medium.

It should be mentioned that the microsomal precursors could only be detected if the microsomes were solublized in the presence of the protease inhibitor, PMSF (phenylmethylsulphonyl fluoride), and in the absence of this inhibitor, smaller polypeptides were identified as precursors of vitellogenin (Penning et al, 1977).

4.2 The Identification of Phosphorylated Precursors of Vitellogenin

4.2.1 The Incorporation of (^{3}H) Leucine and (^{32}P) Phosphate into Secreted, Total Tissue and Microsomal Protein

When liver slices from oestrogen-treated toads were incubated with (^{32}P) phosphate, labelled protein was secreted into the incubation medium as shown in Figure 17. SDS polyacrylamide gel analysis of the incubation medium confirmed that vitellogenin was the only (^{32}P) phosphate-labelled protein secreted during the incubation. The secretion of (^{32}P) phosphate labelled vitellogenin was attended by a 1½-2 hour lag-period, similar to the lag-period characteristic of the incorporation of (^{3}H) leucine into secreted vitellogenin ; this confirmed the results obtained by Merry et al (1973). It was found that by omitting phosphate from the incubation medium and incubating the slices with 20 µCi (^{3}H) leucine and 200 µCi (^{32}P)

Figure 17

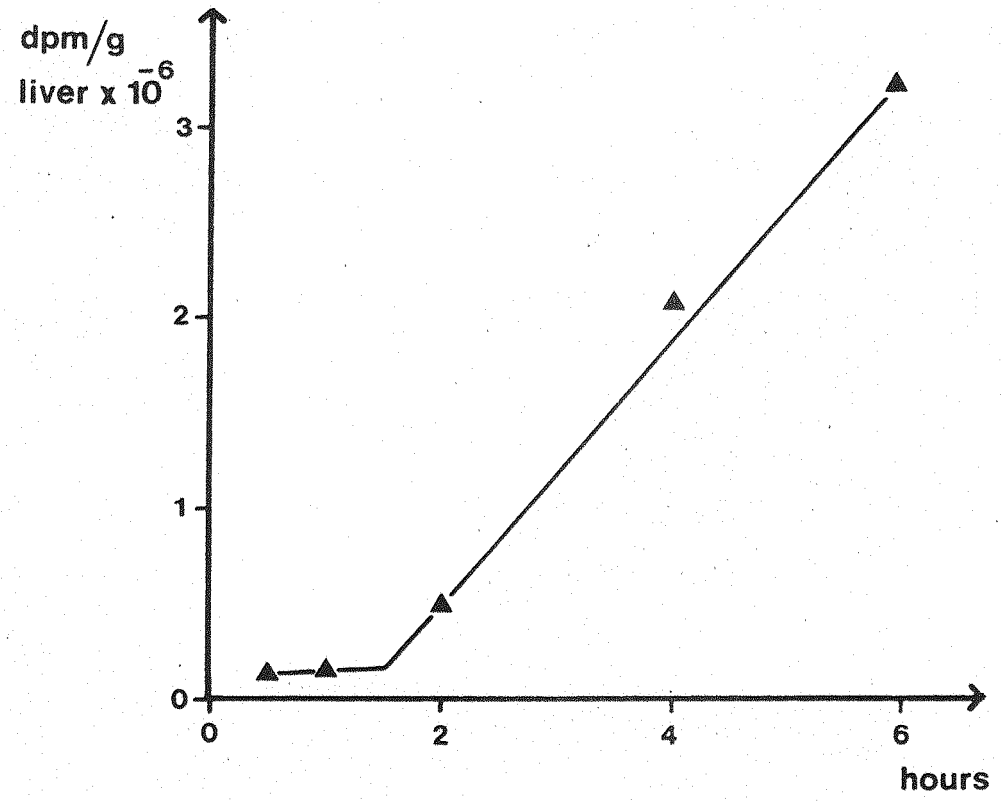


Figure 17 Incorporation of (^{32}P) Phosphate into Secreted Protein

Liver slices (0.5 g) were incubated for 6 hours in 5 mls of incubation medium with 100μ Ci(^{32}P) phosphate. At various times, 0.3 ml samples of the incubation medium were removed and the incorporation of radioactivity into secreted protein was determined by the filter paper disc method as described in Chapter 2.

phosphate, double-labelled vitellogenin was secreted into the incubation medium and SDS polyacrylamide gel analysis showed that the P/T ratio of the secreted vitellogenin was approximately 2 (throughout this section, the term "P/T ratio" refers to the ratio of (^{32}P) dpm to (^{3}H) dpm). Therefore this system was favourable for experiments to compare the level of phosphorylation of the intracellular microsomal precursors of vitellogenin and the final secreted protein. For these experiments, the length of the pulse-period was increased to 2½ hours in order to increase the quantity of radioactivity being analysed.

In initial experiments, samples of 0.5 g of liver slices were incubated with 200 µCi (^{32}P) phosphate and 20 µCi (^{3}H) leucine ; cycloheximide was added at 2½ hours to inhibit further protein synthesis and the incubations were continued for a further 3½ hours (total incubation period 6 hours). Samples of liver slices and incubation medium were taken at 1½, 2½ and 6 hours and the incorporation of radioactivity into secreted, total tissue, and microsomal protein was determined.

Figure 19 shows the results of SDS polyacrylamide gel analysis of samples of incubation medium taken during a (^{3}H) leucine and (^{32}P) phosphate double-labelling experiment ; the analysis indicated that double-labelled vitellogenin was secreted into the incubation medium, and that the P/T ratio of the secreted vitellogenin was approximately 1.8. Figure 18a shows that, as expected, (^{3}H) leucine was incorporated into the secreted protein with a lag-period of approximately 1½ hours. After cycloheximide addition at 2½ hours, the appearance of labelled protein in the incubation medium was accompanied by a concomitant loss of radioactivity from the total tissue and microsomal protein fractions, this represented the loss of (^{3}H) leucine-labelled intracellular precursors of vitellogenin from the tissue (Penning et al, 1977). During the chase-period, the decrease in radioactivity associated with the total tissue protein fraction was approximately equal to the amount of labelled protein secreted into the incubation

Figure 18 Incorporation of a) (^{3}H) Leucine and b) (^{32}P) Phosphate into Secreted, Total Tissue and Microsomal Protein during a 2½ hour Pulse - 3½ hour Cycloheximide Chase Experiment

Liver Slices (4 x 0.5 g) were incubated in 4 mls of incubation medium with 20 µCi (^{3}H) leucine and 200 µCi (^{32}P) phosphate. Cycloheximide (100 µg/ml) was added at 2½ hours (and again at 4½ hours). At various times, incubations were removed and the incorporation of radioactivity into secreted protein (S.P.), total tissue protein (T.T.P) and microsomal protein (M.P.) was determined as described in Chapter 2. (Phosphate was omitted from the incubation medium.)

a) (^{3}H) leucine incorporation

b) (^{32}P) phosphate incorporation

Figure 18

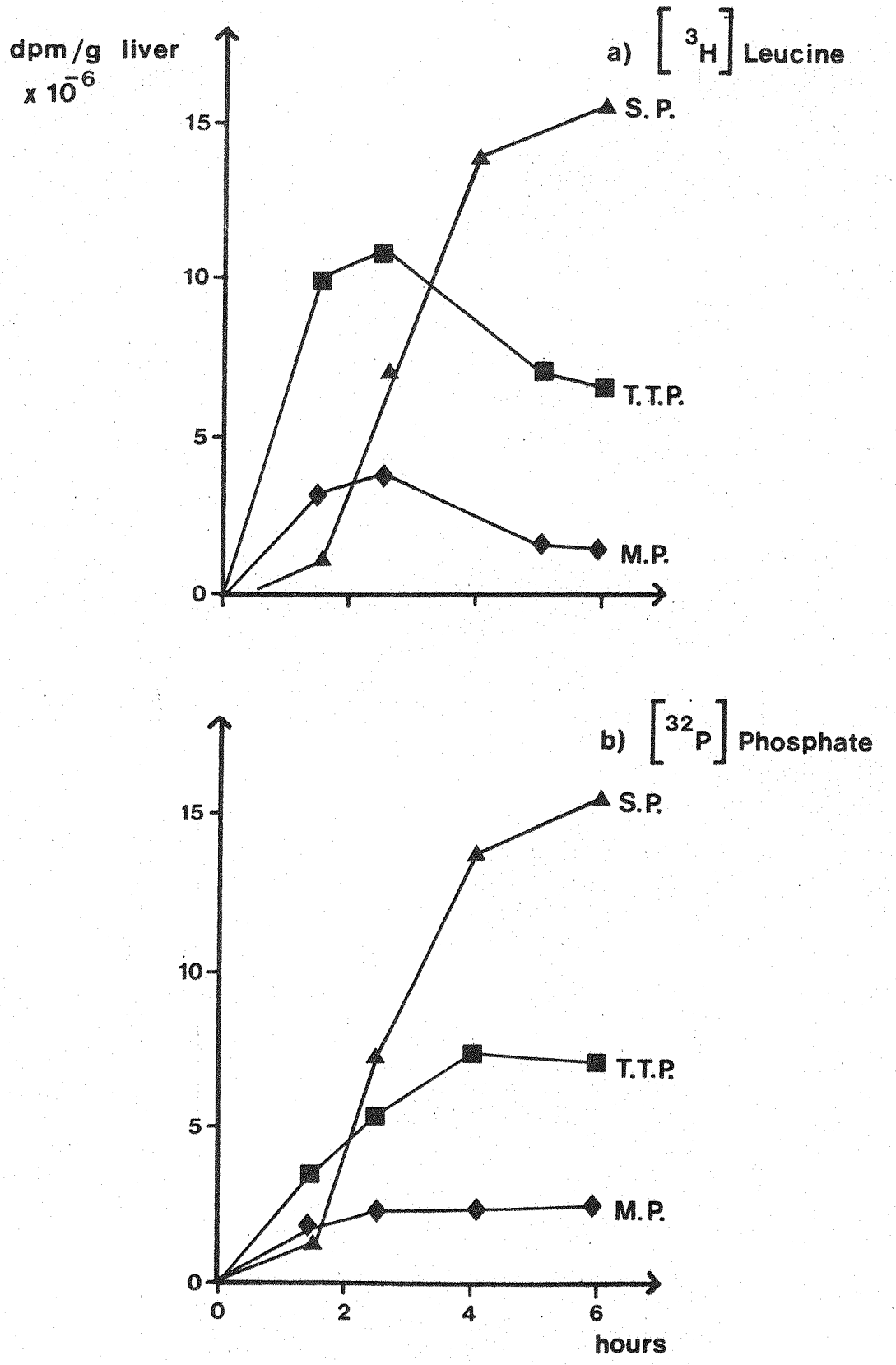
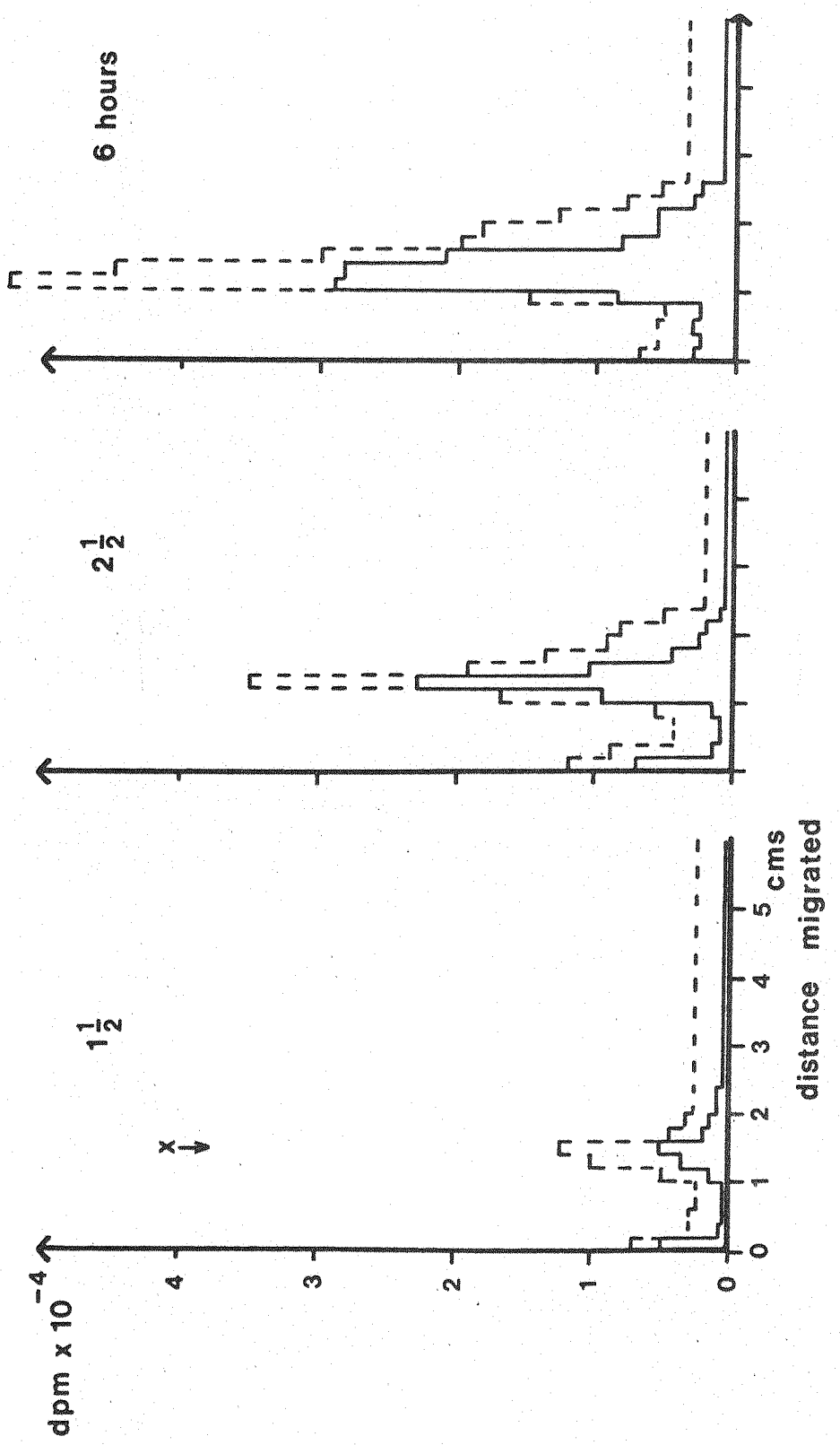

dpm/g liver
x 10⁻⁶
a) [³H] Leucine
S.P.
T.T.P.
M.P.
15
10
5
0
b) [³²P] Phosphate
S.P.
T.T.P.
M.P.
15
10
5
0
0
2
4
6
hours

Figure 19 SDS Polyacrylamide Gel Analysis of (^{3}H) Leucine- and (^{32}P) Phosphate-Labelled Incubation Medium

100 µl samples of the incubation medium obtained at 1½, 2½ and 6 hours in the experiment described in Figure 18 were analysed on 7.5% SDS polyacrylamide gels and the radioactivity in 2 mm slices was determined. 'X' - position of a polypeptide of mol. wt. approx. 200,000.

(^{3}H) ——
(^{32}P) ------

Figure 19


6 hours
2 1/2
1 1/2
x
dpm x 10^-4
0
1
2
3
4
0
1
2
3
4
5 cms
distance migrated

medium ; however, only about 30% of this decrease could be accounted for by the loss of label from the microsomal protein fraction. Since it is well established that only a portion of the microsomal fraction can be isolated by conventional homogenization and centrifugation procedures, and that a high proportion of the fraction is sedimented with the mitochondria during the first centrifugation step (Lewis and Tata, 1973), this discrepancy was not surprising. Indeed, previous workers in this laboratory had also made this observation and had shown that the missing radioactivity could be accounted for by that present in the 4,000 g pellet (Penning et al, 1977).

Figure 18b shows that (^{32}P) phosphate was also incorporated into secreted protein with a lag-period of approximately 1½ hours. However, after cycloheximide addition at 2½ hours, although (^{32}P) phosphate-labelled vitellogenin continued to be secreted into the incubation medium, there was no concomitant loss of (^{32}P) phosphate label from the total tissue or microsomal protein fractions. This suggested that the pool of (^{3}H) leucine-labelled vitellogenin precursors lost from the tissue during the chase period was not fully phosphorylated ; if this was the case, the secretion of (^{32}P) phosphate-labelled vitellogenin into the incubation medium after cycloheximide addition would have been accompanied by a corresponding loss of (^{32}P) phosphate label from the total tissue and microsomal fractions, similar to the observed loss of (^{3}H) leucine label.

It should be stressed that since this was a double-label experiment the same sample of protein was used for the determination of both (^{3}H) leucine and (^{32}P) phosphate incorporation, and therefore any difference in the labelling patterns obtained with the two isotopes were genuine and not due to experimental error.

4.2.2 The Incorporation of (^{32}P) Phosphate into the Microsomal Precursors of Vitellogenin

Samples of the microsomal fractions harvested at 1½, 2½ and 6 hours in the experiment described in 4.2.1 were

solublized and analysed by SDS polyacrylamide gel electrophoresis ; the results are shown in Figure 20. At 1½ and 2½ hours, the (^{3}H) leucine labelled, 200,000 mol. wt. polypeptide precursor of vitellogenin was clearly recognizable, and this was completely lost at 6 hours, thus confirming the previous results. However, when compared with the vitellogenin secreted into the incubation medium (Figure 18), it was clear that the pool of microsomal precursors was only slightly phosphorylated, and it was estimated that the (^{3}H) leucine-labelled precursors included only about 15% of the full complement of (^{32}P) phosphate label associated with the final secreted vitellogenin. The results of two further experiments which also illustrate this point are shown in Figure 21. These results implied that there was little accumulation of phosphorylated precursors of vitellogenin on the microsomes, and thus they were consistent with the observation made in the previous section, that the secretion of (^{32}P) phosphate-labelled vitellogenin into the incubation medium was not accompanied by a concomitant loss of (^{32}P) phosphate label from the total tissue and microsomal protein fractions.

The possibility that the low level of phosphorylation of the microsomal precursors was due to the (^{32}P) phosphate not having reached a steady-state level in the appropriate phosphate-pool was considered. However if this was so it would follow that the vitellogenin secreted during the early part of the time-course would also have a low P/T ratio, but the results of the analysis of the incubation medium (Figure 19) show that this was not the case, and that as early as 1½ hours vitellogenin with a P/T ratio of approximately 2 was detectable.

The low level of phosphorylation of the microsomal precursor compared with the secreted vitellogenin argued against complete phosphorylation taking place soon after the translation of the vitellogenin polypeptide, since this would result in the microsomal precursor having a P/T ratio similar to the secreted protein. It seemed likely that the polypeptides were not being phosphorylated until just prior

Figure 20 SDS Polyacrylamide Gel Analysis of (^{3}H) Leucine- and (^{32}P) Phosphate-Labelled Microsomal Protein (1)

150 µl samples of solublized microsomal protein obtained at 1½, 2½ and 6 hours in the experiment described in Figure 18 were analysed by 7.5% SDS polyacrylamide gels. The radioactivity in 2 mm slices was determined. 'X' - position of a polypeptide of mol. wt. 200,000.

Figure 21 (overleaf) SDS Polyacrylamide Gel Analysis of (^{3}H) Leucine- and (^{32}P) Phosphate-Labelled Microsomal Protein (2)

150 µl samples of solublized microsomal protein obtained at 2½ and 6 hours in two further experiments identical to that described in Figure 18 were analysed on 7.5% SDS polyacrylamide gels. The radioactivity in 2 mm slices was determined. 'X' - position of a polypeptide of mol. wt. approx. 200,000.

(^{3}H) ———

(^{32}P) ------

Figure 20

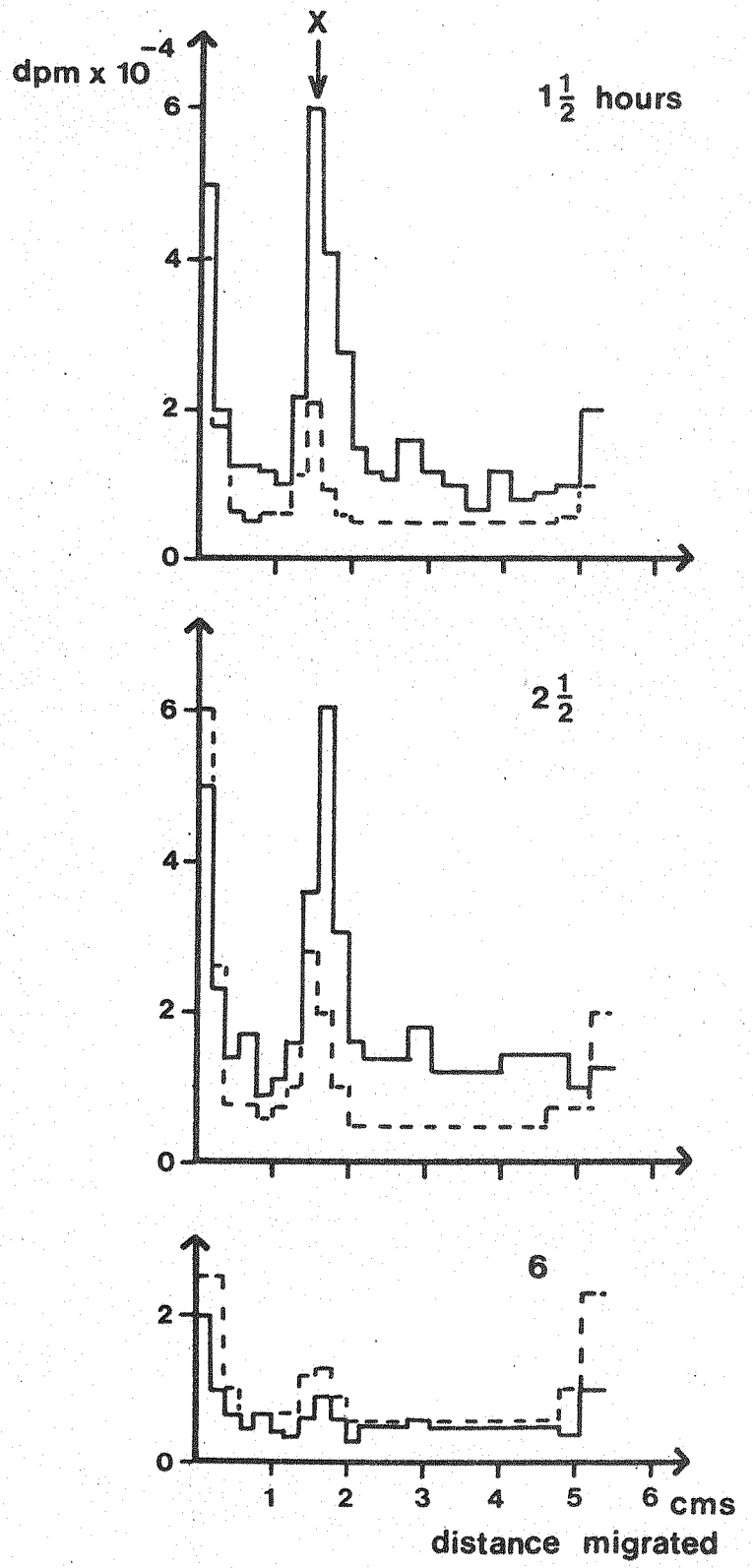
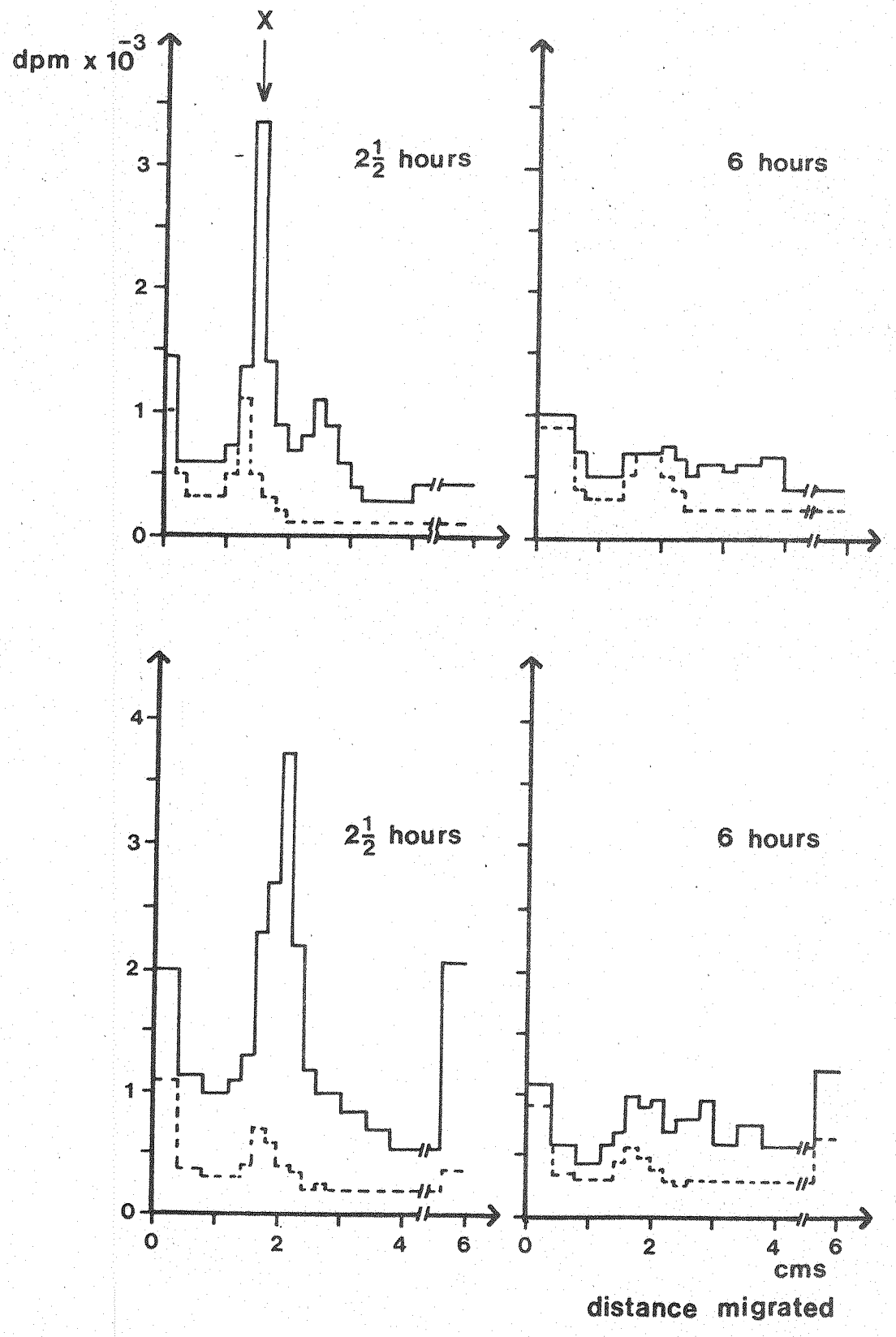


Figure 21


dpm x 10⁻³
X
2½ hours
6 hours
2½ hours
6 hours
0
1
2
3
4
0
2
4
6
cms
distance migrated

to their release from the microsomes, resulting in little accumulation of the phosphorylated species.

4.2.3 The Identification of Possible Phosphorylated Precursors of Vitellogenin in the Post-Microsomal Fraction

The supernatant remaining after the harvesting of the microsomes during the experiment described in sections 4.2.1 and 4.2.2, referred to as the soluble protein or cytosolic fraction, was analysed by SDS polyacrylamide gel electrophoresis ; the results are shown in Figure 22. Somewhat surprisingly, the analysis revealed at 1½, 2½ and 6 hours this fraction contained only one major peak of radioactivity and that this was in the 200,000 mol. wt. region. However, in marked contrast to the microsomal precursors of vitellogenin, this species had a P/T ratio of approximately 2, ie it was phosphorylated to a similar extent to the vitellogenin secreted into the incubation medium (Figure 19). The amount of radioactivity associated with this 200,000 mol. wt. species was maximum at 2½ hours, and at 6 hours the labelled peak was reduced to 50% of its 2½ hour level. The analysis of the post-microsomal supernatant fraction obtained in a similar experiment are shown in Figure 23 ; again the post-microsomal supernatant included the labelled 200,000 mol. wt. species which was phosphorylated to a similar extent to the secreted vitellogenin.

The identification of this labelled phosphorylated species in the post-microsomal supernatant had interesting implications since it suggested that the tissue included two distinct pools of precursor vitellogenin, the microsomal pool, comprising completed 200,000 mol. wt. polypeptide, but including only a fraction of the phosphate associated with the secreted vitellogenin, and a second pool in the soluble protein fraction, comprising fully phosphorylated precursors.

4.2.4 Analysis of the Post-Microsomal Supernatant Fraction

Before continuing with the results of further

Figure 22 SDS Polyacrylamide Gel Analysis of (^{3}H) Leucine- and (^{32}P) Phosphate-Labelled Post-Microsomal Supernatant (1)

150 µl samples of the post-microsomal supernatant obtained at 1½, 2½ and 6 hours from the experiment described in Figure 18 were analysed on 7.5% SDS polyacrylamide gels. The radioactivity in 2 mm slices was determined. 'X' - position of a polypeptide of mol. wt. approx. 200,000

Figure 23 (overleaf) SDS Polyacrylamide Gel Analysis of (^{3}H) Leucine- and (^{32}P) Phosphate-Labelled Post-Microsomal Supernatant (2)

150 µl samples of the post-microsomal supernatant obtained at 1½, 2½ and 6 hours in an experiment similar to that described in Figure 18 were analysed on 7.5% SDS polyacrylamide gels. The radioactivity in 2 mm slices was determined. 'X' - position of a polypeptide of mol. wt. approx. 200,000.

(^{3}H) ——
(^{32}P) - - - - -

Figure 22

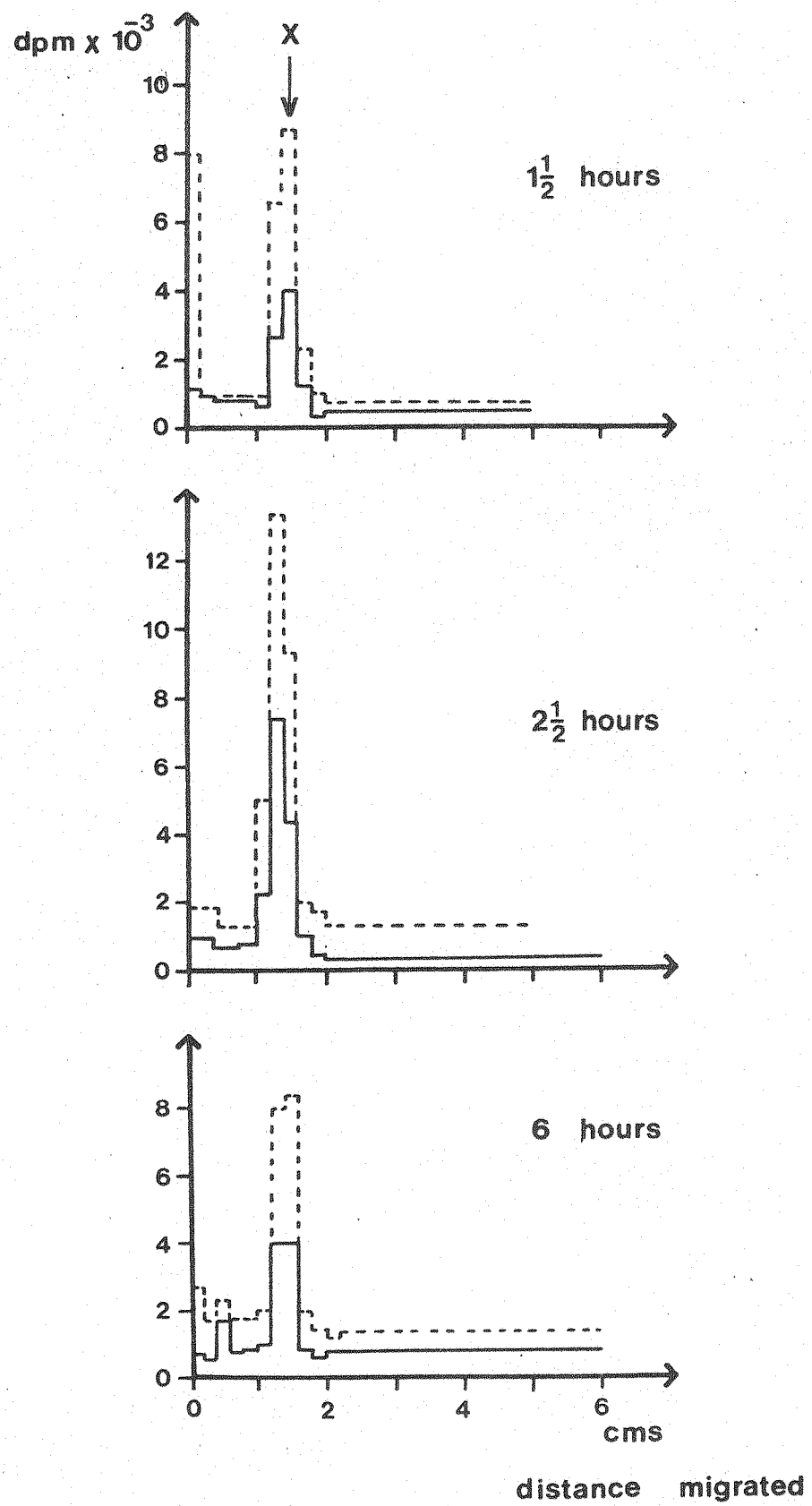
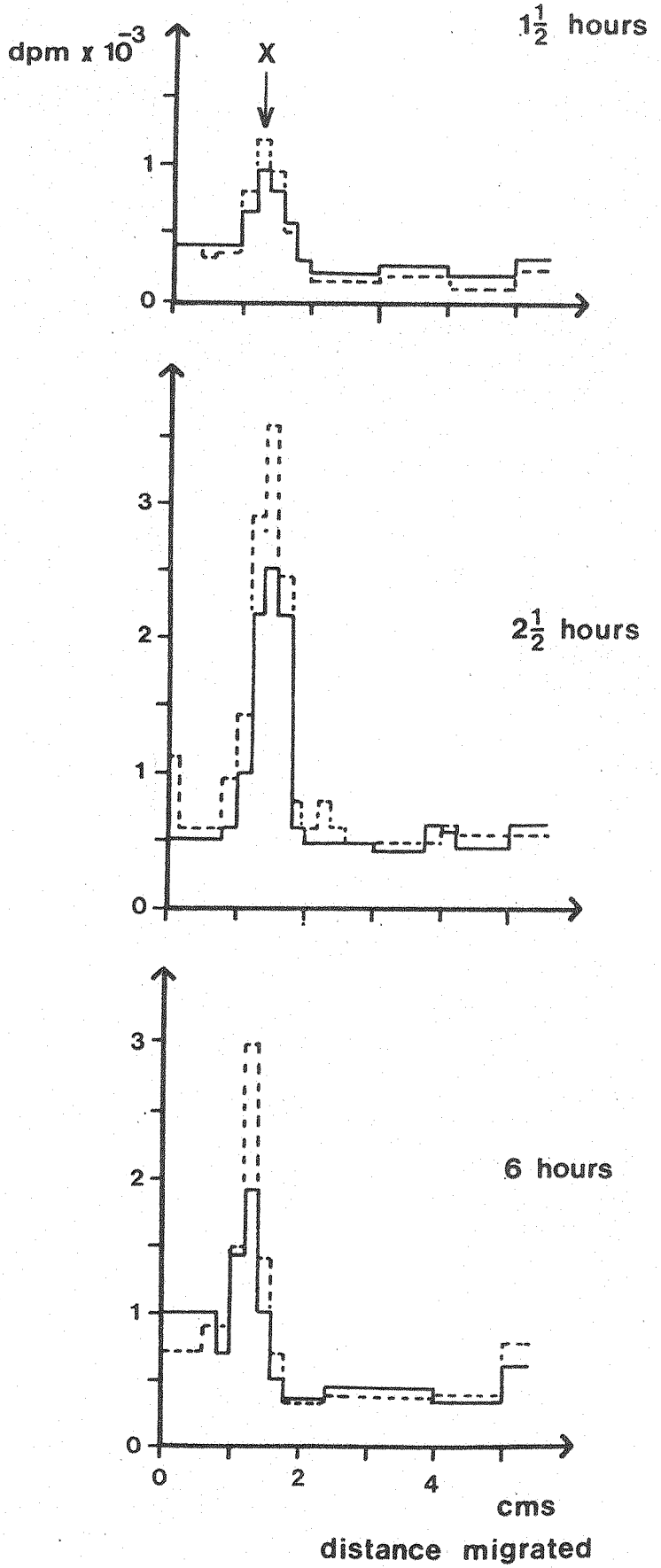


Figure 23


dpm x 10⁻³
X
1½ hours
2½ hours
6 hours
0
1
2
3
4
cms
distance migrated

double-label experiments, the characteristics of the post-microsomal species as determined from single-label experiments with (^{3}H) leucine will be described, since these results have a bearing on the procedures adopted in subsequent experiments.

a) The Effect of Prolonging the Chase Period

In 2½ hour pulse, 3½ hour cycloheximide chase experiments, such as those described in the previous section, the loss of the labelled, 200,000 mol. wt. species from the post-microsomal supernatant during the chase period was variable. Therefore it was decided to increase the length of the chase-period from 3½ hours to 5½ hours, to ensure that the maximum amount of this species was being extruded from the tissue, and consequently that gel analysis would show the maximum loss of the species after cycloheximide addition at 2½ hours.

