BIOSYNTHESIS OF OVOMUCOID

by

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Submitted for the degree of Doctor of Philosophy at the University of Glasgow.

November, 1972.

ACKNOWLEDGMENTS

ACKNOWLEDGMENTS

I am grateful to the late Professor J. N. Davidson and to Professor R. M. S. Smellie for the provision of the facilities of the Biochemistry Department.

I am indebted to Dr. J. G. Beeley for his constant help and guidance throughout this project and for his useful advice at all times.

The technical assistance of Mrs. A. Lindsay is gratefully acknowledged.

I would like to thank my wife, Frances, for her patience and understanding during the period of this project.

My thanks also go to my wife for typing part of the thesis and to Mrs. D. M. Mackenzie who typed the major part of it.

During the course of the work the author was research assistant to Dr. J. G. Beeley who was in receipt of a grant from the Medical Research Council.

INDEX

INDEX

PART 1

INTRODUCTION

Chapter 1 - Glycoproteins

1.1 (1) General Introduction to Glycoproteins

The complexes formed between proteins and polysaccharides have been extensively reviewed (Jeanloz, 1963; Brimacombe & Webber, 1964; Gottschalk, 1966; Sharon, 1966; Ginsburg & Neufeld, 1969; Spiro, 1970).

Protein-polysaccharides have received many names such as mucoproteins, glycoproteins and mucopolysaccharides and various systems of nomenclature have been proposed (see Gottschalk, 1966). There may be some confusion caused by the prefix "muco", which may refer to the protein or the sugar component of protein-polysaccharides. In order to counteract this , Jeanloz (1960) proposed a system of nomenclature where the compounds in which the protein-polysaccharide linkage was thought to be weak (e.g. salt link or hydrogen bond) were known as polysaccharide-protein complexes. The name glycosaminoglycuronoglycans was proposed to cover polysaccharides which had a repeating disaccharide unit of hexosamine and hexuronic acid. Compounds with a covalent linkage between protein and carbohydrate were called glycoproteins. However, most of the polysaccharide-protein complexes, frequently referred to as mucopolysaccharides, have been shown to contain a covalent linkage between their protein and polysaccharide moieties and so can be classed as glycoproteins.

Gottschalk (1966) proposed to update the system of nomenclature of Jeanloz (1960) and to regard mucopolysaccharides

as a specialised class of glycoprotein because of the repeating disaccharide nature of their carbohydrate moiety and the relatively large size of the carbohydrate part. Other glycoproteins do not seem to have a simple repeating unit in their carbohydrate moiety.

In their reviews, Ginsburg and Neufeld (1969) and Spiro (1970) regard mucopolysaccharides as a specialised class of glycoprotein (as these compounds do have a covalent linkage between their protein and polysaccharide portions) in which the carbohydrate moiety is larger than in conventional glycoproteins.

The updated system of nomenclature of Jeanloz (1960) as proposed by Gottschalk (1966) shall be used here.

Glycoproteins have been found to occur widely in the animal and plant kingdoms and more recently have been found in micro-organisms. While the peptide portion reflects the whole spectrum of amino acids, only a limited number of monosaccharides have been found. These characteristic sugars are D-galactose, D-mannose, D-glucose, L-fucose, D-xylose, L-arabinose, N-acetyl-D-glucosamine, N-acetyl-D-galactosamine and the N- and O-acetyl and N-glycolyl derivatives of neuraminic acid. In addition to this the hexuronic acids, D-glucuronic and L-iduronic acid are found as part of mucopolysaccharides.

The carbohydrate portion of glycoproteins may contain from two to six or seven of the monosaccharide types (Gottschalk, 1966; Spiro, 1970) and may represent from less than 1% to more than 80% by weight of the molecule (Spiro, 1970). The number of covalently

bound carbohydrate units in glycoproteins may range from one as in ovalbumin (Johansen, Marshall & Neuberger, 1961), ribonuclease B (Plummer & Hirs, 1964) or deoxyribonuclease (Catley, Moore & Stein, 1969), to higher numbers such as 19 for calf thyroglobulin (Spiro, 1965) up to as many as 800 in the ovine submaxillary glycoprotein (Graham & Gottschalk, 1960). However, there is a wide range of molecular weights of glycoproteins and the degree of glycosylation of the molecule may be visualised by calculating the average number of amino acid residues per covalently bound carbohydrate unit (Spiro, 1970). This spacing varies from an average of 6 amino acids per carbohydrate unit in ovine submaxillary glycoprotein through one carbohydrate group per 507 amino acids for ovalbumin to one carbohydrate group per 1000 amino acids in rabbit scleral collagen (Spiro, 1970).

1.1 (2) Occurence and Biological Activities of Glycoproteins

Proteins that have been found to be glycosylated, as well as being widespread throughout the plant and animal kingdoms have many diverse functions, although in many cases it is difficult to ascribe a particular function to a particular protein. Glycoproteins have been found to be hormones, for example, chorionic gonadotrophin (Bahl, 1969), follicle-stimulating hormone (Cahill, Shetlar, Payne, Endecott & Li, 1968), thyroid-stimulating hormone (Carsten & Pierce, 1963) and thyroglobulin (Spiro & Spiro, 1965) and enzymes, ribonuclease (Plummer & Hirs, 1964), deoxyribonuclease (Catley Moore & Stein, 1969), horse radish

peroxidase (Shannon, Kay & Lew, 1966) and pepsin and pepsinogen (Bohak, 1969). The immunoglobulins IgG (Roseweaver & Smith, 1961). IgA (Dawson & Clamp, 1968) and IgM (Miller & Metzger, 1965) are glycoproteins. Glycoproteins are also involved in transport as in transferrin (Jamieson, 1965) the iron transporting protein of plasma, while other plasma glycoproteins are, for example, fetuin (Spiro, 1960) and fibrinogen (Bray & Laki, 1968). Glycoproteins are found in cellular membranes in platelets (Pepper & Jamieson, 1969), visual pigment (Heller, 1968) and E. Coli cell membrane (Okuda & Weinbaum, 1968) and in extracellular membranes such as glomerular basement membrane (Spiro, 1967 a), lens capsule (Fukushi & Spiro, 1969) plant cell wall (Lamport, 1969) and yeast cell wall (Sentandreau & Northcote, 1968). Phytohaemagglutinins from many sources e.g. soy bean (Lis, Sharon & Katchalski, 1966), black locust (Bourrillon & Font, 1968) and meadow mushroom (Sage & Connett, 1969) are glycoproteins as are the mucins such as ovine submaxillary glycoprotein (Graham & Gottschalk, 1960), sulphated gastric (Pamer, Glass & Horowitz, 1968) and porcine submaxillary glycoprotein (Carlson, 1968). Glycoproteins are also found in urine, Tam and Horsfall glycoprotein (Maxfield & Stefanye, 1962) and in connective tissue as collagens (Spiro, 1969; Butler & Cunningham, 1966) or keratan sulphate-proteins (Bray, Lieberman & Meyer, 1967). Most of the major proteins of hen egg white are glycoproteins with the exception of lysozyme (Montgomery, 1970) although a discussion of egg white glycoproteins

will occur in a later chapter.

So it can be seen that glycoprotein function may be hormonal, enzymatic or structural, or involved in transport, lubrication or cellular adhesion. A discussion of the function of the carbohydrate moiety of glycoproteins will occur in a later chapter.

Chapter 2 Structural Aspects of Glycoproteins

1. 2 (1) Protein - Carbohydrate Linkage

The protein-carbohydrate linkage involves the functional group of an amino acid side chain and the sugar at the reducing terminus of the carbohydrate moiety. The types of protein-carbohydrate linkage which have been reported are as follows:-

(1) A β-N-glycosidic bond between N-acetylglucosamine and asparagine (Marshall & Neuberger, 1964).

(2) An O-glycosidic bond involving serine or threonine with either (a) an N-acetylgalactosamine residue (Graham & Gottschalk, 1960) or (b) a galactose residue (Muir & Lee, 1969).

(3) A β-O-glycosidic bond between serine and xylose (Rodén, 1968).

(4) A β-O-glycosidic bond involving hydroxylysine and galactose (Spiro, 1967c).

(5) An O-glycosidic bond between hydroxyproline and arabinose (Lamport, 1969).

1.2 (2) Carbohydrate Moiety Associated with the Protein-Carbohydrate Linkage

The N-glycosidic link between N-acetylglucosamine and asparagine has been found in ovalbumin and other egg white proteins as well as plasma proteins, hormones and enzymes.

With this type of linkage the carbohydrate moiety may be of a simpler type involving mannose and N-acetylglucosamine residues, or a more complex heteropolysaccharide unit made up of N-acetylglucosamine, mannose, galactose, and sialic acid or fucose. The more complex type of unit is very widespread, being found in plasma glycoproteins (Jamieson, 1965; Spiro, 1962; Dunn & Spiro, 1967; Satake, Okuyama, Ishihara & Schmid, 1959), hormones (Spiro, 1965; Bahl, 1969) and immunoglobulins (Roseweaver & Smith, 1961; Dawson & Clamp, 1968; Clamp & Putnam, 1964), while the simpler unit is found, for example, in ribonuclease B (Plummer & Hirs, 1964), deoxyribonuclease (Catley, Moore & Stein, 1969), thyroglobulin (Spiro, 1965), an immunoglobulin IgA (Dawson & Clamp, 1968) and soy bean phytohaemagglutinin (Lis, Sharon & Katchalski, 1966). Among egg white proteins, ovomucoid (Montgomery & Wu, 1968) has the more complex type of unit while ovalbumin (Johansen, Marshall & Neuberger, 1961), avidin (DeLange, 1969) and ovotransferrin (Williams, 1968) have the simpler type.

A third type of carbohydrate moiety associated with the N-acetylglucosamine-asparagine linkage is found in the mucopolysaccharide, corneal keratan sulphate. This has a carbohydrate

moiety of repeating galactose and N-acetylglucosamine residues with additional sialic acid and fucose (Bhavanandan & Meyer, 1967) and mannose (Baker, Cifonelli, Mathews & Rodén, 1969).

The protein-carbohydrate linkage involving serine or threonine and N-acetylgalactosamine is found in mucins (Graham & Gottschalk, 1960; Carlson, 1968) with the characteristic sugars being sialic acid, fucose, galactose, N-acetylgalactosamine and N-acetylglucosamine. The carbohydrate unit may vary from monosaccharides up to heptasaccharides (Graham & Gottschalk, 1960; Carlson, 1968; Watkins, 1966; Lloyd, Kabat & Licerio, 1968) although larger branched units have been reported for ovarian cyst mucins (Lloyd, Kabat & Licerio, 1968).

The mucins may have blood group activity of the ABH and Lewis systems associated with their carbohydrate moiety depending on the terminal α-linked sugars on the galactose-N-acetylhexosamine core (Watkins, 1966). Terminal N-acetylgalactosamine specifying A-activity, terminal galactose B-activity and the presence of one or two fucose residues determining H- and Lewis activity. The M and N blood group substances isolated from the red blood cell membrane also have their carbohydrate portion attached to the peptide portion by serine or threonine residues (Thomas & Winzler, 1969; Kathan & Adamany, 1967; Winzler, Harris, Pekas, Johnson & Weber, 1967), although M and N blood group antigenicity depends on sialic acid residues (Springer & Ansell, 1958).

It should be noted that in glycoproteins with the asparagine-N-

acetylglucosamine linkage there is considerable variation in the degree of glycosylation from an average of one carbohydrate unit per 41 amino acids for α_1- acid glycoprotein up to one unit per 860 amino acids for soy bean haemagglutinin (Spiro, 1970). In comparison, the degree of glycosylation of mucins with the O-glycosidic linkage involving serine or threonine and N-acetylgalactosamine residues is much higher and more constant, for example, an average of one carbohydrate unit per 6 amino acids in ovine submaxillary glycoprotein and one unit per 8 amino acids in porcine submaxillary glycoprotein (Spiro, 1970). It has been calculated that about 30% of the total amino acid residues in mucins are β-hydroxyamino acids (Hashimoto & Pigman, 1962) and that of these about 50-70% are involved in glycosidic links (Harbon, Herman, Rossignol, Jollès & Clauser, 1964; Tanaka & Pigman, 1965). It is thought that the close spacing of carbohydrate units in mucins with a large number of negatively charged sialic acid residues near each other helps to impart a high viscosity to the glycoprotein through electrostatic repulsion (Gottschalk, 1960).

It has been mentioned that IgA myeloma immunoglobulin has asparagine linked carbohydrate groups, but it also contains a serine linked carbohydrate unit (Dawson & Clamp, 1968) and rabbit IgG has been reported to contain a single N-acetylgalactosamine attached to threonine (Smyth & Utsumi, 1967).

In a similar case to corneal keratan sulphate mentioned above with an asparagine linked carbohydrate moiety, it is believed that

cartilage keratan sulphate has a similar carbohydrate structural pattern although linked to serine and threonine residues by N-acetylgalactosamine residues (Bray, Lieberman & Meyer, 1967; Bhavanandan & Meyer, 1968). A group of sulphated glycoproteins have been reported in gastric mucosa (Pamer, Glass & Horowitz, 1968), colonic mucosa (Inoue & Yosizawa, 1966) and submaxillary gland (Bignardi, Aureli, Balduini & Castellani, 1964) in which the linkage is presumably through serine or threonine residues.

The protein-carbohydrate linkage occuring in collagen from earthworm (Muir & Lee, 1969; Spiro, 1970) and clamworm (Spiro, 1970) cuticles consists of galactose linked to serine and threonine residues. Di- and trisaccharides of galactose apparently represent the main forms of the carbohydrate portion as well as single galatose residues. These are the only collagens found which have serine or threonine links instead of the more usual hydroxylysine linkage. Although no hydroxylysine has been found in these molecules, they contain larger amounts of serine and threonine than other collagens (Spiro, 1970; Josse & Harrington, 1964). It is worth noting also that the body wall of the earthworm has the more usual hydroxylysine type of linkage to a glucosylgalactose disaccharide (Spiro, 1970).

The protein-carbohydrate linkage involving an O-glycosidic bond between serine and xylose occurs in mucopolysaccharides (proteoglycans) such as heparin, heparan sulphate, dermatan sulphate and chondroitin sulphate. As mentioned earlier these may be termed a special

class of glycoprotein as it has been found that they do have a covalent bond between carbohydrate and peptide. The use of mild alkali and proteolytic digestion in the isolation of these compounds from connective tissue, and the repeating unit of the carbohydrate, gave rise to the idea that they were essentially polysaccharides and the precise nature of the protein part has not been determined. The carbohydrate moiety is thought to be larger than in convential glycoproteins. The xylose involved in the peptide-carbohydrate bond is preceded by two galactose residues (Rodén, 1968), then a repeating disaccharide unit of hexuronic acid (iduronic and glucuronic acid) and hexosamine (glucosamine and galactosamine). For a review see Brimacombe and Webber (1964).

The protein-carbohydrate linkage consisting of an O-glycosidic bond between hydroxylysine and galactose is found in collagens (Spiro, 1969; Jeanloz, Bhattacharyya & Roberts, 1969) and basement membranes (Spiro, 1967c; Spiro & Fukushi, 1969) and is unusual as it involves a glycosidic substitution at a hydroxyl group close to an unsubstituted amino group. Usually when an O-glycosidic link takes place near amino groups, these amino groups are blocked. The carbohydrate moiety is an α-(1→2) glucosylgalactose disaccharide (Spiro, 1969; Spiro, 1967c; Spiro & Fukushi, 1969).

In basement membranes nearly all the carbohydrate structural unit is in the form of disaccharide units, while in the fibrillar collagens up to 50% may occur as single galactose residues (Spiro, 1969). The extent of glycosylation varies from an average of one

carbohydrate group per 27 amino acids for bovine lens capsule glycoprotein to one carbohydrate group per 1000 amino acids for rabbit scleral collagen (Spiro, 1970) and in general the carbohydrate is spaced further for collagens and closer for basement membranes. As well as reflecting the high number of hydroxylysines present in basement membranes, about 80% of the hydroxylysines present are involved in protein-carbohydrate linkages while in fibrillar collagens a smaller percentage of hydroxylysines are involved in O-glycosidic linkages (Spiro, 1969).

Although an early report suggested that invertebrate collagens have greater amounts of carbohydrate than vertebrate collagens (Gross, Sokal & Rougwie, 1956), a recent report based on more evidence says that there is no apparent relation between phylogenetic position of a species and percentage carbohydrate content (Katzman, Halford, Reinhold & Jeanloz, 1972). This later paper also has shown an O-α-D-glucopyranosyl -(1→2)-O-β -D- galactopyranosyloxy - (1→5)-lysine unit involved in the protein-carbohydrate linkage (Katzman et al, 1972).

The O-glycosidic linkage between hydroxyproline and arabinose occurs in plant cell wall glycoproteins and the carbohydrate moiety consists of arabinose from a monosaccharide up to a tetrasaccharide (Lamport, 1969) in the linkage region.

It is worth noting that the hydroxyproline of collagen does not seem to take part in the covalent linkage between protein and carbohydrate.

While most glycoproteins contain carbohydrate units of only one type, the occurence of more than one type has been recorded. The proteins in which this occurs have the more complex type of heteropolysaccharide unit linked N-glycosidically to asparagine, but may in addition have the simpler mannose-N-acetylglucosamine unit, for example thyroglobulin (Spiro, 1965). Also, although there is broad similarity of protein-carbohydrate linkage and type of carbohydrate moiety in a biological group of proteins, some proteins contain more than one type of protein-carbohydrate bond. For example, glomerular basement membrane has both hydroxylysine- and asparagine-linked carbohydrate moieties (Spiro, 1967 b), while IgA immunoglobulin (Dawson & Clamp, 1968) and rabbit IgG immunoglobulin (Smyth & Utsumi, 1967; Nolan & Smith, 1962) have asparagine- and serine- or threonine-linked carbohydrate units.

It can be seen therefore, that a glycoprotein may have a carbohydrate unit of one structural pattern or more than one structural pattern which may be linked by the same type of protein-carbohydrate bond or by different protein-carbohydrate linkages.

1. 2 (3) Location of Carbohydrate Moiety on Peptide Chain.

With the exception of asparagine linked to carbohydrate, there is little evidence concerning the amino acid sequence around the types of amino acid involved in protein polysaccharide

linkages. The sequence Ser-Gly-Gly, however, has been reported for mucopolysaccharides (Baker & Rodén, 1970). In the case of N-acetylgalactosamine linked to threonine, there may be a run of two or three proline residues on the carboxyterminal side of the threonine involved in the protein-carbohydrate linkage (Smyth & Utsumi, 1967). The glucosylgalactose disaccharide of skin tropocollagen is linked O-glycosidically to hydroxylysine in the sequence Gly-Met-Hyl-Gly-His-Arg (Butler & Cunningham, 1966). The sequence of amino acids around asparagine residues involved in protein-carbohydrate linkages has led to the suggestion that the sequence Asn-X-Ser/Thr (with X being any amino acid) may be the recognition site for an enzyme attaching N-acetylglucosamine to asparagine (Marshall & Neuberger, 1970). This is further indicated by the complete amino acid sequence of the glycoproteins ribonuclease B (Plummer & Hirs, 1964), porcine ribonuclease (Jackson & Hirs, 1970) and an immunoglobulin IgG (Edelman, Cunningham, Gall, Gottlieb, Rutishauser & Waxdal, 1969) which suggest a specific utilisation of asparagine residues.

Jackson and Hirs (1970) proposed that the nature of the amino acid, X, in the sequence Asn-X-Ser/Thr found in glycoproteins with asparagine linked carbohydrate groups determined the type of oligosaccharide unit synthesised. If X was a polar amino acid, the more complex type of heteropolysaccharide would be synthesised, while if X was non polar the simpler mannose-N-acetylglucosamine moiety would be synthesised. However, ovotransferrin (Williams,

1968) has the more simple type of carbohydrate moiety linked to asparagine in the sequence Asn-Arg-Thr, and one of the more complex type of heterosaccharide units of an IgM immunoglobulin (Shimizu, Putnam, Paul, Clamp & Johnson, 1971) is linked to asparagine in the sequence Asn-Ala-Ser. Apart from these exceptions, most other glycoproteins examined bear out the proposal of Jackson and Hirs (1970), although one of the complex carbohydrate moieties of α_1-acid glycoprotein is reported to be linked to asparagine in the sequence Asn-Thr-Gly (Yamauchi, Makino & Yamashina, 1968) which does not adhere to the sequence Asn-X-Ser/Thr usually found (Marshall & Neuberger, 1970).

In a recent paper, Kabasawa and Hirs (1972) suggest that the nature of residue X in the sequence Asn-X-Ser/Thr must exert an important influence on the configuration of that part of the protein surface recognised by the enzymes responsible for the biosynthesis of the carbohydrate side chain. They further point out that the simpler and more complex heterosaccharide units linked to asparagine in porcine ribonuclease could contain a common core unit made up of α Man(1→3,4)β GlcNAc(1→4)GlcNAc→Asn. In the simpler type of carbohydrate moiety this would be elaborated by the addition of a β-mannosyl residue to the outer of the two N-acetylglucosamines, while in the more complex units it would be elaborated by the addition of an α-mannosyl unit at the inner of the two N-acetylglucosamines (Kabasawa & Hirs, 1972).

1. 2 (4) Microheterogeneity

The structural patterns of the carbohydrate moieties in glycoproteins have been mentioned and the observation that a glycoprotein may have entirely different types of carbohydrate groups attached to its peptide portion has been pointed out. However many glycoproteins have been found to be microheterogeneous in that they contain minor variations in their carbohydrate portion. The ratio of the two constituent sugars, mannose and N-acetylglucosamine, in the single carbohydrate group of ovalbumin varies, even when the ovalbumin is obtained from the eggs of a single hen (Cunningham, Ford & Rainey, 1965; Cunningham, 1968). The heterogeneity would, therefore, seem to have its origins in the biosynthetic process rather than in a genetic phenomenon. The carbohydrate of collagens is a glucosylgalactose disaccharide, however single galactose residues have been reported (Spiro, 1969; Cunningham & Ford, 1968). Studies on human α_2-macroglobulin (Dunn & Spiro, 1967) have shown the presence of 31 heteropolysaccharide units of varying size and composition. From the study of glycopeptides of α_2-macroglobulin, the authors found that the size and composition of the carbohydrate moieties was consistent with the idea of a core of mannose and N-acetylglucosamine residues to which are added sialic acid or fucose, galactose and additional N-acetylglucosamine residues to a varying degree. Kabasawa and Hirs (1972) point out that the observed heterogeneity in glycopeptide fractions originating from the two more complex heteropolysaccharide units of porcine

ribonuclease, may result from the addition of side chains containing N-acetylglucosamine, galactose and N-glycolylneuraminic acid to a common heptasaccharide core consisting of 3 mannose residues, 3 N-acetylglucosamine residues and 1 fucose residue.

In addition to the structural studies above, microheterogeneity may be observed in the intact molecule, in some cases, by electrophoresis or ion exchange chromatography (Reinhold, Dunne, Wriston, Schwarz, Sarda & Hirs, 1968). However charge differences may also arise from differences in amino acid composition such as the replacement of an asparagine residue by an aspartic acid residue, or in a difference in the total basic or acidic amino acid content of species of the molecule.

Although heterogeneity of the carbohydrate portion can be explained on the basis of lack of completion of the carbohydrate moiety, there is the possibility of positional isomerism. In α_1-acid glycoprotein, the sialic acid can be linked to carbon 3,4, or 6 of the galactose (Jeanloz, 1966). It has been proposed that this may explain the polymorphism on starch gel electrophoresis due to the effect of the different linkage positions on the pK values of the sialic acid (Schmid, 1968). Heterogeneity may also be explained by the lack of complete specificity of sugar transferases involved in the synthesis of the carbohydrate portion or by degradation by glycosidases (Gottschalk, 1969).

Chapter 3 Biosynthesis of Glycoproteins

1. 3 (1) General Considerations

Consideration of the biosynthesis of glycoproteins involves, as well as the synthesis of the peptide portion, activation of the monosaccharide residues and their mechanism of assembly to the growing polysaccharide chain. This involves the attachment of the first sugar to the peptide, then elongation of the carbohydrate chain. The sugars must adopt the correct anomeric configuration and the incoming sugar may be linked to, say, one of four or five possible hydroxyl groups. Consideration must also be given to the control of this process, including initiation and termination of chain growth, the intracellular location of the processes and the temporal relationship between the synthesis of the peptide and carbohydrate components of the molecule.

1. 3 (2) Location, Temporal Relationship, and Mechanism of Assembly

It has been found that intracellular proteins are synthesised on free ribosomes, while extracellular proteins are synthesised on membrane bound ribosomes (Birbeck & Mercer, 1961; Redman, Siekevitz & Palade, 1966; Redman, 1969). It is assumed that the peptide portion of glycoproteins is synthesised on ribosomes, coded for by messenger RNA, in the same manner as non-glycosylated proteins. Protein destined for export then passes through the membrane of the endoplasmic reticulum and as it is transported through the spaces of the endoplasmic reticulum to the Golgi apparatus, carbohydrate is added sequentially to the protein under the action of specific

membrane bound sugar transferases. Although most glycoproteins are secreted, not all secreted proteins are glycoproteins, and a discussion of this will follow.

The initial glycosylation, i.e. the attachment of the first sugar to an amino acid side chain, may occur while the protein is still bound to the ribosomes or immediately after its release (Hallinan, Murty & Grant, 1968; Schenkein & Uhr, 1970; Molnar, Robinson & Winzler, 1965; Lawford & Schachter, 1966; Sarcione, Bohne & Leahy, 1964; Sinohara & Sky-Peck, 1965; Molnar & Sy, 1967; Cook, Laico & Eylar, 1965) although this point is not clear at present. Then as the glycoprotein moves through the channels of the endoplasmic reticulum, the sugar portion is built up one sugar unit at a time by the action of specific glycosyltransferases (Ginsburg & Neufeld, 1969). These transferases, which are believed to be attached to the membrane of the endoplasmic reticulum system, have to have specificity for both the donor sugar nucleotide and the acceptor growing glycopeptide. The monosaccharide, its anomeric configuration and the activating base are all specifying factors. In the case of the first attached sugar, the size of the peptide chain and the sequence around the linking amino acid may also offer specificity (Marshall & Neuberger, 1970; McGuire & Roseman, 1967; Hagopian & Eylar, 1969). For the addition of subsequent sugars, the terminal and penultimate sugars as well as their method of linkage may also be specifying factors. As the new sugar-

sugar bond is being formed there is the possibility of linkage to one of four or five hydroxyl groups.

This sequential nature of addition of carbohydrate means that the product of one reaction, becomes the substrate for the next, so that the specificity of the glycosyltransferases determines the type of structural unit synthesised (Roseman, 1970). It is probably not absolutely necessary for the membrane bound transferases to be spatially separated as the specificity of the transferases determines the sequence of the chain.

From studies of subcellular fractionation and autoradiography, it appears that the initial glycosylation and the synthesis of the core portion of plasma glycoproteins occurs on the rough endoplasmic reticulum, while later additions occur in the smooth membranes of the endoplasmic reticulum and in the Golgi apparatus (Schenkein & Uhr, 1970; Schachter, Jabbal, Hudgin, Pinteric, McGuire & Roseman, 1970; Zagury, Uhr, Jamieson & Palade, 1970; Choi, Knopf & Lennox, 1971). Glycosyltransferases involved in the synthesis of glycoproteins are probably located, therefore, on the rough and smooth endoplasmic reticulum as well as on the Golgi complex (Horowitz & Dorfman, 1968) and may be considered to be extracellular, while the sugar nucleotides are synthesised in the cytosol (Winterburn & Phelps, 1971). Polyprenol lipid carriers have been implicated in the translocation of sugar units across the membrane (Tetas, Chao and Molnar, 1970).