The post-microsomal supernatant fraction may include soluble extracellular protein in addition to soluble intracellular protein. In these experiments, there was the possibility of equilibration of the incubation medium with the extracellular fluid of the liver slices. If this was occurring it would mean that the labelled species present in the post-microsomal supernatant could simply be due to the equilibration of the labelled vitellogenin in the incubation medium with the liver slice tissue. Therefore it was necessary to assess the effect of the presence of labelled vitellogenin in the incubation medium on the amount of labelled species recovered in the post-microsomal supernatant fraction.

Three samples of liver slices were incubated with (^{3}H) leucine. One sample was removed at 2½ hours and cycloheximide was added to the remaining incubations. At 6 hours, the slices from one of the remaining flasks were removed, washed and transferred to fresh incubation medium ie transferred from medium containing a large quantity of labelled vitellogenin to medium containing no labelled vitellogenin, and the incubations were continued for a

further 2 hours. The slices were then processed, and samples of the incubation medium and post-microsomal supernatant were analysed by SDS polyacrylamide gel electrophoresis ; the results are shown in Figures 24 and 25.

Analysis of samples of the incubation medium taken at 2½ and 8 hours (Figure 24) confirmed that, during this period, labelled vitellogenin was secreted into the incubation medium. Analysis of the post-microsomal supernatant (Figure 25) showed that at 2½ hours there was a distinct (^{3}H) leucine-labelled peak in the 200,000 mol. wt. region, and that at 8 hours this labelled peak was reduced to 30% of its 2½ hour level. A comparison of the 8 hour post-microsomal fractions obtained from the slices which had remained in the original incubation medium (8a) with that obtained from slices which had been transferred to fresh incubation medium at 6 hours (8b), indicated that there was very little difference (less than 10%) between the amount of 200,000 mol. wt. labelled species recovered. This result ruled out the possibility that the presence of large quantities of labelled vitellogenin in the incubation medium, as was the case in 8a (see Figure 24), was resulting in any significant contamination of the post-microsomal supernatant fraction. However, in subsequent experiments, to ensure that the maximum possible loss of labelled, 200,000 mol. wt. species was observed, two incubations were always included for analysis at 8 hours, and the slices from one were transferred to fresh medium at 6 hours. In each case, post-microsomal fractions from both incubations were analysed, and all of the experiments confirmed that there was very little difference between the amount of the 200,000 mol. wt. labelled species recovered.

An important feature highlighted by these experiments was that at 2½ hours, when there was a maximum amount of labelled 200,000 mol. wt. species in the post-microsomal supernatant fraction (Figure 25), only a small amount of labelled vitellogenin had been secreted into the incubation medium (Figure 24) ; conversely, at 8 hours, when the

Figure 24

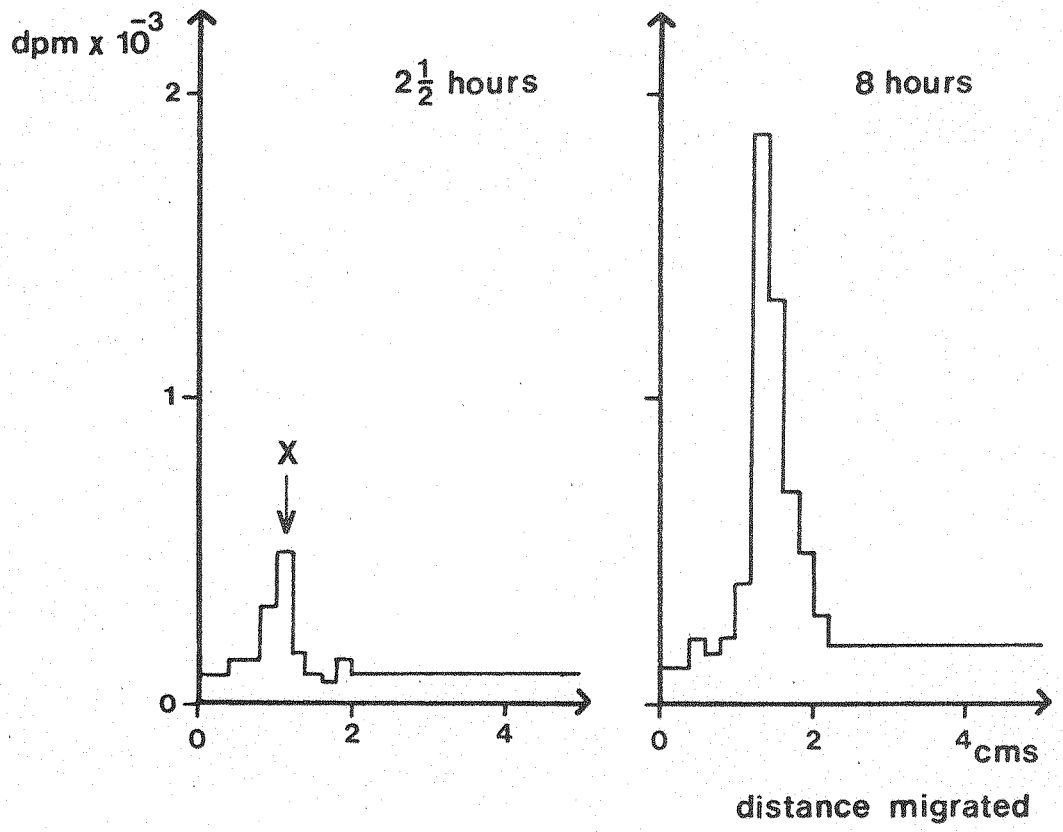


Figure 24 SDS Polyacrylamide Gel Analysis of (^{3}H) Leucine-Labelled Incubation Medium

Liver slices (3 x 0.5 g) were incubated with 10 µCi (^{3}H) leucine. At 2½ hours, one incubation was removed and cycloheximide (100 µg/ml) was added to the remaining flasks. At 6 hours, the slices from one of the remaining flasks, b), were removed, washed and transferred to fresh incubation medium (containing cycloheximide), the slices in the other flask, a), remained in the original medium. The incubations were continued for a further 2 hours. 100 µl samples of the incubation medium obtained at 2½ and 8 hours (from the incubation in which slices remained in the original medium) were analysed on 7.5% SDS polyacrylamide gels. The radioactivity in 2 mm slices was determined. 'X' - position of polypeptide mol. wt. approx. 200,000.

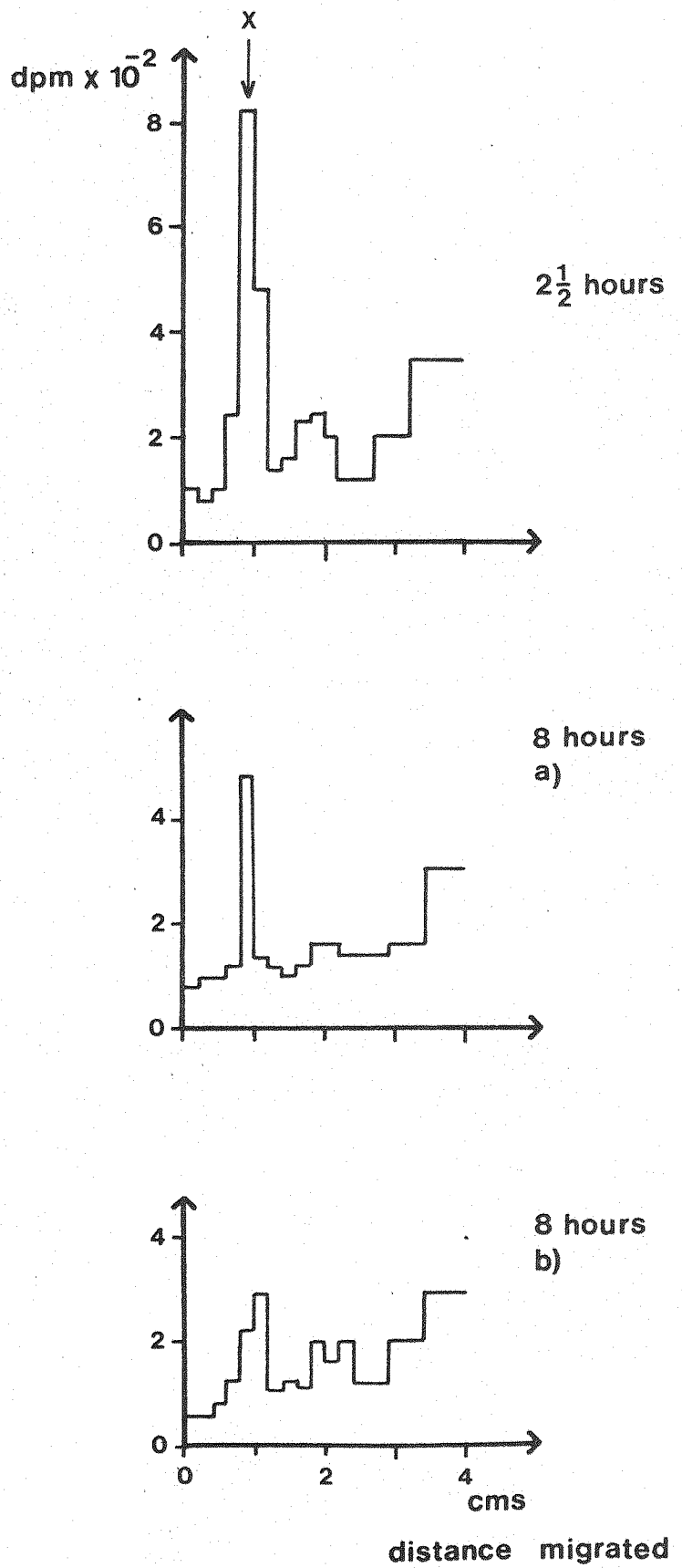
Figure 25 SDS Polyacrylamide Gel Analysis of (^{3}H) Leucine-Labelled Post-Microsomal Supernatant

150 µl samples of the post-microsomal supernatant obtained at 2½ and 8 hours in the experiment described in Figure 24 were analysed on 7.5% SDS polyacrylamide gels. The radioactivity in 2 mm slices was determined.
'X' - position of polypeptide mol. wt. approx. 200,000.

8 hours a) - slices remained in original incubation medium

8 hours b) - slices transferred to fresh incubation medium at 6 hours.

Figure 25


X
dpm x 10^{-2}
8
6
4
2
0
$2\frac{1}{2}$ hours
4
2
0
8 hours
a)
4
2
0
8 hours
b)
0
2
4
cms
distance migrated

maximum amount of labelled vitellogenin had been secreted, the amount of labelled post-microsomal species was reduced by at least 60%. The results of polyacrylamide gel analysis of the incubation medium and post-microsomal supernatant fractions obtained from two more experiments are shown in Figure 26 and they also illustrate this point, as do the results of all subsequent experiments of this type. This relationship provided further evidence that the labelled post-microsomal species was not simply the result of the contamination of the liver slices with labelled vitellogenin from the incubation medium, since if this was the case, the changes in the level of the post-microsomal species would be expected to reflect the amount of labelled vitellogenin secreted into the incubation medium, ie, it would increase between 2½ and 8 hours. The results obtained favoured a precursor-product relationship between the post-microsomal species and the secreted vitellogenin.

Figure 26A SDS Polyacrylamide Gel Analysis of (^{3}H) Leucine-Labelled Incubation Medium (a) and Post-Microsomal Supernatant (b)

100 µl samples of incubation medium, (a), and 150 µl samples of post-microsomal supernatant, (b), obtained at 2½ and 8 hours from an experiment identical to that described in Figure 24 were analysed on 7.5% SDS polyacrylamide gels. The 8 hour post-microsomal supernatant sample was obtained from slices which had been transferred to fresh incubation medium at 6 hours. The radioactivity in 2 mm slices was determined. 'X' - position of a polypeptide mol. wt. approx. 200,000.

Figure 26B (overleaf)

As Figure 26A

Figure 26A

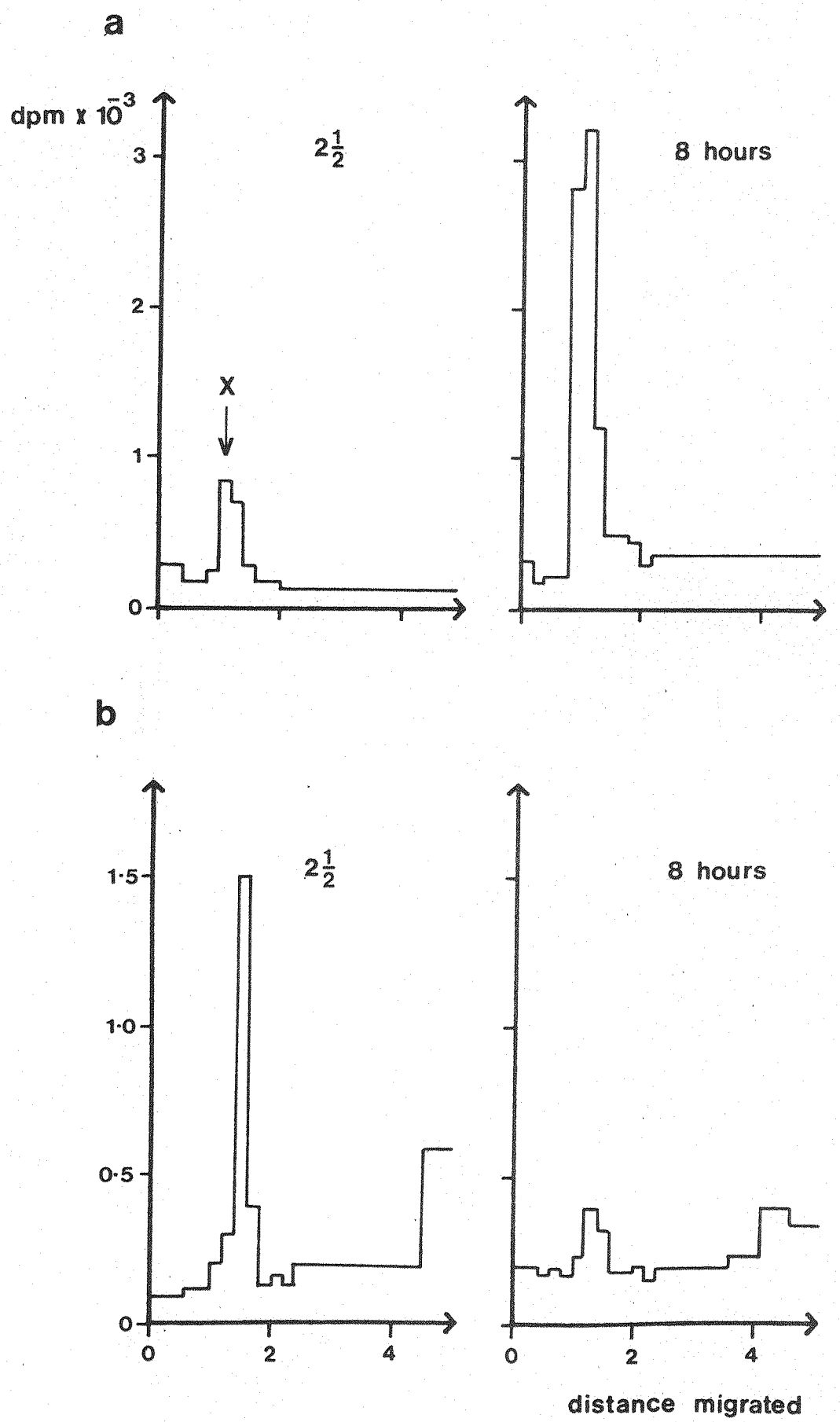
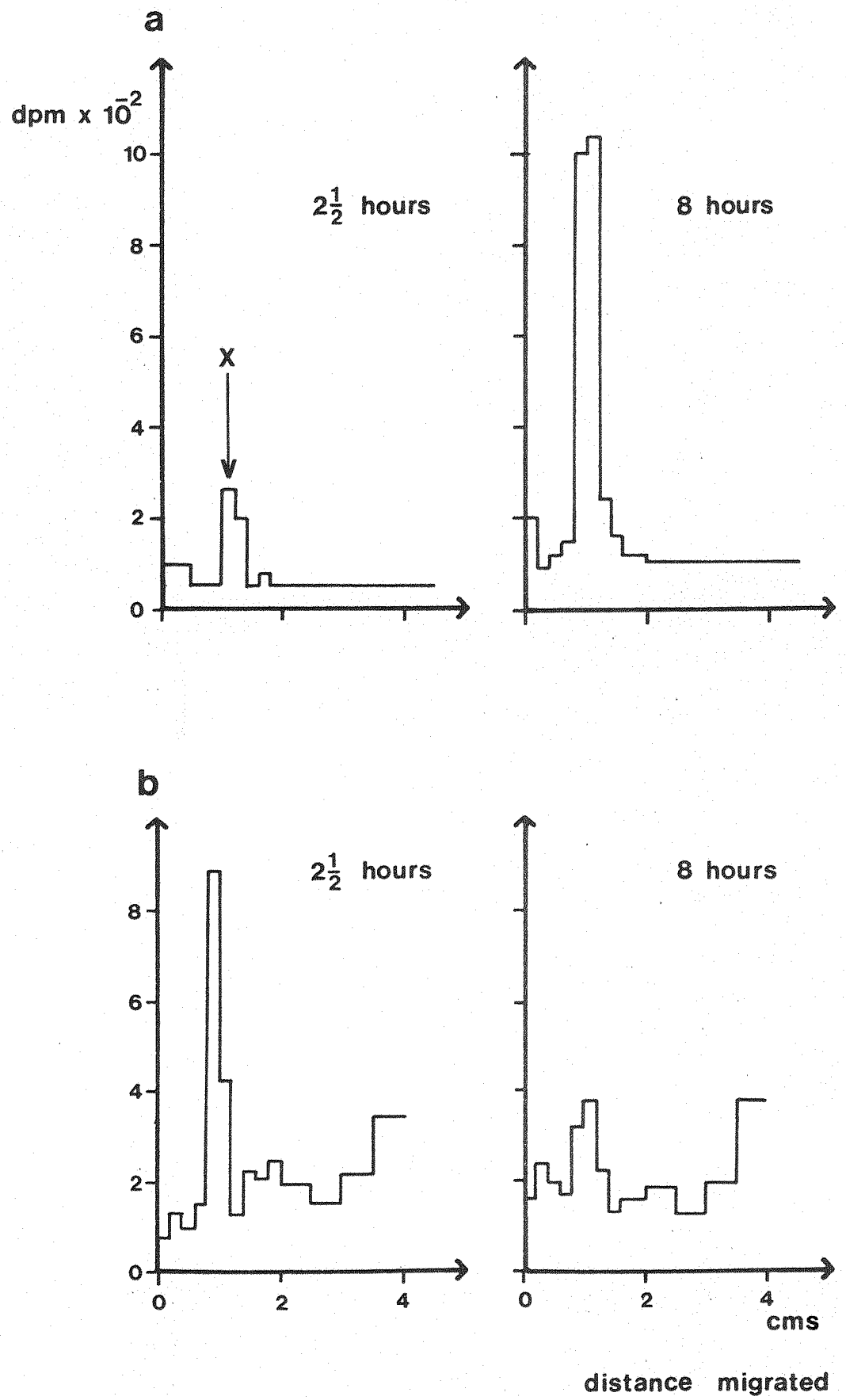

a
dpm x 10⁻³
3
2
1
0
X
2½
8 hours
b
1·5
1·0
0·5
0
2½
8 hours
0
2
4
0
2
4
distance migrated

Figure 26B


a
dpm x 10^{-2}
10
8
6
4
2
0
X
$2\frac{1}{2}$ hours
8 hours
b
$2\frac{1}{2}$ hours
8 hours
8
6
4
2
0
0
2
4
0
2
4
cms
distance migrated

b) The Sedimentation of the 200,000 Molecular Weight Species from the Post-Microsomal Supernatant

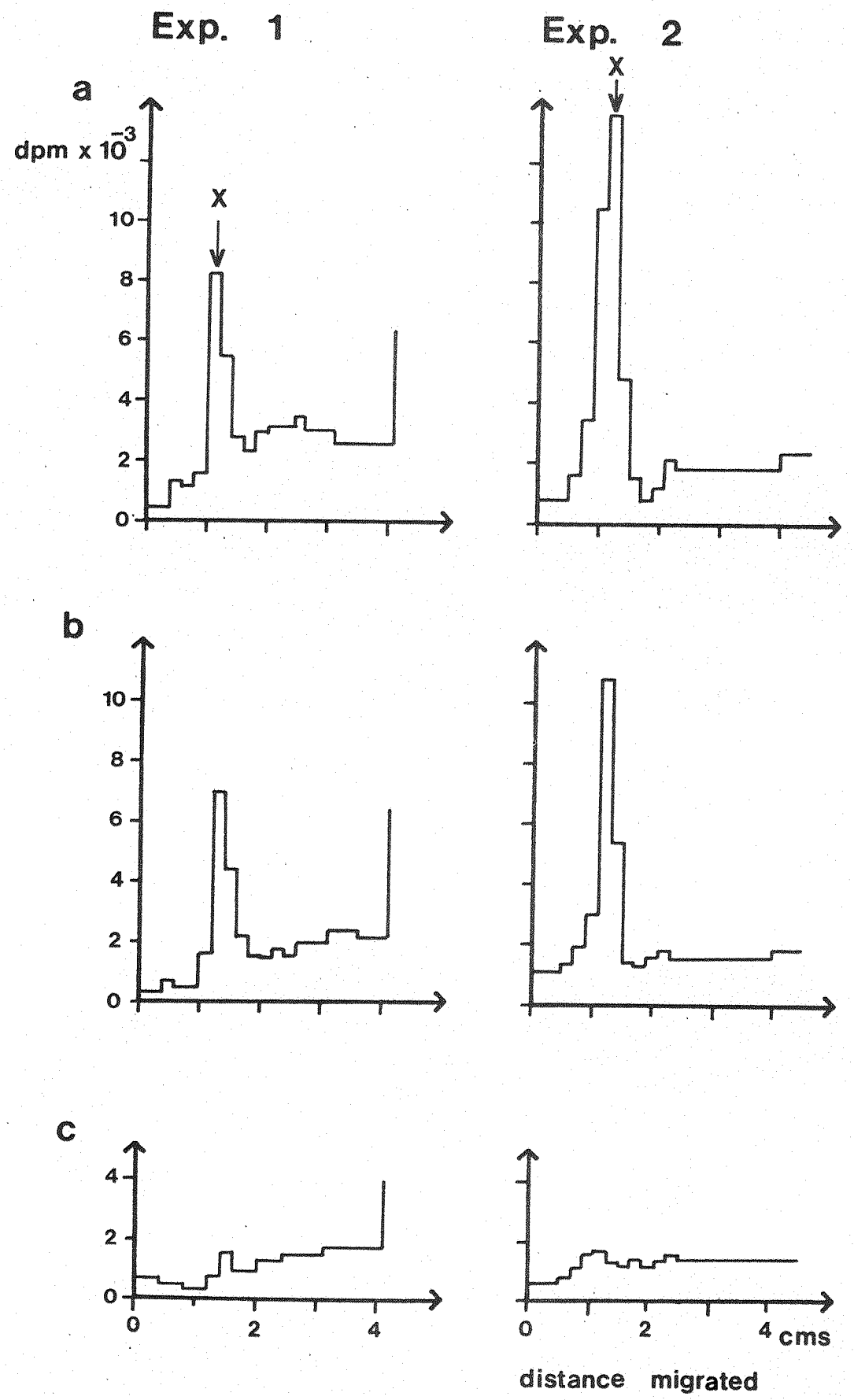
Liver slices were incubated with (^{3}H) leucine for 2½ hours, the microsomes were then harvested and a sample of the post-microsomal supernatant was retained. The remaining supernatant was subjected to a second high-speed centrifugation step for 3 hours at 105,000 g. This centrifugation resulted in a small, red-coloured pellet. The pellet was resuspended in TKMS buffer to give a final volume equal to the volume of the supernatant from which it was harvested and solublized with SDS and 2-mercaptoethanol as described in Chapter 2. Samples of the post-microsomal pellet and the final supernatant were then analysed by SDS polyacrylamide gel electrophoresis. The results of two independent experiments are shown in Figure 27. It should be pointed out that equal volumes of each fraction were analysed, and therefore the counts recovered in each fraction can be compared directly.

Analysis of the post-microsomal supernatant (Figure 27a) showed that it included the familiar, 200,000 mol. wt., labelled species ; however, after the 3-hour centrifugation step, as little as 5-10% of this labelled species was recovered on gels of the final supernatant (Figure 27c). Analysis of the solublized, post-microsomal pellet protein (Figure 27b) revealed that 65-75% of the 200,000 mol. wt., labelled species lost from the supernatant was recovered on gels of this fraction. No corrections were made for any losses occurring during the second centrifugation step or subsequent solublization and analysis. In preliminary experiments it was found that the post-microsomal pellet was difficult to resuspend and careful homogenization was necessary before solublization in order to achieve the maximum recovery of the labelled species on gels of this fraction ; this probably accounts for the observation that the 200,000 mol. wt. labelled species lost from the post-microsomal supernatant was not fully recovered on gels of the post-microsomal pellet fraction.

Figure 27 SDS Polyacrylamide Gel Analysis of (^{3}H) Leucine-Labelled Post-Microsomal Supernatant (a), Post-Microsomal Pellet (b), and Final Supernatant (c)

Liver slices (0.5 g) were incubated with 20 μCi (^{3}H) leucine for 2½ hours. The microsomes were harvested and a sample of the post-microsomal supernatant was retained for gel analysis. The remaining supernatant was centrifuged for a further 3 hours at 105,000 g to obtain the post-microsomal pellet and the final supernatant. The post-microsomal pellet protein was solublized as described in Chapter 2. 150 μl samples of the post-microsomal supernatant, (a), post-microsomal pellet, (b), and final supernatant, (c), were analysed on 7.5% SDS polyacrylamide gels. The radioactivity in 2 mm slices was determined. The results of 2 independent experiments are shown. 'X' - position of polypeptide mol. wt. approx. 200,000.

Figure 27


Exp. 1
Exp. 2
a
dpm x 10⁻³
X
X
10
8
6
4
2
0
b
10
8
6
4
2
0
c
4
2
0
0
2
4
0
2
4 cms
distance migrated

The almost complete loss of the labelled species from the supernatant and its co-sedimentation with the post-microsomal pellet was interesting, since it suggested that it was associated with a particulate fraction and was not truly soluble. The results of electron microscopic analysis of the microsomal and post-microsomal pellets (shown and discussed later) indicated that the two pellets were morphologically distinct. However, vitellogenin is a very large protein, mol. wt. 400-500,000 and the possibility that soluble vitellogenin would be sedimented during a long, high-speed centrifugation step could not be ignored. Therefore, it was decided to look at the behaviour of soluble vitellogenin during an identical fractionation procedure.

Liver slices were incubated with 20 µCi of (^{3}H) leucine for 6 hours, the incubation medium was used as a source of soluble vitellogenin in the following experiment. Two samples of liver slices were incubated for 2½ hours, one with 20 µCi of (^{3}H) leucine and one with no added isotope. At the end of the incubation, the slices incubated with (^{3}H) leucine were homogenized in TKMS buffer as normal, but an aliquot of 6-hour incubation medium (containing labelled, soluble vitellogenin) was added to the buffer before the homogenization of the slices from the incubation with no added isotope, and in this way the behaviour of soluble vitellogenin during the fractionation procedure was followed. The post-microsomal supernatant and post-microsomal pellets obtained were analysed by SDS polyacrylamide gel electrophoresis and the results are shown in Figure 28. Analysis of the post-microsomal supernatant and post-microsomal pellet obtained from the slices incubated with (^{3}H) leucine (Figure 28A) confirmed previous results, approximately 70% of the (^{3}H) leucine-labelled, 200,000 mol. wt. species present on gels of the post-microsomal supernatant was recovered on gels of the solublized post-microsomal pellet protein. In contrast, analysis of the distribution of labelled soluble vitellogenin between the two fractions (Figure 28B) showed that only 20% of the labelled vitellogenin present in the post-microsomal

Figure 28 Comparison of the Sedimentation of the Labelled 200,000 mol. wt. Post-Microsomal Species (Figure 28A) and Soluble Vitellogenin (Figure 28B) During the 3-hour Centrifugation Step

Figure 28A Liver slices (0.5 g) were incubated with 20 µCi (^{3}H) leucine for 2½ hours. The microsomes were harvested and the post-microsomal supernatant was centrifuged for a further 3 hours at 105,000 g to obtain the post-microsomal pellet. 150 µl samples of the post-microsomal supernatant, a), and post-microsomal pellet, b), were analysed on 7.5% SDS polyacrylamide gels and the radioactivity in 2 mm slices was determined. 'X' - position of a polypeptide mol. wt. approx. 200,000.

Figure 28B (overleaf) In a parallel experiment, liver slices (0.5 g) were incubated for 2½ hours but with no added isotope. The slices were then homogenized in TKMS buffer to which 500 µl of 6 hour incubation medium (containing (^{3}H) leucine-labelled, soluble vitellogenin) had been added. The microsomes were harvested and the post-microsomal supernatant further centrifuged to obtain the post-microsomal pellet. 150 µl samples of the post-microsomal supernatant, a), and pellet, b), were analysed on 7.5% SDS polyacrylamide gels and the radioactivity in 2 mm slices was determined. 'X' - position of a polypeptide mol. wt. approx. 200,000.

Figure 28A

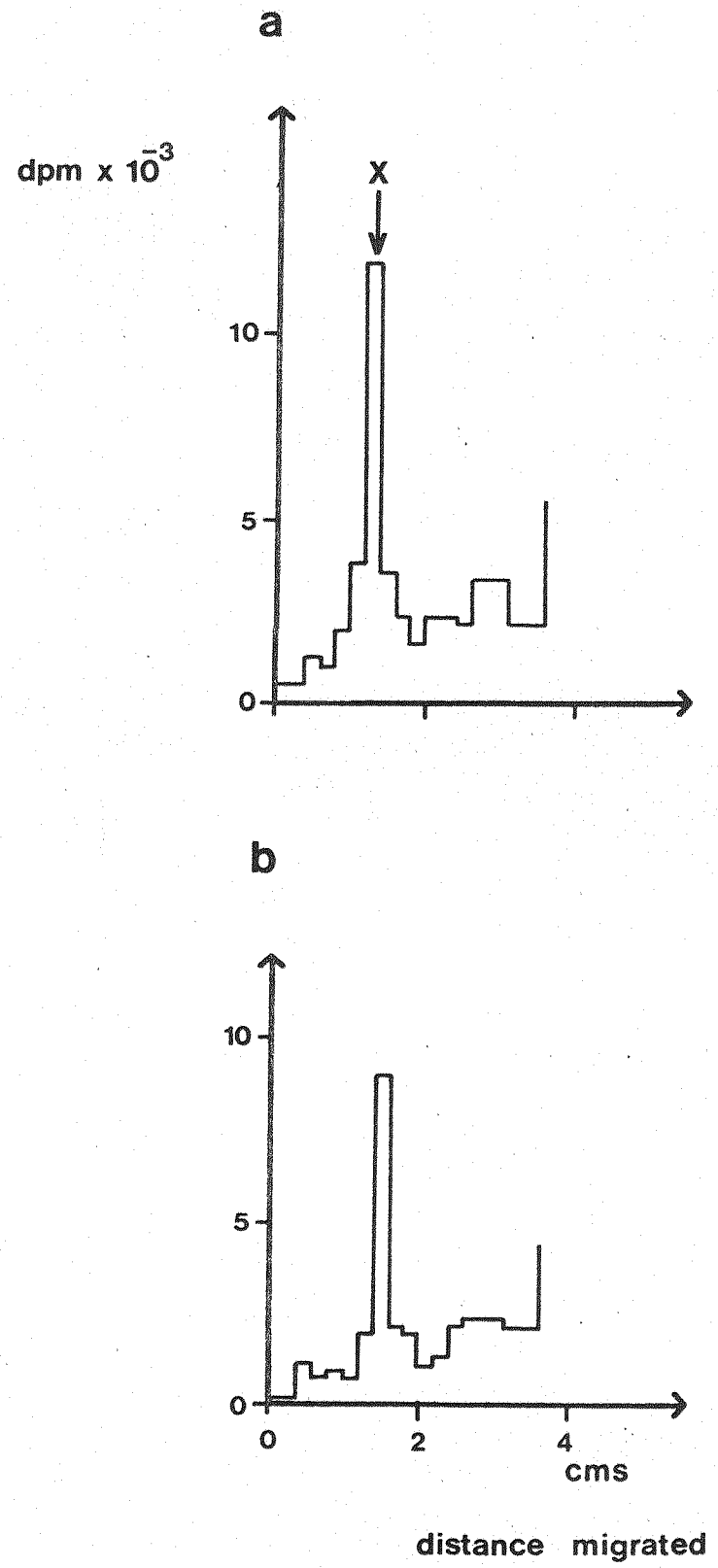
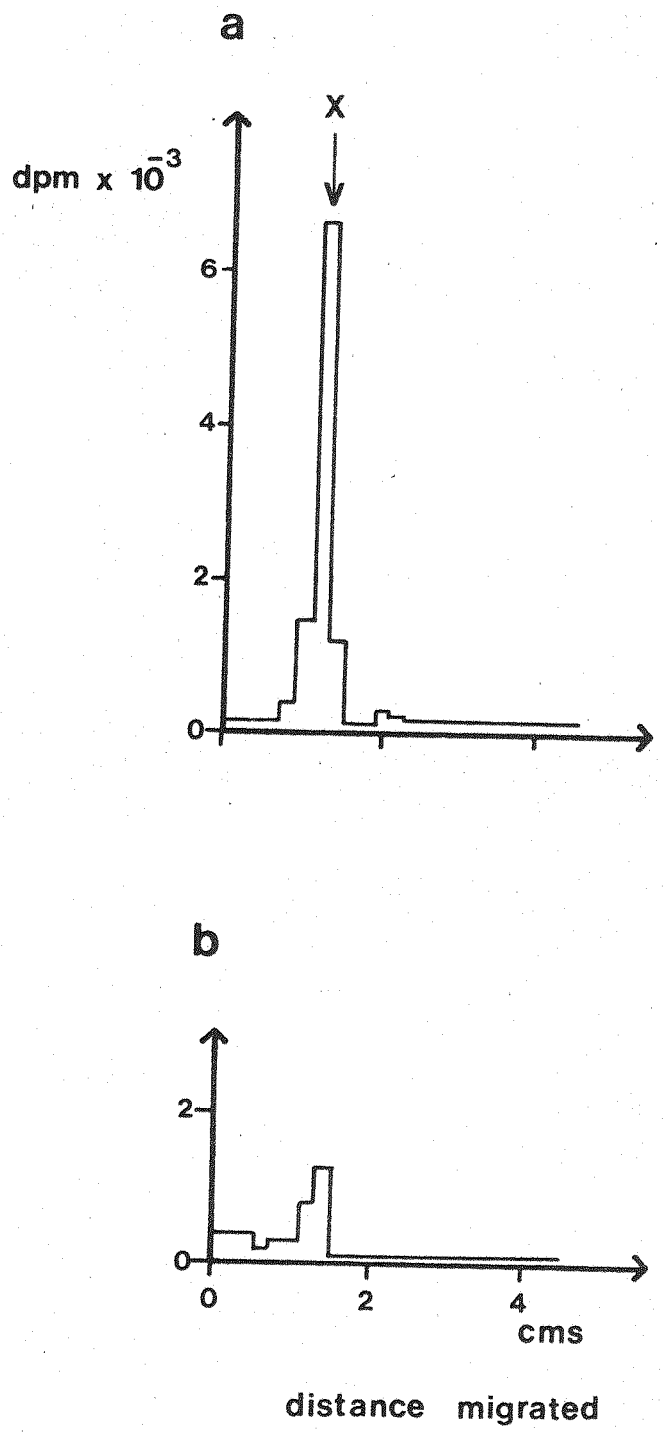


Figure 28B



supernatant was recovered on gels of the post-microsomal pellet.

Although this result did not completely rule out the possibility that soluble vitellogenin present in the post-microsomal fraction would co-sediment with the post-microsomal pellet, it did indicate that there was a clear difference between the behaviour of soluble vitellogenin and the (^{3}H) leucine-labelled, post-microsomal species ; this difference favoured the idea that the post-microsomal species was associated with a particulate fraction and not truly soluble.

In the light of these results, further double-label experiments with (^{3}H) leucine and (^{32}P) phosphate were performed.

4.2.5 Further Investigation of the Phosphorylated Precursors of Vitellogenin

0.5 g samples of liver slices were incubated with (^{3}H) leucine and (^{32}P) phosphate ; one incubation was removed at 2½ hours and cycloheximide was added to the remaining flasks. At 6 hours the slices from one of the remaining incubations were removed, washed and transferred to fresh incubation medium (as described in 4.2.4 a)) and the incubations were continued for a further 2 hours. The microsomal fractions were harvested and samples of the incubation medium, microsomal protein and post-microsomal supernatant were analysed by SDS polyacrylamide gel electrophoresis. The results are shown in Figure 29 and 30. A comparison of the vitellogenin secreted into the incubation medium (Figure 29a) with the microsomal precursors of vitellogenin (Figure 29b) confirmed that, as observed previously, the pool of precursors present on the microsomes at 2½ hours was only partially phosphorylated, and it was estimated that the (^{3}H) leucine-labelled microsomal precursors included only 20% of the (^{32}P) phosphate label associated with the final secreted vitellogenin. The (^{3}H) leucine-labelled microsomal precursors were almost completely lost at 8 hours. Figure 30 shows that at 2½ hours the post-

Figure 29 SDS Polyacrylamide Gel Analysis of (^{3}H) Leucine- and (^{32}P) Phosphate-Labelled Incubation Medium (a) and Microsomal Protein (b) (1)

Liver slices (3 x 0.5 g) were incubated with 20 µCi (^{3}H) leucine and 200 µCi (^{32}P) phosphate. At 2½ hours, one incubation was removed and cycloheximide added to the remaining flasks. At 6 hours, the slices from one of the remaining flasks were removed, washed and transferred to fresh incubation medium, the slices in the other flask remained in the original medium and the incubations continued for a further 2 hours. 100 µl samples of the incubation medium, (a), and 150 µl samples of the microsomal protein,(b), obtained at 2½ and 8 hours (from the incubation in which slices had remained in the original medium) were analysed on 7.5% SDS polyacrylamide gels and the radioactivity in 2 mm slices was determined. 'X' - position of a polypeptide mol. wt. approx. 200,000.

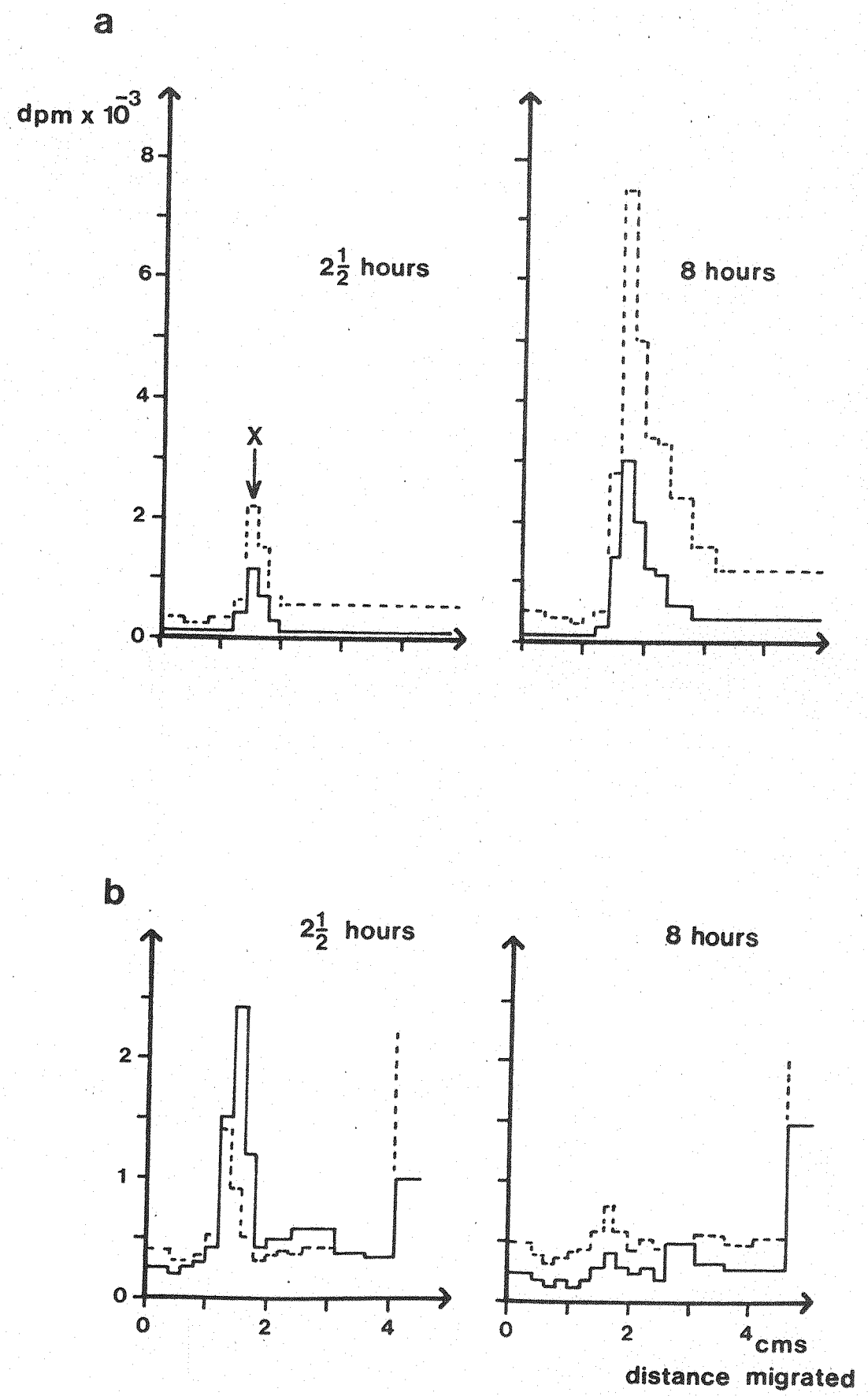
Figure 30 (overleaf) SDS Polyacrylamide Gel Analysis of (^{3}H) Leucine- and (^{32}P) Phosphate-Labelled Post-Microsomal Supernatant

150 µl samples of the post-microsomal supernatant obtained at 2½ and 8 hours in the experiment described in Figure 29 were analysed on 7.5% SDS polyacrylamide gels. The 8 hour sample was obtained from the slices which had been transferred to fresh incubation medium at 6 hours. The radioactivity in 2 mm slices was determined. 'X' - position of a polypeptide mol. wt. approx. 200,000.

(^{3}H) ———

(^{32}P) ------

Figure 29


a
dpm x 10⁻³
8
6
4
2
0
X
2½ hours
8 hours
b
2½ hours
8 hours
2
1
0
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2
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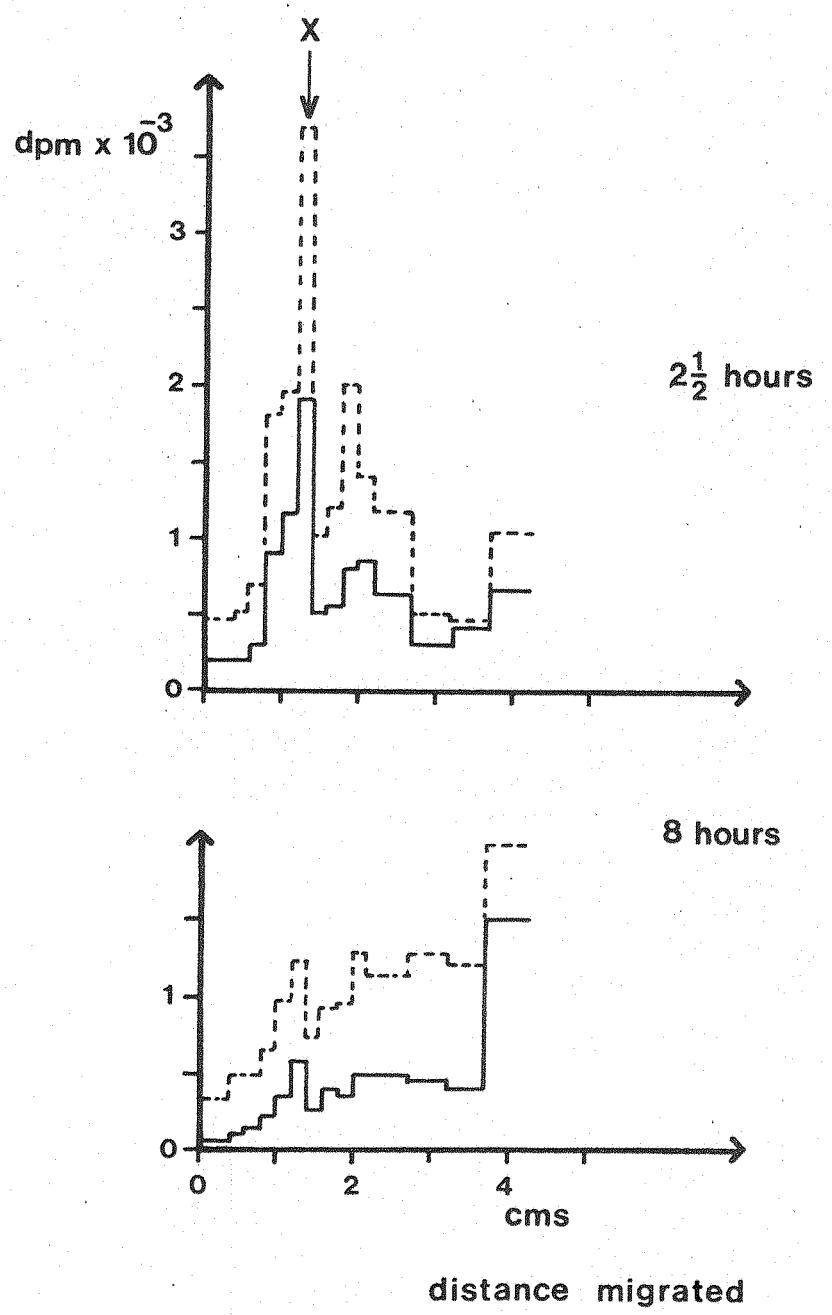
0
2
4 cms
distance migrated

Figure 30



SDS Polyacrylamide Gel Analysis of (^{3}H) Leucine- and (^{32}P) Phosphate-Labelled Post-Microsomal Supernatant

microsomal supernatant included the labelled, 200,000 mol. wt. species and that, in contrast to the microsomal precursors, this had a P/T ratio of approximately 2.5, similar to that of the vitellogenin secreted into the incubation medium. At 8 hours, the labelled post-microsomal species was reduced to 18% of its 2½ hour level.

In a second experiment, the same procedure was adopted, but the post-microsomal supernatant was centrifuged for a further 3 hours in order to obtain the post-microsomal pellet, as described in section 4.2.4 b). The results are shown in Figure 31 and 32. SDS polyacrylamide gel analysis of the incubation medium and the microsomal protein (Figure 31) again confirmed that the microsomal precursors were only partially phosphorylated compared with the final secreted vitellogenin. Figure 32a shows that the labelled 200,000 mol. wt. species present in the post-microsomal supernatant had a P/T ratio of approximately 2.2, again similar to that of the vitellogenin secreted into the incubation medium, and that at 8 hours this labelled species was reduced to 30% of its 2½ hour level. Figure 32c shows that after the 3-hour centrifugation step very little of the double-labelled post-microsomal species remained in the final supernatant, but that approximately 70% was recovered on gels of the post-microsomal pellet (Figure 32b).

Thus, these double-label experiments confirmed the results obtained previously, and indicated that although the pool of microsomal precursors included only a fraction of the (^{32}P) phosphate label associated with the final secreted vitellogenin, the post-microsomal supernatant contained a 200,000 mol. wt. species with a P/T ratio similar to that of the secreted protein. The observed loss of the labelled post-microsomal species during the chase period was consistent with it being a precursor of secreted vitellogenin, and its co-sedimentation with the post-microsomal pellet suggested that it was associated with a particulate fraction and not a true soluble precursor.

Figure 31 SDS Polyacrylamide Gel Analysis of (^{3}H) Leucine- and (^{32}P) Phosphate-Labelled Incubation Medium (a) and Microsomal Protein (b) (2)

Incubations were as described in Figure 29. 100 µl samples of the incubation medium, (a), and 150 µl samples of the microsomal protein, (b), obtained at 2½ and 8 hours (for the incubation in which slices had remained in the original medium) were analysed on 7.5% SDS polyacrylamide gels. The radioactivity in 2 mm slices was determined. 'X' - position of a polypeptide mol. wt. approx. 200,000.

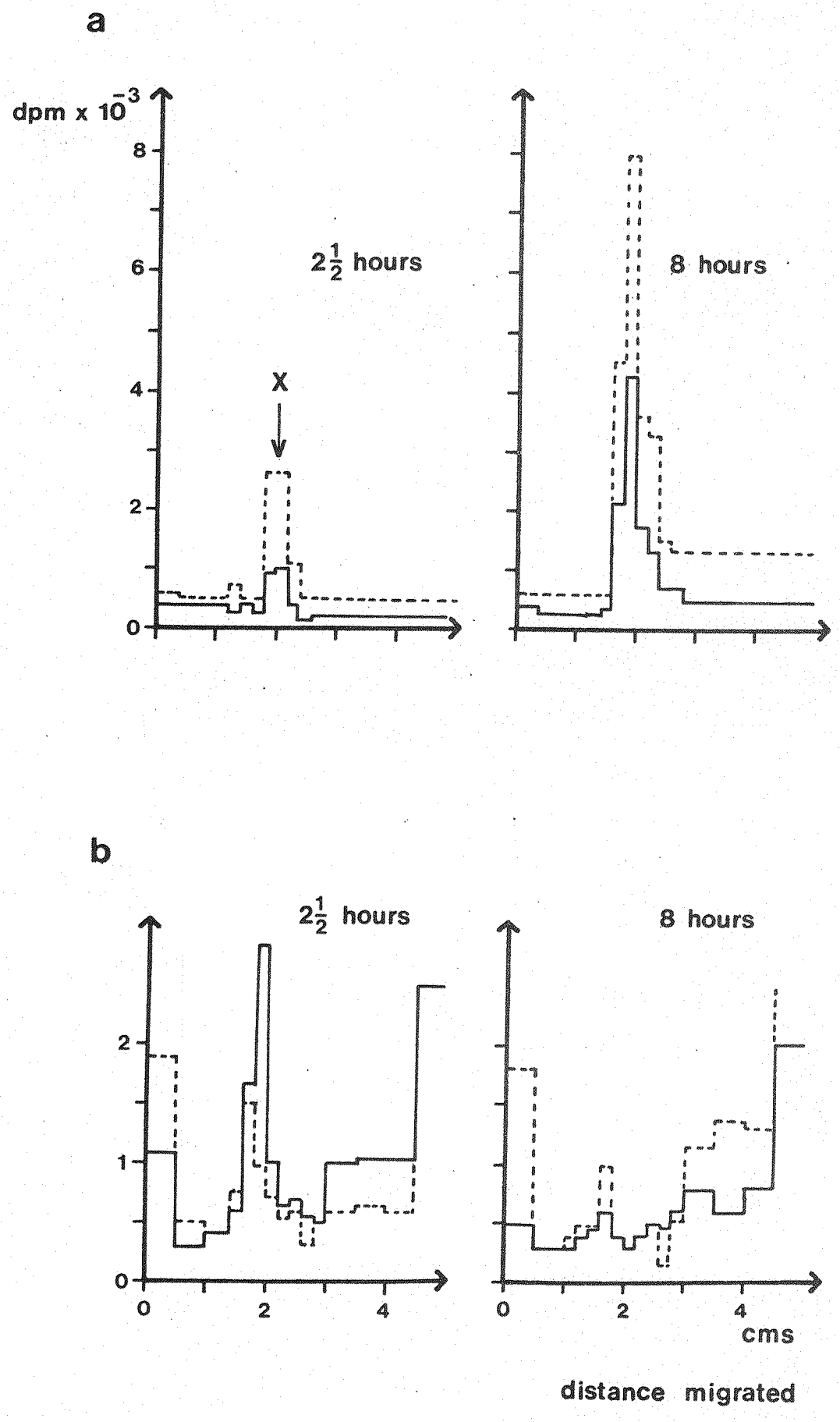
Figure 32 (overleaf) SDS Polyacrylamide Gel Analysis of (^{3}H) Leucine- and (^{32}P) Phosphate-Labelled Post-Microsomal Supernatant (a), Post-Microsomal Pellet (b) and Final Supernatant (c)

150 µl samples of the post-microsomal supernatant (a), post-microsomal pellet, (b), and final supernatant, (c), obtained at 2½ and 8 hours in the experiment described in Figure 31 were analysed on 7.5% SDS polyacrylamide gels. The 8 hour samples were obtained from the slices which had been transferred to fresh incubation medium at 6 hours. The radioactivity in 2 mm slices was determined. 'X' - position of a polypeptide mol. wt. approx. 200,000.

(^{3}H) ——

(^{32}P) ------

Figure 31


a
dpm x 10⁻³
8
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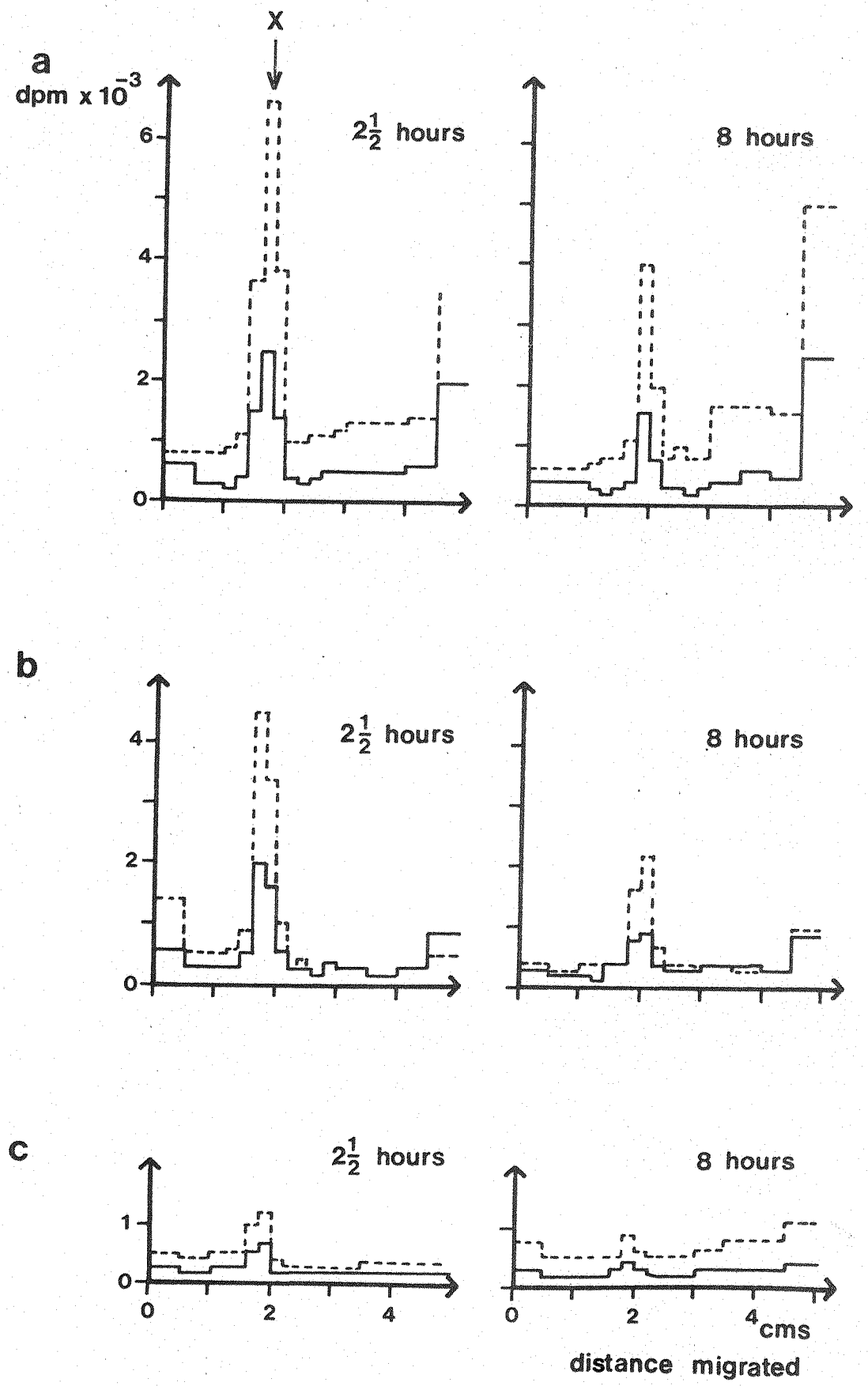
2½ hours
X
8 hours
b
2½ hours
8 hours
2
1
0
0
2
4
0
2
4
cms
distance migrated

SDS Polyacrylamide Gel Analysis of (^{3}H) Leucine- and (^{32}P) Phosphate-Labelled Post Microsomal Supernatant, (a), Post-Microsomal Pellet, (b) and Final Supernatant, (c). Figure 32



The observation that the pool of (^{3}H) leucine-labelled microsomal precursors included only a fraction of the (^{32}P) phosphate label associated with the secreted vitellogenin could imply either, that all of the (^{3}H) leucine-labelled precursors were slightly phosphorylated or, that the majority of the (^{3}H) leucine-labelled precursors were unphosphorylated but that there was a small proportion of fully phosphorylated precursors. When the results of SDS polyacrylamide gel analysis of the microsomal protein obtained from double-label experiments were carefully scrutinized, it was interesting to note that the (^{32}P) phosphate label associated with the 200,000 mol. wt. microsomal precursors of vitellogenin migrated slightly behind the main (^{3}H) leucine-labelled peak (see Figures 20, 21, 29 and 31). This suggested that the (^{32}P) phosphate label was associated with a species of slightly higher mol. wt. than the bulk of the (^{3}H) leucine-labelled precursors, and thus it favoured the idea of a small proportion of fully phosphorylated precursors with a slightly higher mol. wt. being present. To check whether this slight difference in migration was a genuine phenomenon, a sample of (^{32}P) phosphate-labelled incubation medium vitellogenin was mixed with a sample of solublized microsomal protein obtained after the incubation of liver slices with (^{3}H) leucine for 2½ hours and the mixture was analysed on an SDS polyacrylamide gel ; the results of two gel analyses are shown in Figure 33. The (^{32}P) phosphate-labelled incubation medium vitellogenin migrated slightly behind the (^{3}H) leucine-labelled microsomal precursor peak, and the difference in migration was very similar to that observed with double-labelled microsomal protein. This result supported the idea that the microsomal fraction included two distinct species of vitellogenin precursor, the majority being unphosphorylated, but with a small proportion of fully phosphorylated precursors of slightly higher mol. wt. and closely resembling the final secreted vitellogenin. The double-labelled 200,000 mol. wt. species in the post-microsomal fraction did not show the same discrepancy between the migration of the (^{32}P) phosphate and (^{3}H) leucine label,

Figure 33

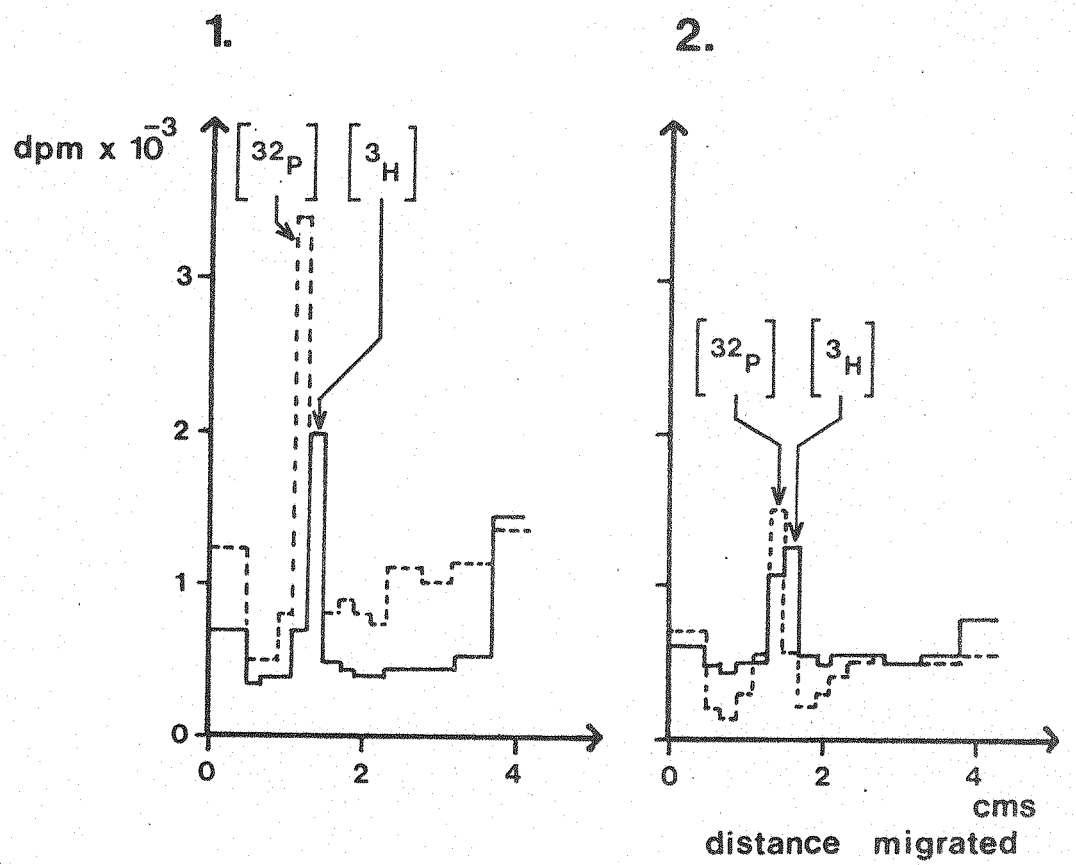


Figure 33 SDS Polyacrylamide Gel Analysis of (^{32}P) Phosphate-Labelled Incubation Medium and (^{3}H) Leucine-Labelled Microsomal Protein

Liver slices (0.5 g) were incubated with 100 µCi (^{32}P) phosphate for 6 hours. In a second incubation, liver slices were incubated with 20 µCi (^{3}H) leucine for 2½ hours and the microsomal fraction obtained. Samples of (^{32}P) phosphate-labelled incubation medium and (^{3}H) leucine-albelled microsomal protein were mixed, and the mixture analysed on a 7.5% SDS polyacrylamide gel. The results of 2 gel analyses (1 and 2) are shown.

(^{3}H) ——
(^{32}P) ------

and this suggested that it only included one species of precursor and that this was very similar to the final secreted vitellogenin.

4.3 The Identification of Glycosylated Precursors of Vitellogenin

4.3.1 The Incorporation of Labelled Sugars into Secreted Vitellogenin

Liver slices from oestrogen-treated animals were incubated with 50 µCi of (^{3}H) glucosamine, (^{3}H) galactose or (^{3}H) mannose for 6 hours and the incorporation of label into protein secreted into the incubation medium was determined. Figure 34 shows that both (^{3}H) glucosamine and (^{3}H) galactose were incorporated into secreted protein, however, the incorporation of (^{3}H) mannose was low and was not studied further. The incorporation of (^{3}H) glucosamine was attended by a 1½-2 hour lag period, similar to that characteristic of (^{3}H) leucine incorporation. SDS polyacrylamide gel analysis of the (^{3}H) glucosamine- and (^{3}H) galactose-labelled incubation medium (Figure 35) showed that in both cases vitellogenin was the only major labelled protein secreted. However, it was consistently found that significant amounts of background radioactivity were associated with the lower mol. wt. regions of the gel and this was probably due to the non-specific binding of the labelled sugars.

Since both (^{3}H) glucosamine and (^{3}H) galactose were incorporated into secreted vitellogenin, experiments were planned to look at the level of glycosylation of the intracellular precursors of vitellogenin. Ideally double-label experiments would have been carried out using (^{14}C)-labelled sugars and (^{3}H) leucine, similar to those performed with (^{32}P) phosphate and (^{3}H) leucine ; however, (^{14}C)-labelled sugars are very expensive and therefore, as a more economical alternative, it was necessary to compare the incorporation of (^{3}H) leucine and either (^{3}H) glucosamine or (^{3}H) galactose in parallel incubations. In order to do this, comparable amounts of (^{3}H) leucine and the

Figure 34

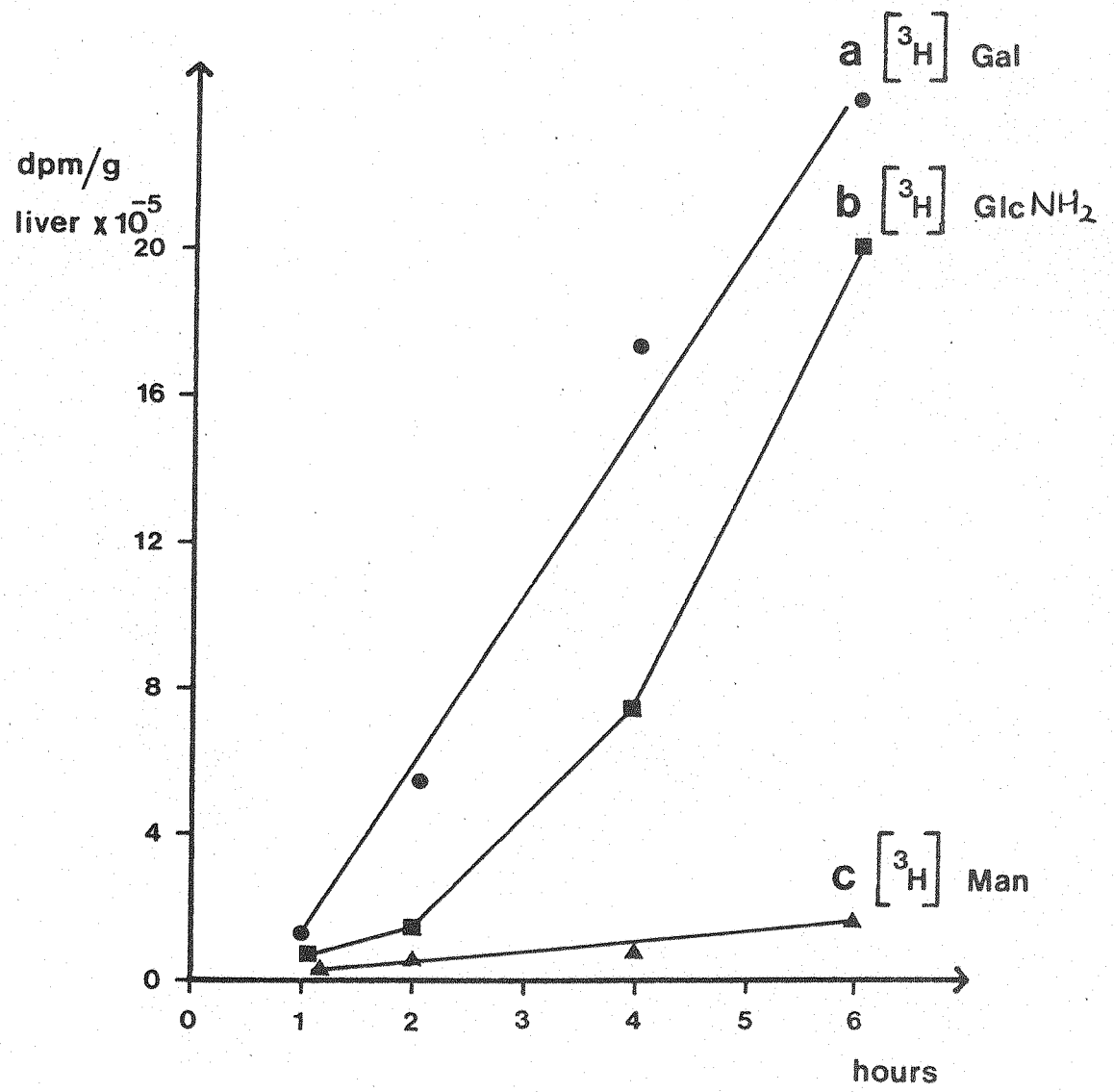


Figure 34 The Incorporation of (^{3}H) Glucosamine, (^{3}H) Galactose and (3H) Mannose into Secreted Protein

Liver slices (0.5 g) were incubated with 50 μCi (^{3}H) glucosamine, (^{3}H) galactose or (^{3}H) mannose for 6 hours in 4.5 ml of incubation medium. At various times, 0.3 ml samples of the incubation medium were removed and analysed for the incorporation of radioactivity into secreted protein by the filter paper disc method as described in Chapter 2.