The Golgi apparatus is believed to concentrate the glyco-

protein and package it for secretion (Rambourg, Hernandez & Leblond, 1969). Terminal fucose and sialic acid residues are added late, perhaps shortly before secretion (Choi, Knopf & Lennox, 1971). The secretory process probably resembling a reverse of pinocytosis involving a fusion of secretory vesicles from the surface of the Golgi and the plasma membrane (Zagury et al, 1970).

Evidence for the above scheme comes from both in vivo and in vitro studies (Molnar, Robinson & Winzler, 1965; Lawford & Schachter, 1966; Spiro & Spiro, 1966; Sarcione, Bohne & Leahy, 1964). Liver particulate fractions contain glycoproteins thought to be precursors of plasma glycoproteins (Sarcione, Bohne & Leahy, 1964; Simkin & Jamieson, 1967; Li, Li & Shetlar, 1968), and studies on the thyroid showed the presence of a particle-bound precursor of soluble thyroglobulin which could be released by the use of sodium deoxycholate (Spiro & Spiro, 1966). This same study (Spiro & Spiro, 1966) demonstrated that the effect of puromycin was to inhibit the synthesis of the peptide portion of soluble thyroglobulin without inhibition of the carbohydrate moiety, indicating that the peptide part was synthesised first. Puromycin also caused some inhibition of the synthesis of the carbohydrate portion of the particle bound thyroglobulin (presumably through depletion of peptide precursors) with the more peripheral sugars being least affected. From the differential effects of puromycin on the incorporation

of the constituent sugars into the particle bound protein, it was concluded that the sugars were added one at a time in a stepwise fashion to a membrane bound precursor of the soluble protein (Spiro & Spiro, 1966). The effect of puromycin on glycoprotein biosynthesis has been studied in other tissues (Molnar & Sy, 1967; Cook, Laico & Eylar, 1965) and it has similarly been found that peptide synthesis was inhibited while carbohydrate addition could continue. The inhibition of carbohydrate synthesis follows the inhibition of peptide synthesis more rapidly in some tissues than in others, presumably reflecting different pool sizes of precursor material with completed peptide chains but incomplete carbohydrate moiety to which sugars may be added, or to a different rate of utilisation of the available precursors.

Several glycosyltransferases have been found in particulate fractions containing membranes of the endoplasmic reticulum (McGuire, Jourdian, Carlson & Roseman, 1965; Johnston, McGuire, Jourdian & Roseman, 1966; Spiro & Spiro, 1968 b,c). It is not thought that the attachment of N-acetylglucosamine to a specific asparagine residue in the peptide chain is under direct genetic control as no transfer RNA containing N-acetylglucosamine has been found (Sinohara & Sky-Peck, 1965). The complex asparagine-linked heteropolysaccharide unit of thyroglobulin is thought to be built up in a sequential fashion by the transferases found in thyroid (Spiro & Spiro, 1968 b,c) as enzymes have been found

which can synthesise the peripheral sequence of sugars sialic acid-galactose-N-acetylglucosamine.

The disaccharide unit of ovine submaxillary glycoprotein can be synthesised by two transferases found in particulate fractions of the gland. The first transfers N-acetylgalactosamine from UDP-N-acetylgalactosamine to serine or threonine residues from which the carbohydrate has been removed (McGuire & Roseman, 1967), while the second transfers sialic acid from CMP-N-acetylneuraminic acid to N-acetylgalactosamine residues already linked to the protein through serine or threonine residues (Carlson, McGuire, Jourdian & Roseman, 1964).

Several glycosyltransferases have been found in gastric mucosa (Ziderman, Gompertz, Smith & Watkins, 1967; Race, Ziderman & Watkins, 1968; Hearn, Smith & Watkins, 1968) and in milk (Kobata, Grollman & Ginsburg, 1968 a,b; Shen Grollman & Ginsburg, 1968) which are involved in the synthesis of oligosaccharides with blood group activity of the ABH and Lewis systems.

A xylosyltransferase involved in the linkage of xylose to serine in the peptide portion of proteoglycans has been described (Grebner, Hall & Neufeld, 1966; Robinson, Telser & Dorfman, 1966). The addition of the two galactose residues found adjacent to the peptide linked xylose proceeds through the action of two distinct galactosyltransferases which have been found on particles of chick embryo cartilage (Helting & Rodén, 1969).

Enzymes involved in the formation of the galactose-hydroxylysine links of basement membranes and collagens have also been described, as has the enzyme responsible for transferring glucose from UDP-glucose to hydroxylysine-linked galactose (Spiro & Spiro, 1968 a). These enzymes have been isolated from both the soluble and the particulate fractions of tissues.

These studies have led to the idea of glycoprotein biosynthesis outlined above. Also, it has been shown recently (Schauer & Wember, 1971), that although N-acetyl-, N-glycolyl- and N-acetyl-O-acetylneuraminic acid may be transferred from their CMP-glycosides by neuraminic acid transferases onto serine or threonine linked N-acetylgalactosamine residues of bovine submaxillary-gland glycoprotein, hydroxylation or O-acetylation of N-acetylneuraminic acid could also occur after incorporation into the growing glycoprotein. It is thought that the enzymes modifying free and membrane bound N-acetylneuraminic acid and the transferases form multienzyme complexes in the membranes of the endoplasmic reticulum (Schauer & Wember, 1971).

Although this discussion has mostly considered secreted proteins, the membrane proteins of the endoplasmic reticulum, Golgi and plasma membrane are synthesised in a similar manner except that they are incorporated into the membrane rather than released into the cisternae (Roseman, 1970; Rambourg,

Hernandez & Leblond, 1969; Peters, Fleischer & Fleischer, 1971). Also, although most extracellular proteins seem to be synthesised by the route described, collagen is not secreted by the Golgi system, but passes directly to the plasma membrane where the carbohydrate residues are added as it is extruded (Bosmann, 1969).

1. 3 (3) Relationship of Biosynthesis to Heterogeneity

From the outline of the biosynthesis of the carbohydrate portion of glycoproteins described, it can be seen that as the protein moves along the channels of the endoplasmic reticulum there might not always be the chance for a glycosyltransferase to act on the growing carbohydrate chain. As long as the secretory process does not require a completed carbohydrate unit, then molecules in varying degrees of completion would be secreted. The observed microheterogeneity might reflect the speed with which the protein moves along the endoplasmic reticulum, steric hindrance of the peptide chain preventing the approach of a glycosyltransferase or perhaps a transfer site being saturated by another peptide molecule.

A lack of complete specificity of a glycosyltransferase could result in positional isomerism or in the substitution of one sugar for another, and positional isomerism could also be explained by a group of transferases forming linkages to different positions on the same terminal sugar. (Gottschalk, 1969).

1. 3 (4) Control of the Biosynthetic Process

As the biosynthesis of the carbohydrate moiety of glycoproteins

is postribosomal, it is presumably under the influence of enviromental factors such as enzyme specificity and availability of substrates rather than direct genetic control, and carbohydrate attachment may therefore be involved in the regulation of the biosynthesis of glycoproteins.

However, the influence of genetic factors can be seen in the case of the glycosyltransferases involved in the synthesis of oligosaccharides with blood group activity (Ziderman et al, 1967; Race et al, 1968; Hearn et al, 1968; Kobata et al, 1968 a,b; Shen et al, 1968). These studies showed that ABH and Lewis activity depend on the inheritance of specific glycosyltransferases responsible for the synthesis of the carbohydrate moiety of all compounds with blood group activity, such as glycoproteins of the gastric mucosa, oligosaccharides of milk and glycolipids of the red cell membrane.

There are three examples of feedback control involved in the regulation of the biosynthesis of the precursor sugar nucleotides. In rat liver: (1) UDP-N-acetylglucosamine inhibits glutamine-fructose-6-phosphate transaminase, which is the first enzyme in the sequence leading to its own synthesis (Kornfield, Kornfield, Neufeld & O'Brien, 1964), and (2) CMP-N-acetylneuraminic acid inhibits UDP-N-acetylglucosamine-2-epimerase which is involved in the formation of N-acetylmannosamine leading to the synthesis of the sialic acid nucleotides (Kornfield et al, 1964). In cartilage: (3) UDP-xylose inhibits UDP-glucose dehydrogenase which forms UDP-glucuronic acid thus inhibiting

the formation of both the glucuronic acid and xylose nucleotides (Neufeld & Hall, 1965). It is possible therefore that glycoprotein biosynthesis may be regulated by the availability of the sugar nucleotides involved in the synthesis of the carbohydrate portion.

Lactose synthetase which transfers galactose residues to glucose to form lactose is made up of two proteins, the A and B protein (Brew, Vanaman & Hill, 1968). The A protein is the catalyst for the reaction which transfers galactose residues to N-acetylglucosamine to form N-acetyllactosamine. The B protein, which was shown to be α-lactalbumin, inhibits this reaction catalysed by the A protein. Thus α-lactalbumin has altered the substrate specificity of the A protein from N-acetylglucosamine to glucose. It is thought (Brew et al, 1968) that this activity of the A protein is the same as the activity of bovine colostrum in incorporating galactose residues into growing oligosaccharides of glycoproteins which contain a terminal β-N-acetylglucosamine residue (McGuire et al, 1965). It is supposed that during lactation, α-lactalbumin, by modifying the acceptor specificity of the A protein, can divert UDP-galactose from its role in the synthesis of glycoproteins to the production of lactose.

The fact that a large number of possible combinations of monosaccharides could exist, but only a small number are found, suggests that the biosynthetic process is non-random. For example, galactose has not been found linked to mannose but is frequently found linked to N-acetylglucosamine.

Although little information is available on chain termination, sialic acid or fucose at the non-reducing terminus may act as signals for cessation (Gottschalk, 1969).

Chapter 4 Catabolism of Glycoproteins

1. 4 (1) Degradative Enzymes

Enzymes having characteristically low pH optima from the lysosomal fraction of the cell have been found to be capable of degrading the carbohydrate unit of glycoproteins (Aronson & de Duve, 1968; Mahadevan, Dillard & Tappel, 1969). These glycosidases include neuraminidase (Mahadevan, Nduaguba & Tappel, 1967), β-galactosidase (Sellinger, Beaufay, Jacques, Doyen & de Duve, 1960), α-N-acetylgalactosaminidase (Weissman & Friederici, 1966), β-N-acetylglucosaminidase (Sellinger et al, 1960), α-fucosidase (Conchie & Hay, 1963) and α-mannosidase (Sellinger et al, 1960; Conchie & Hay, 1963). A β-mannosidase (Muramatsu & Egami, 1967) and an α-galactosidase (Pazur & Kleppe, 1962) are other glycosidases reported. These enzymes are exoglycosidases and act by releasing the non-reducing terminal sugar.

A β-aspartyl-N-acetylglucosamine amidohydrolase has been described which hydrolyses the protein-carbohydrate linkage involving asparagine and N-acetylglucosamine (Mahadevan & Tappel, 1967; Ohgushi & Yamashina, 1968). The products of this reaction are aspartic acid and 1-amino-N-acetylglucosamine, with this latter product hydrolysing nonenzymatically below pH 7 to ammonia and N-acetylglucosamine (Makino, Kojima, Ohgushi & Yamashina, 1968). The enzyme requires that the amino and carboxyl groups of the asparagine residue be unsubstituted (Makino et al, 1968; Ohgushi & Yamashino, 1968; Tarentino & Maley, 1969). However, this amidohydrolase can release N-acetylglucosamine or a whole

carbohydrate unit, e.g. of ovalbumin (Tarentino & Maley, 1969; Makino et al, 1968), transferrin (Tarentino & Maley, 1969) or ribonuclease B (Plummer, Tarentino & Maley, 1968). It appears, therefore, that degradation of the peptide portion would have to precede hydrolysis of the protein-carbohydrate linkage.

The linkage between N-acetylgalactosamine residues and serine or threonine residues may be split by an α-N-acetylgalactosaminidase (Weissmann & Hinrichsen, 1969). Although with ovine submaxillary mucin this enzyme requires the prior removal of external sialic acid residues, it is preferable if the N-acetylgalactosamine is attached to a large segment of the peptide chain (Bhargava, Buddecke, Werries & Gottschalk, 1966).

The linkage between serine and xylose may be hydrolysed by a β-xylosidase (Fukuda, Muramatsu & Egami, 1969).

These enzymes, which have been found from many sources including mammalian liver and kidney and hen oviduct, along with proteolytic enzymes, such as the cathepsins, seem to be able to completely degrade glycoproteins.

1. 4 (2) Relationship of Catabolism to Microheterogeneity

If proteins can be secreted with incomplete carbohydrate moieties, then as well as this arising from biosynthetic reasons as discussed, it could also arise from removal of one or two sugars from the terminal, non-reducing end, of the carbohydrate under the action of some of the exoglycosidases described.

Chapter 5 Function of the Carbohydrate Moiety of Glycoproteins

1. 5 (1) Relationship of the Addition of Carbohydrate to the Secretion of Glycoproteins

Eylar (1965) proposed that the addition of carbohydrate to protein was a signal for export as he found that, in general, extracellular proteins were glycosylated while intracellular protein had no carbohydrate covalently bound. However it is obvious that not all secreted proteins are glycoproteins, as hen egg white lysozyme and bovine pancreatic trypsinogen and chymotrypsinogen have no carbohydrate moiety. Nonetheless, it is still a useful rule of thumb that while not all secreted proteins are glycoproteins, most glycoproteins are secreted.

Winterburn and Phelps (1972) recently discussed Eylar's hypothesis with a redefinition of intracellular and extracellular space. As well as Eylar's definition of extracellular as that which is outside the plasma membrane, they included material in continuity with the extracellular milieu. This covers the cisternae of the endoplasmic reticulum, the inside of Golgi sacs and vesicles and the interior of lysosomes. It is inherent in the definition that an exported protein crosses one membrane, that of the endoplasmic reticulum. Membrane components themselves were also regarded as extracellular. They concluded that carbohydrate was not added as a signal for export but as a means of determining the extracellular fate of the protein molecule.

Their discussion was based on the pattern of glycosylated and non-glycosylated proteins secreted by a single tissue of an

organism, e.g. hen oviduct or bovine pancreas. Pancreas secretes about 5% of its total protein as glycoproteins while oviduct secretes about 97% as glycoprotein and cow's milk contains about 58% of its total protein as glycoprotein. Also, while only two or three of the human plasma proteins are non-glycoproteins, these make up about half the total by weight (Winterburn & Phelps, 1972).

Three of the six well characterised polypeptide hormones secreted by the anterior pituitary are glycosylated, luteinizing hormone, follicle-stimulating hormone and thyrotrophic hormone (Spiro, 1970), while the other three are not, growth hormone, prolactin and adrenocorticotrophic hormone (Nakane, 1970; Li, Dixon, Lo, Schmidt & Pankov, 1970).

As secreted proteins are made on membrane bound ribosomes, the passport for export can be said to lie in the messenger RNA which discriminates for a membrane bound ribosome, and that the decision for export is taken before the addition of carbohydrate and so does not lie in the addition of carbohydrate (Winterburn & Phelps, 1972).

This of course gives rise to a problem, in that some secreted proteins are not glycosylated, and, if they are synthesised and secreted by the same method as glycoproteins, then why do they have no carbohydrate added? It has been mentioned that the amino acid sequence -Asn-X-Ser/Thr may be a recognition site for glycosylation of asparagine residues (Marshall & Neuberger, 1970). If a secreted protein did not contain this

sequence, then perhaps it would not be glycosylated. However proteins having this sequence, but not glycosylated, have been found in secretions (Sinohara, Asano & Fukui, 1971; Bradshaw, Ericsson & Walsh, 1969; Brew, Castellino, Vanaman & Hill, 1970). It is reported that albumin is secreted by the same cytological route as plasma glycoproteins (Peters et al, 1971) and there is no evidence to suggest that albumin was glycosylated but had the carbohydrate removed prior to secretion, and so this problem seems unanswerable at present. However, it has been shown in the magnum region of hen oviduct that there are three types of tubular gland cell involved in the secretory process (Wyburn, Johnston, Draper & Davidson, 1970). It is proposed that one of these cell types is involved in the secretion of lysozyme (the only major egg white protein not glycosylated), while one of the other types is involved in the secretion of ovalbumin (Wyburn et al, 1970). So it is possible that secreted non-glycoproteins, certainly in some cases, may be synthesised and secreted by different cell lines from those involved in the secretion of glycoproteins, even though they originate from the same tissue.

The converse of Eylar's hypothesis is that intracellular proteins are non-glycosylated, and while this is generally true, there are exceptions, for example, kidney γ-glutamyl transpeptidase (Szewczuk & Connel, 1964), and glycoproteins of the mitochondrial (Martin & Bosmann, 1971) and nuclear (Kashnig & Kasper, 1969) membranes.

It is clear from this that the addition of carbohydrate as a signal for export is certainly not the whole explanation for the glycosylation of proteins.

1. 5 (2) Correlation of Carbohydrate with Function of Glycoproteins

While the mucins and glycosaminoglycans may require the polyelectrolyte character conferred on them by the numerous negatively charged groups (Gottschalk, 1960), the inclusion of carbohydrate does not always have obvious justification in glycoproteins. A particular case being that ribonuclease B is glycosylated and has the same activity as ribonuclease A which is non-glycosylated (Plummer & Hirs, 1963). The removal of sialic acid from the plasma glycoproteins haptoglobin, transferrin, thyroxine-binding globulin (Blumberg & Warren, 1961) and corticosteriod-binding globulin (Muldoon & Westphal, 1967) has no effect on their transporting function, and no effect on the tertiary structure of the polypeptide chain of α_1-acid glycoprotein (Schmid & Kamiyama, 1963) or fetuin (Oshiro and Eylar, 1969). Removal of a substantial amount of carbohydrate from thrombin (Skaug & Christensen, 1971), glucoamylase (Pazur, Knull & Simpson, 1970) or chloroperoxidase (Lee & Hager, 1970) was found not to impair their enzymatic function. While, in contrast, the activities of chorionic gonodatrophin (Goverde, Veenkamp & Homan, 1968; Mori, 1969) and follicle stimulating hormone (Mori, 1969; Gottschalk, Whitten & Graham, 1960) are lost upon removal of the terminal sialic acid residues, and it is proposed that modification

of the carbohydrate portion of hormones abolishes the specific interaction of the hormone with its target organ (Montgomery, 1970; Suttajit, Reichert & Winzler, 1971; Van Hall, Vaitukaitis, Ross, Hickman & Ashwell, 1971). Similarly, the removal of carbohydrate affects the activities of blood group specific glycoproteins (Watkins, 1966). However as it is pointed out (Winterburn & Phelps, 1972), these ascribed functions may not represent the complete role of the protein throughout its life, or the "complete biological function" of the protein.

Plasma proteins, except transferrin, are rapidly removed from the circulation by the liver parenchymal cells if the terminal sialic acid residues are removed (Morell, Gregoriadis, Scheinberg, Hickman & Ashwell, 1971). As this activity could be regulated by the activity of neuraminidases, some specificity could be offered here by the type of terminal residue, and catabolic rate would be influenced by the binding of the desialylated protein to the plasma membrane. An extension of these studies using artificially glycosylated albumin (Rogers & Kornfield, 1971) confirmed the idea that the specificity of the response resides in the oligosaccharide unit.

Winterburn and Phelps (1972) conclude that the significance of the glycosyl residues is to impart a discrete recognitional role on the protein and point out the role of the oligosaccharide unit in cellular recognition phenomena as described by Winzler (1970). The conclusion being that terminal sialic acid masks

an underlying antigenic determinant, such that carbohydrate could be added as a code for determining the extracellular fate of a protein molecule within an organism, either to effect recognition of a protein with its target cell or to mask this code until activation by removal of terminal sialic acid. It has been proposed that intracellular adhesions are a result of interactions between oligosaccharides in the membrane of one cell with glycosyltransferases in the membranes of adjacent cells (Roseman, 1970), and a similar mechanism may operate in the catabolism of plasma glycoproteins if the membrane acceptors are sialyltransferases incorporated into the plasma membrane via the Golgi complex (Winterburn & Phelps, 1972).

Before this hypothesis could be generalised, the situation seen for plasma glycoproteins would need to be shown to apply to other groups of glycoproteins as well, but it may help to explain the relatively small number of carbohydrate structural units found in glycoproteins.

Fibroblasts synthesise and secrete collagen, and it is thought, tropoelastin, and although an early report says that carbohydrate is covalently bound to collagen-protein throughout the phylogenetic scale (Gross, 1963), it now seems that the extrusion of collagen through the plasma membrane does not have an obligatory requirement for the addition of the disaccharide unit, as it has been reported that sawfly silk collagen is not glycosylated (Spiro, Lucas & Rudall, 1971) and a similar case exists for porcine tropoelastin

(Grant, Steven, Jackson & Sandberg, 1971). So although the carbohydrate moiety of collagens may be important to the molecule, it does not seem to be compulsory for its function.

A major difficulty in describing the function of the carbohydrate moiety, therefore, lies in the measurement of biological activity, and although the carbohydrate portion may be shown to be unnecessary for a particular activity, it may be necessary for, say, binding to a cell surface or attachment to a membrane. It seems therefore, that even if the carbohydrate moiety is not involved in the major biological function of the glycoprotein, it may play some other supporting role in determining the overall relationship of the glycoprotein to its enviroment.

Chapter 6 Hen Oviduct, and Egg White Proteins

1. 6 (1) General Introduction

The remainder of the discussion will concern egg white glycoproteins, in particular ovomucoid.

Egg white is essentially a mixture of proteins synthesised by the oviduct and secreted around the yolk. The magnum portion of the oviduct is reponsible for the secretion of the protein component of egg white and was used in biosynthetic studies to be described. The oviduct of a laying hen may be about 70cm in length and up to 60g in weight. The portion encompassing the ovary is the infundibulum and is about 10cm long. This gives way to the main region, the magnum, which is about 30cm long and 30g in weight. This leads to the isthmus region, about 14cm long which gives way to the shell gland or uterus region about 7 cm in length. The final 10cm or so is the vaginal region terminating in the cloaca (Wyburn et al, 1970). The weight and size of the oviduct may vary greatly depending on the season, stage of the egg laying cycle and age of the hen, and perhaps reflects the hormone balance of the hen. It is not proposed to discuss these regions in detail, but merely to say that the isthmus-shell gland region is responsible for laying down the shell, while the infundibulum and magnum are responsible for the secretion of most of the proteins of egg white.

The protein component of egg white may be seen from Table 1.

It can be seen that one protein (lysozyme) is non-glycosylated

Table 1. The protein composition of egg white (after Parkinson, 1966; Wyburn et al, 1970).

Protein	% of egg white solids	% carbohydrate in molecule	Biological Activity
Ovalbumin	54	4	Nutritional ?
Ovotransferrin	13	2	Iron transport
Ovomucoid	11	20	Trypsin inhibition
Lysozyme	3.5	None	Lyses bacteria
Ovomucin	1.5	30	Structural ?
Ovoglyceprotein	1	30	?
Apoflavoprotein	0.8	Low	Riboflavin binding
Ovoinhibitor	0.1	6	Protease inhibitor
Avidin	0.05	2	Biotin binding
Globulins	8	—	-

while the rest of the proteins are glycoproteins. The degree of glycosylation varies as can be seen from the % carbohydrate content and number of carbohydrate units. Also, ovalbumin (Johansen et al, 1961), avidin (DeLange, 1969) and ovotransferrin (Williams, 1968) have asparagine linked carbohydrate units, which consist of the simpler mannose and N-acetylglucosamine type, while ovomucoid (Montgomery & Wu, 1963) has the more complex heteropolysaccharide unit consisting of mannose, N-acetylglucosamine, sialic acid and galactose.

Although an activity has been described for those proteins in which it is known, it is not known if this is the precise and complete biological function of the molecule. Ultimately, of course, egg white secretions are nutritive, but initially they may have some anti-bacterial, structural or buffering role to play.

1. 6 (2) Biosynthetic Studies in Oviduct

Hen oviduct is a tissue which is active in protein synthesis and secretion. A hen may lay an egg a day for about a month before pausing, and may lay 250-300 eggs per year. In a standard egg, about 4g of protein are secreted into egg white in the 3 hours that the egg takes to pass along the 30cm magnum portion of the oviduct (Wyburn et al, 1970). As the magnum weighs about 30g, it can be seen that in a week a hen can secrete an amount of protein into egg white, equal to the weight of the magnum itself.

Anfinsen and Steinberg (1951) demonstrated that hen oviduct had the capacity to synthesise ovalbumin in vitro, and Canfield

and Anfinsen (1963) examined the synthesis of lysozyme in this tissue. Hendler (1956; 1957) extended the studies on ovalbumin and found that on tissue fractionation, the microsome-like material sedimented at centrifugal fields of 600g in 10 minutes as opposed to the much higher fields necessary for other tissues, liver, for example. Carey (1966) confirmed this finding although showing that particulate material could be washed out of the 600g pellet to give RNA rich particulate fractions which sedimented at 10,000g in 10 minutes. The results suggested that the microsomal fraction was involved in the synthesis of egg white proteins despite its behaviour in the centrifuge.

By 1962 although the in vitro synthesis of ovalbumin in oviduct tissue had been described (Anfinsen & Steinberg, 1951; Hendler, 1956; 1957) the synthesis of other egg white proteins had not been demonstrated. To examine the possibility that other egg white proteins may be synthesised elsewhere in the hen's body and then concentrated in the oviduct, Mandeles and Ducay (1962) examined the labelling pattern of egg white proteins after in vitro and in vivo studies. (It is perhaps worth recording here that although ovotransferrin has a different carbohydrate moiety from chicken serum transferrin, the protein component of the species is virtually indistinguishable and probably identical (Williams, 1962; 1968). As serum transferrin is thought to be synthesised in the liver, it might be possible for ovotransferrin to be synthesised in the liver and transported to the oviduct via the serum).

Mandeles and Ducay (1962) showed that injection of [^{14}C]-labelled amino acids into laying hens resulted in eggs with ovalbumin and ovotransferrin (formerly known as conalbumin) similarly labelled, but having a higher specific activity than lysozyme. Injection of prelabelled ovotransferrin into a second hen gave a similar pattern, suggesting that ovotransferrin was not concentrated in the oviduct but was broken down and used for the synthesis of ovalbumin and lysozyme as well as ovotransferrin, making an extra-oviducal site of formation of ovotransferrin unlikely. In vitro studies, with and without hormone addition, showed that [^{14}C] glycine could be incorporated into egg white proteins, but that while the specific activities of ovalbumin and ovotransferrin paralleled each other, the specific activity of ovomucoid was sometimes higher, sometimes lower, and the specific activities of the lysozyme and flavoprotein fractions varied considerably when the oviduct was excised at different times in the egg-laying cycle. The results suggested that while the oviduct was the site of egg white protein formation, (a) the rate of formation of each of the proteins is different (b) the proteins may be formed at cytologically exclusive sites, or (c) formation of some of the egg white proteins may require a hormonal cue.

It has been shown that estrogen causes cellular differentiation of immature chicken oviduct epithelial tissue and induces the synthesis of ovalbumin as well stimulating general protein

synthesis (Kohler, Grimley & O'Malley, 1968; O'Malley, McGuire & Korenman, 1967). Progesterone induces the synthesis of avidin in tissue pretreated with estrogen although general protein synthesis remains unaltered (Korenman & O'Malley, 1968). It was concluded from these studies that ovalbumin and avidin were cell specific proteins synthesised by different epithelial cells in oviduct mucosa. The electron microscopic observations of Wyburn et al (1970), suggested that different tubular gland cells were involved in the secretory process of ovalbumin and lysozyme, and so egg white proteins may be synthesised and secreted by different and specific cells. It was further concluded that the selective induction of ovalbumin by estrogen and avidin by progesterone may be through stimulation of specific target cells by these hormones (Kohler et al, 1968).