Figure 35

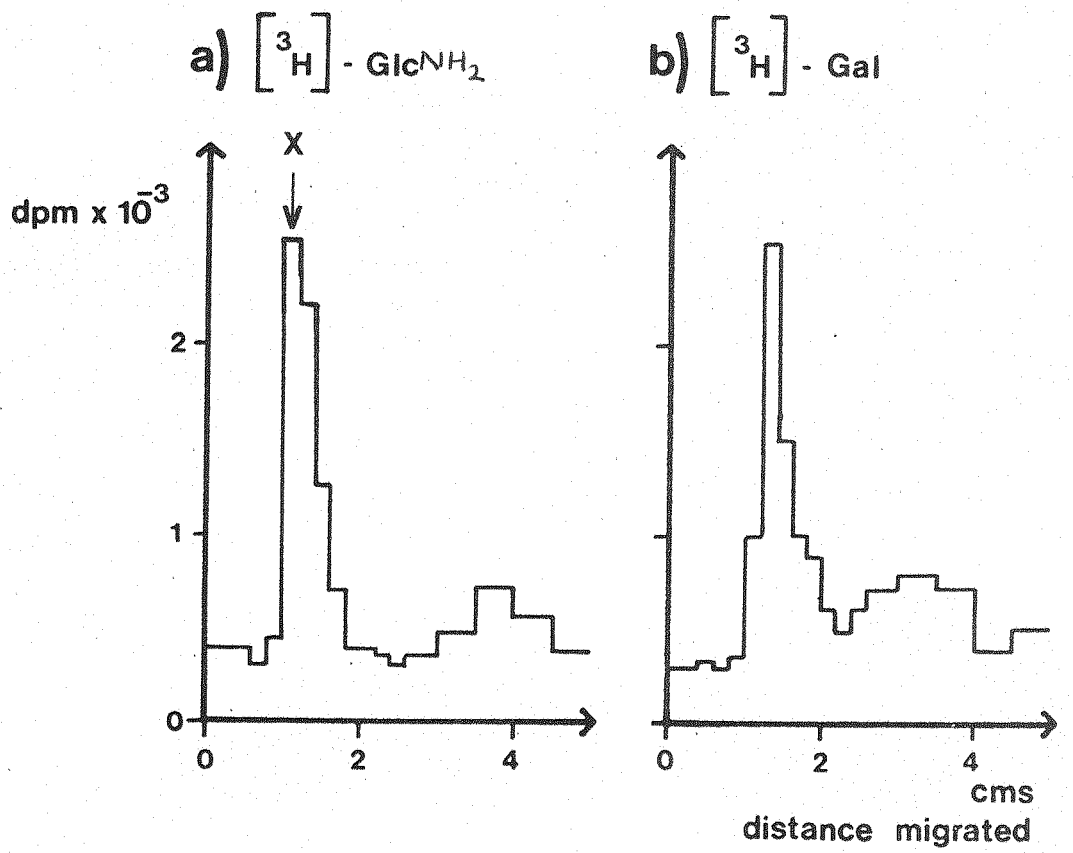


Figure 35 SDS Polyacrylamide Gel Analysis of (^{3}H) Glucosamine- and (3H) Galactose-Labelled Incubation Medium

100 µl sample of (^{3}H) glucosamine-labelled, (a), and (^{3}H) galactose-labelled, (b), incubation medium from the experiment described in Figure 34 were analysed on 7.5% SDS polyacrylamide gels. The radioactivity in 2 mm slices was determined. 'X' - position of polypeptide mol. wt. approx. 200,000.

(^{3}H) sugars had to be incorporated into secreted vitellogenin, and this was achieved by incubating the liver slices with 50 µCi of (^{3}H) sugar and 10 µCi of (^{3}H) leucine.

4.3.2 The Incorporation of (^{3}H) Glucosamine and (^{3}H) Galactose into the Microsomal Precursors of Vitellogenin

0.5 g samples of liver slices were incubated with 50 µCi of (^{3}H) glucosamine and identical samples were incubated with 10 µCi of (^{3}H) leucine. At 2½ hours, one (^{3}H) glucosamine incubation and one (^{3}H) leucine incubation were removed and cycloheximide was added to the remaining flasks. At 6 hours, the slices from one of the remaining incubations with each isotope were removed, washed and transferred to fresh incubation medium (as described in 4.2.4 a)), and the incubations were continued for a further 2 hours. The slices were then fractionated to obtain the microsomes, the post-microsomal supernatant and the post-microsomal pellet as described previously. In an identical experiment, liver slices were incubated with 10 µCi of (^{3}H) leucine and 50 µCi of (^{3}H) galactose.

Figure 36 shows the results of SDS polyacrylamide gel analysis of the (^{3}H) leucine- and (^{3}H) glucosamine-labelled incubation medium, and this confirmed that both were incorporated into secreted vitellogenin. SDS polyacrylamide gel analysis of the (^{3}H) leucine-labelled microsomal protein (Figure 37a) confirmed that at 2½ hours, the familiar (^{3}H) leucine-labelled, 200,000 mol. wt. precursors of vitellogenin were associated with the microsomes and that these were lost during the chase period. However, analysis of the (^{3}H) glucosamine-labelled microsomal protein (Figure 37b) showed that there was very little (^{3}H) glucosamine label associated with the microsomal precursors, and it was estimated that the (^{3}H) leucine-labelled microsomal precursors included only about 10% of the (^{3}H) glucosamine label present in the final secreted vitellogenin.

Figure 38 shows the results of SDS polyacrylamide gel analysis of the (^{3}H) leucine- and (^{3}H) galactose-

Figure 36 SDS Polyacrylamide Gel Analysis of (^{3}H) Leucine-Labelled (a) and (^{3}H) Glucosamine-Labelled (b) Incubation Medium

3 flasks of liver slices (0.5 g) were incubated with 50 μCi ^{3}H(glucosamine and 3 flasks were incubated with 10 μCi (^{3}H) leucine. At 2½ hours, one (^{3}H) glucosamine incubation and on (^{3}H) leucine incubation were removed and cycloheximide was added to the remaining flasks. At 6 hours, the slices from one of the remaining incubations with each isotope were removed, washed, and transferred to fresh incubation medium, the slices in the other flasks remained in the original incubation medium. The incubations were continued for a further 2 hours. 100 μl samples of the (^{3}H) leucine-labelled, (a), and (^{3}H) glucosamine-labelled, (b), incubation medium obtained at 8 hours (from the incubations in which slices had remained in the original medium) were analysed on 7.5% SDS polyacrylamide gels. The radioactivity in 2 mm slices was determined. 'X' - position of polypeptide mol. wt. approx. 200,000.

Figure 37 (overleaf) SDS Polyacrylamide Gel Analysis of (3H) Leucine-Labelled (a) and (3H) Glucosamine-Labelled (b) Microsomal Protein

150 μl samples of (^{3}H) leucine-labelled, (a), and (^{3}H) glucosamine-labelled,(b), microsomal protein obtained at 2½ and 8 hours (from the slices which had remained in the original medium) in the experiment described in Figure 36 were analysed on 7.5% SDS polyacrylamide gels. The radioactivity in 2 mm slices was determined. 'X' - position of polypeptide mol. wt. approx. 200,000.

Figure 36

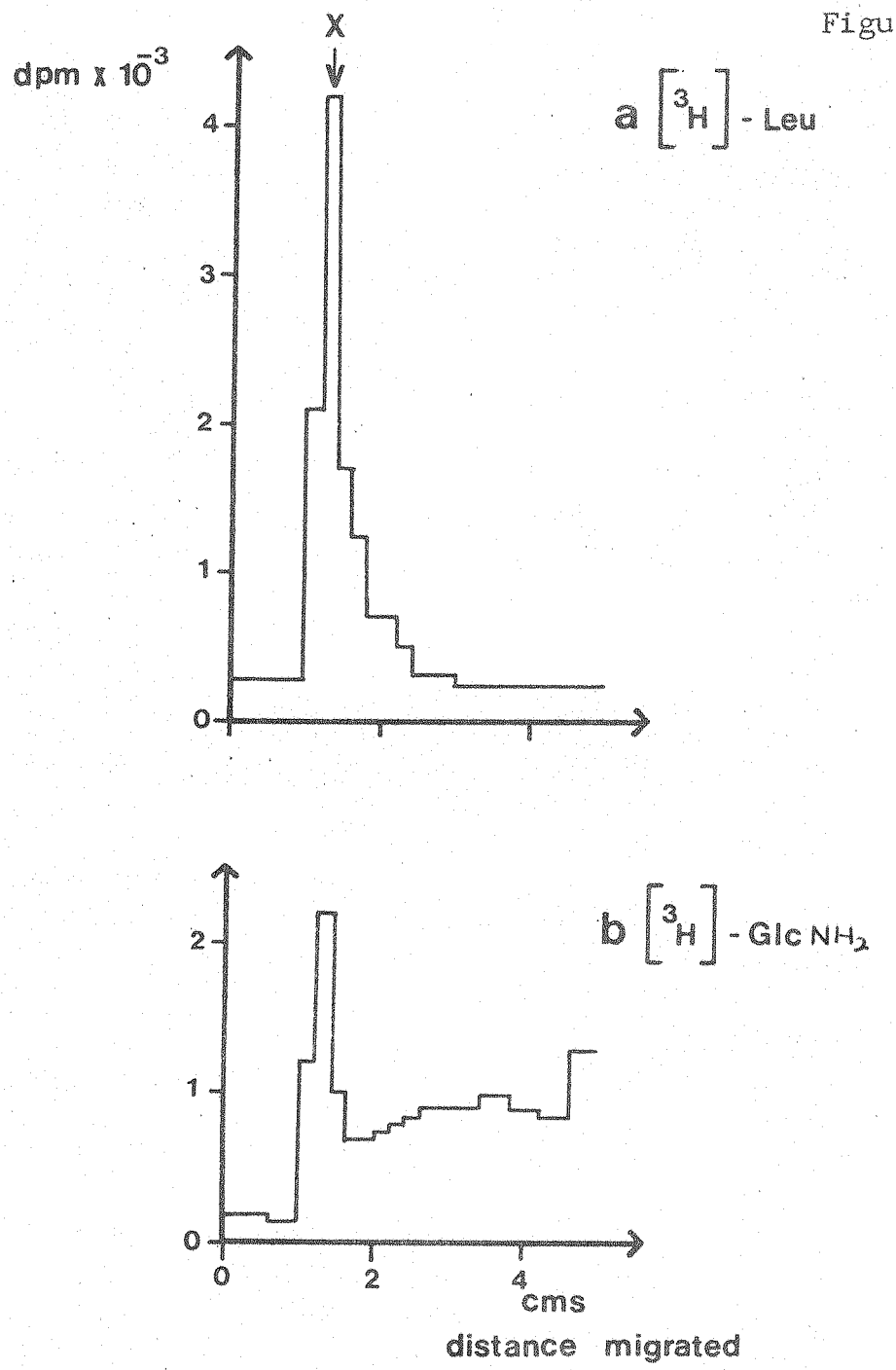
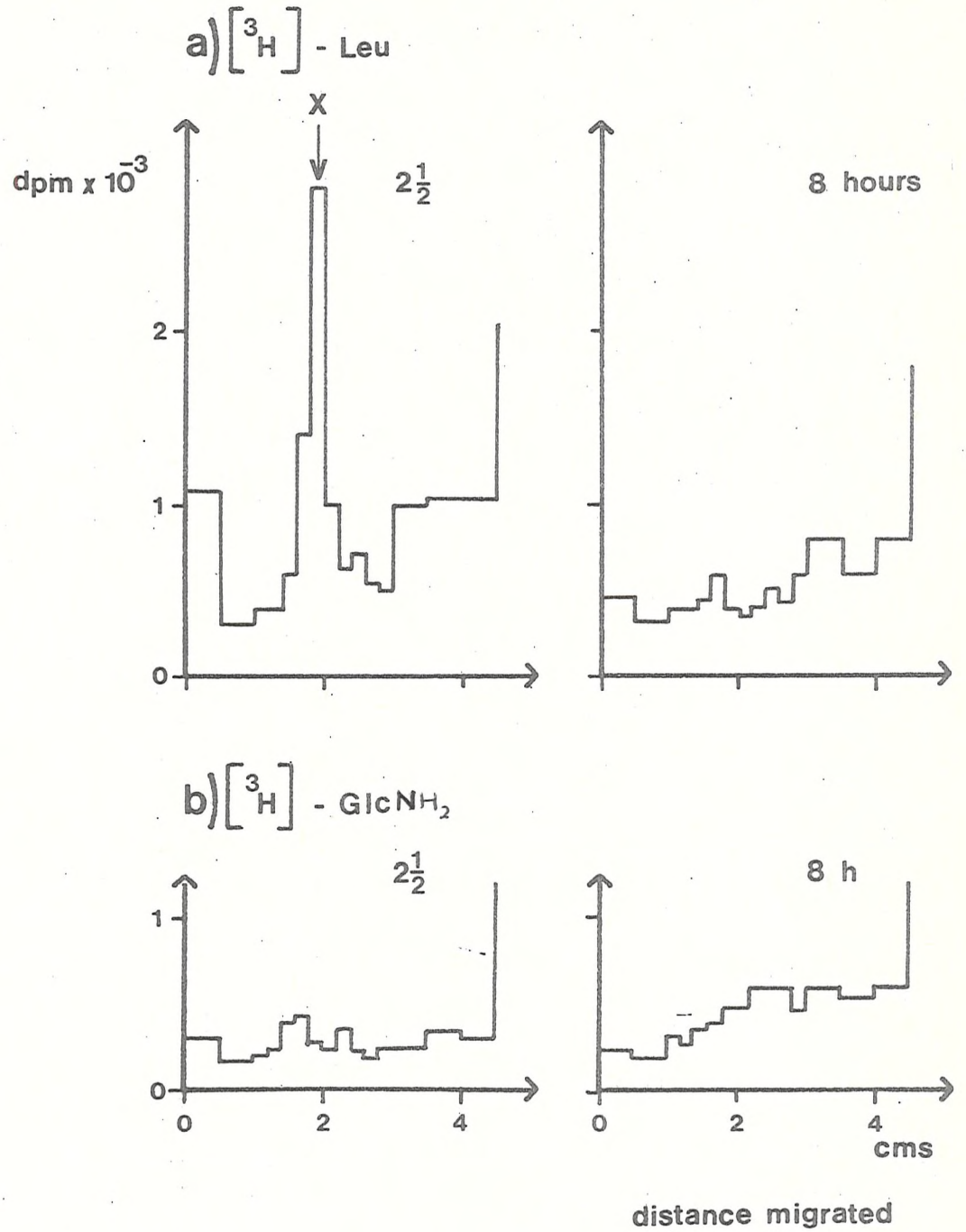

dpm x 10^{-3}
X
4
3
2
1
0
a [^{3}H] - Leu
b [^{3}H] - Glc NH$_2$
2
1
0
0
2
4
cms
distance migrated

Figure 37



SDS Polyacrylamide Gel Analysis of (^{3}H) Leucine-Labelled (a) and (^{3}H) Glucosamine-Labelled (b) Microsomal Protein

Figure 38 SDS Polyacrylamide Gel Analysis of (^{3}H) Leucine-Labelled (a) and (^{3}H) Galactose-Labelled (b) Incubation Medium

The experiment was identical to that described in Figure 36 except that the liver slices were incubated with 50 µCi of (^{3}H) galactose instead of (^{3}H) glucosamine. 100 µl samples of (^{3}H) leucine-labelled, (a), and (^{3}H) galactose-labelled, (b), incubation medium obtained at 8 hours (from the incubations in which slices had remained in the original medium) were analysed on 7.5% polyacrylamide gels. The radioactivity in 2 mm slices was determined. 'X' - position of polypeptide mol. wt. approx. 200,000.

Figure 39 (overleaf) SDS Polyacrylamide Gel Analysis of (^{3}H) Leucine-Labelled (a) and (^{3}H) Galactose-Labelled (b) Microsomal Protein

150 µl samples of (^{3}H) leucine-labelled, (a), and (^{3}H) galactose-labelled, (b), microsomal protein obtained at 2½ and 8 hours (from the incubations in which slices remained in the original medium) in the experiment described in Figure 38 were analysed on 7.5% SDS polyacrylamide gels. The radioactivity in 2 mm slices was determined. 'X' - position of polypeptide mol. wt. approx. 200,000.

Figure 38

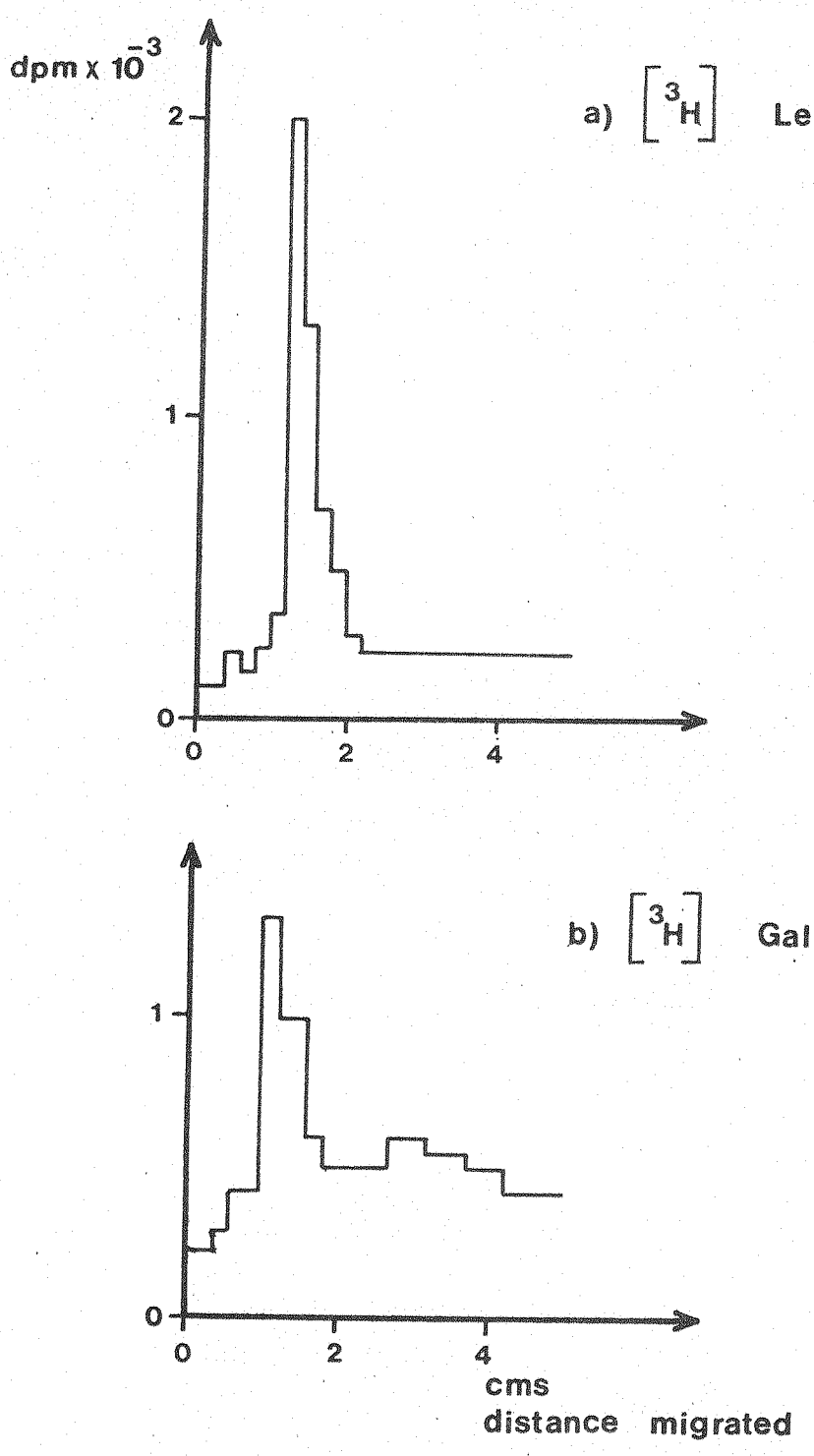
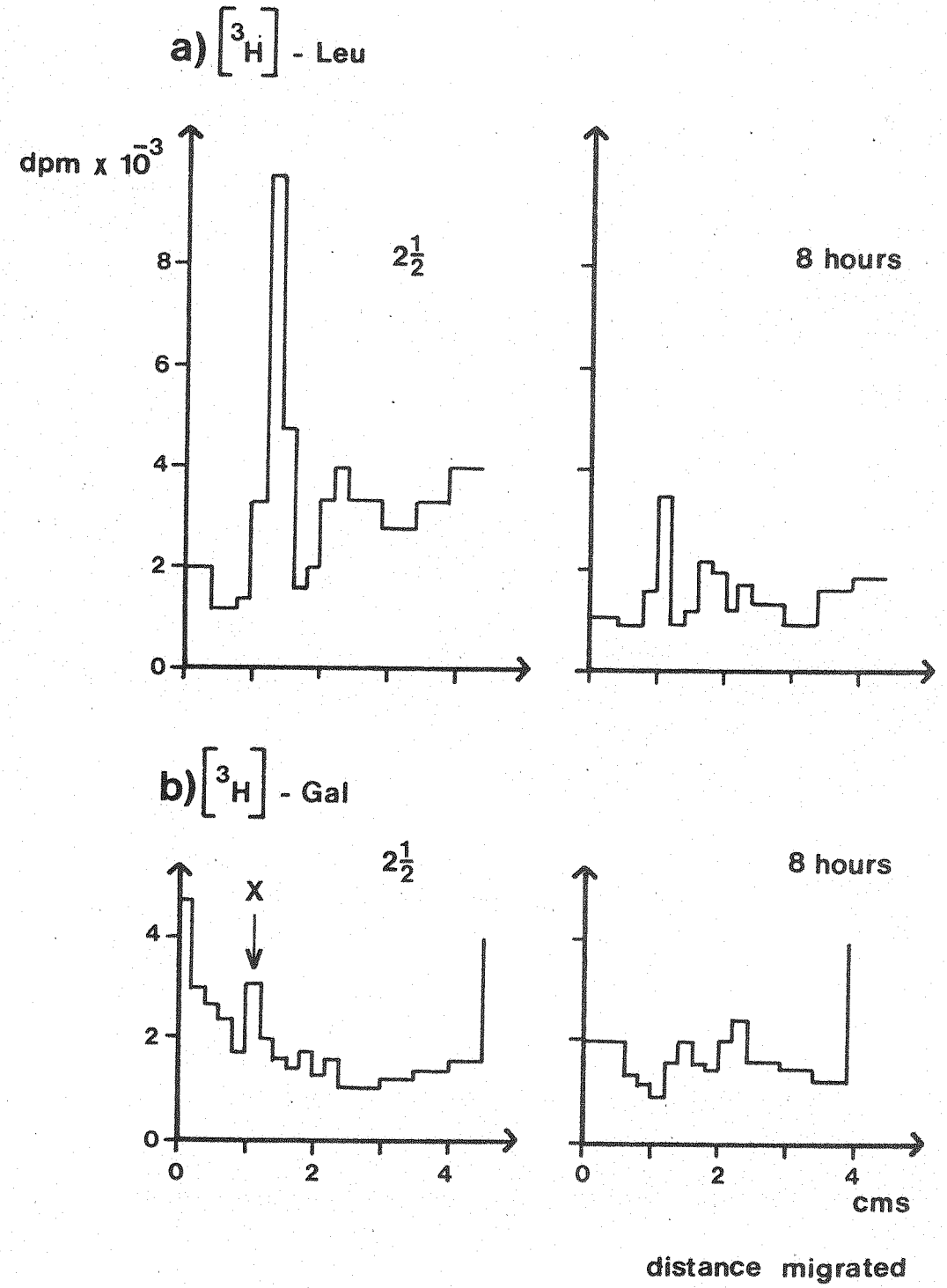


Figure 39



SDS Polyacrylamide Gel Analysis of (^{3}H) Leucine-Labelled (a) and (^{3}H) Galactose-Labelled (b) Microsomal Protein

labelled incubation medium obtained in an identical experiment, and this confirmed that both were incorporated into secreted vitellogenin. SDS gel analysis of the (^{3}H) leucine-labelled microsomal protein (Figure 39a) again confirmed that at 2½ hours, the (^{3}H) labelled, 200,000 mol. wt. precursors of vitellogenin were associated with the microsomes and that these were lost at 8 hours. However, analysis of the (^{3}H) galactose-labelled microsomal protein (Figure 39b) showed that there was very little (^{3}H) galactose label associated with the microsomal precursors, and it was estimated that the (^{3}H) leucine-labelled microsomal precursors included only about 10% of the (^{3}H) galactose label present in the final secreted vitellogenin.

These results indicated that there was little detectable accumulation of (^{3}H)-glucosamine- and (^{3}H) galactose-labelled precursors on the microsomes, and thus they suggested that the microsomal precursors of vitellogenin were relatively unglycosylated.

4.3.3 The Incorporation of (^{3}H Glucosamine and (^{3}H) Galactose into the Post-Microsomal Species

The post-microsomal supernatant and post-microsomal pellet fractions from the experiments described in 4.3.2 were analysed by SDS polyacrylamide gel electrophoresis.

Figures 40 and 41 show the results of SDS polyacrylamide gel analysis of the (^{3}H) leucine- and (^{3}H) glucosamine-labelled post-microsomal supernatant and post-microsomal pellet fractions obtained from parallel incubations. At 2½ hours, the post-microsomal supernatant included the familiar (^{3}H) leucine labelled, 200,000 mol. wt. species, and at 8 hours this was reduced to 30% of its 2½ hour level (Figure 40a). After the three hour centrifugation step, approximately 70% of this labelled species was recovered on gels of the post-microsomal pellet (Figure 40b). Thus, these results confirmed those obtained in previous experiments with (^{3}H) leucine. Analysis of the (^{3}H) glucosamine-labelled post-microsomal supernatant obtained in the parallel experiment (Figure 41a) showed

Figure 40 SDS Polyacrylamide Gel Analysis of (^{3}H) Leucine-Labelled Post-Microsomal Supernatant (a) and Post-Microsomal Pellet (b)

Figure 41 (overleaf) SDS Polyacrylamide Gel Analysis of (^{3}H) Glucosamine-Labelled Post-Microsomal Supernatant (a) and Post-Microsomal Pellet (b)

150 µl samples of (^{3}H) leucine- and (^{3}H) glucosamine-labelled post-microsomal supernatant, (a), and post-microsomal pellet, (b), obtained at 2½ and 8 hours in the experiment described in Figure 36 were analysed on 7.5% SDS polyacrylamide gels. The 8 hour samples were obtained from the slices which had been transferred to fresh incubation medium at 6 hours. The radioactivity in 2 mm slices was determined. 'X' - position of a polypeptide mol. wt. approx. 200,000.

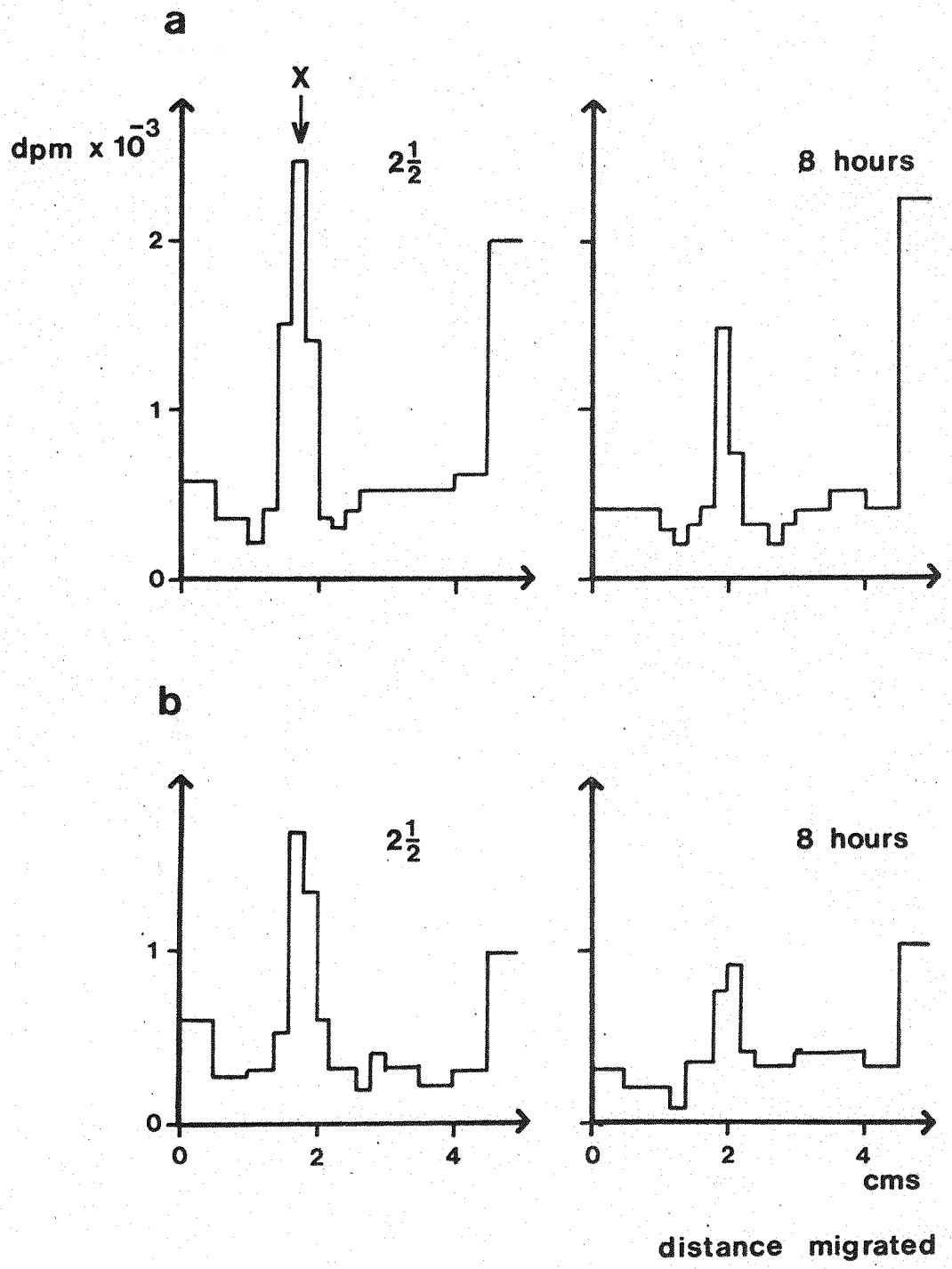
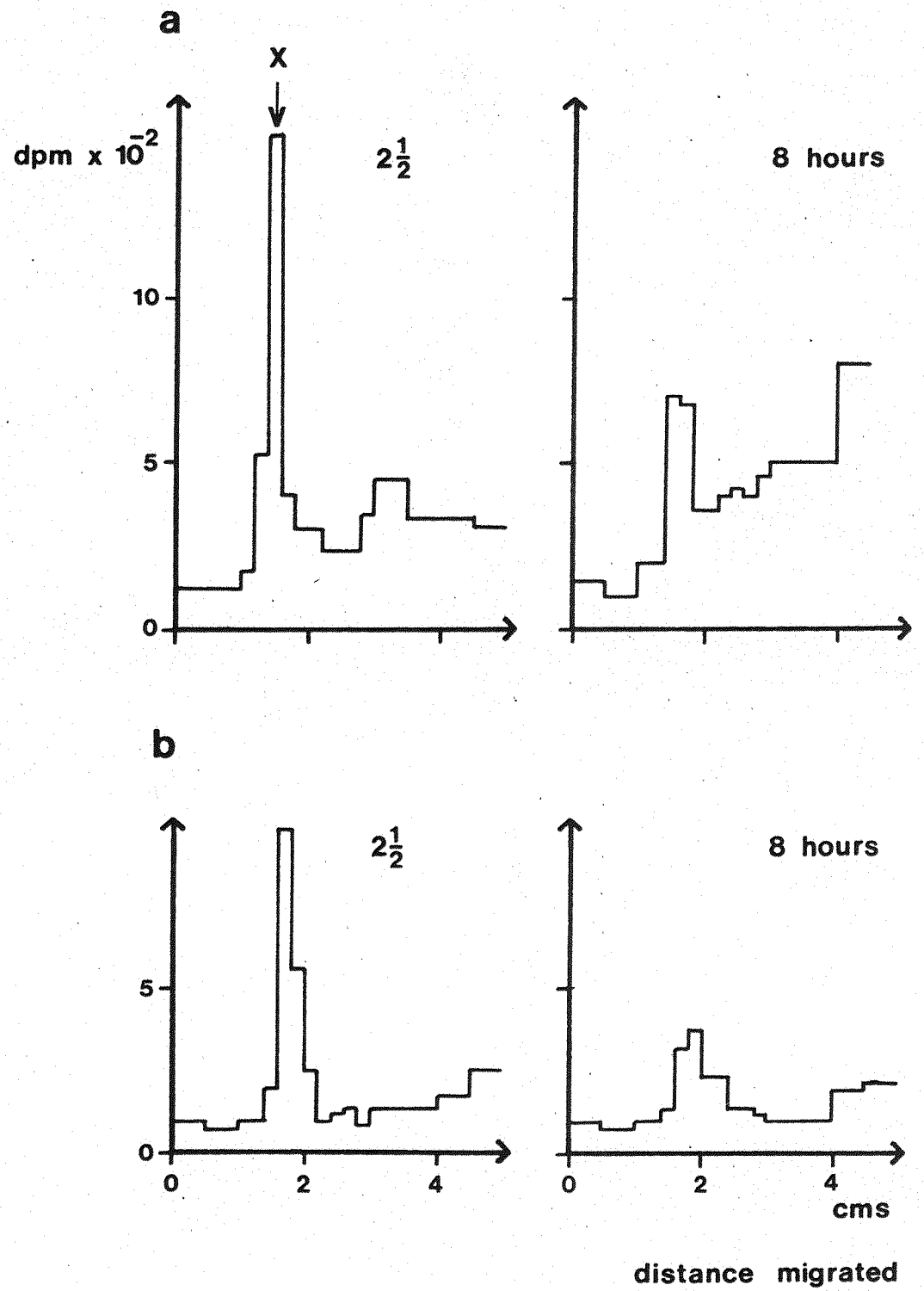

a
X
dpm x 10^{-3}
$2\frac{1}{2}$
8 hours
2
1
0
b
$2\frac{1}{2}$
8 hours
1
0
0
2
4
0
2
4
cms
distance migrated

Figure 41



SDS Polyacrylamide Gel Analysis of (^{3}H) Glucosamine-Labelled Post-Microsomal Supernatant (a) and Post-Microsomal Pellet (b)

that the 200,000 mol. wt. post-microsomal species was also clearly labelled with (^{3}H) glucosamine. However, the loss of this labelled species between 2½ and 8 hours was not so obvious as the loss of the (^{3}H) leucine-labelled species ; this was because of the increase in the background level of radioactivity associated with the gels at 8 hours. Approximately 80% of the (^{3}H) glucosamine-labelled, post-microsomal supernatant species was recovered on gels of the post-microsomal pellet (Figure 41b), and in this fraction the loss of this species between 2½ and 8 hours was clearly shown. A comparison of the relative incorporation of (^{3}H) leucine and (^{3}H) glucosamine into the vitellogenin secreted into the incubation medium (Figure 36) and into the post-microsomal species (Figure 40 and 41) indicated that the post-microsomal species was labelled with (^{3}H) glucosamine to a similar extent to the final secreted vitellogenin.

Figures 42 and 43 show the results of SDS polyacrylamide gel analysis of the (^{3}H) leucine- and (^{3}H) galactose-labelled post-microsomal supernatant and post-microsomal pellet fractions obtained from parallel incubations. At 2½ hours, the post-microsomal supernatant again included the familiar (^{3}H) leucine-labelled, 200,000 mol. wt. species and at 8 hours, this was reduced to 20% of its 2½ hour level (Figure 42a). After the 3-hour centrifugation step, approximately 65% of this labelled species was recovered on gels of the post-microsomal pellet (Figure 42b). These results again confirmed those obtained in previous experiments with (^{3}H) leucine. Analysis of the (^{3}H) galactose-labelled post-microsomal supernatant (Figure 43a) showed that the 200,000 mol. wt. post-microsomal species was also clearly labelled with (^{3}H) galactose, and that at 8 hours this was reduced to about 20% of its 2½ hour level. Approximately 65% of the (^{3}H) galactose-labelled post-microsomal supernatant species was recovered on gels of the post-microsomal pellet (Figure 43b) (In these experiments, the final supernatant remaining after the 3 hour centrifugation step was not analysed, however, earlier experiments had shown that very little of the

Figure 42 SDS Polyacrylamide Gel Analysis of (^{3}H) Leucine-Labelled Post-Microsomal Supernatant (a) and Post-Microsomal Pellet (b)

Figure 43 (overleaf) SDS Polyacrylamide Gel Analysis of (^{3}H) Galactose-Labelled Post-Microsomal Supernatant (a) and Post-Microsomal Pellet (b)

150 µl samples of the (^{3}H) leucine- and (^{3}H) galactose-labelled post-microsomal supernatant, (a), and post-microsomal pellet, (b), obtained at 2½ and 8 hours in the experiment described in Figure 38 were analysed on 7.5% SDS polyacrylamide gels. The 8 hour samples were obtained from the slices which had been transferred to fresh incubation medium at 6 hours. The radioactivity in 2 mm slices was determined. 'X' - position of a polypeptide mol. wt. approx. 200,000.

Figure 42

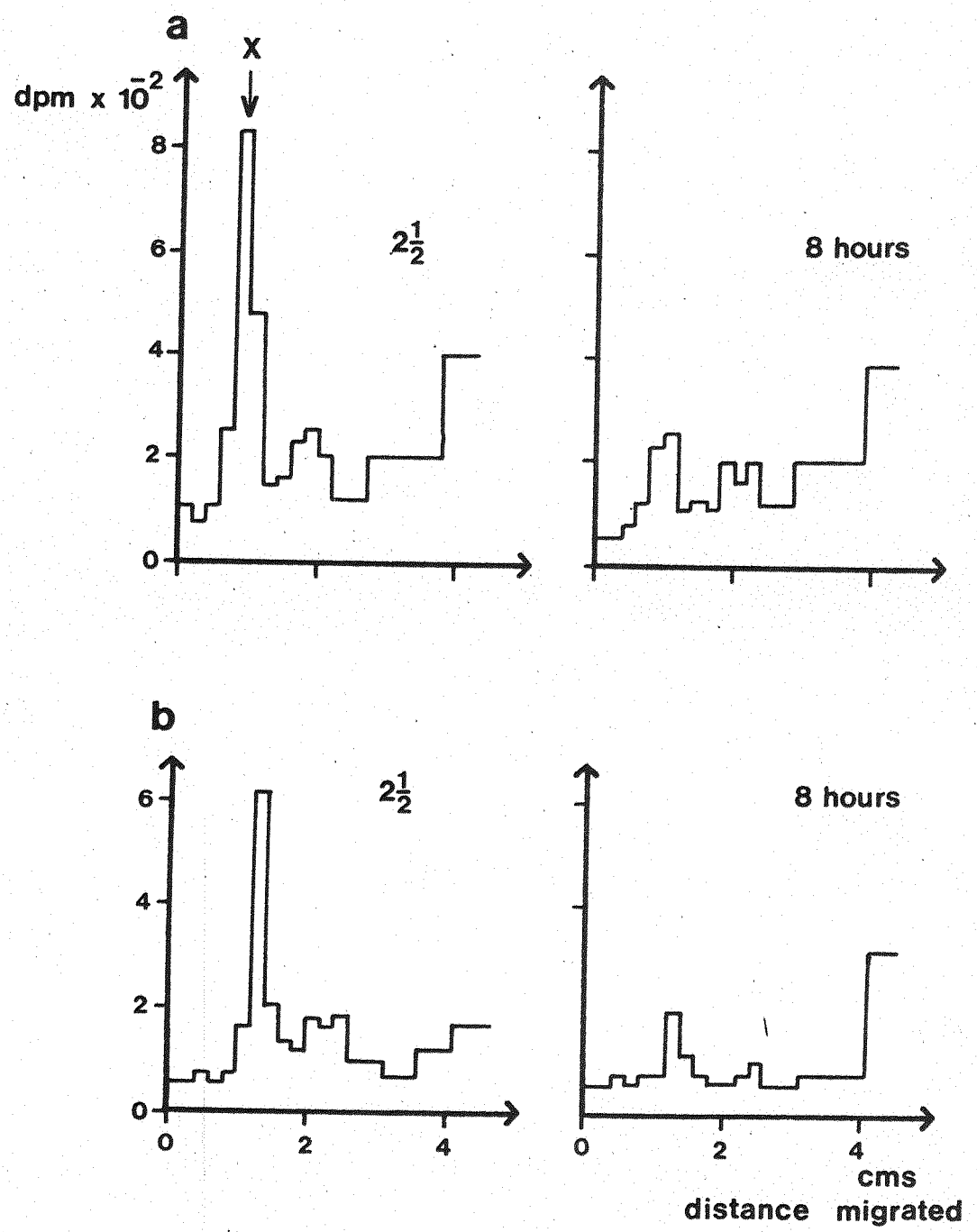
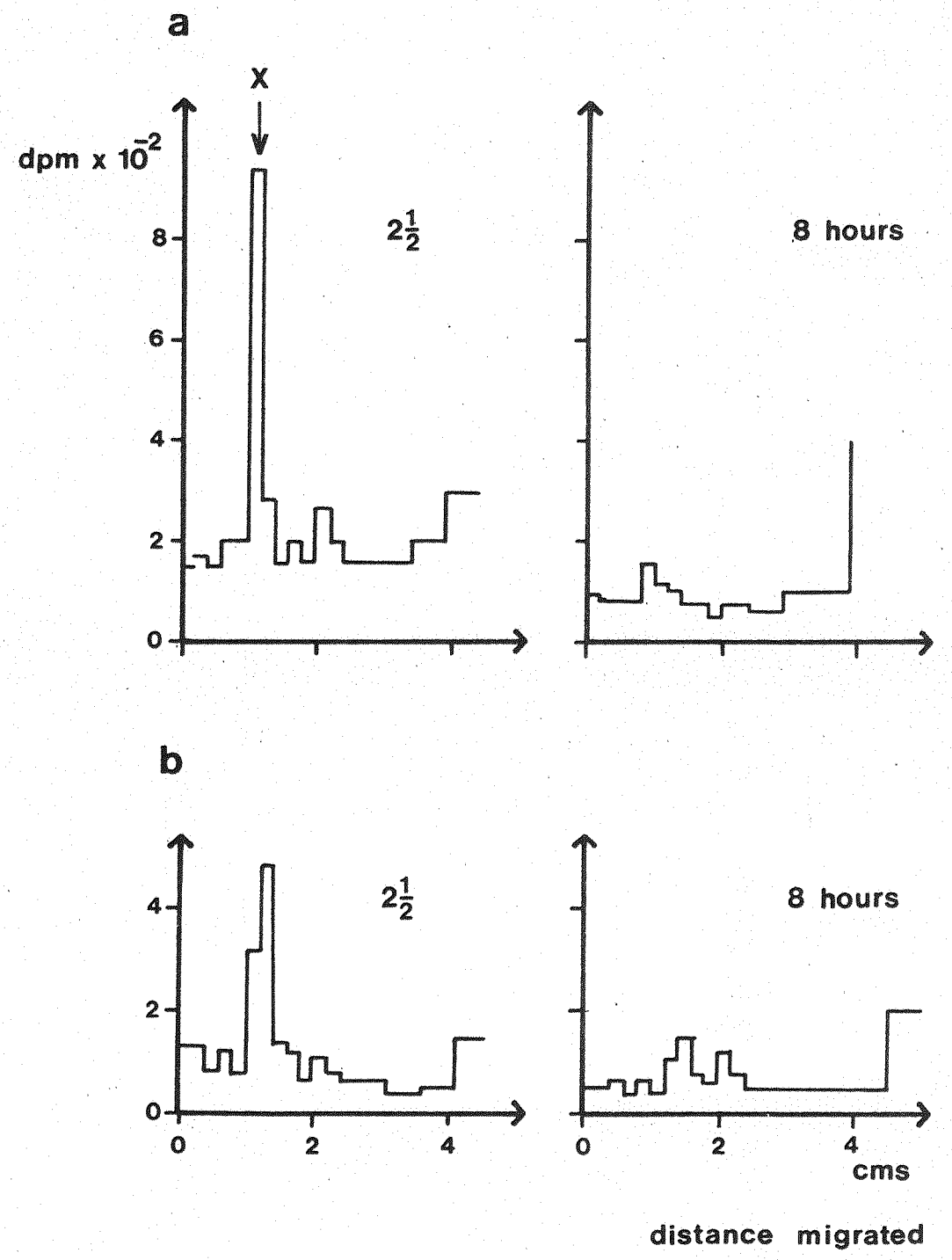

a
X
dpm x 10^{-2}
8
6
4
2
0
$2\frac{1}{2}$
8 hours
b
6
4
2
0
0
2
4
$2\frac{1}{2}$
8 hours
0
2
4
cms
distance migrated

Figure 43



SDS Polyacrylamide Gel Analysis of (^{3}H) Galactose-Labelled Post-Microsomal Supernatant (a) and Post-Microsomal Pellet (b)

labelled, 200,000 mol. wt. species remained in the final supernatant (see sections 4.2.4 b and 4.2.5)). A comparison of the relative incorporation of (^{3}H) leucine and (^{3}H) galactose into the vitellogenin secreted into the incubation medium (Figure 37) and into the post-microsomal species (Figure 42 and 43) indicated that the post-microsomal species was labelled with (^{3}H) galactose to a similar extent to the final secreted vitellogenin.

These results again demonstrated that at 2½ hours, the post-microsomal supernatant contained a (^{3}H) leucine-labelled, 200,000 mol. wt. species which was lost during the chase period, and that this species co-sediment with the post-microsomal supernatant. However, they also showed that, in contrast to the microsomal precursors, the post-microsomal species was labelled with both (^{3}H) glucosamine and (^{3}H) galactose to a similar extent to the secreted vitellogenin and thus they suggested that, unlike the microsomal precursors, the post-microsomal species was relatively highly glycosylated.

CHAPTER 5

Chapter 5

THE INCORPORATION OF LABELLED SUGARS INTO GLYCOLIPID INTERMEDIATES DURING VITELLOGENIN SYNTHESIS

In Chapter 1, the evidence to support the role of glycolipid intermediates during the glycosylation of proteins was discussed. Since vitellogenin is a glycoprotein, it was interesting to see whether there was any evidence to suggest the participation of similar intermediates during oestrogen-induced vitellogenin synthesis in Xenopus laevis. Therefore the experiments reported in this chapter aimed to find out whether oestrogen-treatment resulted in any stimulation of the incorporation of labelled sugars into glycolipid intermediates which could be involved in the glycosylation of vitellogenin. Two types of experiment were performed ; first, microsomal fractions, obtained from control and oestrogen-treated Xenopus, were used to study the transfer of labelled sugars from nucleotide sugars to glycolipid intermediates ; and second, the incorporation of labelled sugars into glycolipid intermediates by liver slices from control and oestrogen-treated Xenopus was investigated.

5.1 The Incorporation of Labelled Sugar from UDP (^{3}H) Galactose, GDP (^{14}C) Mannose and UDP-N-Acetyl (^{14}C) Glucosamine into Glycolipid-Intermediates by Xenopus Liver Microsomes

Microsomal suspensions, prepared from Xenopus liver as described in Chapter 2, were used to study the incorporation of labelled sugar from UDP (^{3}H) galactose, GDP (^{14}C) mannose and UDP N-acetyl (^{14}C) glucosamine into a glycolipid fraction extracted with 2:1 chloroform/methanol. Incubations were in the presence of $MnCl_2$, 2-mercaptoethanol and EDTA and the conditions were as described in Chapter 2.

5.1.1 The Effect of Oestrogen-Treatment on Incorporation

a) UDP (^{3}H) Galactose and GDP (^{14}C) Mannose Incorporation

The microsomal fraction from control and oestrogen-treated animals was incubated with either 1 μCi of UDP (^{3}H) galactose or 0.1 μCi of GDP (^{14}C) mannose. At 10, 20, 30 and 60 minutes samples were taken and lipid extracted with 2:1 chloroform/methanol as described in Chapter 2. The incorporation of radioactivity into the 2:1 chloroform/methanol extract, subsequently referred to as the 2:1 extract, was determined at each time and the results are shown in Figure 44. The incorporation of both UDP (^{3}H) galactose and GDP (^{14}C) mannose into labelled glycolipid present in the 2:1 extract was 3-4 fold higher in the oestrogen-treated incubations than in control incubations. In the oestrogen-treated incubation the incorporation of UDP (^{3}H) galactose was linear, and at 60 minutes the incorporation was 74,000 dpm/g liver, compared with 19,000 dpm/g liver in the control incubation. The incorporation of GDP (^{14}C) mannose in the oestrogen-treated incubation reached a maximum of 81,000 dpm/g liver at 30 minutes, compared with 23,600 dpm/g liver in the control incubation. In subsequent experiments, incubations were for one hour.

In these preliminary experiments, the incorporation data obtained was expressed with reference to the wet

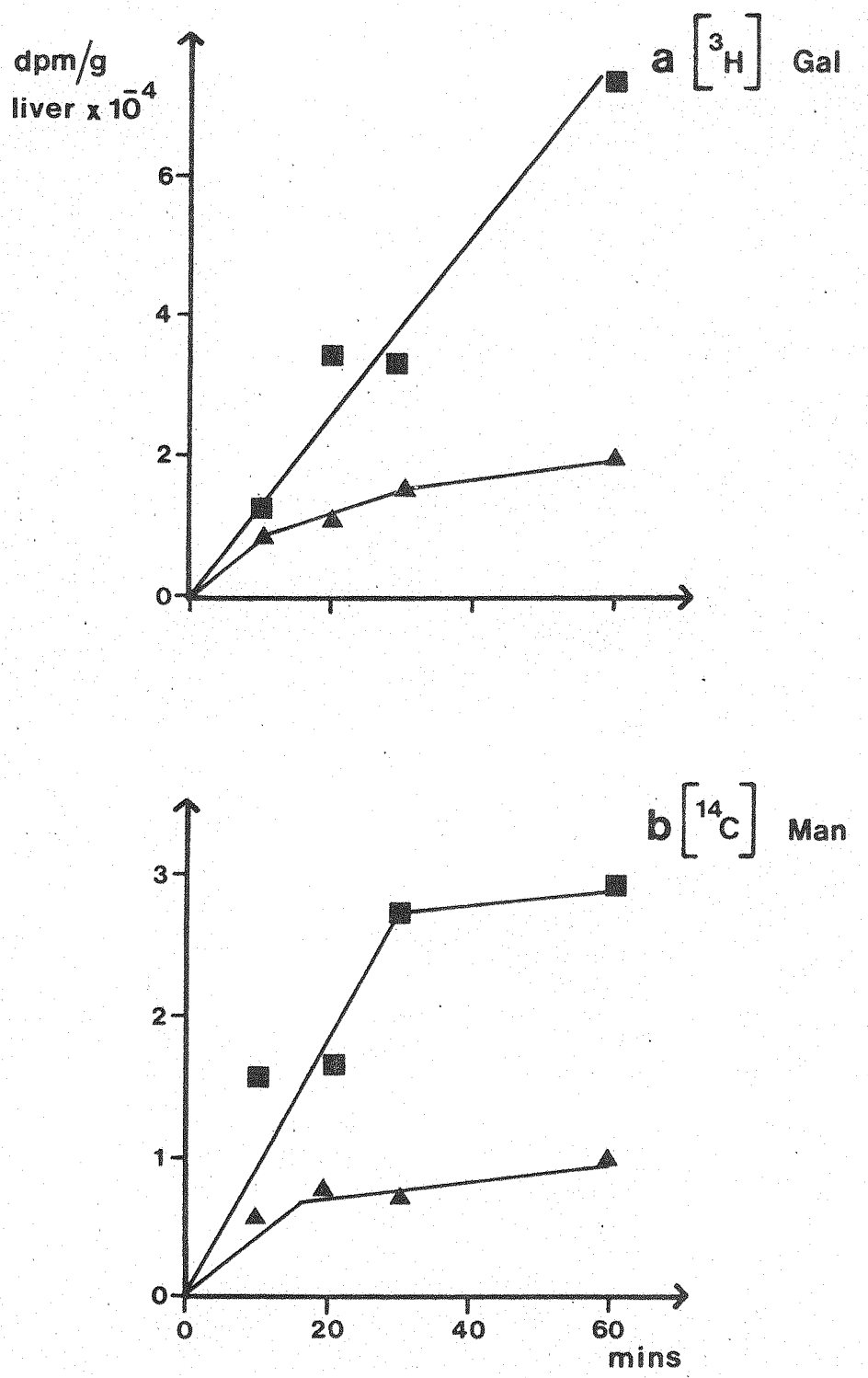
Figure 44 The Incorporation of UDP (^{3}H) Galactose (a) and GDP (^{14}C) Mannose (b) into Glycolipid by Microsomes from Control and Oestrogen-Treated Animals

The microsomal fraction was obtained from the liver of a control and an oestrogen-treated animal. Duplicate 0.8 ml aliquots of microsomal suspension were incubated with either 1 μCi UDP (^{3}H) galactose, (a), or 0.1 μCi GDP (^{14}C) Mannose, (b). At various times, 0.4 ml samples were taken and lipid extracted with 2:1 chloroform/methanol. The incorporation of radioactivity into the 2:1 extract was determined. The results are expressed as dpm/g wet weight liver used for the preparation of the microsomes. Full details are given in Chapter 2.

Control ▲

Oestrogen-treated ■

Figure 44



weight of liver used for the preparation of the microsomes. It has been shown in previous work in this laboratory (Smith et al, 1978) that oestrogen-treatment results in an increase in the microsomal protein content of the liver. Therefore there was the possibility that the increase in incorporation observed after oestrogen-treatment was due, in part, to a higher microsomal protein concentration in the oestrogen-treated incubations, however, the results of subsequent experiments showed that this was not the case.

b) The Time-Course of the Oestrogen-Induced Stimulation of Incorporation

The results of preliminary experiments showed that after oestrogen-treatment there was increased incorporation of labelled sugar from both UDP (^{3}H) galactose and GDP (^{14}C) mannose into a glycolipid fraction by liver microsomes. It was decided to look at the level of stimulation of this incorporation at various times after oestrogen-treatment.

At 3, 7, and 14 days after oestrogen-treatment, microsomes were prepared from 2 experimental animals and one control and incubated with UDP (^{3}H) galactose and GDP (^{14}C) mannose for 1 hour in parallel incubations. The incorporation of radioactivity into the 2:1 extract was determined ; the results are shown in Table 13 and are expressed as incorporation/mg microsomal protein.

At 3 days after oestrogen-treatment the incorporation of UDP (^{3}H) galactose was stimulated to approximately 4 times the control level, and this stimulation was maintained at 7 and 14 days. The stimulation of incorporation of GDP (^{14}C) mannose was slightly less, being approximately 3 times the control level at each day. However, it should be emphasized that the microsomes obtained from individual oestrogen-treated animals were incubated, and therefore, as expected, there was variation between the incorporation levels observed in the 2 experimental animals on each day, this variation in incorporation was most marked on day 14. In Figure 45, the average stimulation over control obtained on each day is plotted.

TABLE 13 THE INCORPORATION OF UDP (^{3}H) GALACTOSE AND GDP (14C) MANNOSE INTO GLYCOLIPID INTERMEDIATES BY MICROSOMES FROM CONTROL AND OESTROGEN-TREATED XENOPUS

Time after Oestrogen-Treatment (days)	Incorporation (dpm/mg protein x 10^{-3})					
	UDP (^{3}H) Gal			GDP (^{14}C) Man		
	Control	1	2	Control	1	2
3	2.1	6.8	9.2	2.4	5.5	7.2
7	1.6	12.2	9.5	2.1	6.6	7.2
14	2.3	13.2	4.9	1.4	7.2	3.3

At 3, 7, and 14 days after oestrogen-treatment, 1 control and 2 oestrogen-treated animals were sacrificed and the microsomal fraction obtained from the liver of individual animals. 0.8 ml aliquots of microsomal suspension were incubated with 1 µCi UDP (^{3}H) galactose or 0.1 µCi GDP (^{14}C) mannose for 1 hour. Lipid was then extracted from the incubations with 2:1 chloroform/methanol and the incorporation of radioactivity into the 2:1 extract determined. Full details are given in Chapter 2.

Figure 45

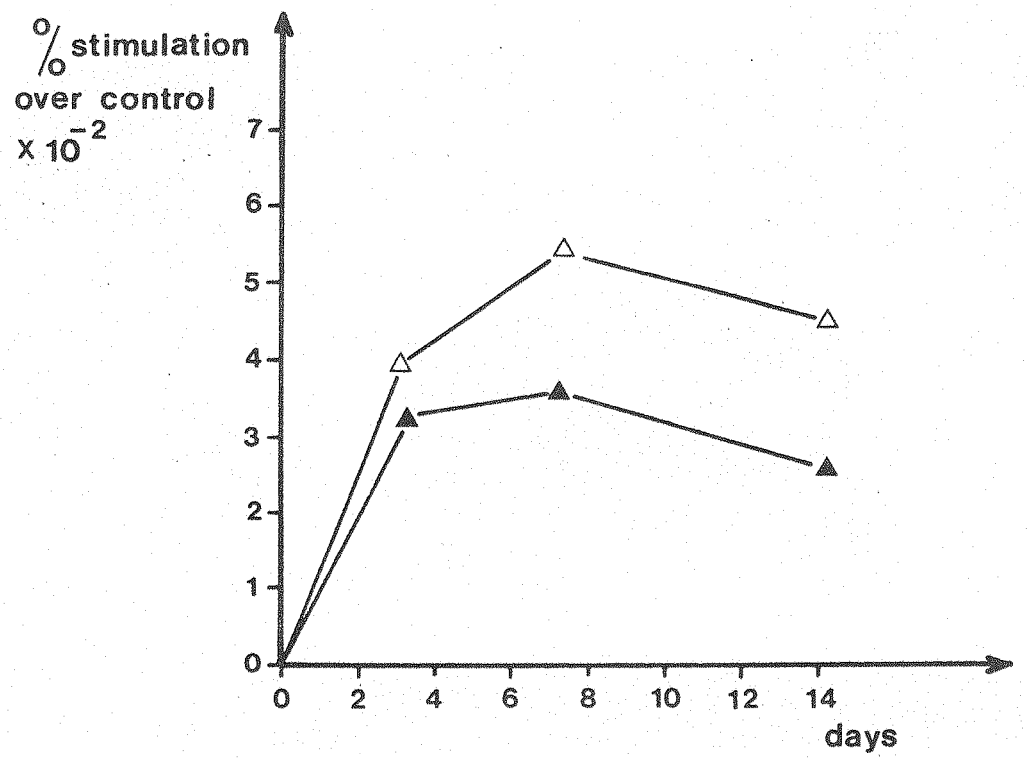


Figure 45 The Effect of Oestrogen-Treatment on the Incorporation of UDP (3H) Galactose and GDP (14C) Mannose into Glycolipid by Microsomes

The incorporation data shown in Table 13 expressed in terms of the average stimulation over control incorporation obtained at each day.

c) UDP N-Acetyl (^{14}C) Glucosamine Incorporation

In a separate set of experiments the effect of oestrogen-treatment on the incorporation of N-acetyl (^{14}C) glucosamine into glycolipid by microsomal fractions was investigated. The microsomal fraction from control and oestrogen-treated animals was incubated with 0.1 μCi of UDP N-acetyl (^{14}C) glucosamine for 1 hour and the lipid was then extracted with 2:1 chloroform/methanol and the incorporation of radioactivity into the 2:1 extract determined. The results of two independent experiments are shown in Table 14, which also shows the incorporation of UDP (^{3}H) galactose obtained in parallel incubations. The incorporation of UDP N-acetyl (^{14}C) glucosamine into labelled glycolipid present in the 2:1 extract was 8-10 fold higher in the oestrogen-treated incubations than in control incubations. The stimulation of the incorporation of UDP (^{3}H) galactose in the parallel incubations confirmed the results obtained previously.

Table 15 shows the calculated values for the amount of each sugar (in nmoles) transferred from the labelled nucleotide sugar to glycolipid during incubations with microsomal fractions obtained from oestrogen-treated animals. Mannose transfer was the highest, being 18 times greater than N-acetyl glucosamine transfer and 65 times greater than galactose transfer. However, since no account was taken of the microsomal pool-size of the relevant derivatives, these values cannot be said to reflect the true level of sugar transfer.

5.2 Analysis of the Labelled Glycolipid Fraction

The results described in the previous section demonstrated that in microsomes prepared from oestrogen-treated animals there was a stimulation of the transfer of labelled sugars, from UDP (^{3}H) galactose, GDP (^{14}C) mannose and UDP N-acetyl (^{14}C) glucosamine, into a glycolipid fraction present in the 2:1 extract. However, the incorporation data gave no indication as to the identity of the glycolipid involved. Therefore the next step was to

TABLE 14 THE INCORPORATION OF UDP N-ACETYL (^{14}C) GLUCOSAMINE INTO GLYCOLIPID INTERMEDIATES BY MICROSOMES FROM CONTROL AND OESTROGEN-TREATED XENOPUS

	Incorporation (dpm/mg protein)	
	1	2
Control	138, (1,380)	174 (4,084)
Oestrogen-treated	1,403 (13,920)	1,904 (40,497)

The microsomal fraction was obtained from the liver of 1 control and 1 oestrogen-treated animal. Duplicate 0.4 ml aliquots of microsomal suspension were incubated with 0.1 μCi UDP N-acetyl (^{14}C) glucosamine for 1 hour. Lipid was then extracted from the incubations with 2:1 chloroform/methanol and the incorporation of radioactivity into the 2:1 extract determined. The results of 2 independent experiments are shown (the experimental animals in 1 and 2 being 10- and 12-days after oestrogen-treatment respectively). The figures in parenthesis show the incorporation of UDP (3H) galactose obtained in parallel incubations (0.5 μCi in 1 and 1.0 μCi in 2). Full details are given in Chapter 2.

TABLE 15 THE CALCULATED TRANSFER OF (^{3}H) GALACTOSE, (^{14}C) MANNOSE AND N-ACETYL (14C) GLUCOSAMINE TO LIPID DURING THE INCUBATION OF MICROSOMES FROM OESTROGEN-TREATED XENOPUS

	Incorporation into Lipid (n moles/g liver)
(^{3}H) Galactose	0.002
(^{14}C) Mannose	0.130
N-Acetyl (^{14}C) Glucosamine	0.007

Knowing the specific activity of the UDP (^{3}H) galactose, GDP (14C) mannose and UDP N-acetyl (14C) glucosamine, the amount of each labelled sugar transferred to lipid during oestrogen-treated incubations was calculated.

establish whether the labelled glycolipid produced had characteristics comsistent with it being a dolichol-sugar.

5.2.1 DEAE-Cellulose Acetate Chromatography

Dolichol monophosphate and diphosphate sugar derivatives can be separated from neutral lipids by anion exchange chromatography using DEAE-cellulose acetate columns. Both dolichol monophosphate- and dolichol diphosphate-sugars bind to DEAE-cellulose acetate by virtue of their negatively charged phosphate groups ; dolichol monophosphate-sugars can be eluted from the column with 0.01M ammonium acetate whereas dolichol diphosphate-sugars bind more strongly, due to the extra negative charge, and are eluted with 0.05M ammonium acetate. Neutral lipids are not retained by the column and are eluted with chloroform/methanol and methanol.

The labelled 2:1 chloroform/methanol extracts, obtained from control and oestrogen-treated incubations in the experiments described in the previous section, were analysed by DEAE-cellulose acetate chromatography as described in Chapter 2 to discover whether the behaviour of the labelled glycolipid fraction was similar to that of a dolichol-sugar derivative ; typical results are shown in Figure 46, 47 and 48.

a) Analysis of the (^{3}H) Galactose-Labelled Glycolipid Fraction (Figure 46)

When the (^{3}H) galactose-labelled 2:1 chloroform extract obtained from oestrogen-treated incubations was analysed, approximately 70% of the radioactivity applied to the column was eluted as a broad peak with 0.01M and 0.05M ammonium acetate. In contrast, when the 2:1 extract obtained from control incubations was analysed, no radioactive peak was eluted with ammonium acetate. The incorporation of radioactivity into neutral lipid, ie, material not retained by the column, was very low in both control and oestrogen-treated incubations.

Figure 46 DEAE-Cellulose Acetate Chromatography of (3H) Galactose-Labelled 2:1 Chloroform/Methanol Extract

Figure 47 (overleaf) DEAE-Cellulose Acetate Chromatography of N-Acetyl (^{14}C) Glucosamine-Labelled 2:1 Chloroform/Methanol Extract

Figure 48 (overleaf) DEAE-Cellulose Acetate Chromatography of (^{14}C) Mannose-Labelled 2:1 Chloroform/Methanol Extract

The labelled 2:1 chloroform/methanol extracts obtained from control and oestrogen-treated incubations in the experiments described in Tables 13 and 14 were analysed by DEAE-cellulose acetate chromatography. The columns were eluted with -

30 ml chloroform/methanol (2:1)

20 ml methanol

50 ml 0.01 M ammonium acetate in chloroform/methanol (2:1)

50 ml 0.05 M ammonium acetate in chloroform/methanol (2:1)

The solvent changes are marked by arrows. 5 or 10 ml fractions were collected and aliquots taken for the determination of radioactive incorporation. Full details are given in Chapter 2.