The effects of progesterone (Korenman & O'Malley, 1968) were confirmed by other workers (Oka & Schimke, 1969), but in both these studies massive amounts of hormone was used. Using lower doses of progesterone, it was shown that progesterone could enhance estrogen induced growth of oviduct and it was concluded that the effect of progesterone was not through increased protein synthesis, but through decreased protein catabolism (Muller, Cox & Carey, 1970).

The above results suggest that hen oviduct is under the control of at least an estrogen and a progesterone, and the synthesis of egg white proteins show a differential response to

these hormones. But, the concentration and proportion of the hormones are probably extremely important, and great care must be taken in interpreting data on the effects of hormones.

Hen oviduct has been shown to be the site of egg white protein formation (Anfinsen & Steinberg, 1951; Canfield & Anfinsen, 1963; Hendler, 1956; 1957; Mandeles & Ducay, 1962; Carey, 1966) and may be used in vitro to study the biosynthesis of egg white proteins. The pattern of proteins from in vivo and in vitro studies is the same on electrophoresis in sodium dodecyl sulphate acrylamide gels (Palmiter, Oka & Schimke, 1971) and the elution profile of proteins from homogenised oviduct and egg white is similar on diethyl-aminoethyl-cellulose chromatography (Mandeles & Ducay, 1962). Hen oviduct may confidently be used, therefore, to examine in vitro biosynthesis of egg white proteins, and was chosen to follow the biosynthesis of ovomucoid and the possible relationship of biosynthesis to the observed microheterogeneity of ovomucoid in studies to be described.

Chapter 7 OVOMUCOID

1. 7 (1) Isolation of Ovomucoid

The presence in egg white of a substance which was not coagulated by heat was recognised in 1890 (Neumeister, 1890). This was subsequently identified as a glycoprotein and named ovomucoid (Mörner, 1894).

Early methods of preparation involved the heat coagulation of other egg white proteins followed by precipitation of ovomucoid with ammonium sulphate or acetone (Longsworth, Cannan & MacInnes, 1940). Other methods have involved the precipitation of other proteins with trichloroacetic acid and precipitation of ovomucoid with acetone at pH 3.5 (Lineweaver & Murray, 1947). Fredericq and Deutsch (1949) used ethanol to precipitate ovomucoid after a preliminary precipitation of other egg white proteins with trichloroacetic acid at pH 3.5.

Carboxymethyl-cellulose ion exchangers have been used in the purification of ovomucoid (Rhodes, Azari & Feeney, 1958; Rhodes, Bennett & Feeney, 1960), and recently a batch technique has been described using an anion and a cation exchanger in which contaminants are absorbed onto ion-exchange resins, but ovomucoid excluded by washing at a pH approximating to the isoelectric point of ovomucoid (Davis, Mapes & Donovan, 1971).

1. 7 (2) Biological Activity of Ovomucoid

The association of the trypsin inhibiting activity of egg white (Delezenne & Pozerski, 1903) with ovomucoid was made by

Lineweaver and Murray (1947). However an additional protein has been found in egg white which is an inhibitor of both trypsin and chymotrypsin (Matsushima, 1958), and probably other proteases, but is present in much lower proportions.

There has been some doubt as to whether the inhibition of trypsin by ovomucoid is competitive or non-competitive. From chemical modification studies and kinetic evidence, Fraenkel-Conrat, Bean and Lineweaver (1949) concluded that ovomucoid did not react with the active centre of trypsin. Green (1953) using the model substrate benzoyl-L-arginine ethyl ester showed that inhibitor could displace substrate and vice versa, and therefore that the inhibition was competitive. The difficulty in demonstrating competitive inhibition when large molecular weight compounds are used to determine trypsin activity (Fraenkel-Conrat et al, 1949), is that the dissociation constant of the enzyme-inhibitor complex is very much lower than the Michaelis constant of the enzyme. The inhibition seems to be in a 1:1 Molar basis.

1. 7 (3) Microheterogeneity

Longsworth et al (1940) found that ovomucoid gave a single component on moving boundary electrophoresis, and calculated the isoelectric point to be 4.3. Heterogeneity was suggested by reversible boundary spreading. Fredericq and Deutsch (1949) showed that ovomucoid has an isoelectric point of 3.9, but that this shifts to 4.2 on heating for one hour at 100°C. Heterogeneity was observed in low ionic strength buffer (0.01M) at a pH near

the isoelectric point (pH 4.5). Bier, Terminiello, Duke, Gibbs & Nord (1953) demonstrated the existance of five species with isoelectric points of 4.41, 4.28, 4.17, 4.01, and 3.83. All had the same activity against trypsin and it was concluded that they were all ovomucoid species with the same biological activity. Rhodes et al (1960) have reported three ovomucoid species isolated by stepwise gradient elution from carboxymethyl-cellulose. Other workers (Chatterjee & Montgomery, 1962) isolated two fractions and when the major one was rechromatographed it gave two components again with the same elution volumes. On electrophoresis at pH 4.6, ionic strength 0.01, material from the major peak showed four components. As the sialic acid content was less than one residue per mole of ovomucoid, it was concluded that there was a mixture of components, some containing sialic acid and some not.

As it has been reported that ovomucoid may have as many as four residues of sialic acid per mole of protein (Rhodes et al, 1960), it is possible to have five molecular species of ovomucoid differing only in having from zero up to four sialic acid residues per mole of protein (Chatterjee & Montgomery, 1962).

On starch gel electrophoresis at pH 4.65, ionic strength 0.015, Melamed (1967) found three components. The two major components had approximately zero, and 0.4 moles sialic acid per mole of protein. The sialic acid containing species separated into two components after neuraminidase treatment. These two components had similar mobilities to the original two major species,

and Melamed concluded that there is a charge difference between the ovomucoid species which resides in some residue other than sialic acid.

Beeley (1971a) reports the isolation of three major and two minor species of ovomucoid by chromatography on sulphoethyl-Sephadex and reports variation in sialic acid, galactose and N-acetylglucosamine content, while the amino acid content and trypsin-inhibiting activity were virtually identical for all the species.

Although these numerous cases of microheterogeneity have been reported, ovomucoid appears homogenous in the ultracentrifuge. For a summary of physical constants see Melamed (1966) and Davis et al (1971).

1. 7 (4) The Peptide Portion of Ovomucoid

The molecular weight of ovomucoid has been reported to be 27,000-28,000 (for a review see Melamed, 1966) and tables of amino acid composition have been reported (Davis et al, 1971; Beeley 1971a). Ovomucoid contains all the usual amino acids except tryptophan and cysteine (but has 8 cystines).

No sequence studies have been carried out on the peptide portion, but the N-terminus is reported to be alanine (Fraenkel-Conrat & Porter, 1952) and the C-terminus phenylalanine (Pénasse, Jutisz, Fromageot & Fraenkel-Conrat, 1952).

1. 7 (5) Carbohydrate Portion of Ovomucoid

Levene and Mori (1929) found mannose and glucosamine in

ovomucoid and Sorensen (1934) also detected galactose. The carbohydrate content was found to be about 20-25%. Early structural studies suggested glucosamine; mannose; galactose in a ratio of 7:3:1 (Stacey & Woolley, 1940; 1942). More recently sialic acid has been found, but this varies with the method of isolation, however only the N-acetyl derivative has been identified (Feeney, Rhodes & Anderson, 1960) and falls in the range 0.4% to 4%.

Methylation and periodate oxidation studies suggested a branched structure with a core of mannose and N-acetylglucosamine to which short chains of mannose and glucosamine were attached, with sialic acid probably protecting galactose residues (Bragg & Hough, 1961; Chatterjee & Montgomery, 1962).

Partial acid hydrolysis suggested that galactose was either terminal, non-reducing, or linked to a terminal sialic acid (Montreuil & Chosson, 1962).

Montgomery and Wu (1963) suggested three similar carbohydrate moieties attached to Asx residues and gave ratios of 8:4:1 for glucosamine: mannose: galactose, with sialic acid in one glycopeptide fraction in the ratio of 1:8 sialic acid: glucosamine, although recovery of sialic acid was not 100%.

Ovoglycoprotein (Ketterer, 1965) which has similar solubility properties to ovomucoid, is a likely contaminant of these preparations and care must be taken in interpreting the results of carbohydrate assays. The highest sialic acid content reported

for an ovomucoid preparation is 4% (Rhodes et al, 1960) while ovoglycoprotein is reported to contain 3% sialic acid (Ketterer, 1965).

The most commonly used method of preparation of ovomucoid in the above structural studies has been that of Lineweaver and Murray (1947), and this preparation may contain up to 20% of other egg white proteins as impurities (Davis et al, 1971). Beeley 1971a) reports values for the carbohydrate composition of ovomucoid isolated by the method of Fredericq and Deutsch (1949) further purified by chromatography on sulphoethyl-Sephadex. This chromatography fractionates ovomucoid into species with varying carbohydrate content. The carbohydrate analysis showed that sialic acid was present in a range of 0-2 moles/mole of protein, galactose in the range 1-6 residues/molecule, mannose 10-13 residues/molecule and glucosamine in the range 14-27 residues/molecule. As the concentration of ovoglycoprotein was found to be of the order of 1-2%, Beeley (1971a) concluded that this was too low to account for the heterogeneity of the carbohydrate moiety of ovomucoid.

1. 7 (6) The Protein-Carbohydrate Linkage of Ovomucoid

Hartley and Jevons (1962) suggested that serine or threonine residues were involved in the protein-carbohydrate linkage of ovomucoid from studies on reducing power. Beeley and Jevons (1963) followed up these studies and cast some doubt on the authenticity of the serine/threonine links. Montgomery and Wu

(1963) proposed three asparagine linked carbohydrate units as they found glycopeptide fractions containing carbohydrate linked to asparagine residues after proteolytic digestion. The linkage would therefore be N-glycosidic, involving asparagine and N-acetylglucosamine.

1. 7 (7) Ovomucoid; Reason for Study

Ovomucoid was chosen for study as it has a fairly well defined structure, and is a major component of egg white, about 11% of the total protein. It has a high carbohydrate content, about 25% by weight of the molecule and can be obtained easily from egg white, isolated with a high degree of purity as will be described. Ovomucoid has the frequently observed asparagine linked carbohydrate moiety with the more complex heteropolysaccharide unit, has more than one carbohydrate unit per molecule and is, in common with many glycoproteins, microheterogeneous. It seemed to be a suitable model compound for studying glycoprotein biosynthesis and for examining the possible connection between biosynthesis and the observed heterogeneity of glycoproteins.

PART 2

MATERIALS AND METHODS

Chapter 8 Materials

Unless otherwise stated reagents were of Analar grade and used without further purification.

2. 8 (1) Chromatographic Media

Sephadex G-series and sulphoethyl (SE)-Sephadex C-50 were obtained from Pharmacia, Uppsala, Sweden. All chromatography columns and accessories and Blue Dextran 2000B were also from Pharmacia. Whatman carboxymethyl cellulose was obtained from W. & R. Balston Ltd., England as were all chromatography and filter papers. Dowex 1 and Dowex 50 were purchased from Sigma Chemical Company, St. Louis, Missouri, U.S.A.

2. 8 (2) Proteins

Trypsin, twice crystallised salt-free and lyophilised was purchased from Koch-Light, Colnbrook, Bucks., U.K. Chymotrypsin, three times crystallised and salt free was obtained from Seravac, Maidenhead, Bercks., U.K. Cytochrome C, ribonuclease A, lysozyme, haemoglobin, subtilisin, pepsin, ovalbumin and bovine serum albumin were from Sigma Chemical Company, St. Louis, Missouri, U.S.A. Conalbumin, six times crystallised, lyophilised, salt-free and iron-free was obtained from Serva, Heidelberg, Germany. Ovoglycoprotein was prepared by the method of Ketterer (1965).

2. 8 (3) Sugars

D-glucose, D-mannose, D-galactose, N-acetyl-D-glucosamine, D-glucosamine . HCl . and N-acetylneuraminic acid (sialic acid) were obtained from Koch-Light, Colnbrook, Bucks., U.K.

2. 8 (4) Amino Acids

All amino acids were of the L-configuration and obtained from British Drug Houses, Poole, Dorset, U.K.

2. 8 (5) Radiochemicals

L- [4, 5-^{3}H] lysine. HCl (250mCi/mmol),

L- [^{14}C] lysine. HCl (12mCi/mmol),

D- [1-^{14}C] glucosamine. HCl (3.0mCi/mmol),

D- [1-^{14}C] galactose (3.0mCi/mmol) and

D- [1-^{14}C] mannose (27.5mCi/mmol)

were all obtained from the Radiochemical Centre, Amersham, Bucks., U.K.

2. 8 (6) Measurement of pH

Determination of pH was carried out on a titrator TTT1C (Radiometer, Copenhagan, Denmark) which had been calibrated at 20°C with standard buffer solution tablets (Soloid brand) pH 4.00, pH 7.0, pH 9.20 (Burroughs Wellcome & Co., London, England).

2. 8 (7) Spectrophotometer

Absorbance was measured on a DB spectrophotometer (Beckman Instruments Ltd., Glenrothes, Fife, Scotland).

2. 8 (8) Dialysis tubing

Visking tubing was obtained from the Scientific Instrument Centre Ltd., London, England, and was boiled three times in distilled water before use.

2. 9 (1) Preparation of G-200-trypsin and G-200-chymotrypsin

Sephadex G-200 was coupled to chymotrypsin by the method of Axén, Porath and Ernback (1967). Insolubilised trypsin was prepared by a slight modification of this procedure. Sephadex G-200 (medium) 10g was allowed to swell in water for 5 days. After decantation, the suspension (800ml) was brought to pH 11 with 1.5M NaOH. Cyanogen bromide (Koch-Light, Colnbrook, Bucks., U.K.), 400ml of a 25mg/ml aqueous solution was added, in a fume cupboard, to the stirred solution which was maintained at pH 11-11.5 by continuous addition of NaOH for 6 minutes. The activated Sephadex was washed on a filter with cold water and cold 0.1M $NaHCO_3$ and then suspended in 200ml of 0.1M $NaHCO_3$ containing 0.05M benzamidine hydrochloride (Ralph Emanuel, Ltd., Wembley, Middlesex, U.K.). Trypsin (1.5g) was added and the suspension stirred for 16 hours at 4°C.

The product was washed 5 times on a centrifuge at 4°C with 5 volumes each of 0.1M $NaHCO_3$, 0.5M NaCl and HCl, pH 1.5. After packing the G-200-trypsin into a column (internal diameter 2.5cm) it was washed overnight with 500ml of 0.5M NaCl adjusted to pH 3 with HCl. The insolubilised trypsin was then washed with 1mM HCl and stored at 4°C under toluene.

2. 9 (2) Enzyme Assays

Proteolytic activity was measured with casein as substrate by a modification of the method of Northrop, Kunitz and Herriott (1948). Samples of insolubilised enzyme in 1ml mM HCl were preincubated for 5 minutes on a rapidly shaking (120 times per minute) water bath at 35°C in conical flasks (25ml). The substrate 1% (w/v) Hammarsten casein

(British Drug Houses, Poole, Dorset, U.K.) in 0.1M sodium phosphate buffer, pH 7.8, was also preincubated at 35°C and the reaction started by the addition of substrate (3ml) to the shaken enzyme solution. After 20 minutes the reaction was stopped by the addition of 5% (w/v) trichloroacetic acid (3ml) and the mixtures transferred to centrifuge tubes. After 1 hour at room temperature all samples were centrifuged at 2500g for 10 minutes. The absorbance of the supernatants was measured at 280nm. A calibration curve was obtained from standards incubated at the same time as unknown samples. Blanks with enzyme or inhibitor added after trichloroacetic acid precipitation were included in all experiments.

To measure trypsin inhibition, insolubilised enzyme and inhibitor were preincubated for 5 minutes in 1ml of 0.1M sodium phosphate, pH 7.8, before addition of casein and assayed as described above. The inhibition of soluble trypsin by ovomucoid preparations was determined similarly.

Esterase activity was determined in 3ml of 0.01M benzoyl-L-arginine ethyl ester (British Drug Houses, Poole, Dorset, U.K.), 0.01M Tris, 0.01M KCl and 0.05M $CaCl_2$, pH 8.0 or pH 9.5 at 25°C. The enzyme was added to the substrate after temperature equilibration and hydrolysis was monitored with a titrator TTT1C coupled to a titrigraph SBR2c (Radiometer, Copenhagan, Denmark) using 0.1M NaOH as titrant.

To measure inhibition of the esterase activity of trypsin, enzyme and ovomucoid were mixed at 4°C before addition to the substrate and assayed as described above.

2. 9 (3) Preparation of "Crude Ovomucoid"

Eggs were obtained from a flock of White Leghorn hens maintained in an animal house. Ovomucoid was isolated from egg white as described by Fredericq and Deutsch (1949) up to the stage of the first ethanol precipitation. This involved a preliminary precipitation of other components with 5% (w/v) trichloroacetic acid at pH 3.5 then precipitation of ovomucoid by adjusting the pH of the trichloroacetic acid supernatant to pH 6 and adding two volumes of ethanol. The ethanol precipitate was then suspended in water and dialysed against water brought to pH 4.6 with acetic acid, with several changes of distilled water over two days. After centrifugation (4000g for 30 minutes) the supernatant was lyophilised to give a "crude ovomucoid" preparation.

2. 9 (4) Batch Isolation of Ovomucoid

Sephadex G-200-trypsin was used to isolate ovomucoid from egg white or oviduct homogenates with or without a preliminary trichloroacetic acid precipitation at pH 3.5.

Before use the insoluble trypsin was washed on a centrifuge successively with HCl, pH 1.5; HCl, pH 3; 0.5M NaCl; water and 0.1M sodium phosphate buffer, pH 7. All solutions were kept on ice and centrifugation was at 600g for 10 minutes at 4°C. Samples from which ovomucoid was to be isolated were adjusted to pH 7 and mixed with an equal volume (settled by centrifugation) of insoluble trypsin. Contaminants were removed by washing successively with 5 volumes of 0.1M sodium phosphate, pH 7 (3 times), 0.5M NaCl (3 times) and water (3 times) in the centrifuge as above. All these washes were discarded, and the

ovomucoid isolated by washing with 2 volumes of HCl, pH 1.5 (2 times), adjusting the pooled acid washes to pH 4.6 with NaOH, dialysing for two days against several changes of distilled water at 4°C and lyophilising. At least 1mg of ovomucoid could be isolated per 1ml (settled volume) of G-200-trypsin.

When the preliminary trichloroacetic acid precipitation step was included, this was carried out at a final concentration of 5% (w/v) trichloroacetic acid at pH 3.5 and 4°C. After 2 hours at 4°C, the precipitate was removed by centrifugation at 4000g for 30 minutes. Sodium phosphate buffer (1M, pH 7) was added to the supernatant to give a final phosphate concentration of 0.1M and ovomucoid was isolated by adsorbtion on and elution from G-200-trypsin as above.

2. 9 (5) Sulphoethyl-Sephadex Chromatography

Ovomucoid was further purified and fractionated by column chromatography on sulphoethyl (SE)-Sephadex C-50. The SE-Sephadex C-50 was allowed to swell in water for 3 days. Before use the chromatographic medium was washed on a Büchner filter funnel under vacuum, using acid hardened filter paper No. 52 (Whatman), with 0.5N HCl then with water until the effluent was above pH 4. The SE-Sephadex C-50 was then washed with 0.5N NaOH followed by water until the effluent was below pH 8. Finally the chromatographic medium was washed with the eluting buffer, 0.014M sodium acetate containing 1mM sodium azide (Koch-Light, Colnbrook, Bucks., U.K.) adjusted to pH 4.85 with acetic acid, until the effluent had the same pH as the buffer solution. The SE-Sephadex C-50 was then added to more eluting buffer, the fines poured off and after degassing

the slurry, it was poured into a column (82cm x 5cm).

The columns were washed at a flow rate of 40ml/hour produced by a peristaltic pump (LKB, Stockholm-Bromma, Sweden) with a further 1 litre of buffer before application of sample. Ovomucoid was either dissolved directly in the eluting buffer (1g in 10ml) or dissolved in water and dialysed overnight against the buffer. After sample application, fractions (15ml) were collected and the extinction at 280nm (E_{280}) read. Samples corresponding to the fractionated ovomucoid species were rechromatographed in the same system.

In this chromatographic system (pH 4.85) there was considerable broadening of the later emerging peaks which limited the usefulness of the method. It was found to be more suitable to separate the sialic acid free ovomucoid variant from the sialic acid containing variants by chromatography in a similar system to that described above, except that columns were equilibrated and samples eluted with buffer at pH 4.90 instead of pH 4.85. Resolution of the sialic acid containing ovomucoid variants could then be achieved by a separate chromatographic step at pH 4.85 (Beeley, 1971a).

The eluting buffers, pH 4.85 and pH 4.90, were made up in batches of 10 litres to ensure that several chromatography runs could be carried out with identical buffer.

2. 9 (6) Carboxymethyl Cellulose Chromatography

Ovomucoid was isolated from egg white by the carboxymethyl cellulose chromatographic method of Rhodes, Azari and Feeney (1958) using carboxymethyl cellulose, CM 23.

2. 9 (7) Protein Determination

Protein concentration of ovomucoid preparations was determined by the method of Lowry, Rosebrough, Farr and Randall (1951) using ovomucoid of known moisture content as reference standard. Moisture content was determined by drying to constant weight at 110°C. A stock solution of ovomucoid (20mg/ml) was made up on this basis and stored at -10°C. Each time a protein determination was made a complete set of blanks and standards were run and the concentration of the stock solution was checked by measuring its extinction at 280nm ($E^{1\%}_{1cm}$ (280nm) = 4, Deutsch & Morton, 1961).

The reagents 2% (w/v) Na_2CO_3 in 0.1N NaOH, 2% (w/v) sodium potassium tartrate and 1% (w/v) $CuSO_4.5H_2O$ (all from British Drug Houses, Poole, Dorset, U.K.) were made up and mixed in the ratio of 100 : 1 : 1 in that order. Folin and Ciocalteu's phenol reagent (British Drug Houses, Poole, Dorset, U.K.) was titrated to pH 7.0, determined on a pH meter, with NaOH. On this basis the reagent was diluted with water to make it 1N with respect to acid (approximately a 2-fold dilution was required).

Samples and standards were in a volume of 1ml, or 0.4ml if less protein was present. The extinction was read at 750nm, or 500nm if the readings at 750nm were too high (Lowry et al, 1951). Ovomucoid concentration could be determined in the range 0.01 - 1mg/ml.

2. 9 (8) Carbohydrate Assays

Glucosamine was determined by the acetylacetone method of Elson and Morgan (1933) using the modification of Boas (1953) or the method of Cessi and Piliego (1960). The acetylacetone (Koch-Light, Colnbrook,

Bucks., U.K.) was redistilled before use and the p-dimethylamino-benzaldehyde (Koch-Light, Colnbrook, Bucks., U.K.) was recrystallised by adding a concentrated alcoholic solution to 5 volumes of water. Optimal hydrolysis conditions for the determination of glucosamine from ovomucoid were found to be 4N HCl for 3 hours at 100°C.

Sialic acid was measured by the thiobarbituric acid assay of Aminoff (1961) or by the resorcinol method of Svennerholm (1958). Sialic acid was released from the glycoprotein by hydrolysis in 0.1N H_2SO_4 for 1 hour at 80°C prior to the thiobarbituric acid assay, prior removal of sialic acid was not necessary in the resorcinol assay method.

Total hexose was determined by the orcinol method (Hartley & Jevons, 1962) or by the anthrone method (Roe, 1955) using mannose as a standard. Orcinol (British Drug Houses, Poole, Dorset, U.K.) was recrystallised from benzene before use.

2. 9 (9) Immunochemical Experiments.

Rabbit antisera to crude ovomucoid were obtained as described by Beeley (1971a), and were found to give precipitates with ovomucoid, ovalbumin and ovoglycoprotein. Antiserum to whole egg white and anti-serum to ovalbumin were obtained from Antibodies Inc., Davis, California, U.S.A.

Double diffusion (Ouchterlony, 1949) was carried out in plastic Petri dishes with 1% (w/v) special agar-noble (Difco Laboratories, Detroit, Michigan, U.S.A.) containing 10mg/100ml merthiolate (Eli Lilley and Co. Ltd., U.S.A.) in 0.9% (w/v) NaCl-0.01M sodium phosphate buffer, pH 7.5. Antiserum was placed in the centre well and the outer wells

were charged with protein solutions at a concentration of 2% (w/v). Diffusion was allowed to go on overnight at 30°C in a humid atmosphere. The gels were then rinsed with 0.9% (w/v) NaCl and photographed.

Immunoelectrophoresis at pH 7.5 was carried out on glass slides (7.5cm x 2.5cm) coated with 1% (w/v) agar containing 10mg/100ml merthiolate in 0.9% (w/v) NaCl-0.01M sodium phosphate, pH 7.5 at 8 volts/cm for 2 hours at room temperature. After electrophoresis troughs were cut out, charged with antiserum and immunodiffusion was carried out overnight at 30°C in a humid atmosphere.

Immunoelectrophoresis at pH 4.9 was carried out similarly except that the agar was made up in 0.01M sodium acetate buffer with the 0.9% (w/v) NaCl omitted. After electrophoresis troughs were cut out and charged with 0.1M sodium phosphate, pH 7.5, which was allowed to diffuse into the agar for 1 hour at room temperature. The phosphate buffer was then removed, antiserum added to the troughs and immunodiffusion carried out overnight at 30°C in a moist atmosphere.

2. 9 (10) Isoelectric Focusing

Purification of ovomucoid was monitored by isoelectric focusing on flat beds of polyacrylamide gel.

Gels were prepared by mixing 2ml 0.8% (v/v) N, N, N', N'-tetramethylethylenediamine (Koch-Light, Colnbrook, Bucks, U.K.), 4ml 28% (w/v) acrylamide (Eastman Organic Chemicals Ltd., Rochester, New York, U.S.A.) containing 1.47% (w/v) N'-N'-methylenebisacrylamide (British Drug Houses, Poole, Dorset, U.K.), 0.4ml 40% (w/v) Ampholine either pH range 3-10 or pH range 3-6 (LKB, Stockholm-Bromma, Sweden), 2ml 0.004% (w/v) riboflavin

(British Drug Houses, Poole, Dorset, U.K.) and 8.6ml water. This mixture was inserted into the space formed between a perspex mould, 130mm x 120mm x 1mm, and a glass plate. The gel was polymerised under the action of daylight. After removing the mould the gel, which adhered to the glass plate, was wrapped in a polythene sheet and equilibrated for 5 hours in a cold room ($4^{o}C$) before use. The gel was placed horizontally in an electrophoresis tank (Shandon Scientific Co. Ltd., London, England) after removing the polythene sheet. The buffer compartments contained 0.5% (v/v) aqueous phosphoric acid (British Drug Houses, Poole, Dorset, U.K.) at the anode and 0.5% (v/v) aqueous ethanolamine (British Drug Houses, Poole, Dorset, U.K.) at the cathode. Continuity of current was made by paper wicks which overlapped the gel by 5mm at each end. Samples of protein (0.1mg - 0.5mg) were applied to pieces of Whatman No.1 paper (5mm x 10mm) and placed on the surface of the gel, approximately $\frac{1}{3}$ of the way along the gel from the cathode, with the 10mm edge parallel to the wicks. Up to 5 samples could be run per gel in this manner. A plastic strip, 130mm x 10mm x 1mm, was placed on top of each wick where it made contact with the gel and a glass plate 130mm x 130mm was placed on top of these strips. Electrolysis was carried out for 16 hours at 100 volts and then for 3 hours at 300 volts with a constant voltage power supply delivered by a Vokam power pack (Shandon Scientific Co. Ltd., London, England). The initial current of 3mA fell to less than 1mA finally. The electrolysis was carried out at $4^{o}C$.