Figure 46

DEAE-Cellulose Acetate Chromatography of (^{3}H) Galactose Labelled Glycolipid Fraction

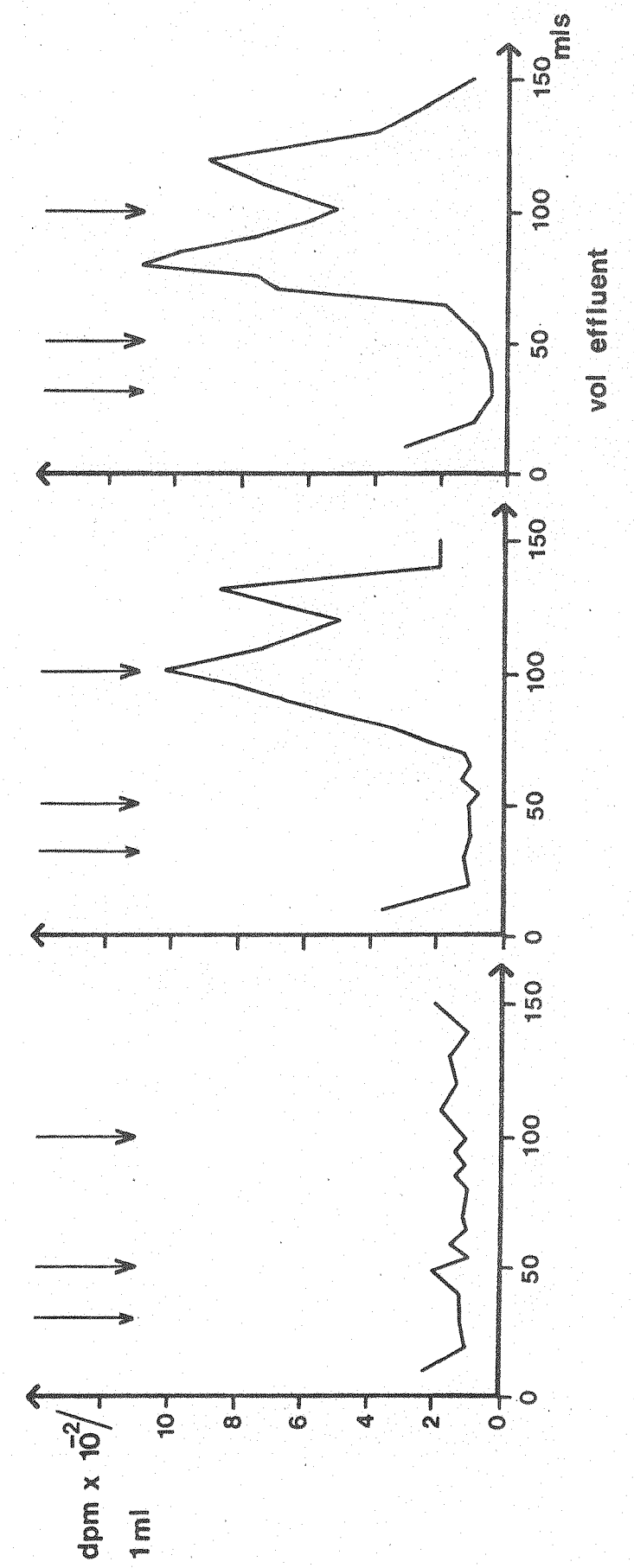
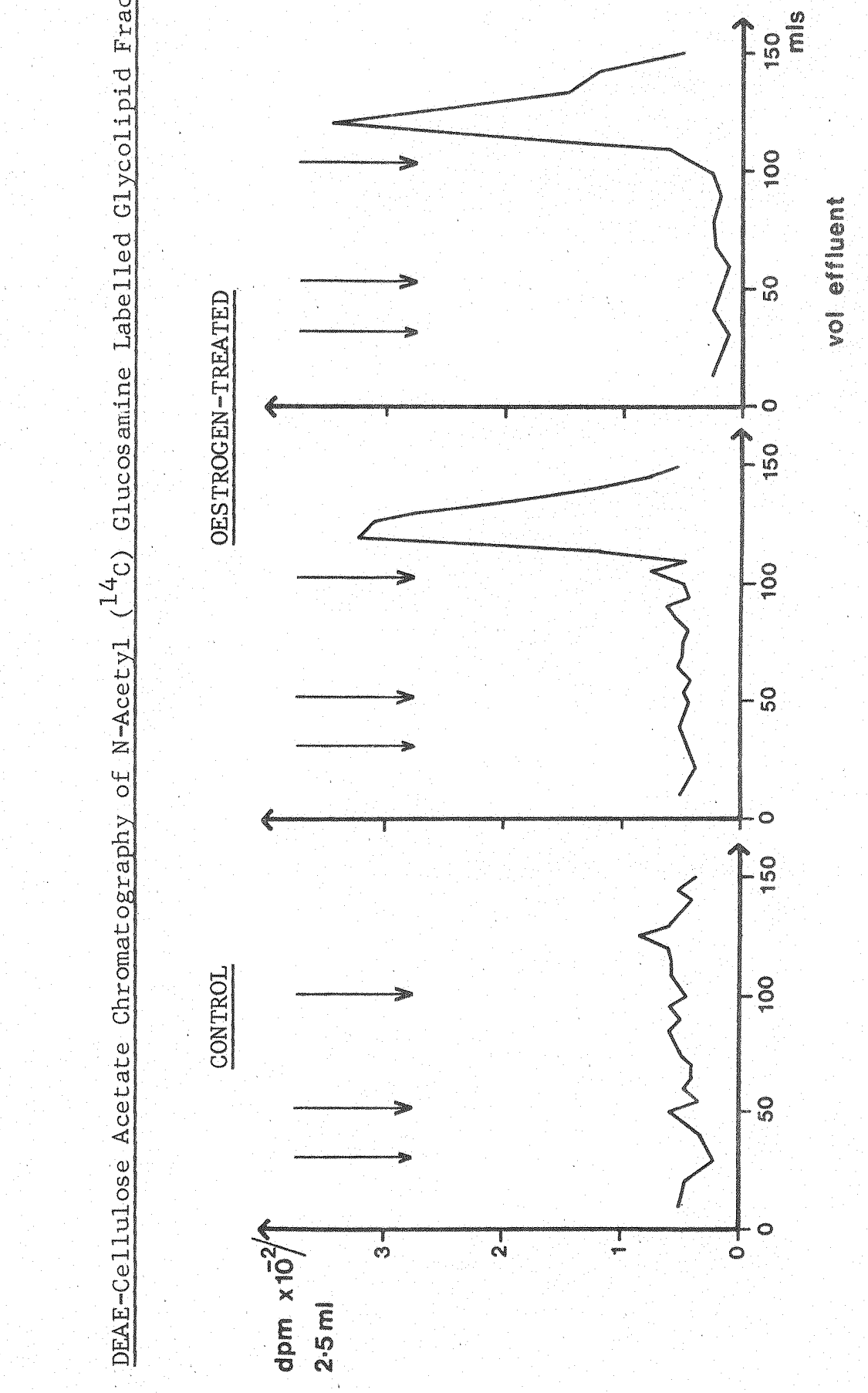


Figure 47

DEAE-Cellulose Acetate Chromatography of N-Acetyl (^{14}C) Glucosamine Labelled Glycolipid Fraction



DEAE-Cellulose Acetate Chromatography of (^{14}C) Mannose Labelled Glycolipid Fraction

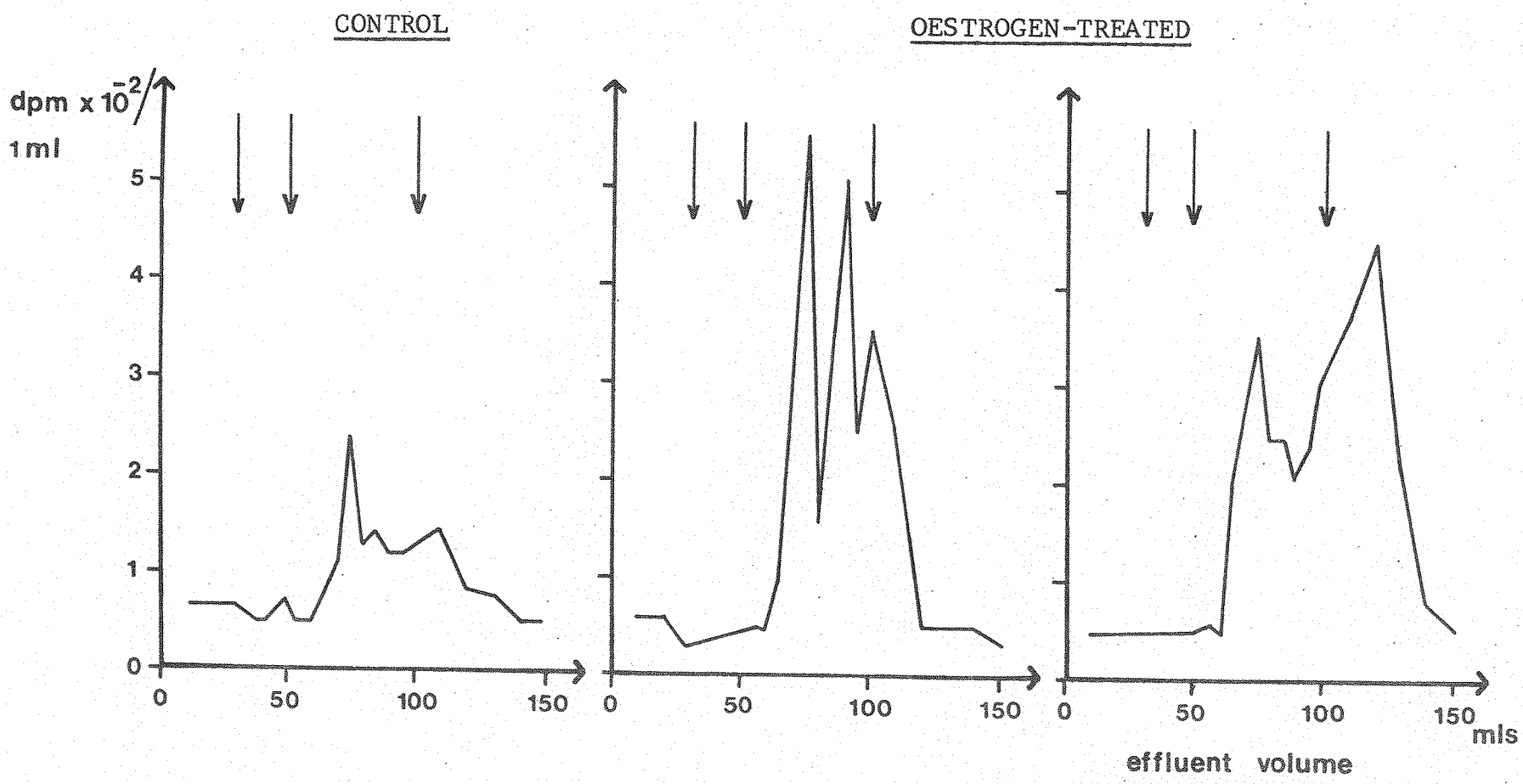


Figure 48

b) Analysis of the N-Acetyl (^{14}C) Glucosamine-Labelled Glycolipid Fraction (Figure 47)

When the N-acetyl (^{14}C) glucosamine-labelled, 2:1 chloroform/methanol extract obtained from oestrogen-treated incubations was analysed, approximately 70% of the radioactivity applied to the column was eluted as a single peak with 0.05M ammonium acetate. In contrast, when the 2:1 extract obtained from control incubations was analysed, no radioactive peak was eluted with ammonium acetate. Again there was no incorporation of radioactivity into neutral lipid in either control or oestrogen-treated incubations.

c) Analysis of the (^{14}C) Mannose-Labelled Glycolipid Fraction (Figure 48)

When the (^{14}C) mannose-labelled, 2:1 chloroform/methanol extract obtained from oestrogen-treated incubations was analysed, approximately 70% of the radioactivity applied to the column was eluted as a broad peak with 0.01M and 0.05M ammonium acetate. When the 2:1 extract obtained from control incubations was analysed, approximately 50% of the radioactivity applied to the column was eluted as a broad peak with 0.01M and 0.05M ammonium acetate. Again, there was no incorporation of radioactivity into neutral lipid in either control or oestrogen-treated incubations.

The most important point to be demonstrated by the DEAE-cellulose acetate chromatographic analysis of the labelled 2:1 extracts was that oestrogen-treatment resulted in the stimulation of incorporation of each of the labelled sugars into a glycolipid fraction eluted with ammonium acetate. The incorporation of labelled sugars into neutral lipid was insignificant in both control and oestrogen-treated incubations.

The effect of oestrogen-treatment on the incorporation of (^{3}H) galactose and N-acetyl (^{14}C) glucosamine, from UDP (^{3}H) galactose and UDP N-acetyl (^{14}C) glucosamine, into labelled glycolipid eluted with ammonium acetate was dramatic. In both cases oestrogen-treatment resulted in the incorporation of labelled sugar into a labelled glyco-

lipid fraction which was not detectable in the 2:1 extract from control incubations. This indicated that the radioactivity present in the 2:1 extract from the control incubations was due to contamination or non-specific incorporation and not due to the specific incorporation of labelled sugar into the glycolipid fraction eluted with ammonium acetate. It followed that the estimates of the effect of oestrogen-treatment made simply by comparing the incorporation of (^{3}H) galactose and N-acetyl (^{14}C) glucosamine into the total 2:1 extracts were actually under estimates of the true stimulation of incorporation of the labelled sugars into the ammonium acetate-eluted glycolipid fraction. This was most apparent in the case of (^{3}H) galactose incorporation ; from a comparison of the incorporation of label into the 2:1 extracts from control and oestrogen-treated incubations the stimulation was estimated to be only 4-5 fold (Table 13 and Figure 45), whereas more rigorous analysis by DEAE-cellulose acetate chromatography revealed that the stimulation of incorporation into the ammonium acetate-eluted glycolipid was actually much greater (Figure 46).

The effect of oestrogen-treatment of the incorporation of (^{14}C) mannose, from GDP (^{14}C) mannose, was less dramatic although still very clear. In both control and oestrogen-treated incubations, (^{14}C) mannose was incorporated into a labelled glycolipid fraction which was eluted with ammonium acetate, however, in the oestrogen-treated incubations the incorporation of label into this fraction was approximately 5 times greater than in control incubations.

The fact that the (^{3}H) galactose-, (^{14}C) mannose- and N-acetyl (^{14}C) glucosamine-labelled glycolipid fractions were retained by the columns and eluted with ammonium acetate was in favour of them being derivatives of dolichol phosphate. The elution of the N-acetyl (^{14}C) glucosamine-labelled fraction as a single peak with 0.05M ammonium acetate suggested that it was a dolichol diphosphate derivative. However, since both the (^{3}H) galactose- and the (^{14}C) mannose-labelled glycolipid fractions were eluted as a broad peak with both 0.01M and 0.05M ammonium acetate, it was not

possible to suggest the exact nature of the derivatives involved.

5.2.2 Thin-Layer Chromatography

Samples of the labelled material eluted from the columns by ammonium acetate were further analysed by silica-gel thin-layer chromatography using chloroform/methanol/water (65:25:4) and isopropanol/ammonia/water (6:3:1) as solvents. Using chloroform/methanol/water as the solvent system, the labelled material migrated as a single species (Figure 49), the Rf values for the (^{3}H) galactose- (^{14}C) mannose- and N-acetyl (^{14}C) glucosamine-labelled glycolipid being 0.31, 0.35 and 0.21 respectively. Reported Rf values of dolichol monophosphate mannose and dolichol diphosphate N-acetyl glucosamine are 0.31 and 0.27 respectively (Richards and Hemming, 1972 ; Cooper and Hemming, 1977). Using isopropanol/ammonia/water as the solvent system, the (^{3}H) galactose- and (^{14}C) mannose-labelled glycolipid migrated as a single species, with an Rf value of 0.72 in both cases. The reported Rf value for dolichol monophosphate glucose is 0.72 (Behrens and Tabora, 1974).

5.2.3 Mild Acid Hydrolysis

When subjected to mild acid hydrolysis, the phosphate-sugar linkage of dolichol phosphate sugar derivatives is hydrolysed and the sugar released, this characteristic distinguishes them from other glycolipids, such as glycerolipids, which are stable to acid treatment. Samples of the (^{3}H) galactose- and (^{14}C) mannose-labelled material eluted from the columns with ammonium acetate were treated with hydrochloric acid as described in Chapter 2. The results are shown in Table 16. After hydrolysis, 83-95% of the label was released from the glycolipid and rendered water-soluble.

5.2.4 Paper Chromatography of the Labelled Sugars Released by Acid Hydrolysis

The labelled sugars released from the labelled glycolipid by acid hydrolysis were analysed by paper chromatography, using butanol/ethanol/water as the solvent

Figure 49 Thin-Layer Chromatography of the Labelled Lipid Eluted from the DEAE-Cellulose Acetate Columns by Ammonium Acetate

Samples of the (^{3}H) galactose-labelled, (a), (^{14}C) mannose-labelled, (b), and N-acetyl (^{14}C) glucosamine-labelled, (c), material eluted from the columns described in Figures 46, 47 and 48 were analysed by thin-layer chromatography with chloroform/methanol/water (65:25:4) as the solvent system. The radioactivity in 1 cm strips was determined. The arrows mark the solvent front.

Figure 49

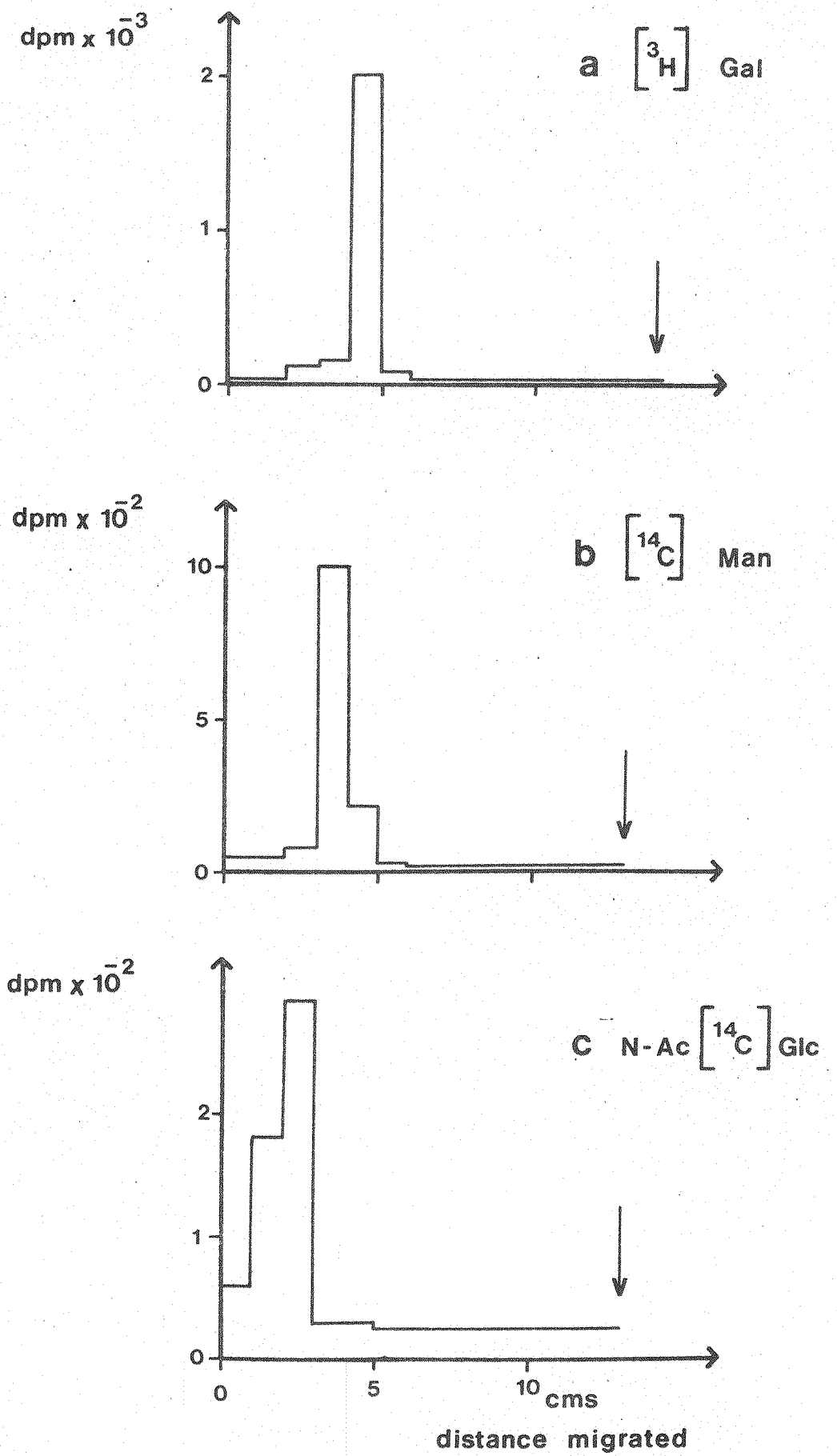

dpm x 10⁻³
2
1
0
a [³H] Gal
dpm x 10⁻²
10
5
0
b [¹⁴C] Man
dpm x 10⁻²
2
1
0
c N-Ac [¹⁴C] Glc
0
5
10 cms
distance migrated

TABLE 16 ACID HYDROLYSIS OF THE LABELLED GLYCOLIPID ELUTED WITH AMMONIUM ACETATE

Lipid Labelled with	dpm recovered in	
	Aqueous (upper)	Organic (lower)
UDP (^{3}H) Gal		
1	9,600 (95%)	480
2	9,592 (96%)	400
GDP (^{14}C) Man		
1	22,000 (84%)	4,000
2	16,000 (93%)	1,200

A sample of the labelled lipid was treated with 0.01 M HCl, at 100°C for 20 min and then neutralized by the addition of 0.01 M NaOH. After the addition of 2 ml of H_2O and 2 ml of chloroform, the mixture was separated into 2 layers by centrifugation. The upper aqueous and lower organic layers were assayed for radioactivity. Full details are given in Chapter 2.

system, to determine whether the label was present in mono-, di-, tri- or oligosaccharide moieties. The results are shown in Figure 50. Most of the radioactivity released from the (^{14}C) mannose-labelled glycolipid ran as a single peak with a mobility similar to glucose, showing that most of the labelled sugar released during the hydrolysis was in the monosaccharide form. Most of the radioactivity released from the (^{3}H) galactose-labelled glycolipid ran as a broad peak with a mobility similar to glucose, but a small amount ran with a mobility close to raffinose and lactose, showing that although most of the labelled sugar released during the hydrolysis was in the monosaccharide form, a small proportion was in the form of di- or trisaccharide. These results indicated that most of the (^{14}C) mannose- and (^{3}H) galactose-labelled glycolipid formed was monosaccharide-lipid (assuming that no hydrolysis of larger sugar units occurred during the acid treatment).

5.3 The Incorporation of (^{3}H) Glucosamine and (^{3}H) Galactose into Glycolipid Intermediates by Xenopus Liver Slices

Liver slices from control and oestrogen-treated animals were incubated with either 50 µCi of (^{3}H) glucosamine or 50 µCi of (^{3}H) galactose for 6 hours. The slices were then homogenized in TKMS buffer and the lipid extracted from the homogenate with 2:1 chloroform/methanol followed by 10:10:3 chloroform/methanol/water as described in Chapter 2. The combined 2:1 and 10:10:3 extracts were then analysed by DEAE-cellulose acetate chromatography, thin-layer chromatography, acid hydrolysis and paper chromatography.

5.3.1 DEAE-Cellulose Acetate Chromatography of the Labelled Glycolipid Extracted from the Total Tissue Homogenate

The combined 2:1 and 10:10:3 extract obtained from control and oestrogen-treated incubations was analysed by DEAE-cellulose acetate chromatography as described in Chapter 2. The results are shown in Figures 51 and 52

Figure 50 Paper Chromatography of the Labelled Sugars Released by Mild Acid Hydrolysis

The water soluble products obtained after the hydrolysis of the (^{3}H) galactose-labelled, (a), and (^{14}C) mannose-labelled, (b), glycolipid eluted with ammonium acetate (see Table 16) were analysed by paper chromatography using butanol/ethanol/water as the solvent system. The radioactivity in 1 cm strips was determined The migration of the standards was determined by staining with silver nitrate.

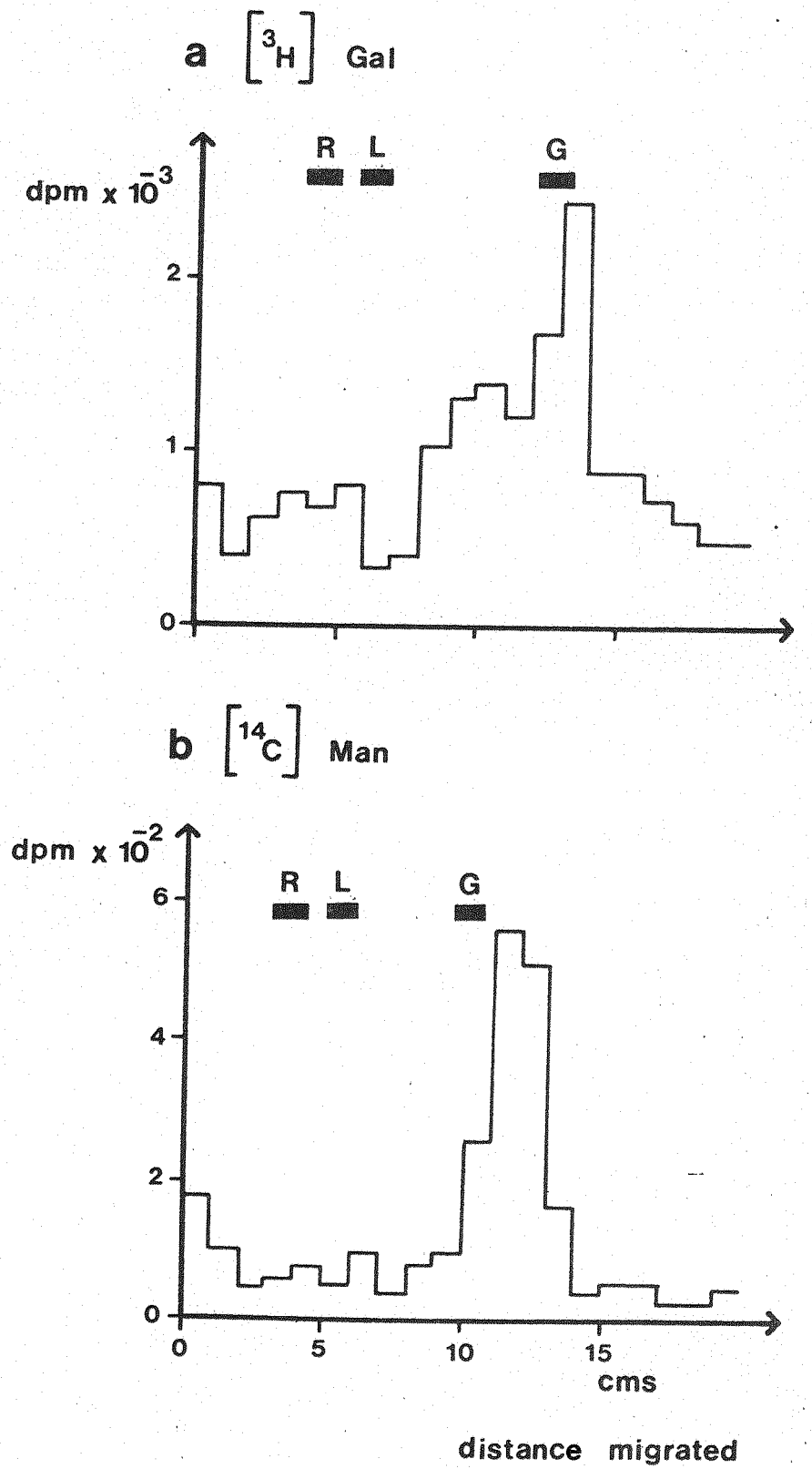
R - Raffinose (trisaccharide)

L - Lactose (disaccharide)

G - Glucose (monosaccharide)

Full details are given in Chapter 2.

Figure 50


a [3H] Gal
R
L
G
dpm x 10-3
2
1
0
b [14C] Man
dpm x 10-2
6
4
2
0
0
5
10
15
cms
distance migrated

and Table 17.

a) Analysis of the (^{3}H) Glucosamine-Labelled Glycolipid Fraction (Figure 51)

When the combined 2:1 and 10:10:3 extract obtained from oestrogen-treated liver slices after incubation with (^{3}H) glucosamine was analysed, approximately 60% of the radioactivity recovered from the column was eluted as neutral lipid with chloroform/methanol and methanol. A second peak of radioactivity was eluted with 0.05M ammonium acetate. When the column was eluted with 0.2M ammonium acetate, no further radioactivity was recovered. The analysis of the lipid extracted from control liver slices gave similar results, with labelled neutral lipid being eluted with chloroform/methanol and methanol, and a second peak of radioactivity being eluted with 0.05M ammonium acetate. The incorporation of radioactivity into neutral lipid and into the fraction eluted with 0.05M ammonium acetate was greater in the oestrogen-treated liver slices, with the incorporation into neutral lipid being approximately 4-fold higher than in the control, and the incorporation into the ammonium acetate-eluted fraction being approximately 3-fold higher than in the control.

b) Analysis of the (^{3}H) Galactose-Labelled Glycolipid Fraction (Figure 52)

When the combined 2:1 and 10:10:3 extract obtained from oestrogen-treated liver slices after incubation with (^{3}H) galactose was analysed, approximately 80% of the radioactivity recovered from the column was eluted as neutral lipid with chloroform/methanol and methanol. As with the (^{3}H) glucosamine-labelled lipid, a second peak of radioactivity was eluted with 0.05M ammonium acetate. The analysis of the lipid extracted from control liver slices gave similar results, with labelled neutral lipid being eluted with chloroform/methanol and methanol, and a second peak of radioactivity being eluted with 0.05M ammonium acetate. The incorporation of radioactivity into the fraction eluted with 0.05M ammonium acetate was approximately 3-fold higher in the oestrogen-treated liver slices than in the control.

Figure 51 DEAE-Cellulose Acetate Chromatography of the Combined 2:1 Chloroform/Methanol and 10:10:3 Chloroform/Methanol/Water Extract Obtained from Liver Slices after Incubation with (^{3}H) Glucosamine

Liver slices (0.5 g) from control, (a), and oestrogen-treated, (b), animals were incubated with 50 µCi (^{3}H) glucosamine for 6 hours. The slices were then homogenized in TKMS buffer and lipid extracted from the homogenate with 2:1 chloroform/methanol followed by 10:10:3 chloroform/methanol/water. The combined 2:1 and 10:10:3 extract was analysed by DEAE-cellulose acetate chromatography. The columns were eluted with -

50 ml chloroform/methanol (2:1)

30 ml methanol

50 ml 0.01 M ammonium acetate in chloroform/methanol (2:1)

50 ml 0.05 M ammonium acetate in chloroform/methanol (2:1)

The solvent changes are marked by arrows. 5 or 10 ml fractions were collected and aliquots taken for the determination of radioactive incorporation. Full details are given in Chapter 2.

Figure 52 (overleaf) DEAE-Cellulose Acetate Chromatography of the Combined 2:1 Chloroform/Methanol and 10:10:3 Chloroform/Methanol/Water Extract Obtained from Liver Slices after Incubation with (^{3}H) Galactose

As Figure 51, except that the liver slices were incubated with (^{3}H) galactose instead of (^{3}H) glucosamine.

Figure 51

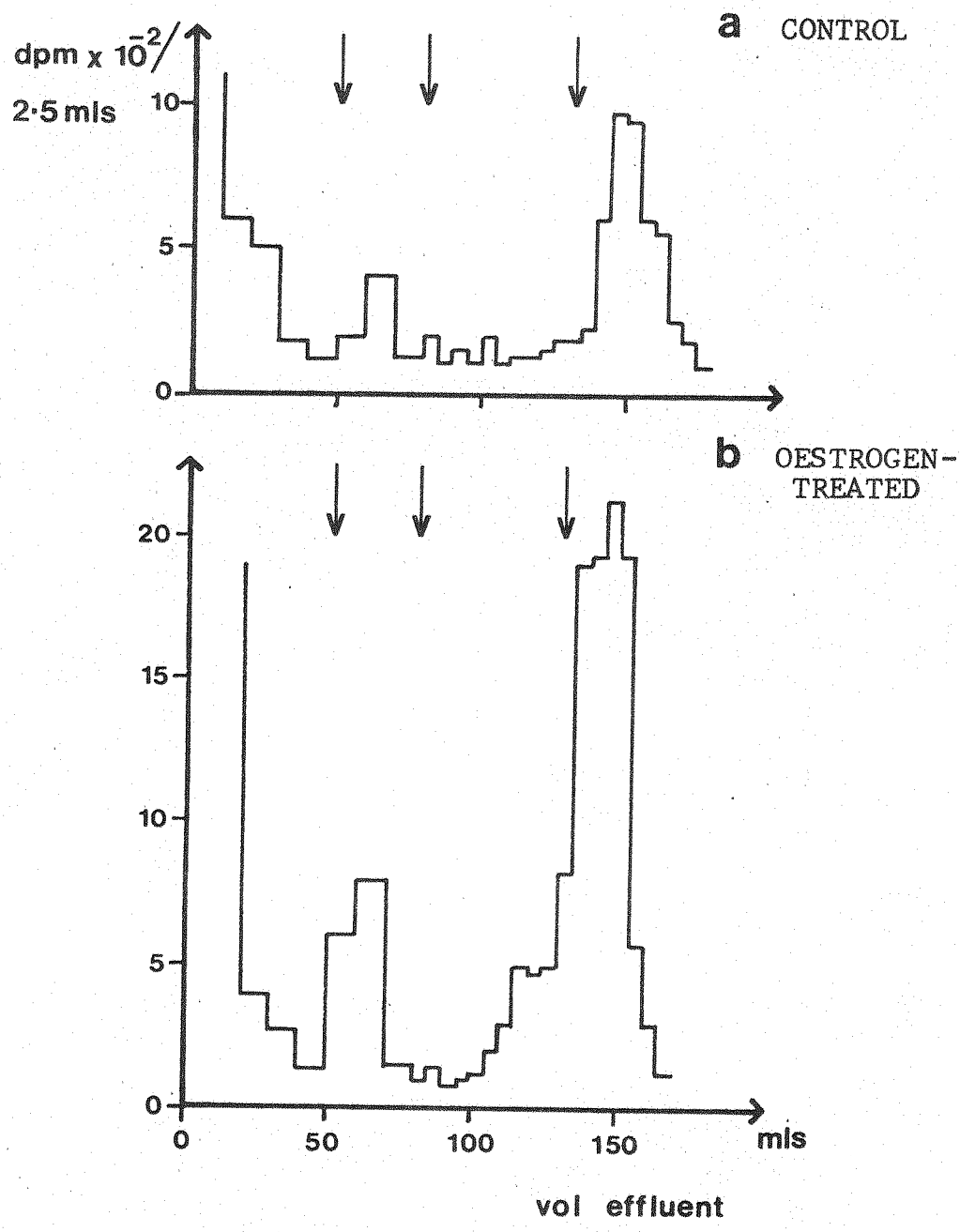
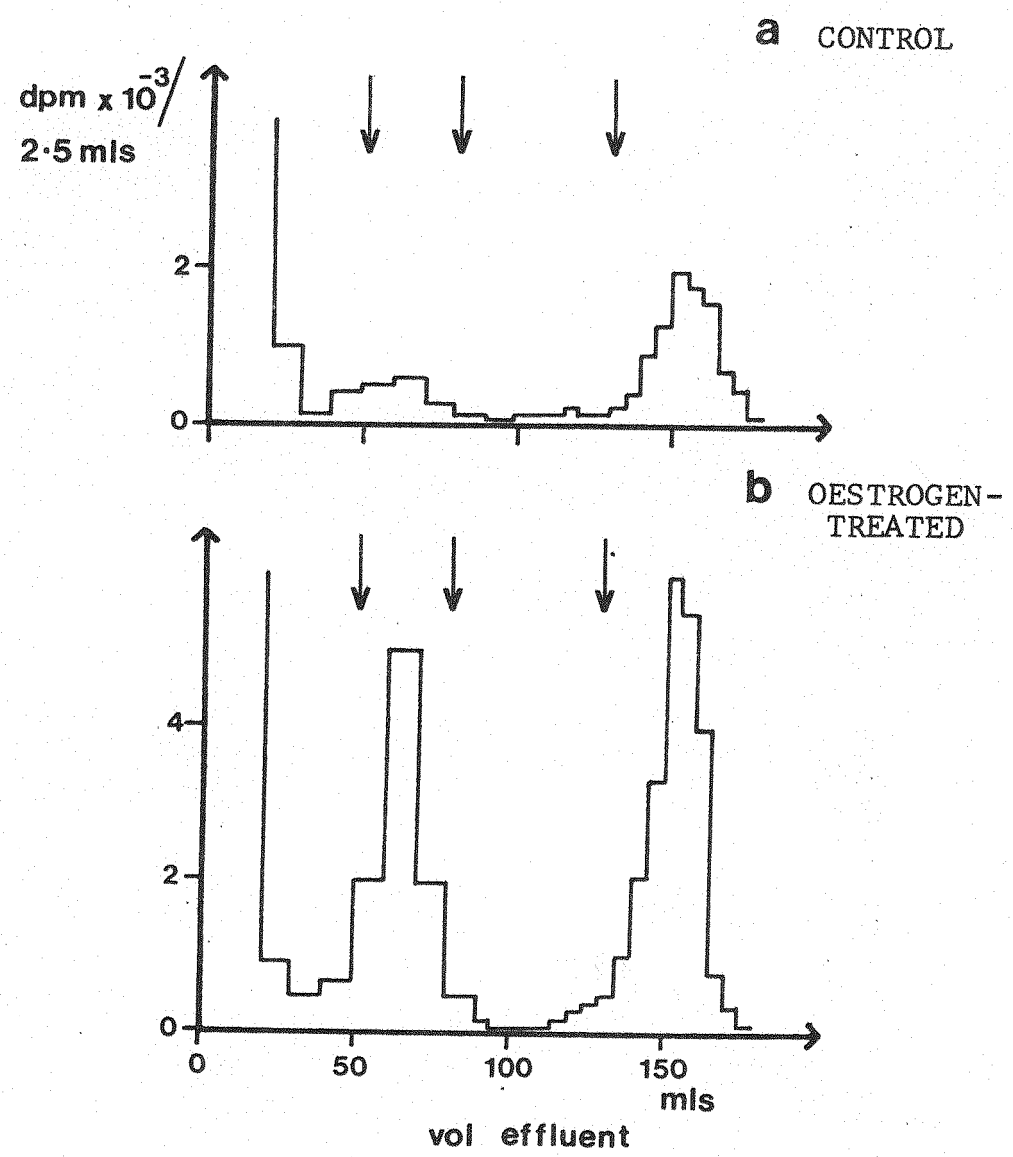

a CONTROL
dpm x 10⁻²/
2·5 mls
10
5
0
b OESTROGEN-
TREATED
20
15
10
5
0
0
50
100
150
mls
vol effluent

Figure 52



DEAE-Cellulose Acetate Chromatography of the Combined 2:1 Chloroform/Methanol and 10:10:3 Chloroform/Methanol/Water Extract Obtained from Liver Slices after Incubation with (^{3}H) Galactose

TABLE 17 THE INCORPORATION OF (^{3}H) GLUCOSAMINE AND (^{3}H) GALACTOSE INTO THE FRACTIONS ELUTED FROM DEAE-CELLULOSE-ACETATE COLUMNS

Lipid Labelled with	Incorporation into Fractions (dpm/g liver x 10^{-3}) eluted with	
	Chloroform/methanol and methanol a)	0.05 M NH_4 Acetate b)
(^{3}H) Glucosamine		
Control	18	14
Oestrogen-treated	76	39 (2.8x)
(^{3}H) Galactose		
Control	140	37
Oestrogen-treated	196	118 (3.2x)

(See Figures 51 and 52)

The incorporation of (^{3}H) glucosamine and (^{3}H) galactose into the fractions eluted with a) chloroform/methanol and methanol and b) 0.05 M ammonium acetate was determined from the elution profiles shown in Figures 51 and 52.

The incorporation by liver slices of both (^{3}H) glucosamine and (^{3}H) galactose into a neutral lipid fraction was in marked contrast to the results obtained with the microsomal system described in the previous section where there was no incorporation of labelled sugars into neutral lipid. However, in the liver slice system there was also incorporation of radioactivity into a glycolipid fraction eluted with 0.05M ammonium acetate, an elution position characteristic of dolichol diphosphate-sugar derivatives, and the incorporation of label into this fraction was increased after oestrogen-treatment.

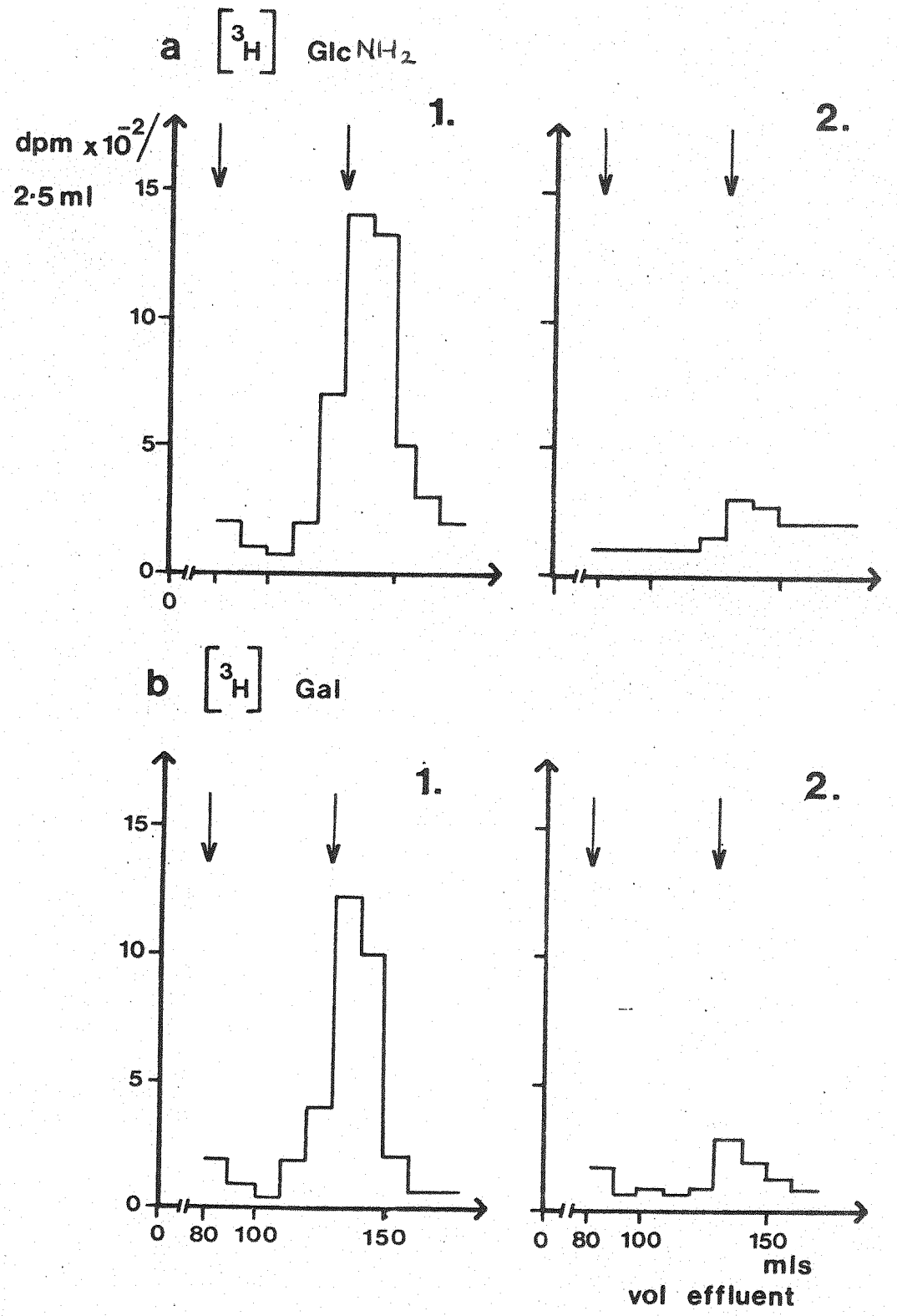
5.3.2 DEAE-Cellulose Acetate Chromatography of the Labelled Glycolipid Extracted from the Microsomes and the Post-Microsomal Supernatant

Liver slices from control and oestrogen-treated animals were incubated with 50 µCi of (^{3}H) glucosamine or 50 µCi of (^{3}H) galactose. The slices were then homogenized in TKMS buffer, the homogenate centrifuged at 6,000 rpm for 10 minutes to remove mitochondria, nuclei and cell debris and the post-mitochondrial supernatant centrifuged to obtain the microsomes and the post-microsomal supernatant as described in Chapter 2. Lipid was extracted from the microsomes and the post-microsomal supernatant with 2:1 chloroform/methanol followed by 10:10:3 chloroform/methanol/water and the combined 2:1 and 10:10:3 extract from each fraction was analysed by DEAE-cellulose acetate chromatography. The results are shown in Figure 53 and they showed that both (^{3}H) glucosamine and (^{3}H) galactose were incorporated into a glycolipid fraction present in the post-mitochondrial supernatant which was eluted with 0.05M ammonium acetate ; at least 85% of this labelled, ammonium acetate-eluted glycolipid was associated with the microsomal fraction and only about 15% remained in the post-microsomal supernatant. Both the microsomal fraction and the post-microsomal supernatant included labelled neutral lipid. In this experiment, no account was taken of any radioactivity incorporated into material sedimented with the mitochondria and cell-debris during the initial centrifugation step.

Figure 53 DEAE-Cellulose Acetate Chromatography of the Combined 2:1 and 10:10:3 Extract Obtained from the Microsomal Fraction (1) and the Post-Microsomal Supernatant (2) after the Incubation of Liver Slices with (^{3}H) Glucosamine (a) or (^{3}H) Galactose (b)

Liver slices (0.5 g) from oestrogen-treated animals were incubated with 50 µCi (^{3}H) glucosamine, (a), or 50 µCi (^{3}H) galactose, (b), for 6 hours. The slices were then fractionated to obtain the microsomes, (1), and the post-microsomal supernatant, (2), and lipid extracted from both fractions with 2:1 chloroform/methanol followed by 10:10:3 chloroform/methanol/water. The combined 2:1 and 10:10:3 extract from each fraction was analysed by DEAE-cellulose acetate chromatography. Elution was as described in Figure 51. The solvent changes are marked by arrows. 10 ml fractions were collected and aliquots taken for the determination of radioactive incorporation.

Figure 53


a [3H] GlcNH2
1.
2.
dpm x 10-2/
2·5 ml
15
10
5
0
0
b [3H] Gal
1.
2.
15
10
5
0
0 80 100 150
0 80 100 150
mls
vol effluent

5.3.3 Thin-Layer Chromatography

Samples of the labelled glycolipid eluted with 0.05M ammonium acetate in the experiments described in Figures 51 and 52 were further analysed by silica-gel thin-layer chromatography using chloroform/methanol/water (65:25:4) as the solvent. The results are shown in Figure 54. The labelled material migrated as a broad peak, the Rf values for the (^{3}H) glucosamine- and (^{3}H) galactose-labelled glycolipid being 0.25 and 0.33 respectively. The Rf values were similar to those reported for dolichol phosphate sugar derivatives (0.21 - 0.35) (Behrens and Tabora, 1974).

5.3.4 Mild Acid Hydrolysis

As described in 5.2.3, dolichol phosphate-sugar derivatives are susceptible to mild acid hydrolysis. Samples of the (^{3}H) glucosamine- and (^{3}H) galactose-labelled material eluted with 0.05M ammonium acetate were treated with hydrochloric acid as described in Chapter 2. The results are shown in Table 18. After hydrolysis, 75-90% of the label was released from the glycolipid and rendered water-soluble.

5.3.5 Paper Chromatography of the Labelled Sugars Released by Acid Hydrolysis

The labelled sugars released from the labelled glycolipid by acid hydrolysis were analysed by paper chromatography using butanol/ethanol/water as the solvent system. The results are shown in Figure 55. The radioactivity released from the (^{3}H) galactose-labelled glycolipid ran as a single peak with a mobility similar to raffinose, showing that most of the labelled sugar released during the hydrolysis was in the form of trisaccharide or larger oligosaccharide moieties. The radioactivity released from the (^{3}H) glucosamine-labelled glycolipid migrated as 2 peaks, one with a mobility similar to raffinose, and a second broader peak with a mobility between lactose and glucose, showing that approximately

Figure 54 Thin-Layer Chromatography of the Labelled Lipid Eluted from DEAE-Cellulose Acetate Columns by 0.05 M Ammonium Acetate

Samples of the (^{3}H) glucosamine-labelled, (a), and (^{3}H) galactose-labelled, (b), material eluted from the columns described in Figures 51 and 52 with ammonium acetate were analysed by thin-layer chromatography with chloroform/methanol/water (65:25:4) as the solvent system. The radioactivity in 1 cm strips was determined. The arrows mark the solvent front.

Figure 54

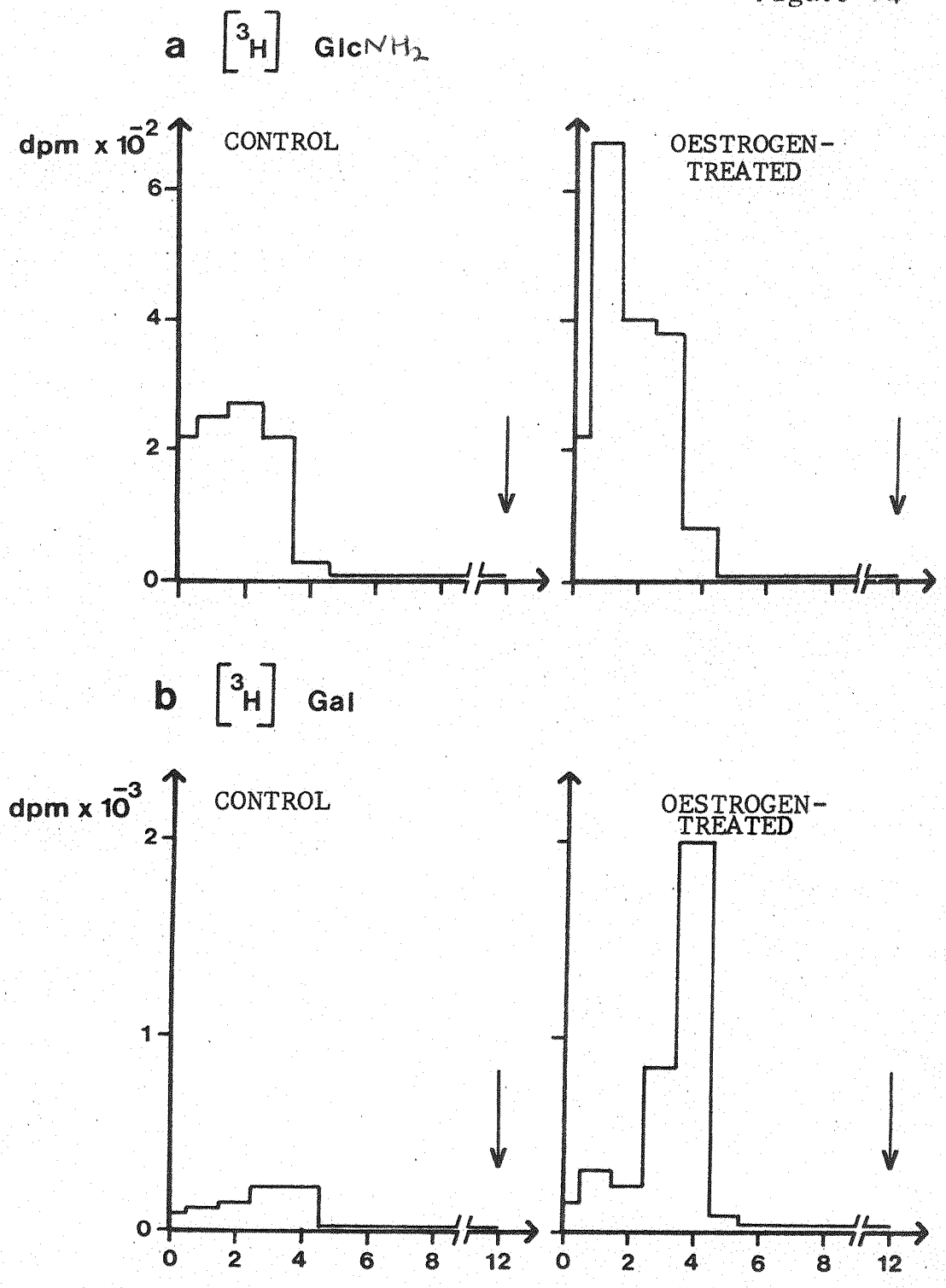

a [3H] GlcNH2
dpm x 10-2
CONTROL
OESTROGEN-TREATED
6
4
2
0
b [3H] Gal
dpm x 10-3
CONTROL
OESTROGEN-TREATED
2
1
0
0 2 4 6 8 12
0 2 4 6 8 12

TABLE 18 ACID HYDROLYSIS OF THE LABELLED GLYCOLIPID ELUTED WITH 0.05 M AMMONIUM ACETATE

Lipid Labelled with	dpm recovered in	
	Aqueous	Organic
(^{3}H) Glucosamine		
1	23,400 (89%)	2,797
2	26,200 (91%)	2,470
(^{3}H) Galactose		
1	28,800 (76%)	8,900
2	24,070 (80%)	5,807

A sample of the labelled lipid was treated with 0.01 M HCl at 100°C for 20 min and then neutralized by the addition of 0.01 M NaOH. After the addition of 2 ml of H_2O and 2 ml of chloroform, the mixture was separated into 2 layers by centrifugation. The upper aqueous and lower organic layers were assayed for radioactivity. Full details are given in Chapter 2.

Figure 55 Paper Chromatography of the Labelled Sugars Released by Mild Acid Hydrolysis

The water-soluble products obtained after the hydrolysis of the (^{3}H) glucosamine-labelled, (a), and (^{3}H) galactose-labelled, (b), glycolipid eluted with 0.05 M ammonium acetate (see Table 18) were analysed by paper-chromatography using butanol/ethanol/water as the solvent system. The radioactivity in 1 cm strips was determined. The migration of the standards was determined by staining with silver nitrate.

R - Raffinose (trisaccharide)

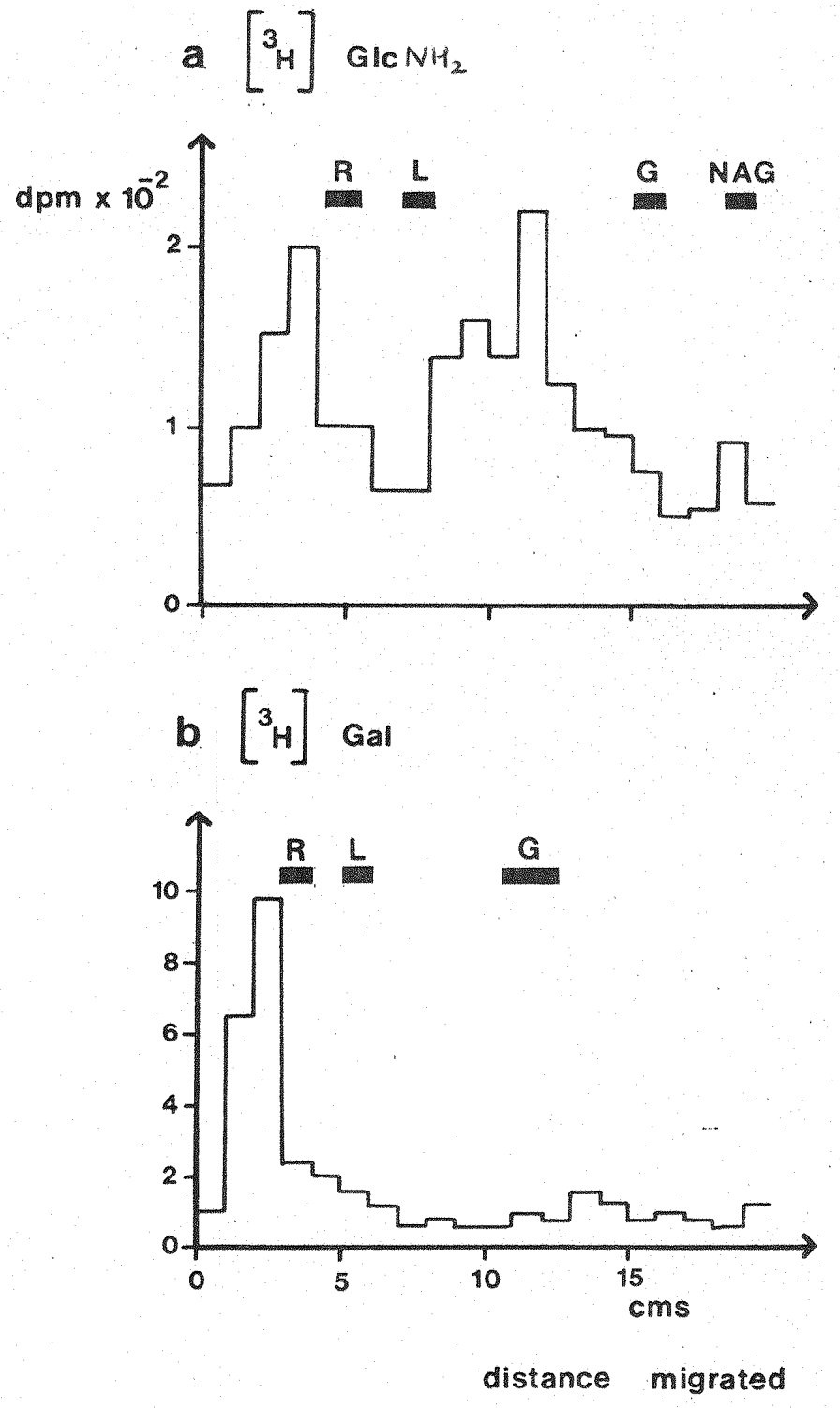
L - Lactose (disaccharide)

G - Glucose (monosaccharide)

NAG - N - acetyl glucosamine

Full details are given in Chapter 2.

Figure 55


a [3H] GlcNH2
dpm x 10-2
R
L
G
NAG
2
1
0
b [3H] Gal
R
L
G
10
8
6
4
2
0
0
5
10
15
cms
distance migrated

40% of the labelled sugar released during the hydrolysis was in the form of trisaccharide or larger oligosaccharide moieties and the remainder in the form of mono- or disaccharide.

CHAPTER 6

Chapter 6

DISCUSSION

6.1 Background

Vitellogenesis in Xenopus laevis involves the oestrogen-induced biosynthesis of vitellogenin in the liver, its secretion into the bloodstream, and subsequently, under the influence of gonadotrophin, its uptake by the ovary and further processing to form the two yolk proteins lipovitellin and phosvitin. A similar basic mechanism for the biosynthesis of the egg-yolk proteins seems to operate in all egg-laying species studies so far, which include amphibians, birds and insects (for reviews see Clemens, 1974 ; Wallace and Bergink, 1974 ; Bergink and Wallace, 1974b ; Engelmann, 1974).

The recent research interest in the field can be traced to the discovery by Urist and Schjeide (1961) that oestrogen-treatment of chickens resulted in the appearance of a new calcium-binding component in the serum ; this phenomenon suggested a possible experimental approach towards the elucidation of the mechanism of oestrogen action. In search of a more convenient experimental animals, workers in this laboratory observed that in Xenopus laevis a single dose of oestradiol-17-β produced a dramatic response with the accumulation of large quantities of a calcium-binding protein in the serum (Munday et al, 1968). The new protein could be isolated from the serum by a single-step manipulation involving precipitation with dimethyl-formamide. The liver was suggested as the site of synthesis of the protein (Munday et al, 1968). During the same period similar observations were made in two other laboratories

(Follett and Redshaw, 1968 ; Wallace and Dumont, 1968 ; Wallace and Jared, 1968). As the technique of polacrylamide gel electrophoresis became available, it was established that the serum protein isolated by the dimethylformamide technique was virtually homogeneous (Ansari et al, 1971), and furthermore this was the only method which allowed the preparation of the protein with its total calcium complement intact.

The easy availability of large amounts of the new protein allowed it to be chemically characterized as a Ca^{2+}-binding glycolipophospho-protein, mol. wt. 450-550,000, containing 0.85% calcium, 1.65% phosphorus, 12% lipid and 1.35% carbohydrate (Ansari et al, 1971 ; Redshaw and Follett, 1971 ; Wallace, 1970). Xenopus vitellogenin also contains 2 moles of the bile pigment, biliverdin, causing it to have a pale green colour (Redshaw and Follett, 1971) ; indeed, a characteristic feature of oestrogen-treatment of Xenopus laevis is that the serum becomes green due to the acuumulation of vitellogenin. Meanwhile, studies by Wallace and co-workers (Wallace and Dumont, 1968 ; Wallace and Jared, 1968) established that vitellogenin, biosynthesized in the liver and released into the blood, was sequestered by growing oocytes in the ovary, and then through a series of translocation and proteolytic cleavage processes, was converted into its two component egg-yolk proteins, lipovitellin and phosphovitin.

It is now established that the vitellogenin molecule is a dimer, made up of 2 similar sized polypeptide chains of mol. wt. approximately 200,000 (Bergink and Wallace, 1974a ; Clemens et al, 1975). Recent work by Wallace has indicated that serum vitellogenin includes 3 distinct forms of polypeptide subunit which differ slightly in mol. wt. (Wiley and Wallace, 1978). The results imply that, depending on the way in which the 3 subunit types combine to form dimers, between 3 and 6 different species of vitellogenin may exist in the serum. The heterogeneity in the vitellogenin polypeptide subunit may result either from multiple gene copies or from differential post-

translational processing of a common gene product.

In laying hens, the egg-yolk proteins are also biosynthesized as components of a large precursor vitellogenin molecule (Deeley et al, 1975). However, this has only been demonstrated relatively recently and previously it was believed that phosvitin and lipovitellin were synthesized as separate moities.

The enormity of the response of Xenopus laevis to a single dose of oestrogen suggested that the process could be exploited for the study of several fundamental biochemical processes in the fields of the mechanism of hormone action and developmental biology ; so, in the decade following the original observations, increasing research interest has been focused on oestrogen-induced vitellogenesis in Xenopus laevis. During this period, major advances have been made in the area of the isolation of vitellogenin mRNA and the regulation of its biosynthesis by oestrogen (Baker and Shapiro, 1977 ; Ryffel et al, 1977 ; Farmer et al, 1978). Vitellogenin synthesis has been successfully induced in vitro in tissue culture (Wangh and Knowland, 1975 ; Green and Tata, 1976) and, more recently, DNA sequences derived from vitellogenin mRNA have been cloned and characterized (Wahli et al, 1978 ; Smith et al, 1978). Related studies have also been carried out in the chicken system (Deeley et al, 1977). (For reviews see Tata, 1976 ; Ryffel, 1978 ; Tata and Smith, 1979.)

We have concentrated on several other aspects of the response. After oestrogen-treatment the incorporation of radioactive precursors into vitellognnin synthesized by the liver increases linearly, reaching a maximum at 9 - 16 days after hormone-treatment (Dolphin et al, 1971), and at the peak of the response at least 40-60% of liver protein synthesis is directed towards vitellogenin production (Merry et al, 1973). Electron microscopic evidence has shown that the early biochemical changes following hormone treatment involve a marked proliferation of the endoplasmic reticulum in the hepatocytes (Nicholls et al, 1968 ; Lewis et al, 1976). Workers in this laboratory have provided

quantitative evidence to support these ultrastructural studies, showing that oestrogen treatment resulted in a 120-160 fold stimulation of hepatic cholesterol and fatty acid synthesis, and that the newly synthesized lipids were destined to become components of the cell membranes (Smith et al, 1978). It has also been demonstrated that the synthesis of the other principal component of microsomal membranes, rRNA, is oestrogen-induced (Smith, 1977). Such a proliferation of the endoplasmic reticulum is in keeping with the synthesis of large quantities of a secretory glycolipophosphoprotein. When liver slices from oestrogen-treated Xenopus laevis are incubated with labelled amino-acids, there is a 1½-2 hour lag period before the appearance of labelled vitellogenin in the incubation medium, this represents the time taken for the biosynthesis of the vitellogenin polypeptide, its subsequent modification through glycosylation, phosphorylation, Ca^{2+} and lipdi addition and, finally the secretion of the completed molecule (Merry et al, 1973 ; Penning et al, 1976). The work presented in Chapter 4 aimed to look at the temporal relationship between the synthesis of the vitellogenin polypeptide, its post-translation modification, and secretion.

6.2 The Post-Translational Phosphorylation and Glycosylation of Vitellogenin

The preliminary experiments described in Chapter 4 confirmed previous work from this laboratory (Dolphin et al, 1971 ; Merry et al, 1973). When liver slices from oestrogen-treated Xenopus were incubated with (^{3}H) leucine, there was a 1½-2 hour lag period before the appearance of labelled vitellogenin in the incubation medium (Figure 15a). If at 1 hour further translation was inhibited by the addition of cycloheximide, labelled vitellogenin was still secreted into the incubation medium as normal for about 1½ hours after the end of the lag period (Figure 15b), showing that the processing events occurring during the second hour of the lag period did not involve further peptide bond formation. Analysis of the microsomal protein fraction

by SDS polyacrylamide gel electrophoresis (Figure 16) showed that during the lag period, (^{3}H) leucine-labelled, 200,000 mol. wt. polypeptide precursors of vitellogenin accumulated on the microsomes, and that at the end of the lag period, the loss of these precursors was concomitant with the secretion of vitellogenin into the incubation medium. The ability to incorporate impressive amounts of radioactivity into intracellular precursors of vitellogenin, and to reliably identify these precursors by SDS polyacrylamide gel electrophoresis suggested a way of studying the post-translational modification of the vitellogenin molecule. The subsequent experiments were designed to investigate the level of phosphorylation and glycosylation of the vitellogenin precursors.

6.2.1 The Identification of Phosphorylated Precursors of Vitellogenin

When liver slices from oestrogen-treated Xenopus were incubated with (^{32}P) phosphate there was a lag period of 1½-2 hours before the appearance of labelled vitellogenin in the incubation medium (Figure 17) ; thus the time-course followed a pattern identical to that for (^{3}H) leucine incorporation. The same time-course for the incorporation of (^{32}P) phosphate had been demonstrated by previous workers in this laboratory (Dolphin *et al*, 1971 ; Merry *et al*, 1973). This result suggested that the addition of phosphate was occurring very soon after the translation of the vitellogenin polypeptide. In order to establish more firmly whether this was indeed the case, it was necessary to perform double-label, pulse-chase experiments with (^{3}H) leucine and (^{32}P) phosphate and to compare the amount of (^{32}P) phosphate-label associated with the microsomal precursors of vitellogenin and with the completed vitellogenin molecule, ie, secreted vitellogenin.

When liver slices were incubated with (^{3}H) leucine and (^{32}P) phosphate, SDS polyacrylamide gel analysis confirmed that double-labelled vitellogenin was secreted into the incubation medium throughout the time

course (Figure 19). However, SDS polyacrylamide gel analysis of the microsomal fraction (Figure 20) showed that the 200,000 mol. wt. (^{3}H) leucine-labelled microsomal precursors included only a fraction (10-15%) of the (^{32}P) phosphate label associated with the final secreted vitellogenin. If, as suggested by the time-course of the incorporation of (^{32}P) phosphate into secreted protein, phosphorylation was occurring very soon after the translation of the vitellogenin polypeptide, the microsomal precursors would have been labelled with (^{32}P) phosphate to a similar extent to the secreted vitellogenin.

It may be argued that the observed low level of incorporation of (^{32}P) phosphate into the (^{3}H) leucine-labelled microsomal precursors could be due to the time taken for the added (^{32}P) phosphate-label to reach a steady-state level in the appropriate phosphate-pool, rather than reflecting the non-phosphorylation of the precursors per se ; a similar explanation could account for the lag-period prior to the appearance of (^{32}P) phosphate-labelled vitellogenin in the incubation medium. However, the results shown in Figures 19 and 20 indicate clearly that this was not the case, since even at 2½ hours, when impressive amounts of (^{32}P) phosphate and (^{3}H) leucine-labelled vitellogenin were present in the incubation medium, the level of phosphorylation of the microsomal precursors was still low ; indeed, at as early as 1½ hours, (^{3}H) leucine-labelled vitellogenin with its full complement of (^{32}P) phosphate was detectable in the incubation medium.

Thus the results of polyacrylamide gel analysis of the microsomal protein suggested that there was little accumulation of fully phosphorylated polypeptide precursors of vitellogenin on the microsomes, and therefore that phosphate addition was not occurring until just prior to, or after, the release of the precursors from the microsomes. This contrasted with the earlier suggestion, based on the time-course of incorporation of (^{32}P) phosphate into secreted vitellogenin, that phosphorylation was occurring very soon after translation, and highlights the problems involved in interpreting simple kinetic data in

the absence of additional evidence.

Since the results suggested that the pool of microsomal precursors comprised mainly non-phosphorylated or only slightly phosphorylated vitellogenin polypeptides, they raised the question as to whether phosphorylated precursors were accumulating in any other intracellular pool prior to secretion? SDS Polyacrylamide gel analysis of the supernatant remaining after the harvesting of the microsomal fraction, hereafter referred to as the "post-microsomal supernatant", revealed that, like the microsomal fraction, it included a single-labelled polypeptide species with a mol. wt. of approximately 200,000 (see Figure 22). However, what made this observation particularly interesting was that, in marked contrast to the microsomal precursors, the (^{3}H) leucine-labelled post-microsomal 200,000 mol. wt. species was labelled with (^{32}P) phosphate to a similar extent to the final secreted vitellogenin molecule. However, the problem was whether the 200,000 mol. wt. post-microsomal species was a genuine phosphorylated precursor of secreted vitellogenin or whether it was just an artefact due to the experimental procedure. The possibility that the recovery of this species in the "post-microsomal supernatant" was due to the contamination of this fraction with double-labelled vitellogenin derived from the incubation medium had to be considered ; but the experiment described in Figure 24 indicated that this was not the case. The kinetic relationship between the amount of the 200,000 mol. wt. labelled species recovered in the microsomal and the post-microsomal fractions and the secretion of labelled vitellogenin during the time-course (summarized in Figure 56) was consistent with a precursor product relationship between the microsomal and post-microsomal species and secreted vitellogenin ; during the lag-period prior to secretion, the labelled species accumulated first in the microsomal fraction and then in the post-microsomal fraction, and the secretion of labelled vitellogenin into the medium during the chase period correlated with the loss of

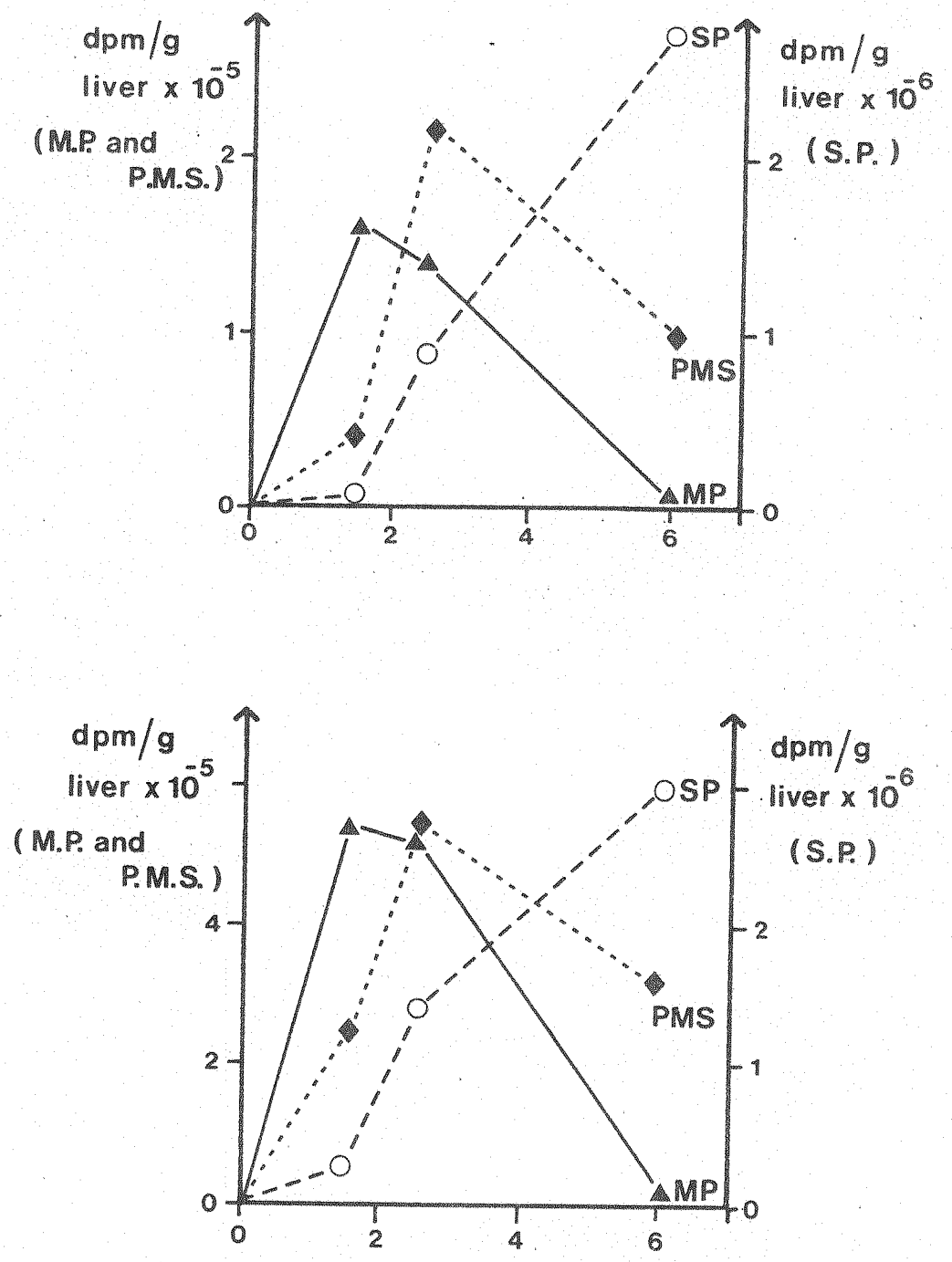


Figure 56 The Kinetic Relationship between the (^{3}H) Leucine-Labelled 200,000 mol. wt. Microsomal and Post-Microsomal Species and Secreted Vitellogenin

The amount of (^{3}H) leucine-labelled 200,000 mol. wt. species recovered in the microsomal and post-microsomal fractions and in the incubation medium was determined from the SDS polyacrylamide gels described in Figures 19-23. (M.P. - microsomal protein, P.M.S. - post-microsomal supernatant, S.P. - secreted protein.)

the labelled species from the microsomal and "post-microsomal supernatant" fractions. The results of polyacrylamide gel analysis indicated that at 2½ hours similar amounts of the (^{3}H) leucine-labelled, 200,000 mol. wt. species were associated with both the microsomal and "post-microsomal supernatant" fractions (see Figure 56). However, it is known that only 20-30% of the total microsomes are recovered in the microsomal pellet fraction (Lewis and Tata, 1974), and this was confirmed in this work (see later, Table 19), therefore it seems probable that in the intact liver tissue, considerably more of the labelled 200,000 mol. wt. species is associated with the microsomes than with the "post-microsomal supernatant".