After electrolysis the gel was fixed and Ampholine removed by soaking in a solution containing 5% (w/v) trichloroacetic acid in 50% (v/v) aqueous

ethanol over a period of two days with one change of fixing solution. The gels were then stained for 30 minutes with 0.1% (w/v) amido black (G.T. Gurr Ltd., London, England) in 50% (v/v) aqueous ethanol, and destained by soaking in several changes of a solution containing 10% (v/v) acetic acid in 50% (v/v) aqueous ethanol. Ovomucoid is soluble in trichloroacetic acid if the ethanol is omitted. The gels shrink to half their original size following ethanol treatment.

When it was desired to elute ovomucoid samples from the gel a more rapid staining procedure was used. In this case the segments of gel containing the samples to be eluted were cut out and kept at 4^{o}C while a reference standard was stained as follows. The gel was immersed for 1 hour in a solution containing 4% (v/v) 2-mercaptoethanol (Koch-Light, Colnbrook, Bucks., U.K.) and 50% (v/v) ethanol in 0.1M tris pH 8.5. The gel was then transferred to a solution containing 10% (v/v) acetic acid in 50% (v/v) aqueous ethanol for a further 1 hour. The gel was then stained in a solution containing 0.05% (w/v) bromophenol blue (British Drug Houses, Poole, Dorset, U.K.) and 1% (w/v) mercuric chloride (British Drug Houses, Poole, Dorset, U.K.) in 2% (v/v) aqueous acetic acid for 10 minutes (Bailey, 1962) and destained with 7.5% (v/v) acetic acid. When the gel had regained its original size (30 minutes) ovomucoid samples from an unstained segment of the gel were cut out by reference to this stained standard and the ovomucoid species allowed to diffuse out of the gel into 1ml distilled water or 1ml 0.1N acetic acid overnight.

In some cases the segments of gel containing the ovomucoid species were placed directly onto a second gel and refocused under the same con-

ditions described above.

The pH gradient established by the ampholytes was checked by removing portions of the gel at 5 or 10mm intervals along the gel prior to fixation. The pieces of gel were either removed with a cork borer or strips of gel 10mm x 5mm were taken. The pH at each part of the gel was then checked by laying the gel portions directly onto narrow range indicator paper (British Drug Houses, Poole, Dorset, U.K.) or by immersing the gel portions in 1ml of distilled water for 4 or 24 hours and then measuring the pH as described earlier. All the methods of pH determination gave similar results. A graph was then drawn of pH versus distance along the gel (from either electrode). By measuring the position of the ovomucoid variants (from either electrode) the isoelectric points of the ovomucoid species were determined by reference to this graph.

2. 9 (11) Separation of Ovomucoid from Carrier Ampholytes

A mixture of ovomucoid and ampholytes could be completely separated by gel filtration on Sephadex G-50 by a modification of the method of Vesterberg (1969) for the separation of proteins and ampholytes.

Three samples were prepared by mixing (1) ovomucoid 20mg/ml (0.2ml) and 0.15N acetic acid (0.4ml), (2) Ampholine 40% (w/v), pH 3-10 (0.2ml) and 0.15N acetic acid (0.4ml) and (3) ovomucoid 20mg/ml (0.2ml), Ampholine 40% (w/v), pH 3-10 (0.2ml) and 0.3N acetic acid (0.2ml). The samples were applied individually to a column (30cm x 1.5cm) of Sephadex G-50 and eluted with 0.1N acetic acid. Fractions (1ml) were collected at a flow rate of 30ml/hour at room temperature. It was found that Ampholine gives a deep blue colour in the protein determination method of Lowry, Rosebrough,

Farr and Randall (1951) and so the column effluents were monitored by this method.

By running ovomucoid and Ampholine on their own their respective elution volumes could be determined and when a mixture of ovomucoid and Ampholine was applied to the column it was found that they could be completely separated. The separation of ovomucoid and Ampholine was not complete on columns (30cm x 1.5cm) of Sephadex G-25 nor on shorter columns of Sephadex G-50. Dialysis was also an ineffective separation method as ampholytes remained inside the dialysis sac even after extensive dialysis. Although it was possible to precipitate ovomucoid with acetone, the gel filtration method gave a quick and complete separation of ovomucoid from ampholytes. This also meant that protein content of ovomucoid separated from Ampholine could confidently be determined by the method of Lowry et al (1951) without the possibility of ampholytes contributing to the colour yield.

2. 9 (12) Sodium Dodecyl Sulphate Gel Electrophoresis

Sodium dodecyl sulphate (SDS) was obtained from British Drug Houses, Poole, Dorset, U.K., all other chemicals were from sources already indicated.

The molecular weight of ovomucoid was determined by polyacrylamide gel electrophoresis in the presence of SDS as described by Weber and Osborn (1969). However their staining and destaining procedure was modified as ovomucoid is soluble in acetic acid and washes out of the gel.

After electrophoresis the length of the gel and the distance moved by the marker dye (bromophenol blue) were measured (Weber & Osborn, 1969).

The gels were then placed upright in tubes containing 0.1% (w/v) amido black in 50% (v/v) aqueous methanol and stained for 2 hours. The staining solution was then poured off and the gels destained by soaking in several changes of a solution containing 7.5% (v/v) acetic acid in 50% (v/v) aqueous methanol. The length of the gel and the position of the protein bands were determined by scanning with a densitometer UFD 100 (Vitatron Instruments Ltd., Dieren, Netherlands) and the mobilities of the proteins calculated (Weber & Osborn, 1969). The mobilities of standard proteins were plotted against the logarithm of their known molecular weights and the molecular weight of ovomucoid determined from its mobility using this standard curve (Weber & Osborn, 1969).

Ovomucoid was prepared from egg white or oviduct homogenates by the method of Fredericq and Doutsch (1949) further purified and fractionated by chromatography on SE-Sephadex C-50. Ovomucoid was also isolated from these sources by adsorbtion on and elution from G-200-trypsin and was used without further purification. The marker proteins were cytochrome C, ribonuclease, lysozyme, haemoglobin, trypsin, subtilisin, pepsin, ovalbumin and bovine serum albumin. The marker proteins and ovomucoid were run individually or in various combinations. Each combination of proteins or single protein was run in 4 individual gels.

2. 9 (13) Incorporation Studies using Oviduct Tissue

All the $[^{14}C]$ labelled precursors (mannose, galactose, glucosamine and lysine) were obtained as solids and were dissolved in the incubation medium (Krebs & Henseleit, 1932) to give a solution containing 50μCi/ml. The $[^{3}H]$ lysine was purchased as a 1ml sterilised solution containing 1mCi.

To this was added an equal volume of a solution containing unlabelled lysine at the same concentration so that the specific radioactivity of the isotope was halved to 125mCi/mmol. As the specific radioactivity of [^{14}C] lysine was 12mCi/mmol, this meant that 1μCi [^{14}C] lysine gave the equivalent amount of lysine in the incubation medium as 10μCi [^{3}H] lysine.

Oviduct tissue was removed from regularly laying White Leghorn hens killed by decapitation. The albumen secreting magnum of the oviduct was transferred to a buffered salts medium (Krebs & Henseleit, 1932) at 4°C. The composition of this medium is given in Table 2. The oviduct tissue was then dried by blotting with filter paper, weighed, finely chopped with scissors and portions (1g) suspended in fresh salts medium (5ml) which had the additions shown in Table 3. The tissue portions were incubated at 37°C, with continuous gentle shaking, for periods of time up to 8 hours with [^{14}C] glucosamine (4μCi/g of tissue) and [^{3}H] lysine (40μCi/g of tissue) and gassed continuously with O_2 + CO_2 (95:5).

For single label incorporation experiments, 1μCi/g of tissue of [^{14}C] lysine or [^{14}C] glucosamine was used. In this latter case, L-lysine.HCl (73.1mg/litre) was added to the incubation medium. In one experiment the labelled precursors were [^{14}C] mannose (4μCi/g of tissue) and [^{3}H] lysine (40μCi/g of tissue) and in another they were [^{14}C] galactose (4μCi/g of tissue) and [^{3}H] lysine (40μCi/g of tissue).

After incubation the tissue samples were plunged into ice and centrifuged at 600g for 10 minutes at 4°C. The supernatants were collected and the tissue was washed twice more with cold incubation medium (1g : 5ml) and the supernatants combined. The washed tissue samples were then

Table 2. The composition of the incubation medium (Krebs & Henseleit, 1932).

The salts medium was prepared by mixing in the following proportions:

Proportion	Salt	Concentration (g/100ml)
100	NaCl	4.5
4	KCl	5.75
3	$CaCl_2.6H_2O$	12.05
1	KH_2PO_4	10.55
1	$MgSO_4.7H_2O$	19.0
21	$NaHCO_3$	6.5

The medium was now at 5x the required strength. The salts were made up individually at the stated concentrations and stored separately in a refrigerator.

(1932). (amino acids, Eagle's Minimum Essential Medium; CoA, Hendler, 1957; antibiotics, Carey, 1966).

Ingredient	Final Concentration (mg/L)
L-arginine.HCl	126.2
L-cystine	24.0
L-histidine.HCl	38.3
L-isoleucine	52.4
L-leucine	52.4
L-methionine	14.9
L-phenylalanine	33.0
L-threonine	47.6
L-tryptophan	10.2
L-tyrosine	36.2
L-valine	46.8
L-glutamine	292.0
D-glucose	2000
Coenzyme A	300
Penicillin G	100
Streptomycin sulphate	100

The ingredients were made up at 100x their final strength and stored at -10°C. All the amino acids were made up together in water except glutamine which was kept separate and cystine and tyrosine which were made up together in 0.1N HCl.

Coenzyme A, penicillin G.and streptomycin sulphate were obtained from Sigma Chemical Co., St. Louis, Missouri, U.S.A.

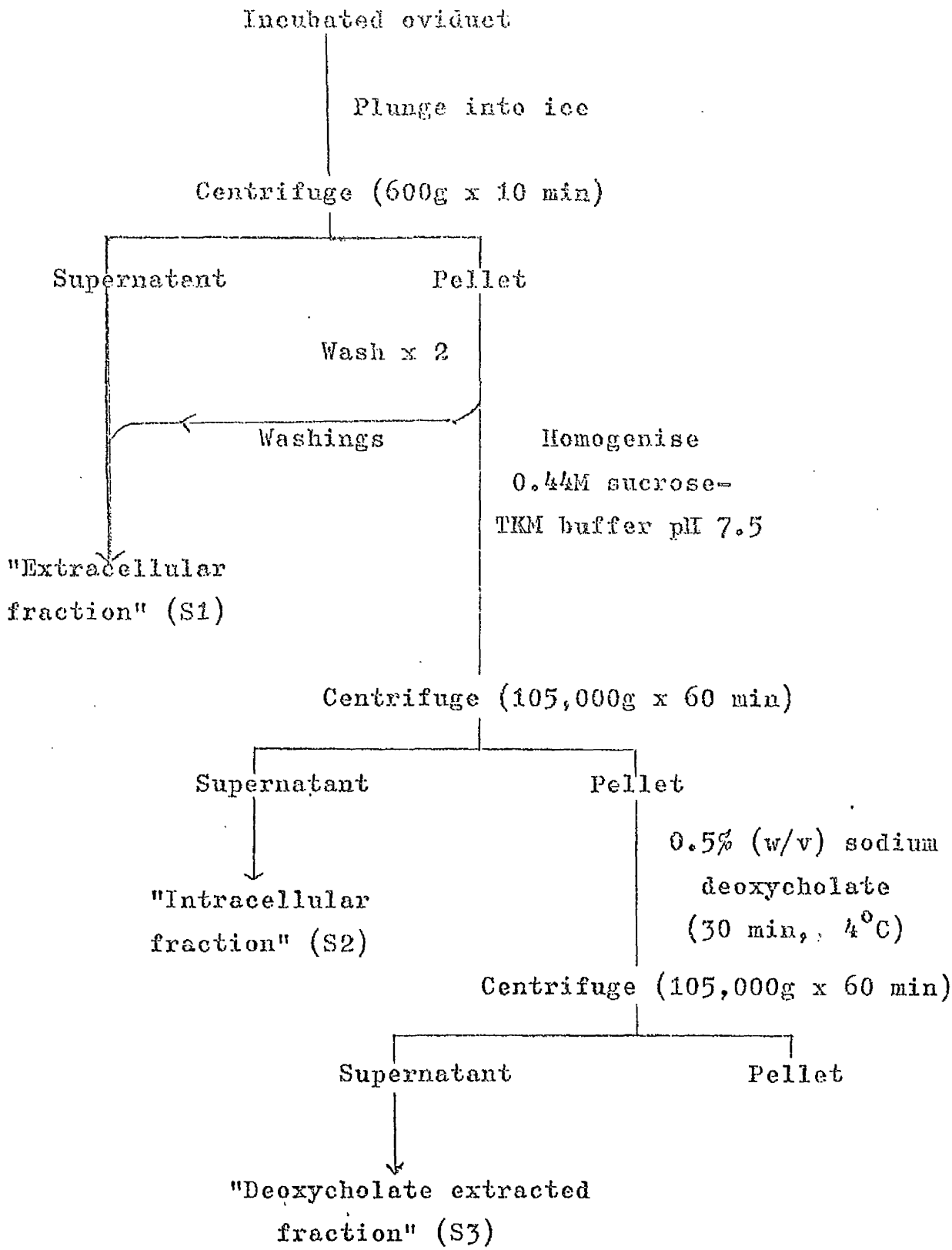
suspended in 0.44M sucrose containing 0.05M tris, 0.025M KCl and 5mM $MgCl_2$ (TKM), pH 7.5 (5ml/g tissue) and homogenised using a Dounce homogeniser with a teflon pestle. After centrifugation for 1 hour at 105,000g the supernatant was used for the isolation of "intracellular" ovomucoid (Fig. 1). Ovomucoid isolated from the combined washings of the tissue was considered to be "extracellular" (Fig. 1). The 105,000g pellet was extracted with 0.5% (w/v) sodium deoxycholate for 30 minutes at 4°C and after recentrifugation at 105,000g for 1 hour ovomucoid was isolated from the "deoxycholate extracted" supernatant (Fig. 1).

Although this was the normal incubation method used, in preliminary incorporation studies the medium of Hendler (1956; 1957) with the phenol red indicator omitted and with the addition of streptomycin and penicillin (Carey, 1966) was also employed. In early experiments the method of homogenising the tissue was also investigated by homogenising the tissue in cold incubation medium or in buffered sucrose in an all glass Potter-Elvehjem type homogeniser driven by a power drill (Black and Decker) or in a Dounce type homogeniser.

2. 9 (14) Isolation of Ovomucoid from Tissue Fractions

The tissue fractions illustrated in Fig. 1 were brought to pH 3.5 and to them was added an equal volume of 10% (w/v) trichloroacetic acid at pH 3 or, more usually 0.1 volume of 55% trichloroacetic acid pH 2, and the pH readjusted to pH 3.5. After 2 hours at 4°C, the precipitate was removed by centrifugation at 4000g for 30 minutes. The supernatant was brought to pH 7, sodium phosphate buffer (1M, pH7) was added to give a final concentration of 0.1M and ovomucoid isolated by adsorbtion on and

Fig. 1. Tissue fractionisation



elution from G-200-trypsin as described earlier. For a comparison of the methods of isolation of ovomucoid, in early studies the trichloroacetic acid supernatant was brought to pH 6 and ovomucoid precipitated by adding two volumes of ethanol (Fredericq & Deutsch, 1949). The ovomucoid samples were dialysed, lyophilised then taken up in water (1ml) and aliquots removed for assay of protein content and radioactivity determinations.

In order to determine the specific radioactivity of ovomucoid after additional purification steps had been included in the isolation, ovomucoid from the intracellular fraction (150mg) was passed through a column of Sephadex G-75 (28cm x 2.5cm), eluted with 0.025M sodium acetate pH 4.7. The flow rate was 12.5ml/hour and 3ml fractions were collected. The fraction corresponding to ovomucoid was pooled and an aliquot chromatographed on carboxymethyl cellulose CM-23 by the method of Rhodes et al (1958). Ovomucoid which had been prepared by the ethanol precipitation method of Fredericq and Deutsch (1949) was then treated with G-200-trypsin. Similarly ovomucoid which had been isolated using insolubilised trypsin was precipitated by adding two volumes of ethanol (Fredericq & Deutsch, 1949). Ovomucoid isolated using G-200-trypsin was also treated with G-200-chymotrypsin using the same washing conditions as in the batch isolation of ovomucoid by adsorbtion on and elution from G-200-trypsin. However as ovomucoid is not adsorbed on insolubilised chymotrypsin, the washings at pH 7 were collected as the ovomucoid fraction.

Although intracellular and extracellular ovomucoid were routinely isolated by adsorbtion on and elution from G-200-trypsin following a preliminary precipitation stage with trichloroacetic acid at pH 3.5, it was

found to be more suitable to omit the trichloroacetic acid precipitation stage in the isolation of deoxycholate extracted ovomucoid as inclusion of this preliminary step resulted in very low yields of ovomucoid from this fraction.

2. 9 (15) Additions to the Incubation Medium

Cycloheximide (Calbiochem, Los Angeles, California, U.S.A.) at a concentration of 50µg/ml or 100µg/ml was added to the incubation medium 5 minutes prior to the addition of labelled lysine and glucosamine to determine its effect on the incorporation of precursors into ovomucoid. Similar experiments were carried out with puromycin dihydrochloride (Nutritional Biochemicals Corporation, Cleveland, Ohio, U.S.A.).

The effect of hormones was investigated by the addition of 1µM or 0.01µM progesterone (Δ^4 pregnen 3,-20 dione) or of 1µM or 0.01µM β-estradiol (both from Sigma Chemical Co., St. Louis, Missouri, U.S.A.) to the incubation medium. A combination of both hormones at 1µM or both at 0.01µM was also tested. The hormones were made up at a concentration of 0.02M in ethanol. To an aliquot (0.25ml) of the stock solution was added 15ml 0.5M NaOH, this was neutralised with 0.5M HCl and made up to 50ml with water. This gave a solution of either hormone at a concentration of 0.1mM in phisiological saline. These solutions were used to obtain the final concentrations of 1µM and 0.01µM of the hormones in the incubation medium.

The possible effects of 2-deoxyglucose (Koch-Light, Colnbrook, Bucks., U.K.) at a concentration of 10mM and 0.1mM on the incorporation of labelled lysine and glucosamine into ovomucoid was also investigated. In one

experiment in the presence of 2-deoxyglucose (10mM), mannose was added to the incubation medium at the same concentration as 2-deoxy-D-glucose.

2. 9 (16) Liquid Scintillation Counting

Aliquots (0.1ml - 0.5ml) of a solution of labelled ovomucoid from incubation studies were made up to 0.5ml with water and incubated at 37°C for 10 minutes with 0.5ml hyamine hydroxide (a 1M solution in methanol obtained from Nuclear Enterprises, Edinburgh, Scotland) in scintillation vials. Then 10ml of scintillation fluid containing 0.7% (w/v) 2,5-diphenyloxazole (Koch-Light, Colnbrook, Bucks., U.K.) and 10% (w/v) napthalene (Nuclear Enterprises, Edinburgh, Scotland) in 1,4-dioxan (Koch-Light, Colnbrook, Bucks., U.K.) was added. The dioxan based scintillator was made up in batches of 1 litre at a time and stored under (oxygen free) nitrogen to prevent peroxide formation. The vials were counted in a liquid scintillation analyser (Philips, Netherlands). As the liquid scintillation counter can discriminate between pulses of different energy, and ^{3}H is a lower energy emitter than ^{14}C, it is possible by selection of the energy levels in which the counts are to be recorded to count ^{3}H and ^{14}C simultaneously. The efficiency of counting was determined by the quenching of an external standard which allowed d.p.m. to be calculated from the observed c.p.m. The efficiency of counting of single label ^{14}C was 62%, while the efficiency of dual label counting was 33% for ^{14}C and 15% for ^{3}H. Background radiation was automatically subtracted from the counts by the analyser.

2. 9 (17) Determination of Fate of Precursor.

After tissue incubations with $[^{14}C]$ glucosamine and $[^{3}H]$ lysine as

precursors, samples of ovomucoid were hydrolysed in 6N HCl for 18 hours at 105°C in sealed tubes under vacuum. The HCl was removed by rotary evaporation at 40°C and the hydrolysed protein taken up in 1ml water. A portion was prepared for liquid scintillation counting and an aliquot fractionated in an amino acid analyser JLC-5AH (Japanese Electron Optics Laboratory Co. Ltd., Tokyo, Japan) using a 0.8cm x 15cm column of LCR-1 resin (J.E.O.L. Co. Ltd., Tokyo, Japan). The amino acid peaks were collected by splitting the stream so that 0.42ml/min of the sample went to the ninhydrin detector and 0.80ml/min was collected in 2ml fractions. Portions (0.5ml) of these 2ml fractions were prepared for liquid scintillation counting.

Glucosamine was liberated from labelled ovomucoid by hydrolysis in 4N HCl at 100°C for 3 hours. The HCl was removed by rotary evaporation at 40°C and the residue taken up in 2ml water. An aliquot was prepared for liquid scintillation counting and the rest was passed through a column (11cm x 1cm) of Dowex 50 X2, H^+ form. The effluent was discarded and the column washed with 25ml of water which was also discarded. The glucosamine was eluted by washing the column with 25ml 2N HCl and the HCl removed by rotary evaporation at 40°C. The residue was taken up in 1ml water, 0.4ml was prepared for liquid scintillation counting and the rest used for the assay of glucosamine by the acetylacetone reaction (Elson & Morgan, 1933). The Dowex 50 was washed with 2N NaOH, water, 3N HCl and water again before use.

Sialic acid was released from ovomucoid by hydrolysis in 0.1N H_2SO_4 at 80°C for 1 hour in a volume of 1ml. The acid was neutralised with

NaOH and a further 2ml of water added. The sample was applied to a column (9cm x 1cm) of Dowex 1 X8, formate form. The effluent was discarded and the column washed with a further 25ml of water which was also discarded. Sialic acid was eluted by washing the column with 30ml of 0.3N HCOOH. The formic acid was removed in a rotary evaporator at 40°C and the sample taken up in 1ml water. A portion (0.4ml) was prepared for liquid scintillation counting and the rest assayed for sialic acid content by the thiobarbituric acid method of Aminoff (1961). Dowex 1 formate was generated from Dowex 1 chloride by washing the resin with 3M sodium formate. This was done after washing the chloride form resin with 2N NaOH, water, 2N HCl and water again.

2. 9 (18) Estimation of the Specific Radioactivity of the Total Trichloroacetic Acid Precipitable Protein

In the isolation of intracellular and extracellular ovomucoid from oviduct there was a preliminary precipitation step with 5% (w/v) trichloroacetic acid at pH 3.5 included. The precipitate from this stage was washed twice with 5% (w/v) trichloroacetic acid and dissolved in 0.5N NaOH (1ml/original g of tissue) at room temperature for 1 hour. Aliquots (5µl to 20µl) were assayed for protein content by the method of Lowry et al (1951) using ovalbumin as the reference standard. As the samples were in NaOH the Na_2CO_3 reagent was made up in a reduced strength of NaOH to account for the NaOH which was being added to the assay in the protein samples (Lowry et al, 1951). To a further aliquot (10µl) was added 10µl of potassium phosphate buffer, 0.1M pH 7 and just enough HCl to neutralise the sample. The samples were then prepared for liquid scintillation counting as described.

The specific radioactivities of the intracellular and extracellular trichloroacetic acid precipitable proteins determined in this way were the same as those determined when aliquots of the extracellular and intracellular fractions were precipitated with 5% (w/v) trichloroacetic acid not brought to pH 3.5. It was much more convenient therefore to routinely use the trichloroacetic acid, pH 3.5, precipitates for the determination of the specific radioactivities of total protein fractions of the tissue.

2. 9 (19) Sulphoethyl-Sephadex Chromatography of Labelled Ovomucoid

Labelled ovomucoid was fractionated on columns (60cm x 1cm) of sulphoethyl-Sephadex C-50 by elution with 0.014M sodium acetate containing 1mM sodium azide, pH 4.90 at a flow rate of 4ml/hour. Fractions (1.6ml) were collected and 0.4ml portions used for the estimation of protein content (Lowry et al, 1951) and 0.4ml portions prepared for liquid scintillation counting. The columns were run with buffer maintained under nitrogen to avoid uptake of CO_2 from the atmosphere.

2. 9 (20) Isoelectric Focusing of Ovomucoid after Neuraminidase Treatment

Neuraminidase (E.C. 3. 2. 1. 18) was obtained from Worthington Biochemical Corporation, Freehold, New Jersey, U.S.A. It was described that 1 unit of enzyme activity hydrolysed 1 μmole of N-acetylneuraminic acid from a glycoprotein substrate in 1 minute. The activity of the neuraminidase was 0.7 unit/mg neuraminidase, and a 1ml vial contained 2mg.

Ovomucoid was incubated with neuraminidase (1.4m units/mg of ovomucoid) for 24 hours at 37°C in 0.05M sodium acetate buffer pH 5.5.

The samples were dialysed, lyophilised and the band pattern on isoelectric focusing compared to the band pattern of unhydrolysed ovomucoid.

2. 9 (21) Fractionation of Oviduct Homogenate by Differential and Sucrose Density Gradient Centrifugation

(a) Differential Centrifugation

A portion of oviduct (8g) was homogenised in 40ml 0.44M sucrose in a Dounce homogeniser at 4°C. All subsequent steps were carried out at 4°C. The homogenate was centrifuged at 600g for 10 minutes in an MSE Major centrifuge (Measuring and Scientific Equipment Ltd., Crawley, Sussex, England) using the 8 x 50ml swing out rotor. The pellet was washed three times in fresh 0.44M sucrose by resuspending and recentrifuging. The washed pellet was called P1. The combined supernatants were centrifuged at 10,000g for 10 minutes in an MSE Highspeed 18 centrifuge. The pellet was washed once with sucrose and the washed pellet called P2. The combined supernatants were centrifuged at 105,000g for 60 minutes in an MSE Superspeed 50 centrifuge using the MSE 50 rotor. The pellet was washed once with sucrose and the washed pellet called P3. The combined supernatants were called S3.

The cell fractions were washed with 5% (w/v) trichloroacetic acid at room temperature. The precipitate was extracted with 5% (w/v) trichloroacetic acid at 90°C for 10 minutes and centrifuged. The extraction was repeated twice. The combined extracts were considered to contain the nucleic acids and the residue to contain the protein (Carey, 1966). The cell fractions were also made 0.2N with respect to perchloric acid and the nucleic acids fractionated by the method of Fleck and Munro (1962).

(b) Discontinuous Sucrose Gradient Centrifugation

A discontinuous sucrose density gradient was prepared by layering the following solutions on top of each other. First 4.5ml of saturated sucrose containing 0.05M tris, 0.025M KCl and 0.005M $MgCl_2$ (TKM buffer) pH 7.5, then 4.5ml 2M sucrose-TKM buffer pH 7.5 followed by 9ml 0.5M sucrose-TKM buffer pH 7.5. A sample of oviduct homogenate (0.4g) in 0.25M sucrose-TKM buffer pH 7.5 was layered on top of the gradient. The samples were centrifuged at 80,000g for 10 hours in an MSE Superspeed 50 centrifuge using the MSE 40 rotor. Fractions (1.5ml) were collected from the bottom of the tube by inserting a capillary right to the bottom of the gradient and drawing off the sucrose.

The extinctions at 280nm and at 260nm of the fractions were determined. An equal volume of 10% (w/v) trichloroacetic acid was added to the fractions to precipitate the protein and nucleic acids, and the precipitate was washed with cold 5% (w/v) trichloroacetic acid. The nucleic acids were extracted from the trichloroacetic acid precipitate by incubating at 70°C for 30 minutes in 0.5N perchloric acid.

(c) Linear Sucrose Density Gradient Centrifugation

Linear sucrose density gradients were prepared by filling one limb of a gradient mixing vessel with 0.5M sucrose-TKM buffer pH 7.5 and the other limb with an equal volume of 2.5M sucrose-TKM buffer pH 7.5. The mixer limb which contained the denser sucrose had a bar magnet driven by an electric magnetic stirrer. The limbs were connected at the bottom and the solutions allowed to mix while drawing off the mixture to fill a centrifuge tube with the gradient. Each limb contained half the total

volume of gradient required to fill the centrifuge tube. One sample of oviduct homogenate (0.8g) in 0.25M sucrose-TKM buffer pH 7.5 was applied to a 20ml gradient and centrifuged at 20,000g for 16 hours in an MSE Superspeed 50 centrifuge using the MSE 40 rotor. Another sample was applied to a 15ml gradient and centrifuged at 5,000g for 16 hours in an MSE Highspeed 18 centrifuge. Another sample of oviduct homogenate was applied to a 20ml gradient and centrifuged at 2000g for 16 hours in an MSE Mistral 4L centrifuge using the 8 x 50ml swing out rotor with rubber adaptors to take the 20ml MSE tubes. Fractions were collected from each gradient as described and their extinctions at 260nm and 280nm determined. Protein and nucleic acids were precipitated with 5% (w/v) trichloroacetic acid and the nucleic acids extracted by incubating the trichloroacetic acid precipitate with 0.5N perchloric acid for 30 minutes at 70°C.

In both the sucrose gradient centrifugation methods portions of the oviduct were homogenised with a Dounce homogeniser and portions with a Potter-Elvehjem homogeniser.

2. 9 (22) Chemical Determinations on Oviduct Cell Fractions

The total protein content and the radioactivity of trichloroacetic acid precipitates were determined as described above (section 2. 9 (18)).

The DNA content of hot trichloroacetic or hot perchloric acid extracts and DNA isolated by the method of Fleck and Munro (1962) was estimated by the method of Burton (1956) or by the method of Ceriotti (1952) using calf thymus DNA (Sigma Chemical Co., St. Louis, Missouri, U.S.A.) as a standard.

The RNA content of hot trichloroacetic acid or hot perchloric acid

extracts and RNA isolated by the method of Fleck and Munro (1962) was determined by the orcinol reaction (Dische, 1955) after destruction of hexoses by heating in 0.1N NaOH at 100°C for 15 minutes (Solomon, 1957). Yeast RNA (British Drug Houses, Poole, Dorset, U.K.) was used as reference standard.

PART 3

RESULTS

Chapter 10 Isolation of Ovomucoid

The first part of this section concerns the isolation, fractionation and characterisation of ovomucoid. The later section is concerned with studies on the biosynthesis of ovomucoid.

For biosynthetic studies it was necessary to develop a specific isolation method which could isolate small quantities of ovomucoid with a high degree of purity. The use of an insolubilised derivative of trypsin in the isolation of ovomucoid was investigated and some properties of the insoluble trypsin are included.

The homogeneity of ovomucoid isolated by use of insolubilised trypsin was investigated by isoelectric focusing and immunochemical techniques and the isolation method compared to that of Fredericq and Deutsch (1949). Ovomucoids from egg white and the oviducts of laying hens were also compared by these above techniques and by their carbohydrate content and trypsin inhibiting activity.

Further fractionation of ovomucoid was carried out by chromatography on columns of sulphoethyl-Sephadex C-50 and the sialic acid content and trypsin inhibiting activity of the ovomucoid species determined.

The isoelectric points of the ovomucoid variants were determined by isoelectric focusing. The molecular weight of ovomucoid was estimated by gel electrophoresis in the presence of sodium dodecyl sulphate.

3. 10 (1) Properties of Insolubilised Trypsin

The use of an insolubilised trypsin derivative in the isolation of ovomucoid was examined as part of a collaborative project with Dr. J.G. Beeley. Trypsin was bound to Sephadex G-200 as described in the Methods section and a description of the properties of the insolubilised enzyme have been published (Beeley, 1970; Beeley & McCairns, 1972).

The proteolytic activity of the preparation of G-200-trypsin used in these studies towards casein and the esterase activity towards benzoyl-L-arginine ethyl ester (BAEE) are given in Table 4.

TABLE 4

The activity of G-200-trypsin towards BAEE and casein

The activity is related to the µg of soluble trypsin giving an equivalent rate of hydrolysis of substrate per mg dry weight of insolubilised enzyme.

Substrate	Activity (µg soluble trypsin/mg dry weight)
BAEE	43.6
Casein	4.3

Of the activities expressed in Table 4, 71% of the proteinase activity and 53% of the esterase activity of insolubilised trypsin were inhibitable by ovomucoid (Beeley & McCairns, 1972).

3. 10 (2) Batch Isolation of Ovomucoid using Insolubilised Trypsin

Ovomucoid binds to G-200-trypsin at pH 7 but dissociates readily

as the pH is lowered. The ovomucoid bound to the insoluble enzyme may be liberated by washing at pH 1.5 (Beeley & McCairns, 1972).

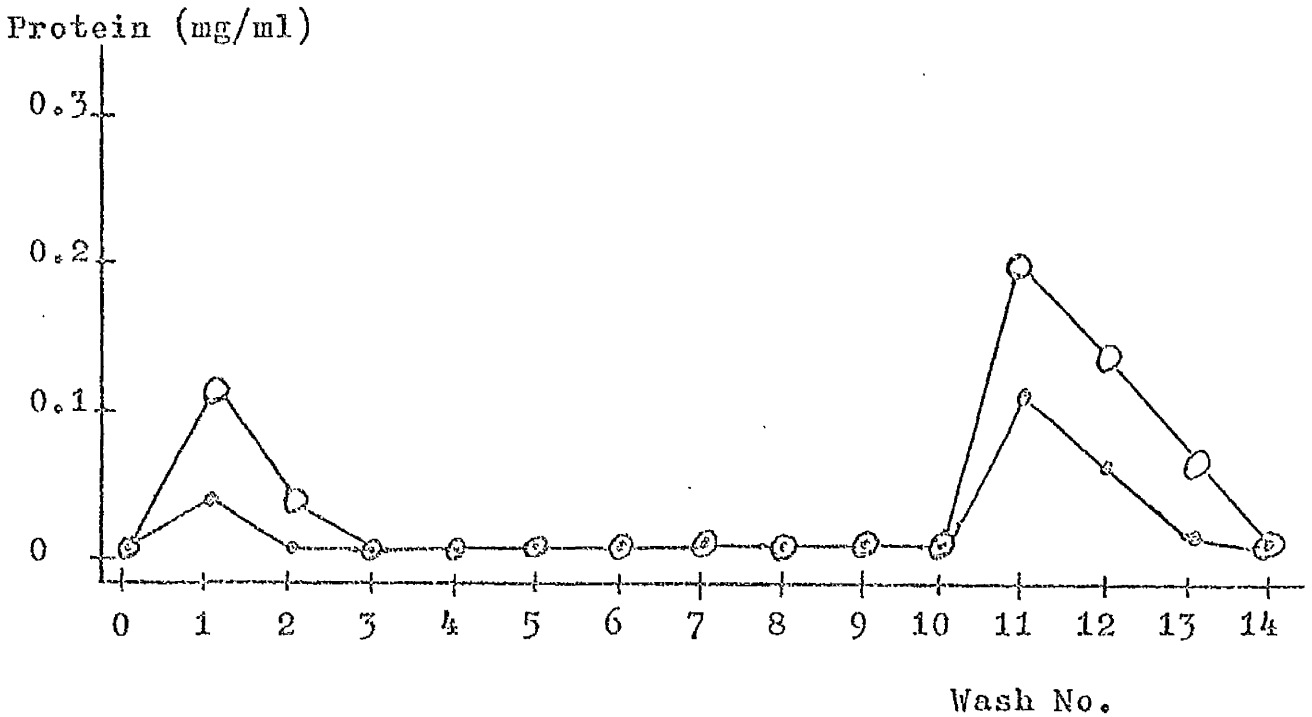
An example of the batch isolation of ovomucoid using an insolubilised trypsin derivative is given in Fig. 2. The ovomucoid was a "crude preparation" of ovomucoid from egg white isolated by the method of Fredericq and Deutsch (1949).

It was found that 10 ml (settled volume) of G-200-trypsin had the capacity to isolate a maximum of 10mg of ovomucoid. If less than 10mg of ovomucoid was added to 10ml (settled volume) of insoluble trypsin, then 80% was adsorbed at pH 7 and eluted at pH 1.5. If more than 10mg ovomucoid was added to 10ml (settled volume) of G-200-trypsin, then the maximum isolated by washing at pH 1.5 was 10mg.

3. 10 (3) Isoelectric Focusing of Ovomucoid

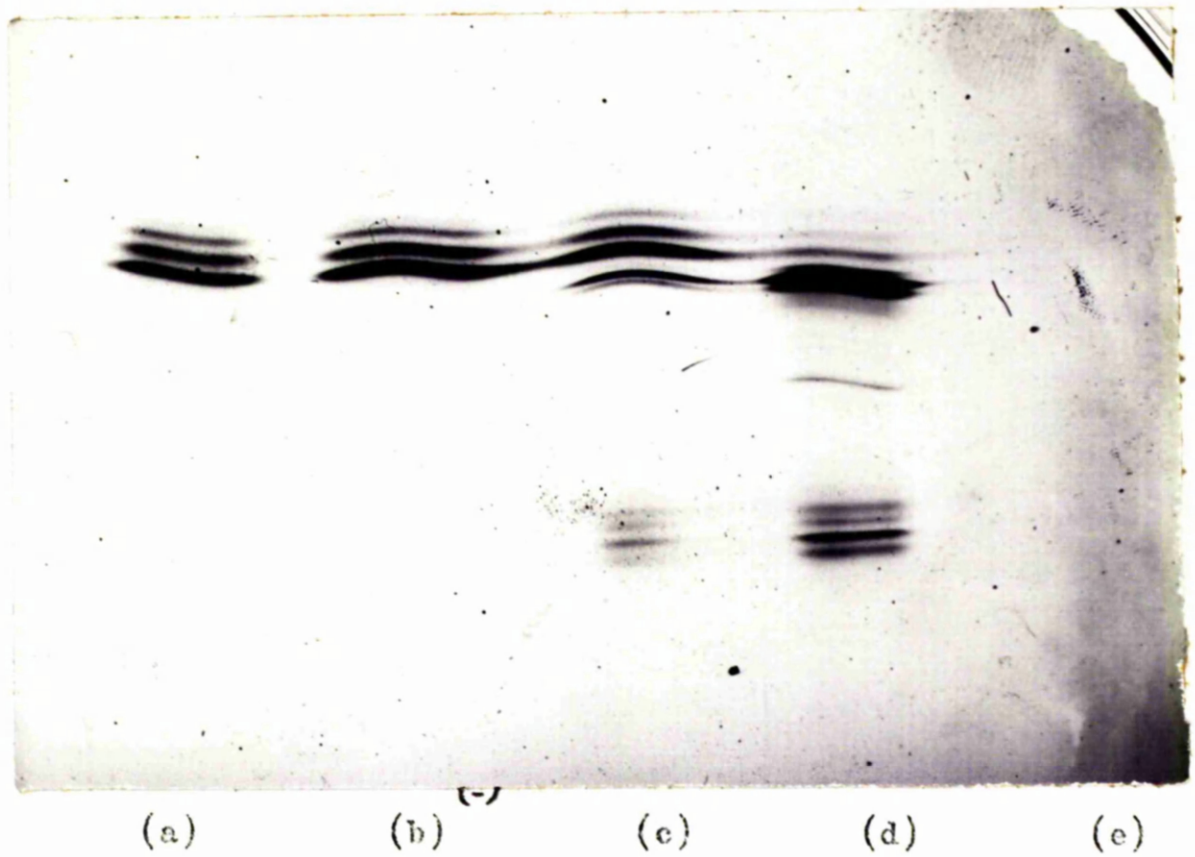
Ovomucoid was isolated from egg white and oviduct homogenate using insolubilised trypsin. A parallel experiment was carried out at the same time with insoluble chymotrypsin as a control. The isoelectric focusing band pattern of the material which eluted at pH 1.5 is shown in Fig. 3. Adsorbtion of egg white or the supernatant from oviduct homogenate directly onto G-200-trypsin led to the isolation of components having the same isoelectric points as the major ovomucoid species. However, additional components were observed, one group with isoelectric point slightly higher than that of the most basic ovomucoid species and a much more basic series of components. This more basic series of components was also observed in the eluate from G-200-chymotrypsin. There is an inhibitor of both trypsin and chymotrypsin present in egg white

Fig. 2. Batch isolation of ovomucoid using G-200-trypsin



Samples of ovomucoid (●—● 2.5mg ; o—o 5mg) in 10 ml 0.1M sodium phosphate, pH 7 isolated from 10 ml (settled volume) of G-200-trypsin. Wash 1 was the supernatant, Wash 2,3,4; 0.1M sodium phosphate pH 7, Wash 5,6,7; 0.5M NaCl, Wash 8,9,10; water and Wash 11,12,13 and 14; HCl pH 1.5. The ovomucoid was a crude preparation isolated from egg white by the method of Fredericq and Deutsch (1949). Details of the washing procedure are given in the text.

Fig. 3. Isoelectric focusing of ovomucoid



(a) Ovomucoid isolated from oviduct by adsorbtion on and elution from G-200-trypsin following pH 3.5 trichloroacetic acid precipitation of other components. (b) Same method applied to egg white ovomucoid. (c) Proteins isolated from egg white by direct adsorbtion on and elution from G-200-trypsin. (d) Proteins isolated by direct adsorbtion on and elution from G-200-chymotrypsin. (e) Sample obtained by trichloroacetic acid precipitation at pH 3.5 of egg white followed by adsorbtion on and elution from G-200-chymotrypsin.

named ovoinhibitor (Feeney, Stevens & Osuga, 1963).

Contaminating proteins were removed by a preliminary precipitation step with 5% (w/v) trichloroacetic acid at pH 3.5 before adsorbtion with G-200-trypsin (Fig. 3a and 3b). After trichloroacetic acid precipitation, no material remained in the supernatant which would bind to chymotrypsin (Fig. 3e).

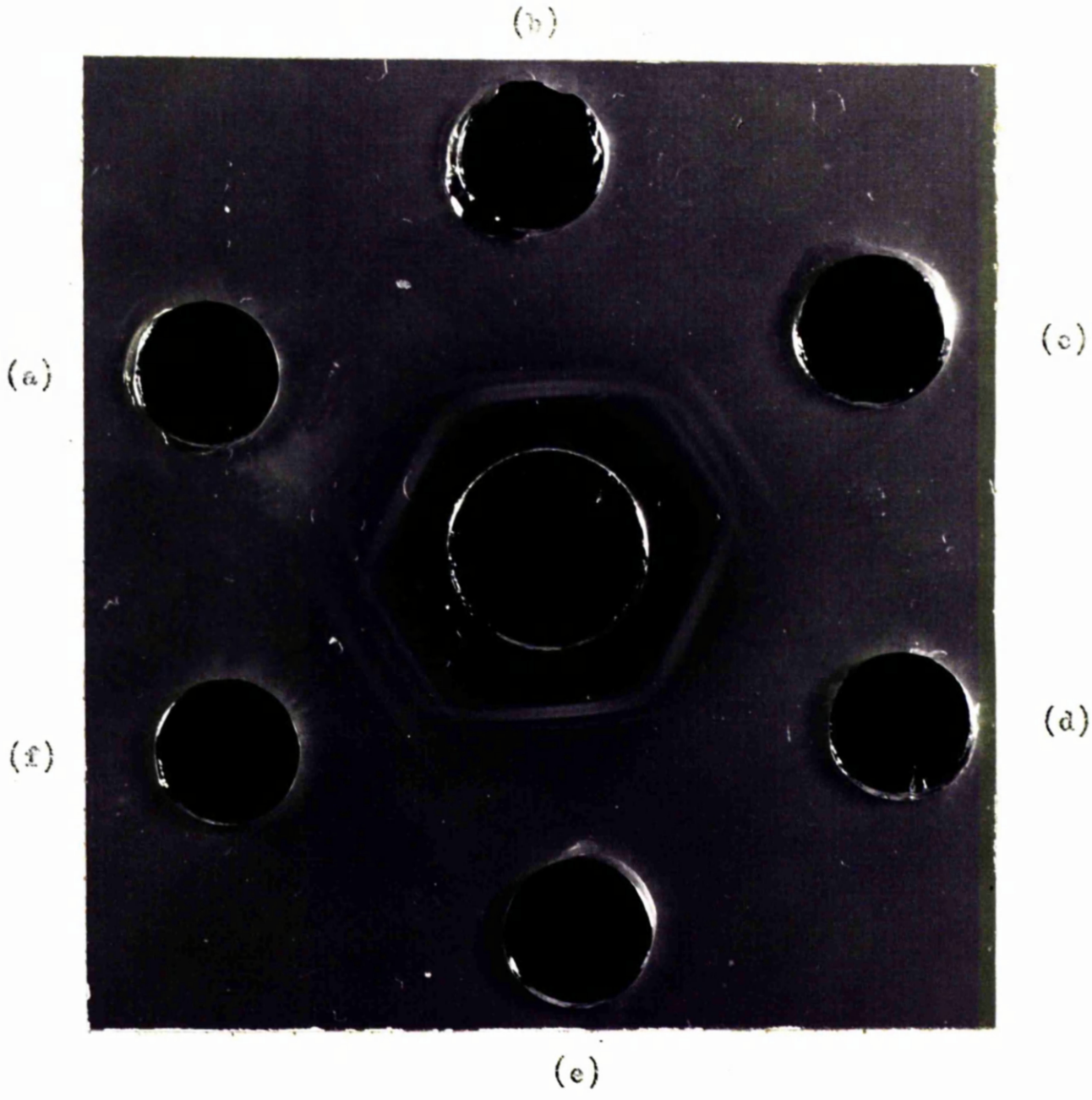
Ovomucoid isolated from the supernatant resulting from trichloroacetic acid precipitation of egg white or oviduct homogenate by use of an insolubilised trypsin derivative showed an isoelectric focusing pattern similar to that of the best conventional preparations, for example, ovomucoid isolated by the method of Fredericq and Deutsch (1949). The isoelectric focusing pattern of ovomucoid may be used as an indication of the homogeneity of the preparation. A comparison of ovomucoid isolated from oviduct or egg white (Fig. 3a and 3b) showed that the same charged species were present.

3. 10 (4) Immunochemical Experiments

The homogeneity of ovomucoid isolated by adsorbtion on and elution from G-200-trypsin following a preliminary trichloroacetic acid precipitation was further examined by immunoelectrophoresis (at pH 7.5 and pH 4.9) and immunodiffusion using antisera to whole egg white, ovalbumin or crude ovomucoid.

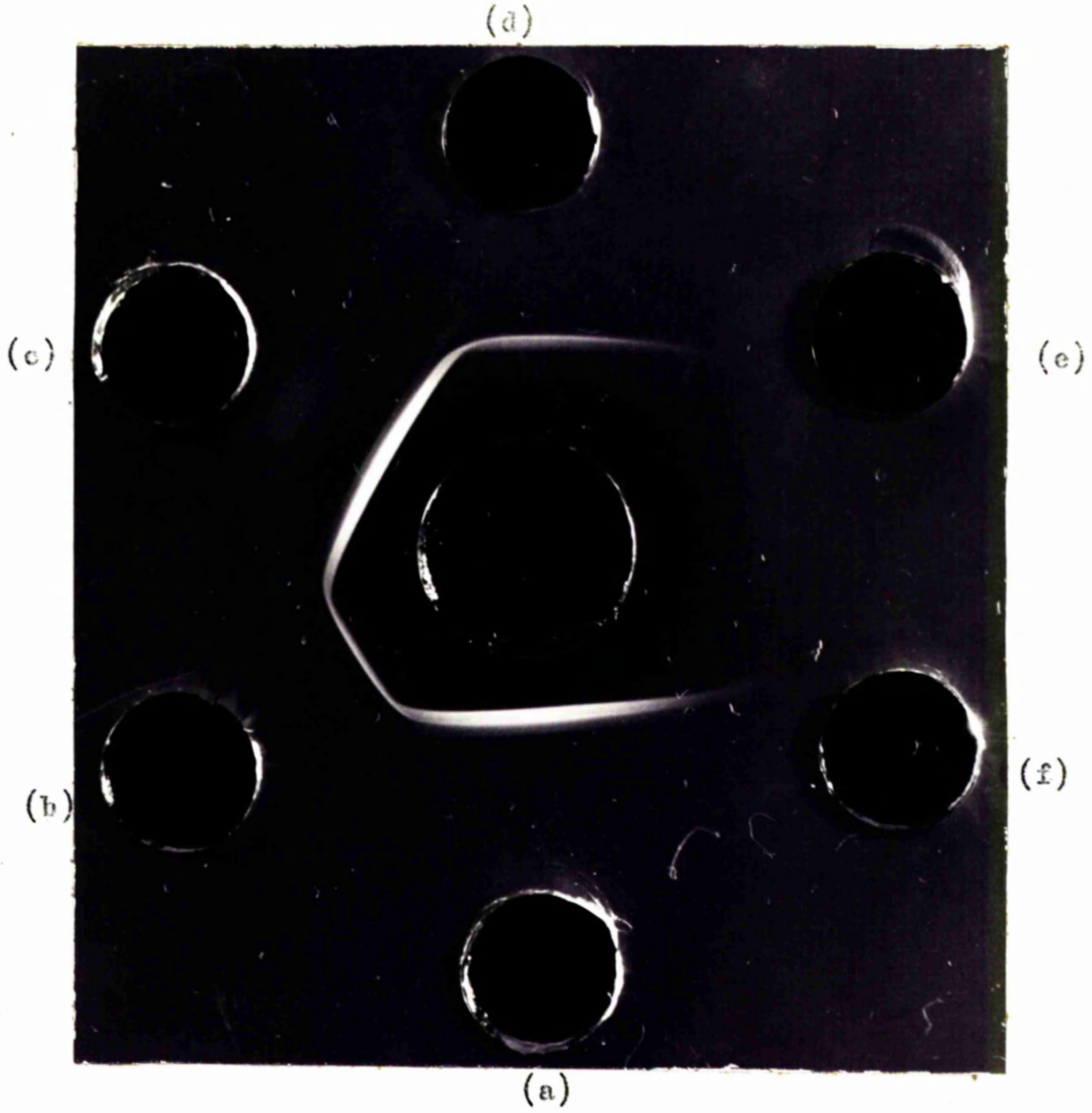
From the results of double diffusion using antiserum to crude ovomucoid (Fig. 4) it can be seen that crude ovomucoid (prepared by the method of Fredericq & Deutsch, 1949) contained a second antigenic species which could be completely removed by adsorbtion on and elution from

Fig. 4. Immunodiffusion of ovomucoid using anti-crude ovomucoid antiserum



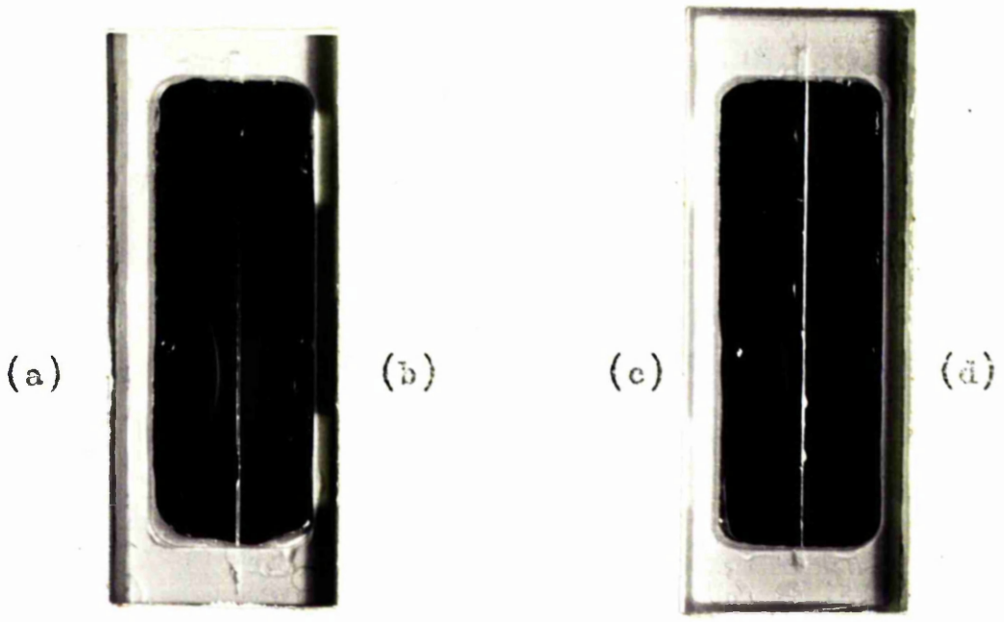
(a) & (d) Ovomucoid isolated by adsorbtion on and elution from G-200-trypsin following trichloroacetic acid pH 3.5 precipitation of other components. (b) & (e) Ovomucoid isolated by the method of Fredericq & Deutsch (1949). Samples (a) & (b) were from egg white while samples (d) & (e) were isolated from oviduct homogenate. (c) & (f) Ovoglycoprotein isolated from egg white by the method of Ketterer (1965).

Fig. 5. Immunodiffusion of ovomucoid using anti-ovalbumin antiserum



(a) & (c) Whole egg white. (b) & (d) Ovalbumin. (e) Ovomucoid from egg white isolated by adsorbtion on and elution from G-200-trypsin following precipitation of other components with pH 3.5 trichloroacetic acid. (f) Ovomucoid isolated from oviduct homogenate by the same method.

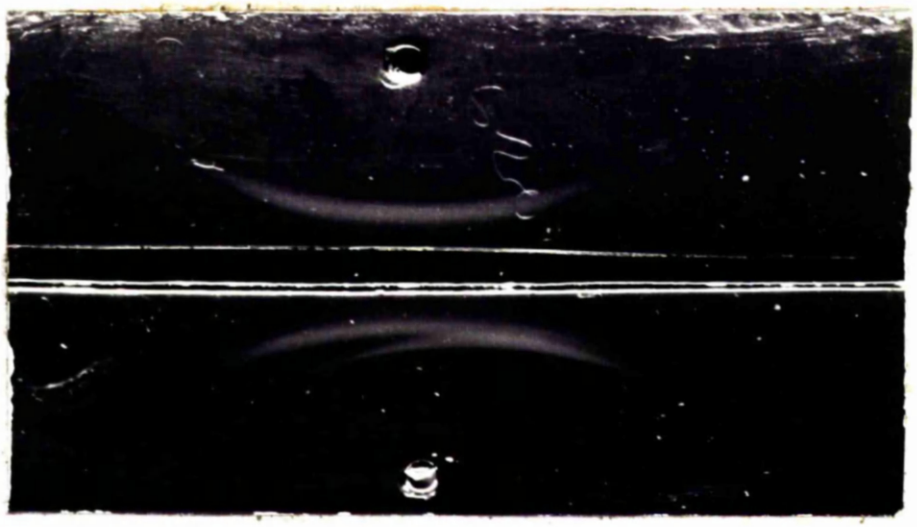
Fig. 6. Immunoelectrophoresis of ovomucoid at pH 7.5 using anti-crude ovomucoid antiserum



(a) & (c) Ovomucoid isolated by adsorbtion on and elution from G-200-trypsin following a preliminary precipitation of other components with pH 3.5 trichloroacetic acid. (b) & (d) Ovomucoid isolated by the ethanol precipitation method of Fredericq and Deutsch (1949). Samples (a) & (b) were from oviduct homogenate while samples (c) & (d) were isolated from egg white.