Further single-label experiments with (^{3}H) leucine- revealed that when the "post-microsomal supernatant" was subjected to a second 3-hour centrifugation step, the labelled, 200,000 mol. wt. post-microsomal species was almost quantitatively co-sedimented with a "post-microsomal pellet", and that very little remained in the final supernatant (see Figure 27). This suggested that the post-microsomal species was not soluble, but associated with a "light" particulate fraction. The observation that soluble vitellogenin was not similarly sedimented during a parallel fractionation procedure supported this idea (see Figure 29). In the light of these observations, further experiments with (^{3}H) leucine and (^{32}P) phosphate were performed, and the "post-microsomal pellet" fraction analysed ; these confirmed that the double-labelled "post-microsomal" species was co-sedimented with the "post-microsomal pellet" (see Figure 32).

Thus the evidence suggested that prior to secretion polypeptide precursors of vitellogenin were associated with two distinct intracellular pools ; a microsomal pool, comprised of completed polypeptide precursors of vitellogenin, but including only a fraction of the (^{32}P) phosphate label found in secreted vitellogenin, suggesting that the average level of phosphorylation of the precursors was low, and a post-microsomal pool, comprised of precursors

labelled with (^{32}P) phosphate to a similar extent to secreted vitellogenin, thus suggesting that they were fully phosphorylated.

The observation that the (^{32}P) phosphate label associated with the microsomal precursors always migrated slightly behind the main (^{3}H) leucine-labelled peak (see Figures 20, 21, 29 and 31), indicated that the ^{32}P) phosphate label was present in a species with a slightly higher mol. wt. than the bulk of the (^{3}H) leucine-labelled microsomal precursors ; it was also shown that the mol. wt. of secreted vitellogenin was slightly higher than that of the microsomal precursors (Figure 33). Thus the results favoured the idea that the low average level of (^{32}P) phosphate labelling of the microsomal precursors reflected the presence of two distinct populations of precursors the majority being non-phosphorylated, with a mol. wt. slightly lower than secreted vitellogenin, but with a small proportion, about 15-20% of fully phosphorylated precursors, with a mol. wt. similar to secreted vitellogenin.

6.2.2 The Identification of Glycosylated Precursors of Vitellogenin

In view of the evidence suggesting the presence of two distinct intracellular pools of vitellogenin precursors, it was interesting to look at the level of glycosylation of the precursors associated with the microsomal and post-microsomal fractions. Both (^{3}H) glucosamine and (^{3}H) galactose were incorporated into vitellogenin secreted by liver slices (Figures 35, 36 and 38). However, a comparison of the relative incorporation of (^{3}H) leucine and the labelled sugars into secreted vitellogenin and into the (^{3}H) leucine-labelled microsomal precursors of vitellogenin revealed that the microsomal precursors included only a fraction of the (^{3}H) glucosamine and (^{3}H) galactose present in the secreted vitellogenin (Figures 37 and 39) ; it was estimated that the microsomal precursors included a maximum of 15% of the (^{3}H) glucosamine and (^{3}H) galactose label associated with

the final secreted vitellogenin. In contrast, analysis of the post-microsomal fraction showed that the (^{3}H) leucine-labelled, 200,000 mol. wt. species was labelled with (^{3}H) glucosamine and (^{3}H) galactose to the same extent as the secreted vitellogenin (Figures 40-41). These experiments again demonstrated that the labelled species present in the "post-microsomal supernatant" was co-sedimented with the "post-microsomal pellet", and showed the loss of this species between 2½ and 8 hours. Thus the results suggested that the average level of both phosphorylation and glycosylation of the microsomal precursors was low compared with secreted vitellogenin, but that the post-microsomal species was both phosphorylated and glycosylated.

6.2.3 Electron Microscopic and Enzymic Analysis of the Microsomal and Post-Microsomal Fractions

The microsomal and "post-microsomal pellet" fractions were examined by electron microscopy (Plates 1-3). The microsomal fraction (Plate 1) was heterogeneous, consisting mainly of rough surfaced vesicles, derived from the rough endoplasmic reticulum, and also some smooth surfaced vesicles and other membrane fragments, thus it represented a typical microsomal fraction. In contrast, the "post-microsomal pellet" fraction consisted of much finer particulate material, with no clear vesicular structures or membrane fragments. The results showed convincingly that the microsomal and "post-microsomal pellet" fractions obtained were morphologically distinct, and that the "post-microsomal pellet" was not contaminated with fragments derived from the microsomal fraction. Plate 3 is an electron micrograph of a section through the microsomal pellet, and this shows clearly that although most of the pellet was predominantly made of vesicular structures as described above, a layer of smaller particulate material, resembling that found in the "post-microsomal pellet", was sedimented on the surface. The microsomal fraction was obtained by performing a 1 hour centrifugation step, and since the "post-microsomal pellet"

Plate 1 Electron Micrograph of the Microsomal Fraction Derived from Xenopus Liver

Magnification Factor : 10,000 x

Plate 2 Electron Micrograph of the Post-Microsomal Pellet Fraction Derived from Xenopus Liver

Magnification Factor : 10,000 x

Plate 3 (overleaf) Electron Micrograph of the Microsomal Fraction Derived from Xenopus Liver

Magnification Factor : 3,600 x

The fractions were obtained, fixed and processed for electron microscopy as described in Chapter 2.

Plate 1

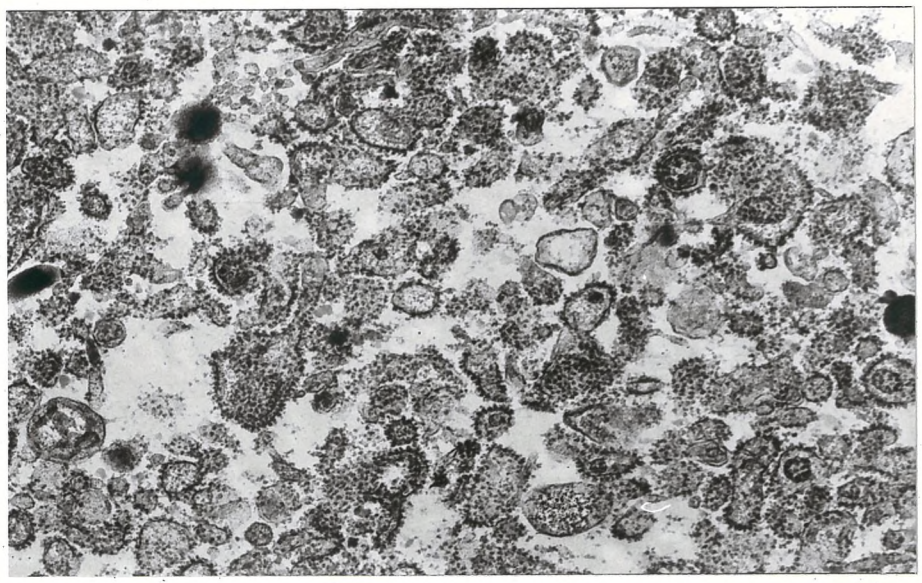


Plate 2

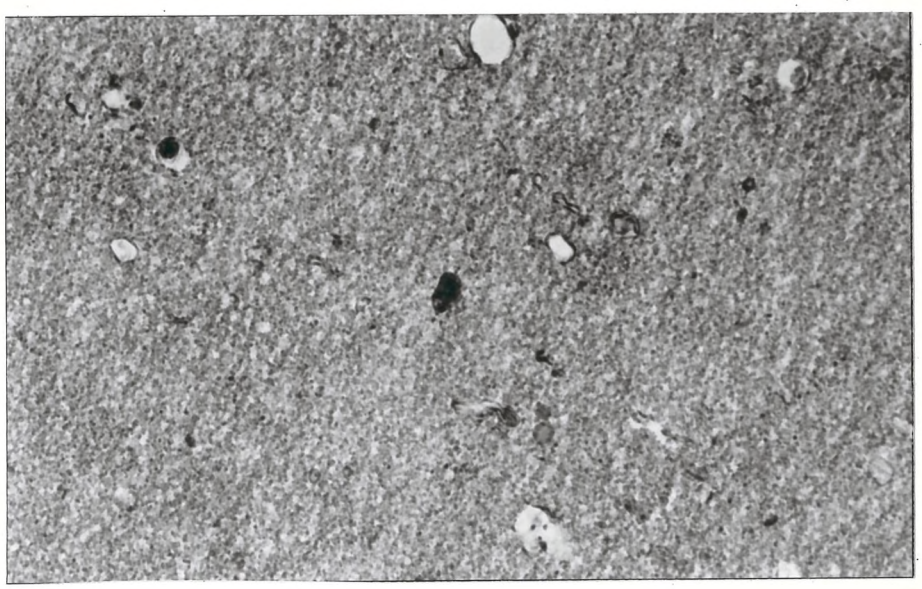
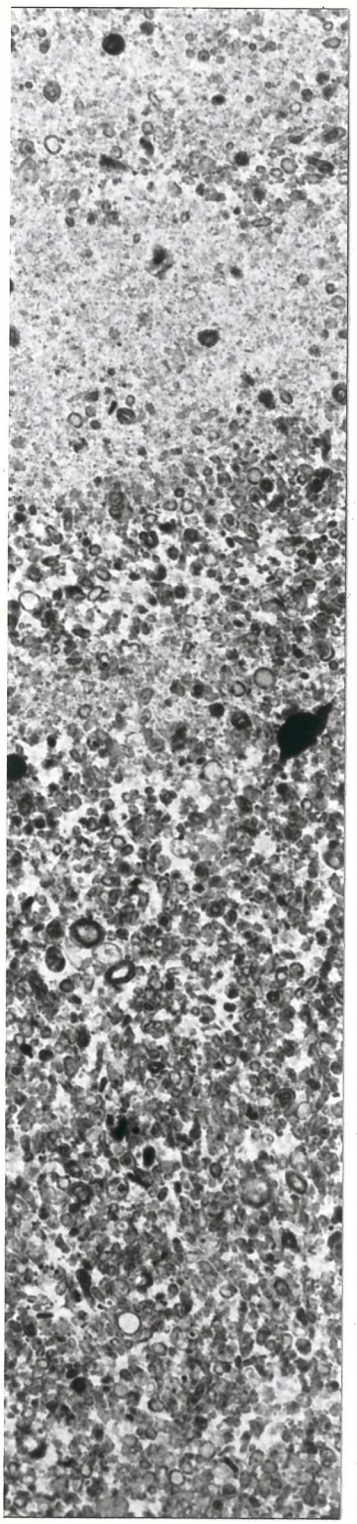


Plate 3



was isolated simply by prolonged centrifugation, it was not surprising that the microsomal fraction was contaminated with some of the lighter material. However, this observation does not affect the interpretation of the results obtained, on the contrary, it implied that at least some of the (^{32}P) phosphate associated with the (^{3}H) leucine-labelled microsomal precursors was due to the presence of some of the double-labelled species derived from the lighter fraction and that therefore the level of phosphorylation of the microsomal precursors was even less than previously estimated ; some of the (^{3}H) glucosamine and (^{3}H) galactose label associated with the microsomal precursors could also be due to the contamination of the microsomal pellet with the lighter fraction. Conversely, the observation that the "post-microsomal pellet" was completely free from microsomal contamination showed unambiguously that the "post-microsomal" labelled species was not derived from the microsomes.

It was thought that the light membrane fraction could possibly be derived from fragments of the smooth endoplasmic reticulum. Therefore to complement the electron microscopy, the distribution of glucose-6-phosphatase activity, the marker enzyme for the endoplasmic reticulum, between the microsomal and post-microsomal pellets was investigated ; the results are shown in Table 19 (overleaf).

TABLE 19 Distribution of Glucose-6-Phosphatase Activity in Microsomal and Post-Microsomal Fractions from Xenopus Liver

Fraction	Specific Activity (μ mole Pi/mg/hour)	
	Control	Oestrogen-Treated
Total Tissue Homogenate	0.43 (100%)	0.42 (100%)
Microsomes	1.30 (27%)	0.81 (21%)
Post-Microsomal Pellet	0.06 (0.4%)	0.04 (0.4%)
Final Supernatant	-	-

The fractions were obtained and G6Pase activity determined as described in Chapter 2. The values in parenthesis give the recovery of activity in each fraction as a percentage of the total activity.

Approximately 25% of the total G6Pase activity present in the tissue homogenate was recovered in the microsomal fraction, and the specific activity in this fraction was 2-3 times higher than in the homogenate. The observation that only a fraction of the total G6Pase activity was recovered in the microsomal fraction emphasized the point made earlier, that the yield of microsomes obtained during the fractionation procedure was low due to the sedimentation of a high proportion during the 6,000 g centrifugation step. However, compared with the microsomal fraction, the specific activity of G6Pase in the "post-microsomal pellet" fraction was negligable, and the percentage recovery of total activity was also very low (0.4%) ; the very low G6Pase specific activity strongly suggested that the "post-microsomal pellet" was not derived from the endoplasmic reticulum. To confirm this, it was decided to compare the G6Pase activity of the "post-microsomal pellet" with that of the smooth microsomal fraction (including the smooth endoplasmic reticulum)

obtained by a discontinuous sucrose gradient procedure ; the distribution of G6Pase activity between the rough and smooth microsomal fractions obtained and the microsomal and "post-microsomal pellet" fractions is shown in Table 20 (overleaf). The specific activity of G6Pase in the smooth microsomal fraction was 7 times greater than in the "post-microsomal pellet", and thus the two fractions were enzymically distinct (see also footnote*). Electron micrographs of the rough and smooth microsomal fractions are shown in Plates 4 and 5. In contrast to the "post-microsomal pellet", the smooth microsomal fraction (Plate 5) was made up of distinct smooth-surfaced vesicles. These results showed conclusively that the "post-microsomal pellet" fraction was not synonymous with the smooth microsomal fraction, and that it was not derived from the smooth endoplasmic reticulum.

The distribution of 5'-nucleotidase activity, the marker enzyme for the plasma membrane, between the fractions was also investigated ; the results are shown in Table 22 (overleaf). 5'-nucleotidase activity was present in both the microsomal and the "post-microsomal pellet" fractions, and also in the final supernatant, but the specific activity was highest in the microsomal fraction, as would be expected since the plasma membrane is a heavy fraction. This result ruled out the possibility that the "post-microsomal pellet" fraction was primarily derived from plasma membrane fragments.

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* The specific activity of G6Pase in the rough microsomal fraction was approximately twice that found in the smooth microsomal fraction. Lewis and Tata (1974) reported a similar difference between the G6Pase specific activity of rough and smooth microsomal fractions obtained from rat liver, and this was confirmed in this study (see Table 21 overleaf). It was interesting that in the fractions obtained from Xenopus liver, the specific activity of the G6Pase was at least ten times lower than in fractions obtained from rat liver ; the reason for this could be that the Xenopus fractions were incubated at 22°C, whereas the rat fractions were incubated at 37°C.

TABLE 20 A Comparison of the Distribution of Glucose-6-Phosphatase Activity in Rough and Smooth Microsomes (a) and in Microsomal and Post-Microsomal Fractions (b) from Xenopus Liver

Fraction	Specific Activity (μ mole Pi/mg/hour)
(a)	
Total Tissue Homogenate	0.30 (100%)
Total Microsomes	0.48 (12.5%)
Rough Microsomes	0.62 (8.7%)
Smooth Microsomes	0.28 (1.2%)
(b)	
Total Tissue Homogenate	0.32 (100%)
Microsomes	0.59 (21.5%)
Post-Microsomal Pellet	0.04 (0.1%)

TABLE 21 Distribution of Glucose-6-Phosphatase Activity in Rough and Smooth Microsomal Fractions from Rat Liver

Fraction	Specific Activity (μ mole Pi/mg/hour)
Total Tissue Homogenate	4.0 (100%)
Total Microsomes	8.0 (25%)
Rough Microsomes	9.2 (13%)
Smooth Microsomes	5.1 (5%)

The fractions were obtained and the G6Pase activity determined as described in Chapter 2, except that incubation of rat liver fractions was performed at 37°C. The values in parenthesis give the recovery of activity in each fraction as a percentage of the total activity.

Plate 4 Electron Micrograph of the Rough Microsomal Fraction Derived from Xenopus Liver

Magnification Factor : 10,000 x

Plate 5 Electron Micrograph of the Smooth Microsomal Fraction Derived from Xenopus Liver

Magnification Factor : 10,000 x

The fractions were obtained, fixed and processed for electron microscopy as described in Chapter 2.

Plate 4

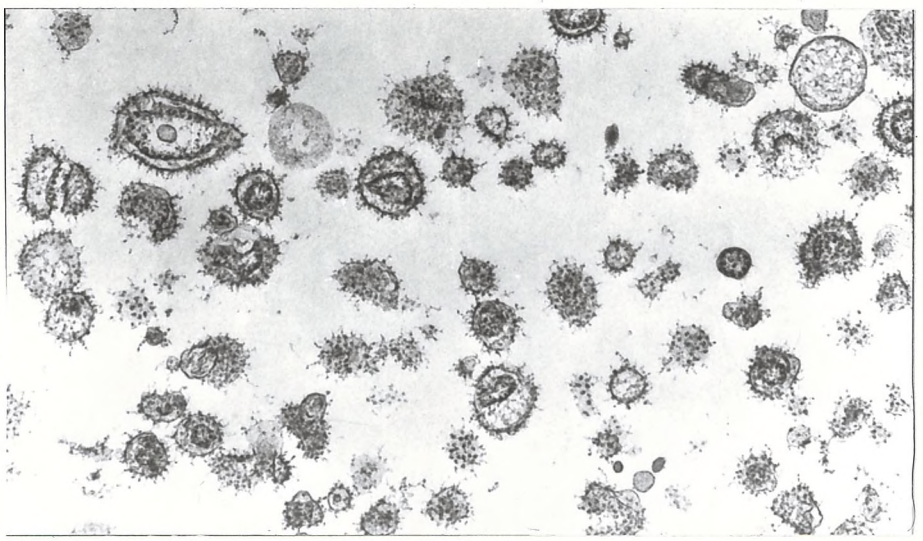


Plate 5

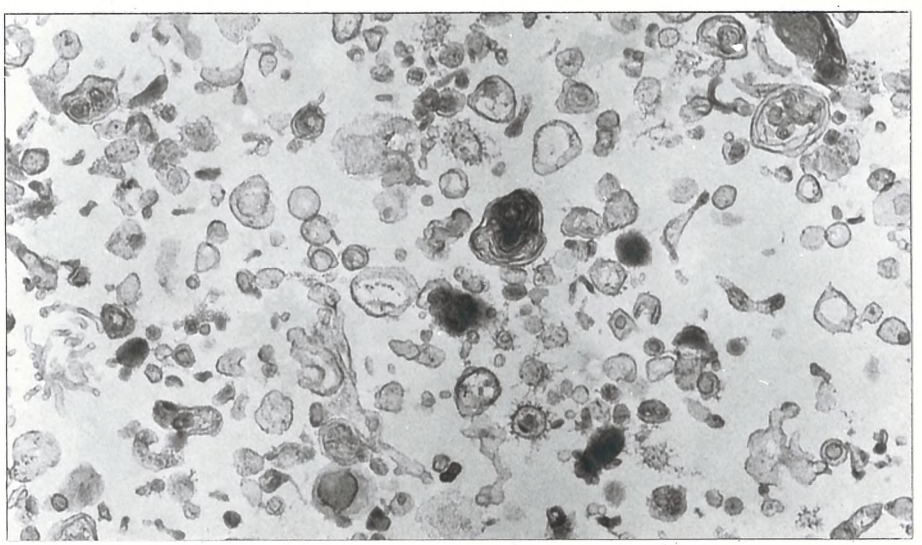


TABLE 22 Distribution of 5'-Nucleotidase Activity in Microsomal and Post-Microsomal Fractions from Xenopus Liver

Fraction	Specific Activity (μ mole Pi/mg/hour)	
	control	Oestrogen-treated
Total Tissue Homogenate	0.30 (100%)	0.29 (100%)
Microsomes	0.53 (15.6%)	0.83 (24%)
Post-Microsomal Pellet	0.32 (4.8%)	0.11 (1.6%)
Final Supernatant	0.23 (12.0%)	0.15 (6.5%)

The fractions were obtained and the 5'-nucleotidase activities determined as described in Chapter 2. The values in parenthesis give the recovery of activity in each fraction as a percentage of the total activity.

6.3 The Effect of Oestrogen-Treatment on the Synthesis of Lipid-Sugar Intermediates in Xenopus Liver

In Chapter 1, the evidence to suggest the role of glycolipid intermediates during glycoprotein synthesis was discussed. The oestrogen-induced synthesis of vitellogenin in Xenopus liver offers a particularly interesting system in which to study the possible role of lipid-intermediates during the synthesis of a specific secretory glycoprotein, since after oestrogen-treatment up to 70% of liver protein synthesis is directed towards vitellogenin, a protein not synthesized prior to hormone treatment. The experiments described in Chapter 5 looked at the effect of oestrogen-treatment in the synthesis of lipid-sugar intermediates in Xenopus liver.

When liver microsomal fractions were incubated with UDP (^{3}H) galactose, GDP (^{14}C) mannose or UDP N-acetyl (^{14}C) glucosamine, it was found that oestrogen-treatment resulted in a stimulation of the incorporation of each of the labelled sugars into glycolipid extrable in 2:1

chloroform/methanol (Figure 44, Tables 13 and 14). Analysis of the labelled lipid extract by DEAE-cell chromatography (Figures 46, 47 and 48) showed that the increased incorporation could be accounted for by an increase in the incorporation of labelled sugar into a glycolipid fraction eluted with ammonium acetate ; the effect on the incorporation of (^{3}H) galactose and N-acetyl (^{14}C) glucosamine was dramatic, since in both cases oestrogen-treatment resulted in the formation of a labelled glycolipid fraction which was not detectable in lipid extracted from control incubations. The elution of the labelled glycolipid fraction with ammonium acetate was consistent with them being derivatives of the dolichol phosphate type ; the results of thin-layer chromatography (Figure 49) and the susceptibility of the glycolipid to acid hydrolysis (Table 16) were also consistent with this. Paper chromatography of the labelled sugars released from the (^{3}H) galactose- and (^{14}C) mannose-labelled glycolipid during acid hydrolysis (Figure 50) indicated that the intermediates formed were monosaccharide lipid derivatives. (When the residue remaining after the 2:1 chloroform/methanol extraction was re-extracted with 10:10:3 chl ro-form/methanol/water, a solvent system favouring the extraction of more polar oligosaccharide lipid derivatives, and the extract analysed by DEAE-cellulose acetate chromatography, it was found that the amount of labelled, ammonium acetate-elutable material extracted in this fraction was less than 10% of that present in the 2:1 chloroform/methanol extract. This indicated that the incorporation of labelled sugars into oligosaccharide lipid was very low in this system.)

An especially interesting aspect of these results is that substantial amounts of (^{3}H) galactose were trans-ferred from UDP (^{3}H) galactose to endogenous lipid by microsomal preparations from oestrogen-treated animals, since although galactose containing lipid intermediates are known to be involved in the synthesis of antigenic determinants in the bacterial cell wall (Wright et al, 1965), the transfer of galactose to endogenous lipid in

other liver microsomal preparations has usually been shown to be negligable (Janowski and Chojnacki, 1972 ; Behrens et al, 1971). However, it should be pointed out that after oestrogen-treatment, Xenopus liver represents a unique situation, in which the majority of the protein synthetic capacity of the cell is devoted towards vitellogenin synthesis ; the microsomal systems previously investigated were only carrying out general hepatic protein synthesis, and thus are only comparable with the situation in control Xenopus liver, where there was no detectable transfer of (^{3}H) galactose to endogenous glycolipid intermediates.

In Chapter 4 it was shown that liver slices from oestrogen-treated Xenopus incorporated both (^{3}H) galactose and (^{3}H) glucosamine into secreted vitellogenin. Therefore in this system it was possible to determine whether there was also an increase in the formation of glycolipid intermediates in a situation in which the completed vitellogenin molecule was being synthesized. Liver slices from control and oestrogen-treated animals were incubated with (^{3}H) galactose or (^{3}H) glucosamine, and at the end of the incubation lipid was extracted from the tissue with 2:1 chloroform/methanol followed by 10:10:3 chloroform/methanol/water and the extracts pooled and analysed by DEAE cellulose acetate chromatography. In both control and oestrogen-treated incubations, (^{3}H) galactose and (^{3}H) glucosamine were incorporated into a glycolipid fraction which was eluted with 0.05 M ammonium acetate (Figures 51 and 52), but after oestrogen-treatment, the incorporation of label into this fraction was 3-4 fold greater than in the control incubations. The results of thin-layer chromatographic analysis (Figure 54) and the susceptibility of the ammonium acetate-eluted glycolipid fraction to acid hydrolysis again favoured the (^{3}H) galactose- and (^{3}H) glucosamine-labelled glycolipid being derivatives of the dolichol-phosphate-sugar type. However, in contrast to the derivatives obtained from the microsomal system, paper chromatography of the labelled sugars released by acid hydrolysis (Figure

55) suggested that both (^{3}H) glucosamine- and (^{3}H) galactose were incorporated into oligosaccharide-lipid during liver slice incubations.

Thus the results obtained in both microsomal and liver slice systems showed that after oestrogen-treatment there was an increase in the synthesis of glycolipid intermediates of the type suggested to be involved in glycoprotein biosynthesis. However, a problem with the interpretation of results obtained in tissues which synthesize secretory proteins is that it is difficult to distinguish between the glycolipid intermediates which may be involved in the glycosylation of the secretory protein and those involved in the glycosylation of endogenous membrane proteins. It has been shown that glycolipid intermediates are involved in the synthesis of the structural glycoproteins of the endoplasmic reticulum (Nilsson et al, 1978), and since the response of Xenopus liver to oestrogen involves a proliferation of the endoplasmic reticulum, this must require a considerable increase in the synthesis of structural glycoproteins destined to become components of the new membranes. However, in this system it has already been shown that after the incubation of liver slices with (^{3}H) leucine, the only major labelled polypeptide associated with the microsomal fraction was the 200,000 mol. wt. polypeptide precursor of vitellogenin (for example see Figures 16, 20 and 21), and it follows that the synthesis of any new structural protein was negligable compared with vitellogenin synthesis. Further to this, there was no evidence for significant incorporation of (^{3}H) glucosamine or (^{3}H) galactose into microsomal structural protein (see Figures 37 and 39). Therefore it is unlikely that the increase in the formation of glycolipid intermediates observed is involved with the synthesis of new structural glycoproteins. It is interesting that in chick oviduct, the tissue most closely similar to the yolk protein synthesizing system in its response to oestrogen, it has been shown that there is an increase in the synthesis of glycolipid intermediates

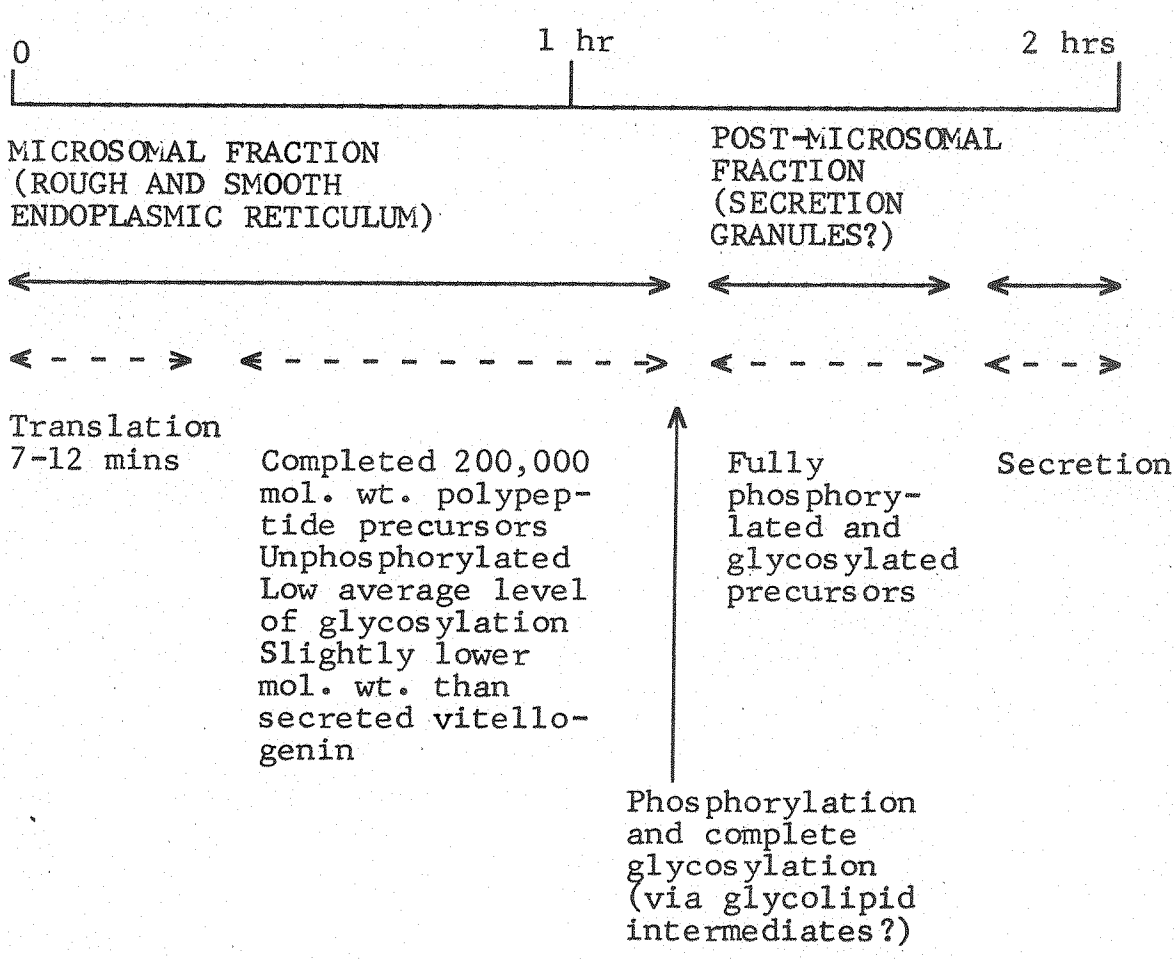
following oestrogen-treatment (Lucas, 1979), and that these may be involved in the development of the tubular gland cells and/or the glycosylation of the secretory protein, ovalbumin. It is proposed that the rate limiting step for the formation of glycolipid intermediates is the availability of dolichol phosphate (Harford et al, 1977), and that the level of dolichol phosphate may play a role in regulating the rate of glycoprotein synthesis. More recent reports have indicated that the synthesis of dolichol phosphate is regulated by the enzyme 3-hydroxy-3-methyl-glutaryl CoA reductase, the enzyme responsible for the regulation of cholesterol biosynthesis (Mills and Adamany, 1978). It is interesting to speculate that in Xenopus liver, an increase in the activity of 3-hydroxy-3-methyl-glutaryl CoA reductase following oestrogen-treatment could result in a co-ordinated increase in the synthesis of cholesterol, glycoproteins destined to become components of new cell membranes, and the secretory glycoprotein vitellogenin.

6.4 General Conclusions

The procedures adopted in this study enabled the direct identification of two distinct populations of vitellogenin precursors. The population associated with the microsomal fraction - which includes the membranes of the rough and smooth endoplasmic reticulum - was predominantly non-phosphorylated with a very low average level of glycosylation compared with secreted vitellogenin ; the observation that the mol. wt. of these precursors was slightly lower than that of secreted vitellogenin was consistent with the absence of the phosphate and carbohydrate groups. In contrast, the population of precursors associated with the "post-microsomal pellet" was phosphorylated and glycosylated to the same extent as secreted vitellogenin. The results suggested that phosphorylation and glycosylation were not being completed until just prior to the release of the precursors from the endoplasmic reticulum membranes.

However, what is the nature of the precursors which are associated with the "post-microsomal pellet" fraction? A possibility is that the precursors present in this fraction are in the form of secretion granules or aggregates of vitellogenin molecules assembled prior to secretion. Such granules may be homogeneous, made up entirely of vitellogenin molecules, or heterogeneous, consisting of vitellogenin molecules in association with other proteins. Aggregation of the precursor molecules may be necessary for the formation of the vitellogenin dimer from the two subunits, or for the inclusion of calcium and the bile pigment biliverdin into the molecule.

On the basis of the results obtained it is possible to propose a tentative scheme to explain the intracellular distribution of the precursors of vitellogenin prior to secretion and to indicate the stages at which phosphate and sugar addition are likely to occur.



There is little accumulation of phosphorylated and glycosylated precursors of vitellogenin on the microsomes. It is interesting to speculate that the complete modification of the polypeptide precursors causes a change in the physical characteristics or conformation of the molecules and favours their release from the endoplasmic reticulum and their conversion into a form suitable for secretion. Both the phosphate groups and the oligosaccharide chain are added to the region of the parent molecule which is destined to become phosvitin ; therefore, just as the hydrophobic "signal-peptide" allows the transport of the newly translated secretory polypeptide across the endoplasmic reticulum membrane and into the intercisternal space, so the extensive modification of a limited region of the vitellogenin molecule may "trigger" the release of the precursors from the membranes of the endoplasmic reticulum and their formation into secretion granules.

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