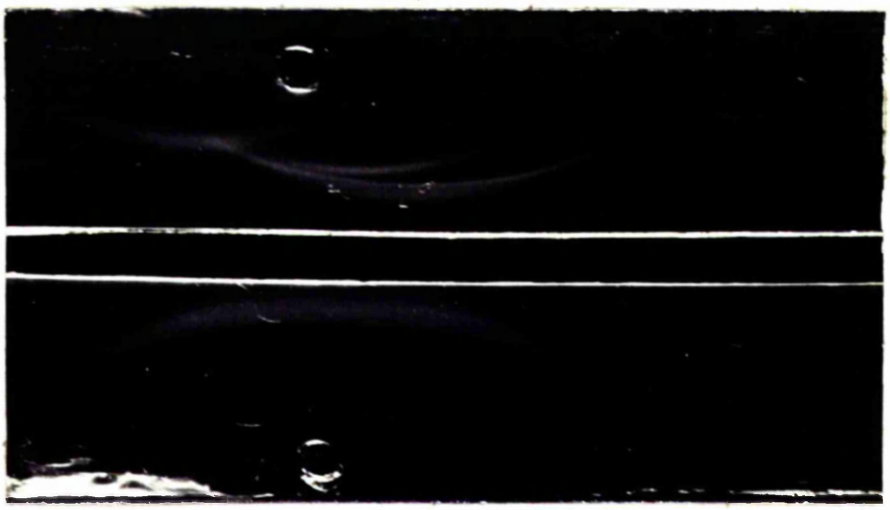
Fig. 7. Immunoelectrophoresis of ovomucoid at pH 4.9 using anti-crude ovomucoid antiserum

(a)



(b)

(c)



(d)

(a) & (d) Ovomucoid isolated from oviduct(a) or egg white(d) by adsorbtion on and elution from G-200-trypsin after pH 3.5 trichloroacetic acid precipitation of other components. (b) Ovomucoid isolated by the method of Fredericq & Deutsch (1949). (c) Ovoglycoprotein prepared by the method of Ketterer (1965).

G-200-trypsin. Ovoglycoprotein prepared by the method of Ketterer (1965) gave three precipitin arcs.

On using an antiserum to ovalbumin it is seen (Fig. 5) that ovomucoid isolated by use of an insolubilised trypsin derivative contains no species corresponding to ovalbumin. The results with anti-egg white antiserum were similar.

On immunoelectrophoresis at pH 7.5 using antiserum to crude ovomucoid (Fig. 6), ovomucoid prepared by the method of Fredericq and Deutsch (1949) gave two precipitin arcs, while ovomucoid prepared by adsorbtion on and elution from G-200-trypsin gave one precipitin arc indicating homogeneity under these conditions. When the electrophoresis was carried out at pH 4.9 prior to immunodiffusion at pH 7.5 using antiserum to crude ovomucoid, the same pattern was found (Fig. 7).

It was shown earlier (Beeley, 1971a) that rabbit antisera raised to crude ovomucoid gave three precipitin arcs corresponding to ovomucoid, ovalbumin and ovoglycoprotein. From the results described here (Figs. 4, 5, 6 and 7) it appears that the major contaminant of this preparation of crude ovomucoid is ovoglycoprotein. Ovomucoid isolated by the G-200-trypsin method gave one precipitin arc, indicating homogeneity, under all the conditions employed. Ovomucoids from egg white or oviduct homogenates were indistinguishable in these studies.

3. 10 (5) Analytical Determinations of Carbohydrate

The carbohydrate content of a crude preparation of ovomucoid from a mixture of egg whites and the same preparation after adsorbtion on and elution from G-200-trypsin is illustrated in Table 5.

TABLE 5

The hexose and hexosamine content of egg white ovomucoid before and after adsorbtion on and elution from G-200-trypsin.

Preparation of ovomucoid	Hexose (g/100g)	Hexosamine (g/100g)
(a) Crude ovommucoid	8.85	13.3
(b) Ovomucoid after adsorbtion on and elution from G-200-trypsin	8.75	13.1

It can be clearly seen from Table 5 that the hexose and hexosamine content of ovomucoid isolated by both techniques are indistinguishable.

In Table 6, the carbohydrate content of ovomucoid isolated from a single egg is compared with the carbohydrate content of the ovomucoid isolated from the oviduct of the hen laying that egg.

TABLE 6

The carbohydrate content of egg white and oviduct ovomucoid isolated by use of G-200-trypsin.

Ovomucoid	Hexose (g/100g)	Hexosamine (g/100g)	Sialic acid (g/100g)
(a) Egg white	8.80	13.6	0.46
(b) Oviduct	8.75	14.1	0.50

The values of the carbohydrate assays are virtually identical for ovomucoid from oviduct and egg white and compare well with carbohydrate assays on ovomucoid isolated from egg white by alternative methods (Beeley 1971a; Montgomery & Wu, 1963).

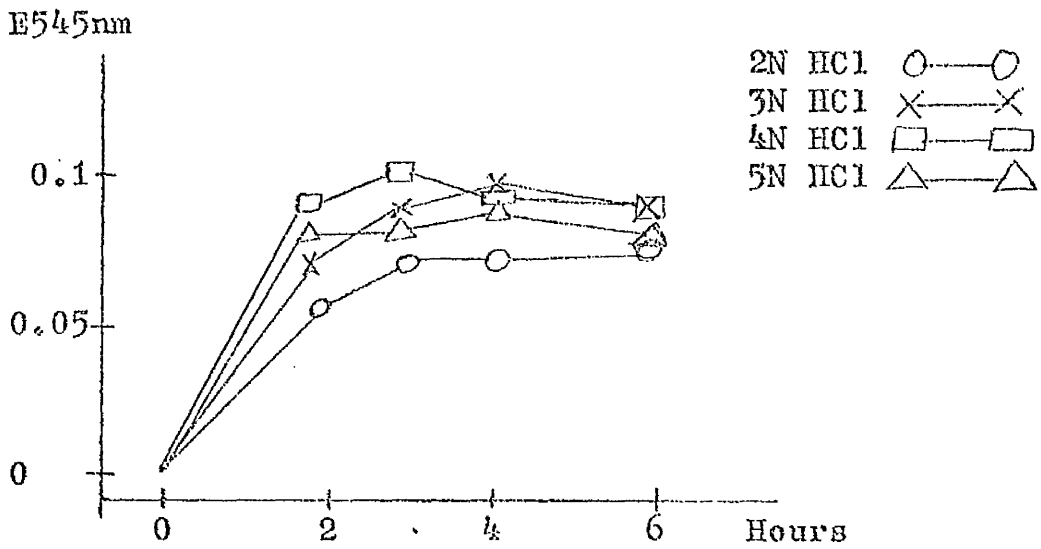
The optimal conditions for the hydrolysis of ovomucoid for the assay of glucosamine were determined. In a preliminary experiment, hydrolysis at room temperature with varying strengths of HCl for periods up to 6 hours was found to be ineffective in liberating glucosamine from ovomucoid. Hydrolysis at 100°C with 3N HCl was effective in liberating glucosamine and Fig. 8 illustrates the hydrolysis of ovomucoid (at 100°C) with varying strengths of HCl over a 6 hour period. Optimal hydrolysis conditions were found to be 4N HCl at 100°C for 3 hours. Incubation of glucosamine under these conditions caused no destruction of glucosamine as the values for the optical density after hydrolysis agreed with those before hydrolysis. A wavelength scan of glucosamine assayed before and after hydrolysis also suggested that there was no destruction of glucosamine under these conditions as the scans were indistinguishable.

The protein content of ovomucoid preparations was determined by the method of Lowry et al (1951) using ovomucoid of known moisture content as the reference standard and this is illustrated in Fig. 9. The carbohydrate content was related to this.

3. 10 (6) Trypsin Inhibiting Activity of Ovomucoid Preparations

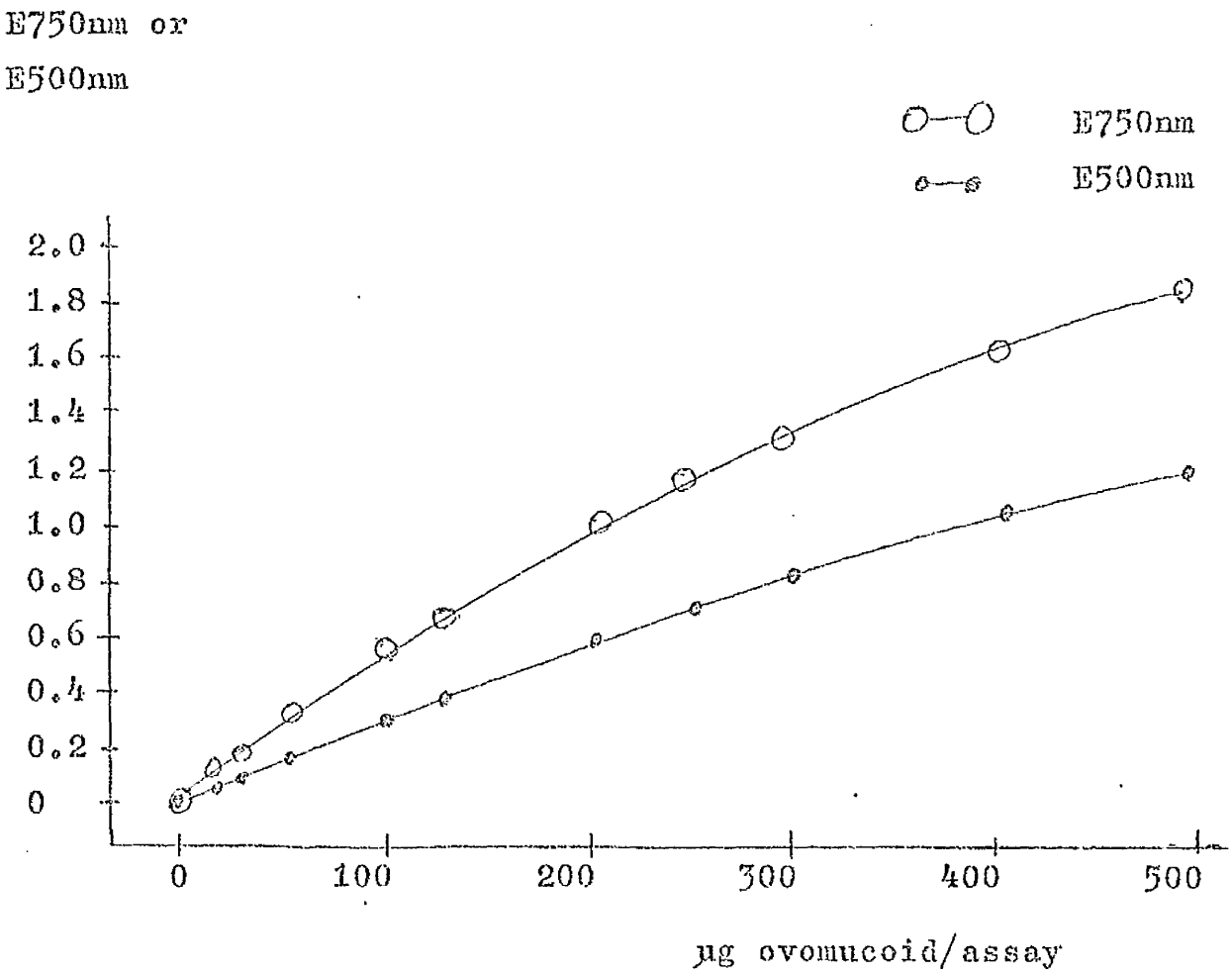
No difference could be found in the trypsin inhibiting activity of ovomucoid from egg white or oviduct isolated by either the method of Fredericq and Deutsch (1949) or by the use of G-200-trypsin. When assayed with casein as substrate it was found, in all cases, that 1µg ovomucoid inhibited 1µg trypsin. If all the trypsin was active, then taking the respective molecular weights of trypsin (23,300) and ovomucoid (27,000-30,000) into account, this might suggest that inhibition was in a 1:1 molar basis.

Fig. 8. Optimal hydrolysis conditions for the determination of glucosamine from ovomucoid



Hydrolysis of ovomucoid (0.1mg/ml) at 100°C with varying concentrations of HCl. Glucosamine was assayed by the method of Cessi and Piliego (1960). Optimal hydrolysis conditions were determined to be 4N HCl for 3 hours. There was no destruction of glucosamine under these conditions.

Fig. 9. Protein estimation by the method of Lowry et al (1951) using ovomucoid as reference standard.



Samples were assayed by the method of Lowry et al (1951) using the 0.4ml assay volume. In the 1ml assay volume ovomucoid at a concentration of 100µg/ml had an extinction (750nm) of approximately 0.22, while ovalbumin or bovine serum albumin had extinctions of approximately 0.32.

3. 11 (1) Sulphoethyl-Sephadex Chromatography

Ovomucoid, from either egg white or oviduct tissue, could be further fractionated by chromatography on columns of sulphoethyl (SE)-Sephadex C-50 as illustrated by Fig. 10. Rechromatography of the peaks labelled B, C, D and E in the same system is illustrated in Fig. 11. Peak B and peak C both rechromatographed as single peaks emerging in the expected positions. Peak D rechromatographed as a major peak emerging in the same position as previously but also had a small later emerging peak. Peak E on rechromatography had an asymmetrical profile with material overlapping with the major and minor peaks of rechromatographed component D.

The isoelectric focusing pattern of components B, C, D and E is represented by Fig. 12. Peak D and peak E corresponded to ovomucoid species O_1 on isoelectric focusing, peak C corresponded to ovomucoid O_2 and peak B to ovomucoid O_3.

The trypsin-inhibiting activities of the chromatographic components are shown in Table 7.

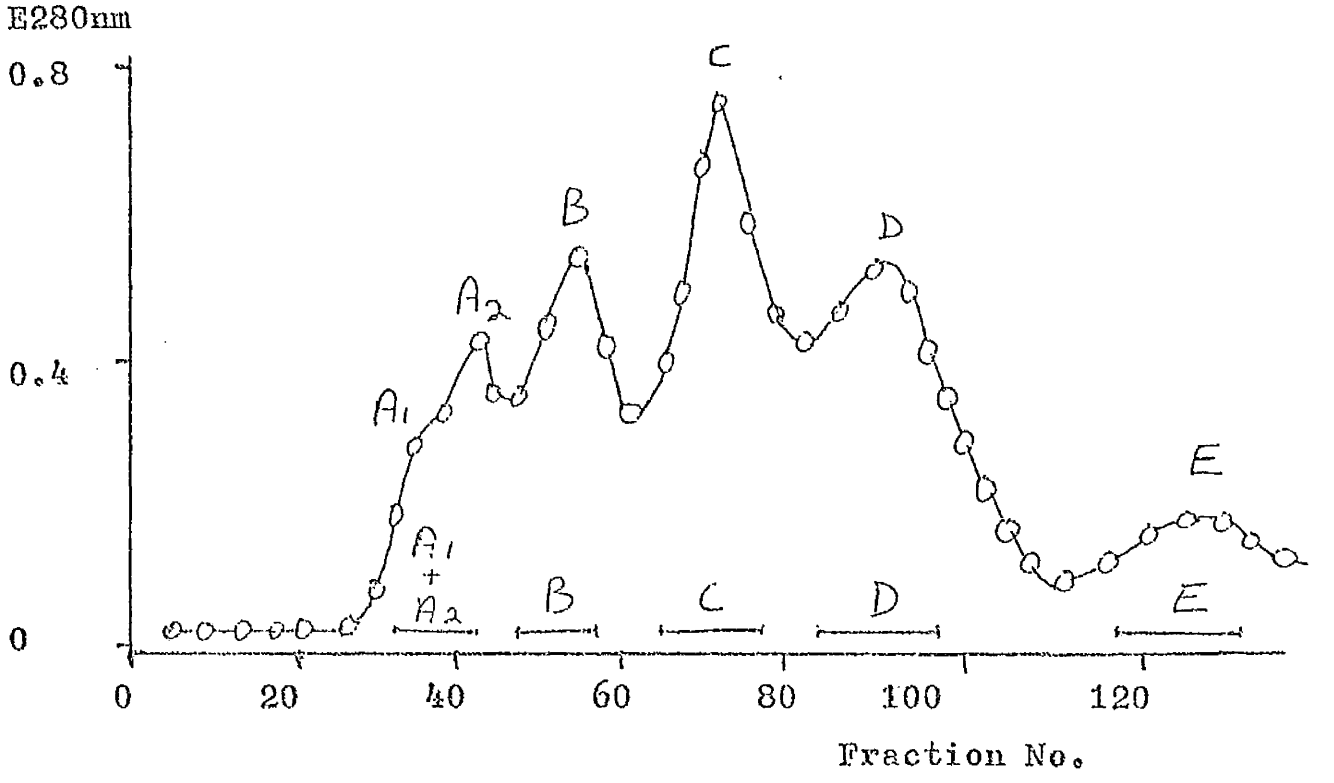
TABLE 7

Relative trypsin-inhibiting activity of ovomucoid variants resulting from SE-Sephadex C-50 chromatography

Trypsin inhibition was measured with casein as substrate, values are expressed as % inhibitory activity of fraction C.

Component	A_1+A_2	B	C	D	E
% Trypsin inhibition	95	96	100	102	102

Fractions were pooled as illustrated



Chromatography of ovomucoid on SE-Sephadex C-50 (82cm x 5cm), 40ml/hour flow rate, 15ml fractions collected, Eluant 0.014M sodium acetate, 1mM sodium azide pH 4.85. Sample was 1.2g of ovomucoid in 10ml of buffer, equilibrated by dialysis.

Fig. 11. Rechromatography of the ovomucoid species in the same system

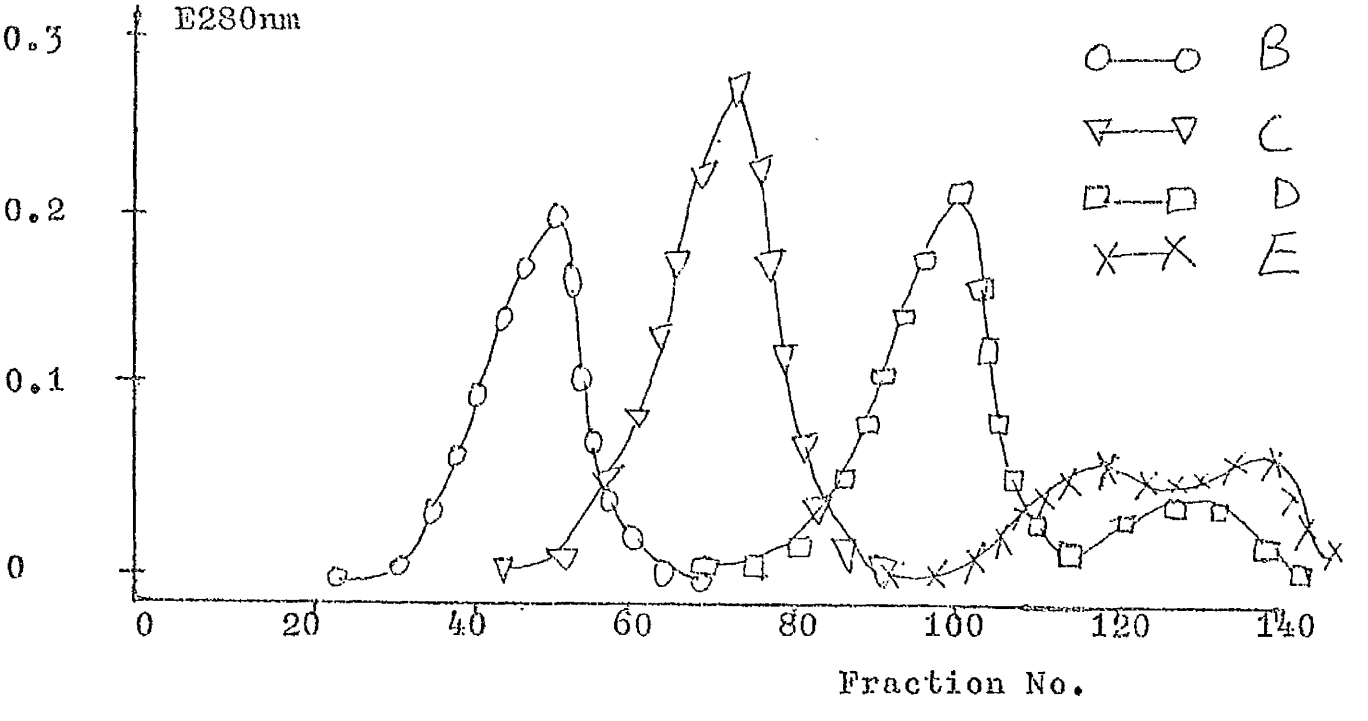
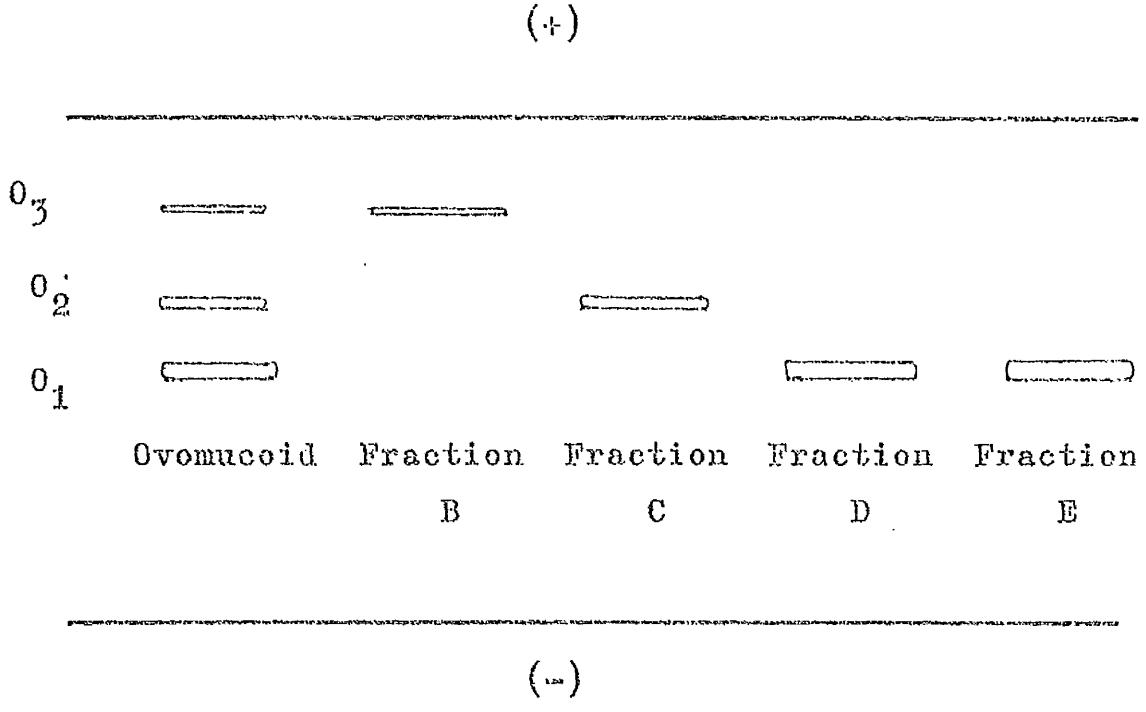


Fig. 12. Isoelectric focusing of rechromatographed peaks from SE-Sephadex C-50 chromatography



The ovomucoid variants have been labelled O_1, O_2, O_3, in order of decreasing isoelectric point (Beeley, 1971a).

It can be seen that the trypsin inhibiting activities of the species were virtually identical (Table 7).

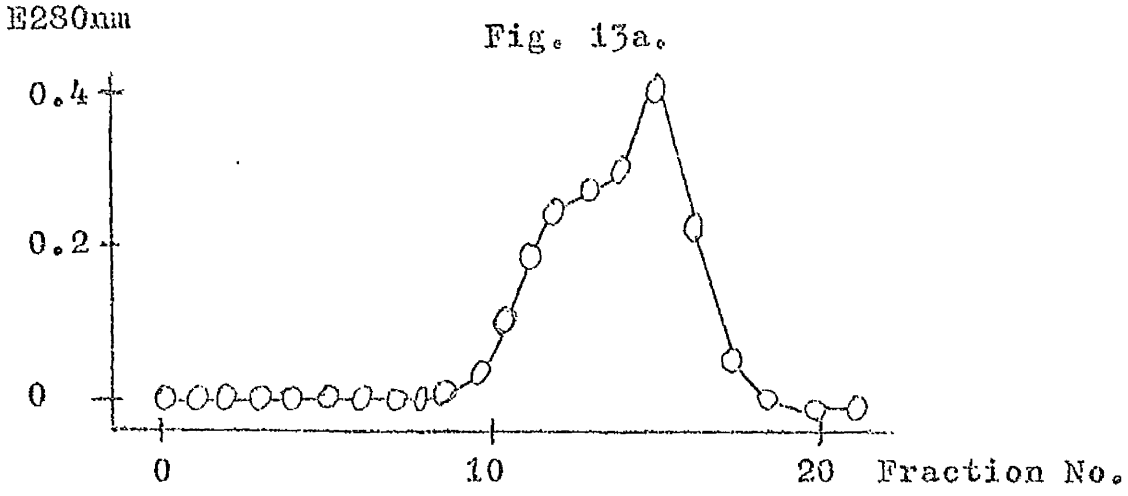
The peaks D and E (which both corresponded to the most basic ovomucoid variant O_1 on isoelectric focusing) were not completely resolved in this system. The pH of the eluting buffer in the above experiments was pH 4.85. If the pH of the eluting buffer was raised to pH 4.90, then ovomucoid, from either egg white or oviduct, could be resolved into two components (Fig. 13a). If the pH was raised above pH 4.90, e.g. pH 4.92, ovomucoid was unfractionated (Fig. 13b). On rechromatography of fractions I and II at pH 4.90, both emerged as single peaks with the expected elution volumes (Fig. 14).

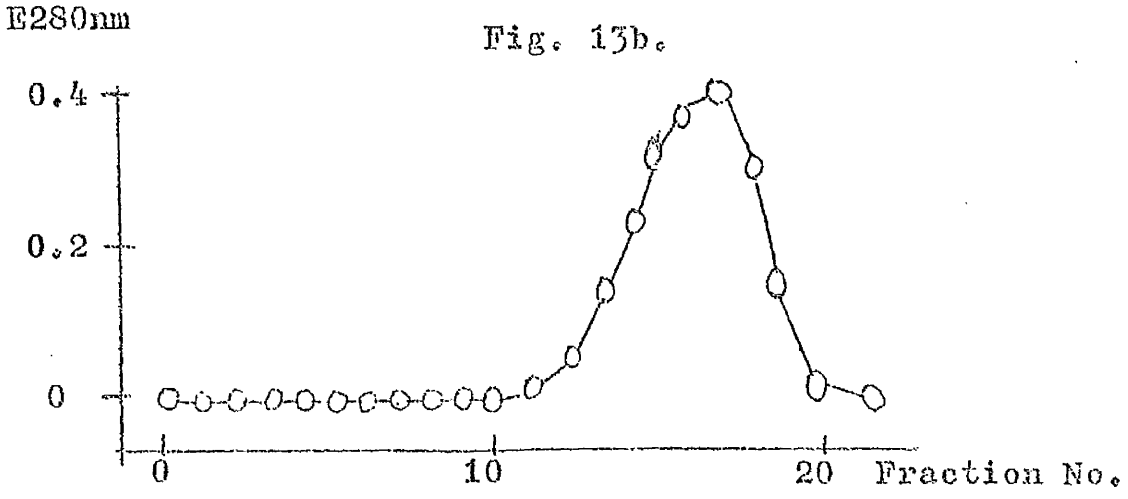
The sialic acid content of fractions I and II is illustrated in Fig. 15 and it can be seen that sialic acid content decreased with increasing elution volume. On isoelectric focusing it was found that fraction I contained ovomucoid variants O_2 to O_5, while fracton II contained the sialic acid free variant O_1.

Due to the broadening of the later peaks in the pH 4.85 chromatographic system, the pH 4.90 system, which separated the sialic acid free ovomucoid variant from the sialic acid containing variants, was found to be the more useful fractionation method.

There was no difference in elution profile in the pH 4.90 chromatographic system or in the pH 4.85 system between ovomucoids from egg white or oviduct.

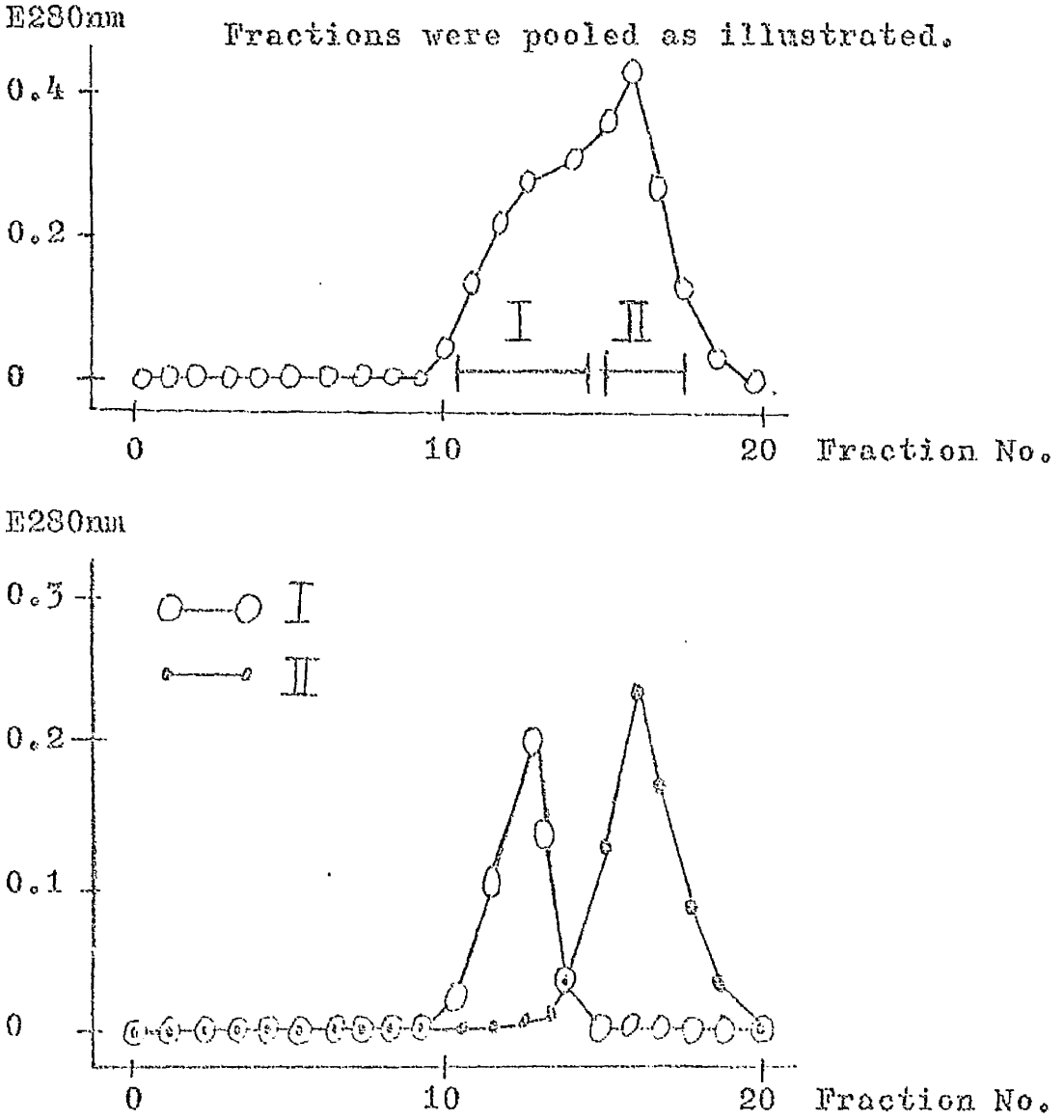
Fig. 13. SE-Sephadex C-50 chromatography of ovomucoid at pH 4.90 (Fig. 13a) and pH 4.92 (Fig. 13b)





Chromatography of ovomucoid on columns (90cm x 1.5cm) of SE-Sephadex C-50. Flow rate was 5.44ml/hour, 3.4ml fractions collected. Sample was 15mg of ovomucoid in 1ml eluting buffer, pH 4.90 (Fig. 13a) or pH 4.92 (Fig. 13b). Eluant 0.014M sodium acetate containing 1mM sodium azide.

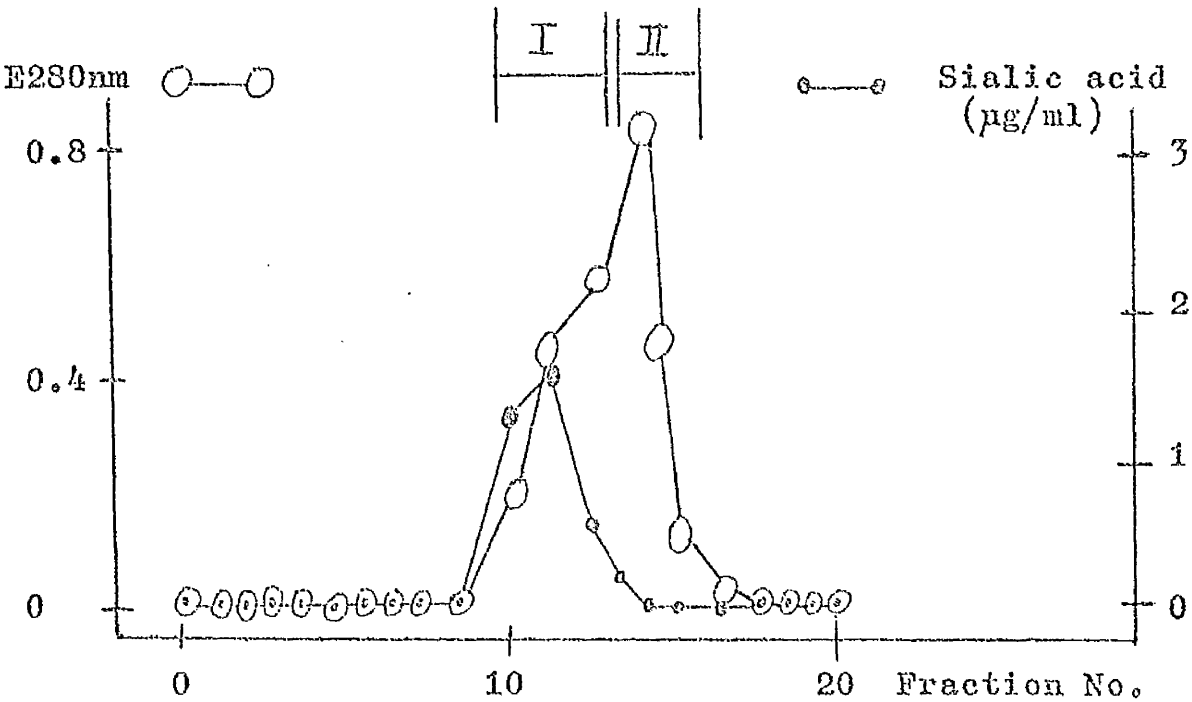
Fig. 14. Rechromatography of the ovomucoid species fractionated by SE-Sephadex C-50 chromatography at pH 4.90



Upper graph : SE-Sephadex chromatography of ovomucoid (20mg) under the same conditions as in Fig. 13a.

Lower graph : rechromatography of the fractions I and II (pooled as illustrated) in the same system.

Fig. 15. The sialic acid content of ovomucoid species fractionated by SE-Sephadex chromatography



Ovomucoid(30mg) was fractionated by SE-Sephadex chromatography at pH 4.90 under the same conditions as described in Fig. 13a and sialic acid content determined by the method of Aminoff (1961).

Fraction No.	Sialic acid(g/100g of ovomucoid)
10	2.6
11	1.6
12	0.6
13	0.06
14	0
15	0

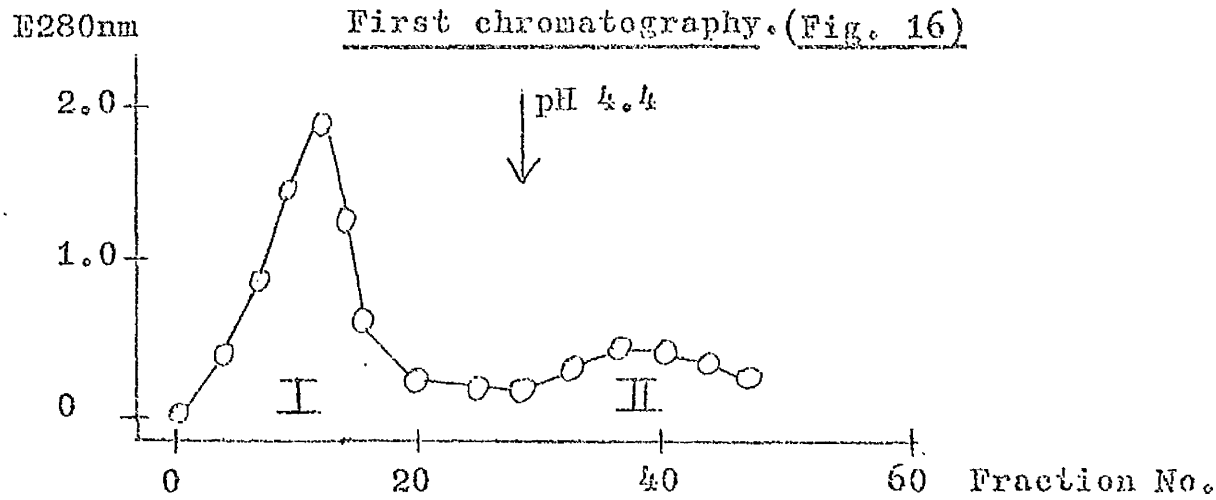
On isoelectric focusing fraction I corresponded to the sialic acid containing variants(O_2-O_5) while fraction II corresponded to the sialic acid free ovomucoid O_1.

3. 11 (2) Carboxymethyl Cellulose Chromatography

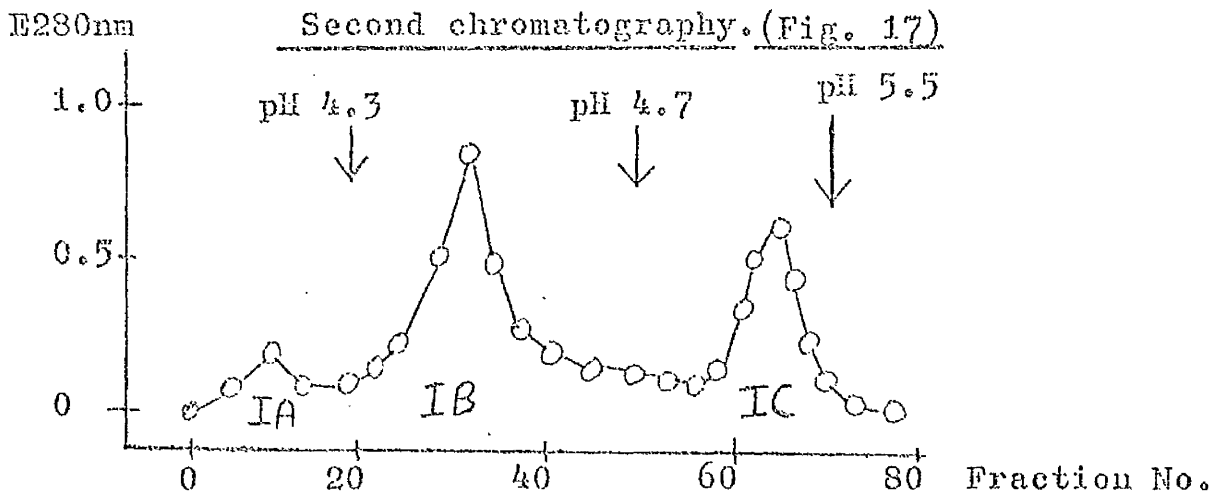
Blended egg white can be fractionated on columns of carboxymethyl cellulose (Rhodes, Azari & Feeney, 1958). An example of the separation of ovomucoid from other egg white proteins is given in Fig. 16. Fraction I from this preliminary chromatography when chromatographed in a second system (Rhodes et al, 1958) gave rise to three components (Fig. 17). As this two-stage chromatography gave the same elution pattern as that of Rhodes et al (1958), it was attempted to isolate ovomucoid from oviduct homogenate by this method. The elution profile of fractionated oviduct homogenate, equilibrated by dialysis, was the same as that for egg white illustrated in Figs. 16 and 17.

The peaks IA, IB and IC (Fig. 17) from the second chromatographic step in the fractionation of ovomucoid from oviduct homogenate were collected for assay of trypsin inhibition. The results of the trypsin inhibition studies are presented in Fig. 18. Fraction IA showed almost no trypsin-inhibiting activity. Fractions IB and IC both had about half the activity towards trypsin shown by stock ovomucoid. By reference to Rhodes et al (1958), it would be expected that fraction IA corresponded to riboflavin, fraction IB to ovomucoid and fraction IC to apoflavoprotein. In view of the low trypsin-inhibiting activity of ovomucoid isolated by carboxymethyl cellulose chromatography, this method of isolation of ovomucoid was discontinued. It is not clear whether fractions IB and IC were both a mixture of components, such as ovomucoid and apoflavoprotein, or whether there had been some denaturation of ovomucoid during the isolation. Either of these alternatives could explain the low trypsin-inhibiting activity found.

Figs. 16 & 17. Isolation of ovomucoid from egg white by carboxymethyl cellulose chromatography

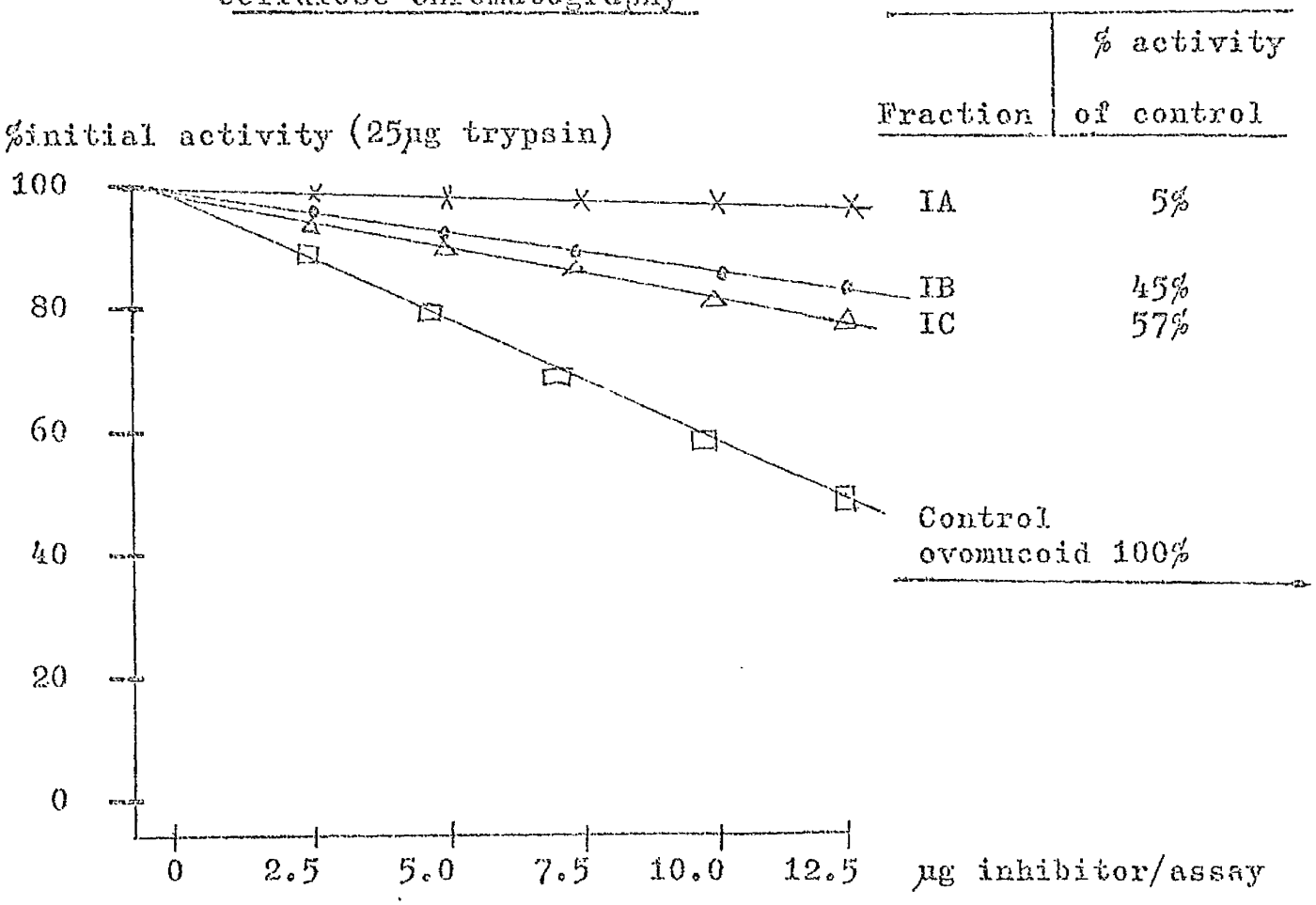


Initial fractionation of egg white on a column(16.5 x 2.5cm) of CM 23. Eluant was 0.1M acetic acid brought to pH 4.3 with NH_4OH, pH changed to pH 4.4 at position shown by arrow. Sample was 30ml blended egg white dialysed for 2 days against the starting buffer. Flow rate was 150ml/hour and 15ml fractions were collected. Fraction I was chromatographed in a second system described below.



Peak I from the first step was dialysed overnight against 0.025M sodium acetate pH 3.7 and fractionated on CM 23. The arrows indicate the pH and point of change of the buffer.

Fig. 18. The trypsin inhibiting activities of fractions isolated from oviduct homogenate by carboxymethyl cellulose chromatography



An oviduct homogenate was fractionated by carboxymethyl cellulose chromatography (Rhodes et al, 1958). The trypsin inhibiting activities of the fractions (Fig. 17) was measured using casein as substrate in the assay of Northrop et al (1948). The activity is expressed as a percent of that of ovomucoid isolated from oviduct homogenate by use of G-200-trypsin. The activity of oviduct ovomucoid isolated this way was the same as that of egg white or oviduct ovomucoid isolated by the method of Fredericq and Deutsch (1949).

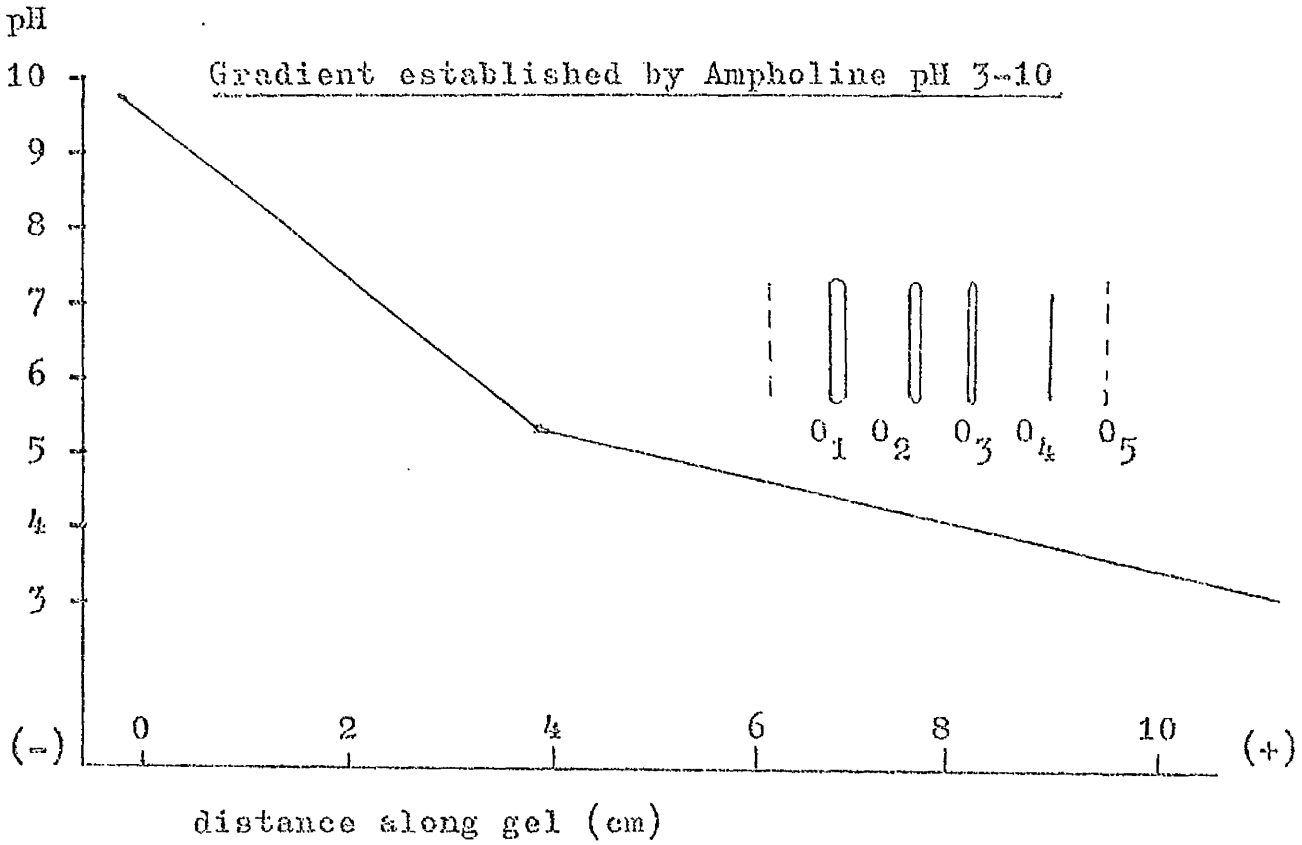
3. 12 (1) Isoelectric Points of the Ovomucoid Variants.

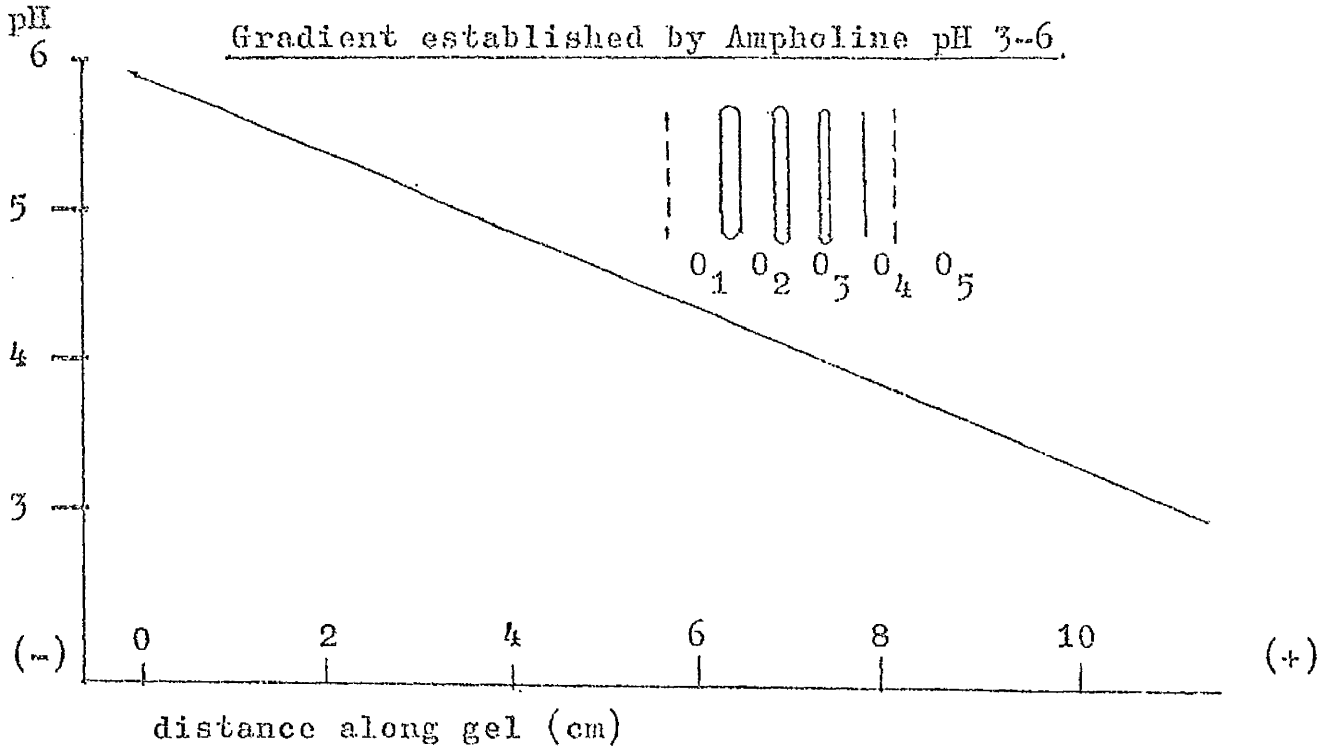
Isoelectric focusing is a separation method based on establishing a pH gradient in which a protein migrates to a point corresponding to its isoelectric point. The isoelectric points of the ovomucoid variants were determined by measuring the pH profile of the gradient established by Ampholine and reading off the pH at the points corresponding to the ovomucoid species O_1 to O_5. This is illustrated by Fig. 19. Ovomucoid variants O_4 and O_5 did not stain well as they were present in much smaller proportions than O_1, O_2 or O_3.

The isoelectric points of the major ovomucoid variants were determined to be; O_1, 4.45; O_2, 4.30; O_3, 4.15. From the values found in several experiments, these readings were $\pm$ 0.2pH unit. The isoelectric point of ovomucoid O_4 was estimated to be 4.0 and the isoelectric point of O_5 would have been less than this although it was not determined directly because of difficulties with staining. It was not possible to distinguish the isoelectric points of the ovomucoid variants derived from egg white from those derived from oviduct.

In the experiment represented by Fig. 19 the ovomucoid was a "crude" preparation isolated by the method of Fredericq and Deutsch (1949). The minor component with an isoelectric point higher than that of the most basic ovomucoid species, O_1, was sometimes present in ovomucoid isolated by this method. The isoelectric point of this component is approximately 4.6 and it focuses in the same position as ovalbumin. It is possible that this represents a trace of ovalbumin contamination of this

Fig. 19. Isoelectric points of the ovomucoid variants





The pH was determined as described in the text. The positions of the ovomucoid species are shown. The ovomucoid used was a crude preparation from egg white which contains a contaminant more basic than O_1.

Fig. 20. Refocusing of the ovomucoid variants

(+)

(a) (b) (c) (d) (e) (f) (g) (h)

(-)

After isoelectric focusing in a first gel, the location of the ovomucoid variants was determined by staining a reference standard of a crude preparation of ovomucoid focused at the same time. Pieces of gel were cut out, applied to a second gel and refocused under the same conditions. (a) Ovomucoid O_1. (b) Ovomucoid O_2. (c) Ovomucoid prepared by the method of Fredericq and Deutsch (1949). (d) Portion of the original gel containing ovomucoid prepared by the method of Fredericq and Deutsch (1949). (e) Ovomucoid O_3. (f) Ovomucoid O_4. (g) Ovomucoid O_5. (h) Ovomucoid prepared as in sample (c). The ovomucoid variants have been labelled O_1-O_5 in order of decreasing isoelectric point (Beeley, 1971a).

crude preparation of ovomucoid. When ovomucoid was isolated by use of G-200-trypsin following a trichloroacetic acid precipitation step, the basic component was eliminated, and the isoelectric points of the ovomucoid variants were found to be indistinguishable from those given above.

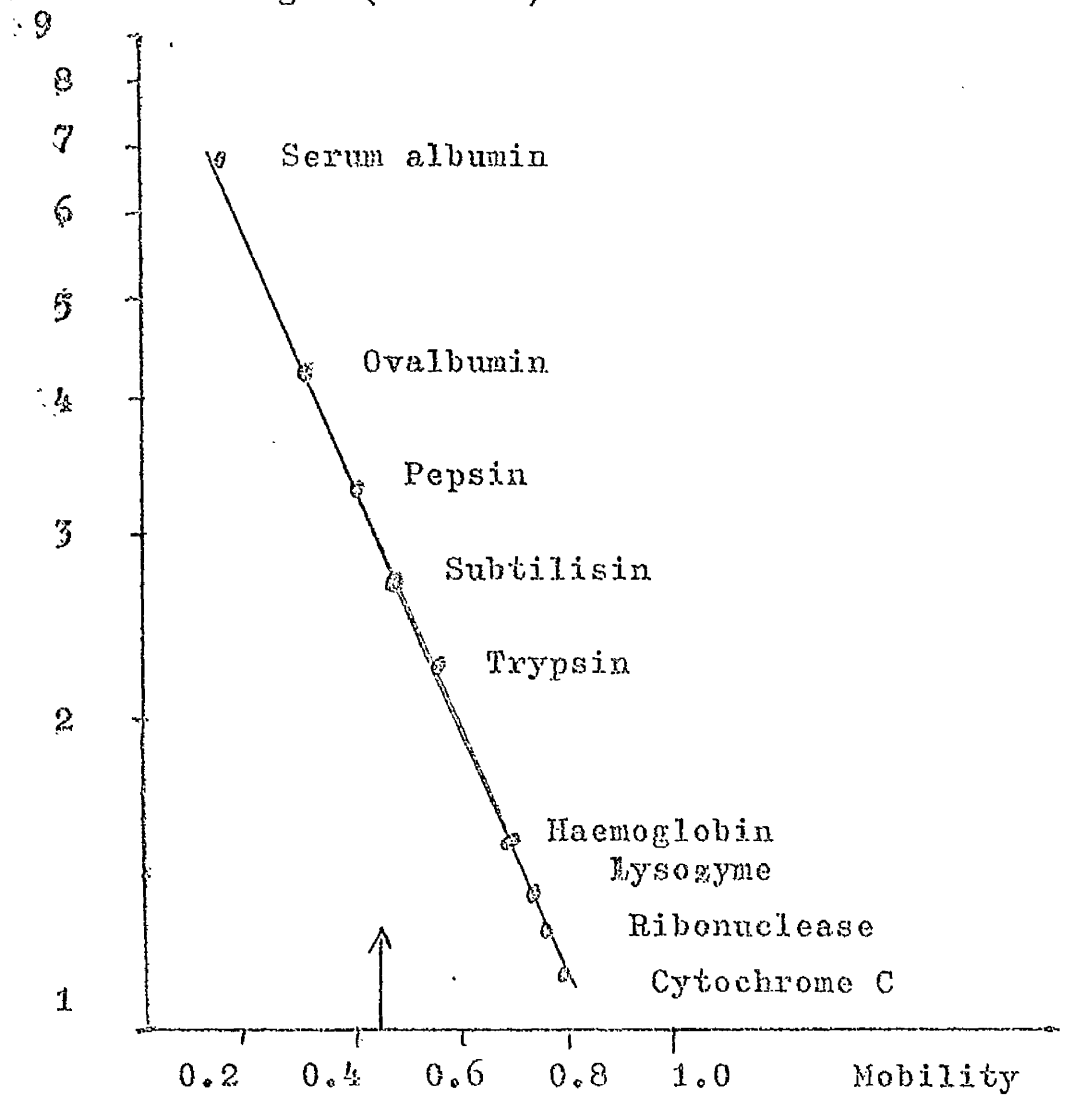
If a piece of gel corresponding to one of the ovomucoid variants was cut out and applied to a second gel, then under the same conditions of focusing, one protein band with the same isoelectric point as previously was found (Fig. 20). As this was true of all the ovomucoid variants (neglecting trace contamination of a neighbouring species) it illustrates that the multiplicity of bands found on isoelectric focusing is not an artifact of the focusing procedure and suggests that there is a genuine charge difference between the ovomucoid variants.

3. 12 (2) Molecular Weight Estimation by Sodium Dodecyl Sulphate Gel Electrophoresis

The estimation of molecular weights of proteins by electrophoresis in polyacrylamide gels in the presence of the detergent sodium dodecyl sulphate (SDS) was reported to be quick, easily performable and accurate to within $\pm$ 10% (Weber & Osborn, 1969). The molecular weight and homogeneity of ovomucoid preparations were checked by this technique.

The molecular weight of egg white and oviduct ovomucoid was calculated to be 30,000 $\pm$ 2,500 (Fig. 21). When ovomucoid was isolated by adsorbtion on and elution from G-200-trypsin, following a preliminary trichloroacetic acid precipitation step, no trace of any additional protein component could be found, indicating homogeneity. When ovomucoid

Fig. 21. Molecular weight of ovomucoid estimated by Sodium dodecyl sulphate gel electrophoresis



The electrophoretic mobilities of the marker proteins are shown as a linear function of their respective molecular weights (logarithm). The arrow indicates the mobility of ovomucoid from which the molecular weight was calculated to be 30,000 ± 2,500.

was further fractionated by chromatography on columns of SE-Sephadex C-50, the molecular weights of the charged variants were indistinguishable from each other or from unfractionated ovomucoid.

The above results have shown that ovomucoid may be isolated from egg white or oviduct homogenates by use of an insolubilised trypsin derivative. Egg white ovomucoid and oviduct ovomucoid have identical trypsin-inhibiting activities and their hexose, hexosamine and sialic acid content are indistinguishable. The isoelectric focusing patterns of ovomucoids from both sources resemble each other as do the elution profiles on SE-Sephadex C-50 chromatography. The molecular weights of ovomucoid from both sources are indistinguishable. Furthermore, ovomucoid isolated by use of G-200-trypsin resembles ovomucoid isolated by the method of Fredericq and Deutsch (1949) by all the above criteria. However, the sensitive techniques of immunodiffusion and immunoelectrophoresis suggest that ovomucoid isolated by use of G-200-trypsin is immunologically pure, while ovomucoid isolated by ethanol precipitation, following a preliminary trichloroacetic acid precipitation step, contains more than one antigenic species. The major contaminant of ovomucoid prepared by ethanol precipitation seems to be ovoglycoprotein, although traces of ovalbumin may also be present. In view of this, isolation of ovomucoid by adsorbtion on and elution from G-200-trypsin, following a preliminary trichloroacetic acid precipitation stage, would be the preferred method. There was no evidence of chymotrypsin inhibiting activity present in ovomucoid isolated by this method, indicating the absence of ovoinhibitor.

The previous section described methods of isolation of ovomucoid and illustrated that ovomucoid could be isolated from oviduct and that ovomucoid from this source had the same properties as egg white ovomucoid.

In early biosynthetic studies a further appraisal of the methods of isolation of ovomucoid was made by comparing the specific radioactivities of ovomucoid isolated by the method of Fredericq and Deutsch (1949) and by the use of G-200-trypsin. Radiological purity of ovomucoid isolated by these techniques was examined by determining the specific radioactivity of ovomucoid after additional purification steps had been included in the isolation methods.

Methods of preparation of oviduct tissue for in vitro biosynthetic studies and the nature of the biosynthetic medium will be discussed as will the methods of homogenisation of the labelled tissue.

The time course of incorporation of glucosamine and lysine into "intracellular" and "extracellular" ovomucoid was followed and compared to the incorporation of glucosamine and lysine into the total, trichloroacetic acid precipitable, protein intracellularly and extracellularly. An examination was made of the possibility that ovomucoid synthesis or secretion might be stimulated in vitro by the addition of hormones to the incubation medium.

The presence of possible precursors to soluble intracellular ovomucoid was examined by extracting the pellet which remained after centrifugation of oviduct homogenates with deoxycholate. Attempts were also made to fractionate oviduct into subcellular components by centri-

fugation in sucrose density gradients.

In order to investigate the temporal relationship between the biosynthesis of the peptide and carbohydrate portions of ovomucoid, tissue incubations were carried out in the presence of inhibitors of protein synthesis.

Labelled ovomucoid was fractionated by chromatography on sulphoethyl-Sephadex C-50 and the specific radioactivities of the ovomucoid variants compared. The possible relationship of these observations to the microheterogeneity of ovomucoid is discussed.

3. 13 (1) Methods of Preparation of Tissue for Incubation Studies

As the method of preparation of the tissue might affect its ability to incorporate precursors into ovomucoid, the oviduct was prepared for *in vitro* studies as follows: (1) the tissue was finely chopped with scissors, (2) the tissue was put once through a mincing machine, (3) a section of tissue was stretched over a glass ring so that both sides of the tissue were bathed in incubation medium or (4) a piece of tissue was turned inside out as a sac.

The resultant specific radioactivities of ovomucoid isolated from the tissue after a 2 hour incubation period with [^{14}C]. lysine are shown in Table 8. It can be seen that compared to chopping the tissue with scissors (Hendler, 1956) mincing the tissue with a mincer has greatly reduced the incorporation of lysine into ovomucoid. Stretching the tissue or turning it inside out as a sac have resulted in ovomucoid with a higher specific radioactivity. However, as protein content may vary along the magnum with the position of an egg in the oviduct (Wyburn

Table 8. Methods of preparation of tissue for incubation studies.

Method of preparation of tissue	Specific radioactivity d.p.m./mg of ovomucoid
(1)Finely chopped with scissors	520
(2)Minced in mincer	147
(3)Stretched over glass ring	2030
(4)Turned inside out as sac	1220

Oviduct portions (3g) were incubated in 7.5ml of the medium of Hendler (1956; 1957) supplemented with antibiotics (Carey, 1966). Incubation was for 2 hours with [^{14}C] lysine (1μCi/g of oviduct). Ovomucoid was isolated from the intracellular fraction using G-200-trypsin after homogenisation of the tissue in 0.44M sucrose-TKM buffer, pH 7.5, in a Dounce homogeniser.

et al, 1970), in studies where several pieces of tissue were required, it was considered to be preferable to chop the tissue with scissors as this would distribute the tissue more evenly between different samples. In the following incorporation experiments the oviduct was finely chopped with scissors prior to incubation.

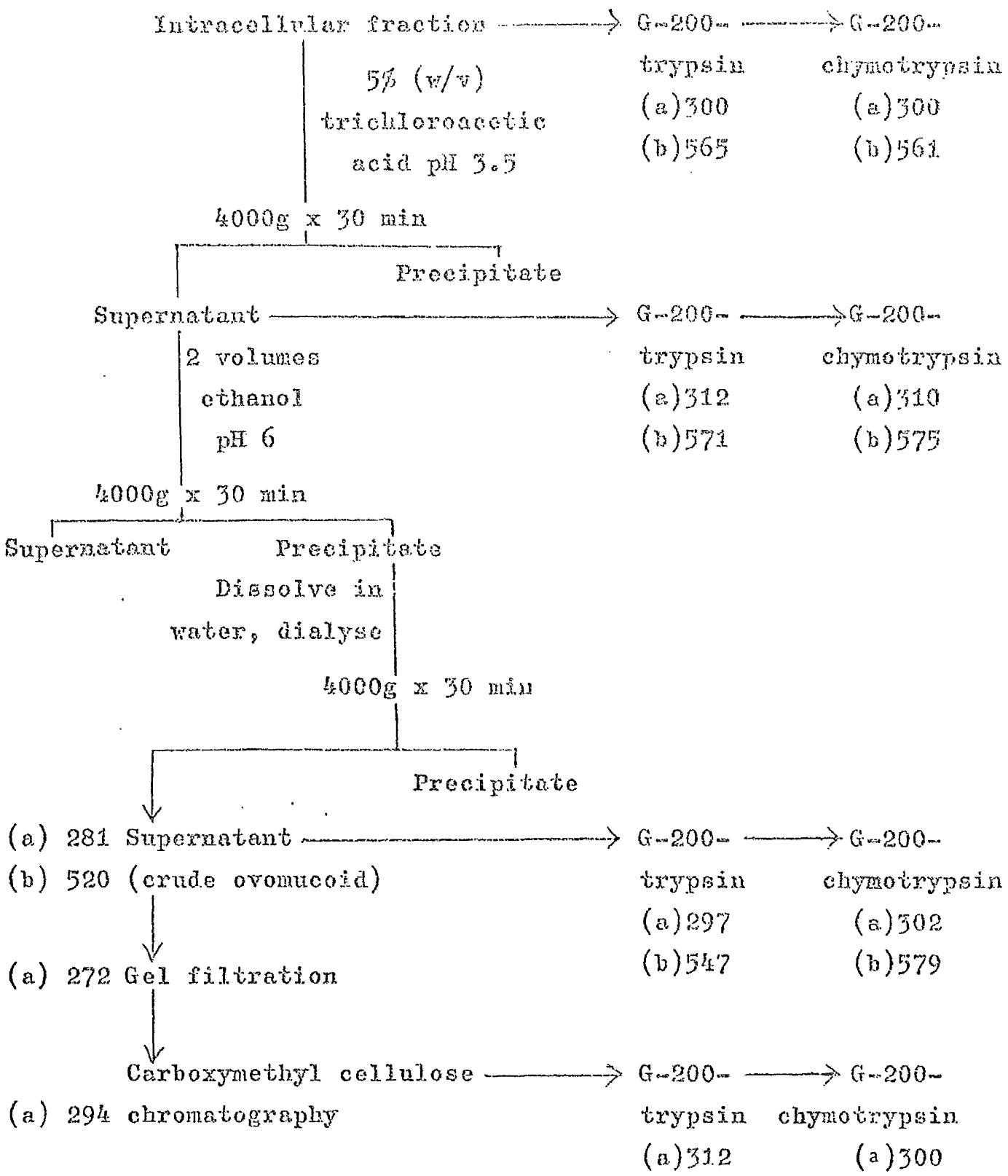
3. 13 (2) Radiological Purity of Isolated Ovomucoid, and Comparison of Isolation Methods

After incubation of oviduct with [^{14}C] lysine for 2 hours, ovomucoid was isolated from the 105,000g supernatant of oviduct homogenate by the method of Fredericq and Deutsch (1949) or by adsorbtion on and elution from G-200-trypsin. In Fig. 22 the specific radioactivities of ovomucoid from two separate experiments are shown along with the specific radioactivities after additional purification steps. It can be seen that when ovomucoid prepared by ethanol precipitation was further purified by gel filtration and carboxymethyl cellulose chromatography, the specific radioactivity of the ovomucoid was essentially unaltered. The specific radioactivity of ovomucoid isolated by use of G-200-trypsin was similar to that of ovomucoid prepared by ethanol precipitation. This was true no matter at what stage in the purification G-200-trypsin was used to isolate ovomucoid. In Fig. 22 it can be seen that G-200-trypsin was used to isolate ovomucoid prior to trichloroacetic acid precipitation, following trichloroacetic acid precipitation, following ethanol precipitation and after the chromatographic steps in the purification of ovomucoid from oviduct homogenate and in all cases the specific radioactivities were comparable.

Fig. 22. Radiological purity of ovomucoid (1)

Whole oviducts(40g) were incubated in 100ml of the medium of Hendler (1956;1957) supplemented with antibiotics (Carey, 1966) for 2 hours with [^{14}C]lysine, 1μCi/g of oviduct as precursor. Ovomucoid was isolated from the supernatant resulting from centrifugation (105,000g x 60 min) of oviduct homogenate by the methods shown. The numbers refer to the specific radioactivities (d.p.m./mg) of ovomucoid at each stage in the isolation. In Fig. 22(a) the oviduct was homogenised in fresh incubation medium in a Potter-Elvehjem homogeniser. In Fig. 22(b) the oviduct was homogenised in 0.44M sucrose-TKM buffer, pH 7.5 in a Dounce homogeniser. The material which adsorbed on G-200-trypsin at pH 7 and eluted at pH 1.5 was treated with G-200-chymotrypsin, collecting the non-adsorbed material. A portion of ovomucoid isolated by ethanol precipitation (Fredericq & Deutsch, 1949) was passed through columns of Sephadex G 75 and carboxy-methyl cellulose CM 23 as described in the text.

(105,000g x 60 min supernatant)



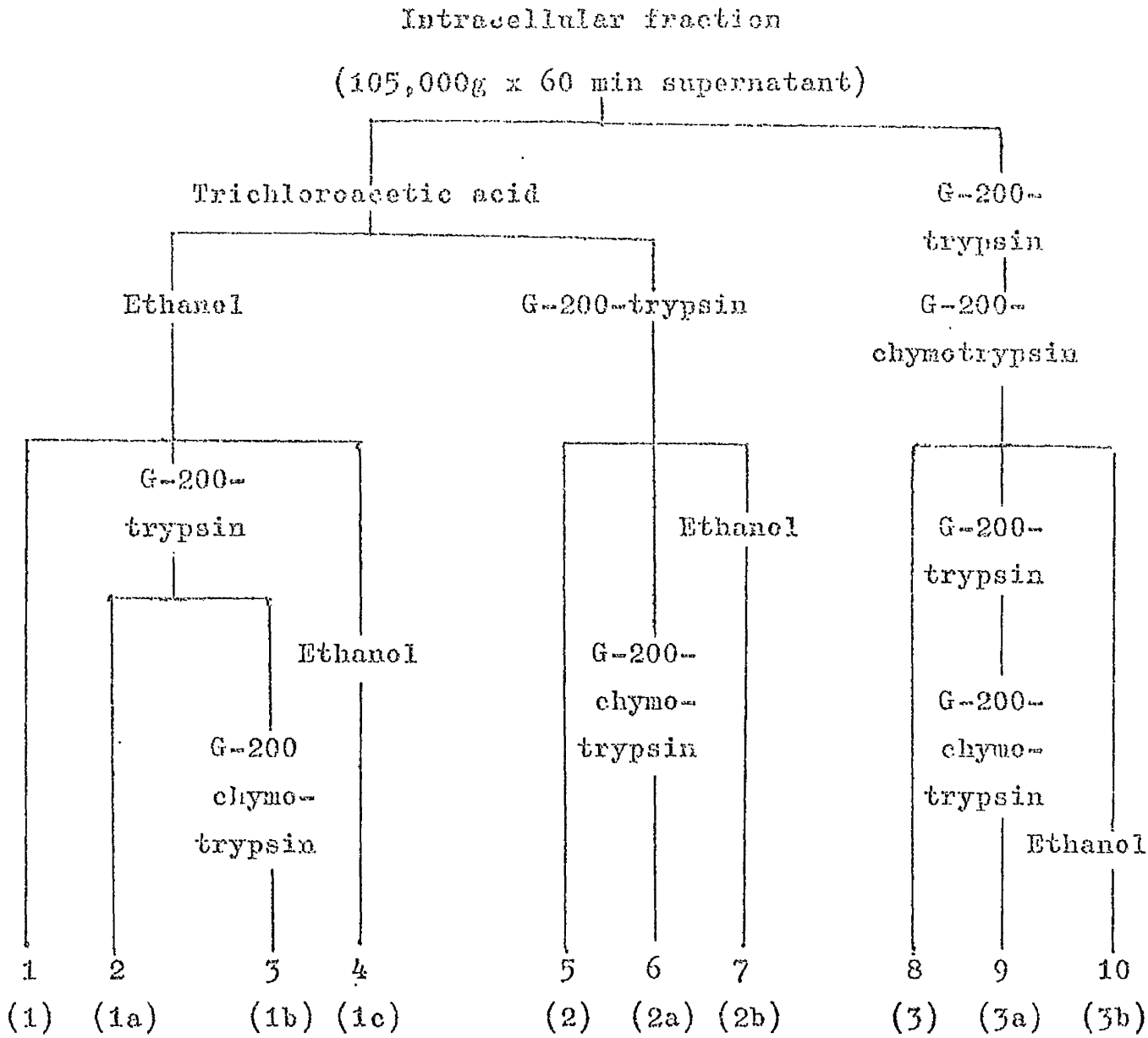
When ovomucoid isolated by use of G-200-trypsin was passed over G-200-chymotrypsin and the non-adsorbed material collected, there was essentially no change in the specific radioactivity of ovomucoid. This particular step would be expected to remove ovoinhibitor by adsorbtion on G-200-chymotrypsin, especially in the case of ovomucoid isolated by use of G-200-trypsin without a preliminary trichloroacetic acid precipitation stage.

This observed lack of change in specific radioactivity of ovomucoid when additional purification steps were included in the isolation of ovomucoid from oviduct was further investigated in the experiment represented by Fig. 23. In this case the order of the purification steps, ethanol precipitation, adsorbtion on and elution from G-200-trypsin and the coupling of G-200-trypsin to G-200-chymotrypsin in the isolation of ovomucoid from oviduct homogenate was varied. The results again suggested that ovomucoid prepared by ethanol precipitation had a similar specific radioactivity to ovomucoid isolated by use of G-200-trypsin, and that on additional purification steps the specific radioactivity of ovomucoid was virtually unchanged. It was also indicated that ovomucoid may be isolated directly from the 105,000g supernatant of oviduct homogenate by use of G-200-trypsin without a preliminary trichloroacetic acid precipitation step.

On comparing the yields of ovomucoid isolated by the various methods (Fig. 23), it can be seen that the amount of ovomucoid isolated in sample (2b), i.e. ovomucoid prepared by ethanol precipitation of ovomucoid isolated by use of G-200-trypsin following a preliminary trichloro-

Fig. 23. Radiological purity of ovomucoid (2)

A whole oviduct (30g) was incubated in 75ml of the medium of Hendler (1956;1957) supplemented with antibiotics (Carey, 1966) for two hours with [^{14}C] lysine, 1μCi/g of oviduct as precursor. Ovomucoid was isolated from the intracellular fraction of oviduct homogenate by the methods shown. The tissue was homogenised in a Potter-Elvehjem homogeniser in fresh incubation medium. Trichloroacetic acid precipitation was at 5% (w/v), pH 3.5, the supernatant collected after centrifugation (4000g x 30 minutes). Precipitation of ovomucoid was achieved by adding 2 volumes of ethanol to a solution of ovomucoid at pH 6 (Fredericq & Deutsch, 1949). The material which adsorbed on G-200-trypsin at pH 7 and eluted at pH 1.5 was treated with G-200-chymotrypsin, collecting the non-adsorbed material. The specific radioactivity (d.p.m./mg) of ovomucoid is shown along with the yield of ovomucoid (mg/g of tissue) for each isolation method.



Sample

1	1a	1b	1c	2	2a	2b	3	3a	3b
d.p.m./mg of ovomucoid									
1798	1740	1499	1791	1785	1750	1440	1470	1511	1532
mg of ovomucoid/g of oviduct									
1.94	1.87	1.70	1.72	1.55	1.12	0.24	1.51	1.53	1.50

acetic acid precipitation stage, is much lower than the yields of ovomucoid isolated in all the other samples. Difficulty was frequently found in precipitating small quantities of ovomucoid with ethanol. As the ovomucoid isolated by the methods shown in Fig. 23 was split equally among all the fractions, the yield should have been the same in all the samples. The normal range of ovomucoid isolated from oviduct homogenates was 1.5-7mg ovomucoid/g (wet weight) tissue.

There is a large volume increase in the isolation of ovomucoid by the method of Fredericq and Deutsch (1949), e.g. adding an equal volume of 10% (w/v) trichloroacetic acid then 2 volumes of ethanol produced a 6 fold increase in volume of the sample. In order to counteract this, 0.1 volume of 55% (w/v)trichloroacetic acid was used to give the correct final concentration of trichloroacetic acid, but some difficulty was still found on attempting to precipitate small quantities of ovomucoid with ethanol.

The three experiments represented by Figs. 22 and 23 were performed with three separate oviducts. Although the incubation conditions in the experiments were the same, the resultant specific radioactivities of ovomucoid from each experiment were different. This illustrates the difficulty in interpreting and comparing the results from experiments performed on different oviducts.

In the experiment illustrated by Table 9, portions of oviduct were incubated with $[^{14}C]$ lysine or $[^{14}C]$ glucosamine for varying lengths of time and the specific radioactivities of ovomucoid isolated by ethanol precipitation and by use of G-200-trypsin coupled to G-200-chymotrypsin

Table 9. Comparison of the specific radioactivities of ovomucoid isolated from the tissue at different times by two isolation methods.

Oviduct portions (3g) were incubated in 7.5ml of the medium of Hendler (1956; 1957) supplemented with antibiotics (Carey, 1966). Precursors were [^{14}C] lysine (1μCi/g of tissue) or [^{14}C] glucosamine (1μCi/g of tissue). The tissue portions were homogenised in a Potter-Elvehjem homogeniser in fresh incubation salts (Hendler, 1956; 1957) and after centrifugation (105,000g for 60 minutes) the supernatant was used to isolate intracellular ovomucoid by the methods described. In one incubation with lysine, puromycin (100μg/ml) was added to the medium.

Column (A) represents ovomucoid isolated by the method of Fredericq and Deutsch (1949). Column (B) represents ovomucoid isolated using G-200-trypsin (without a preliminary trichloroacetic acid precipitation step) then treated with G-200-chymotrypsin, keeping the non-adsorbed material.

Isotope	Incubation time (hours)	Specific radioactivity d.p.m./mg of ovomucoid	
		(A)	(B)
Lysine	0	0	0
Lysine	0.4	94	80
Lysine	0.7	200	175
Lysine	5	1250	1200
Lysine (+Puromycin)	5	62	0
Lysine	16	560	480
Glucosamine	0	0	0
Glucosamine	5	1000	920
Glucosamine	16	1200	878
Glucosamine	18	1004	700

compared. It can be seen that the specific radioactivities of ovomucoid isolated by either method were similar for any one incubation time, both for lysine labelled material and glucosamine labelled material. The specific radioactivity of lysine labelled ovomucoid increased up to 5 hours, but showed no further increase at 16 hours. Likewise, the specific radioactivity of glucosamine labelled ovomucoid was similar at 16 or 18 hours to that of ovomucoid isolated after a 5 hour incubation period.

Incubation in the presence of puromycin (100µg/ml) has caused at least 95% inhibition of the incorporation of lysine into ovomucoid after 5 hours.

This experiment as well as suggesting that lysine and glucosamine are incorporated into ovomucoid, forms the basis for a more detailed study of the time course of incorporation of precursors into ovomucoid.

The ovomucoid from one part of this experiment had been isolated using G-200-trypsin, without a preliminary trichloroacetic acid precipitation stage, but further purified by passing over G-200-chymotrypsin and collecting the non-adsorbed material. Any material which did adsorb on G-200-chymotrypsin was also collected, by washing with HCl, pH 1.5, and its radioactivity determined to check on the presence of the protein, ovoinhibitor. No radioactivity above blank, 30c.p.m., was found in the pH 1.5 HCl washes from G-200-chymotrypsin. As ovoinhibitor is a minor component of egg white, it may have been present in low proportions.

Although it has been shown that ovomucoid could be isolated from the 105,000g supernatant of oviduct homogenate without a preliminary trichloroacetic acid precipitation, the high speed supernatant contains

a substantial amount of non-ovomucoid protein. In early studies, it was found that some ovomucoid could be adsorbed on G-200-chymotrypsin and some material corresponding to ovalbumin on isoelectric focusing could be adsorbed on G-200-trypsin if the 0.5M NaCl washes were omitted from the washing procedure. It was considered to be preferable, therefore, to remove the bulk of the high molecular weight contaminants by including this preliminary trichloroacetic acid precipitation stage in the isolation of ovomucoid.

As the specific radioactivity of ovomucoid isolated by adsorbtion on and elution from G-200-trypsin is similar to that of ovomucoid isolated by the method of Fredericq and Deutsch (1949), either method could be used in the isolation of labelled ovomucoid from oviduct. However, as the ethanol precipitation method was found to be unsuitable for isolating small quantities of ovomucoid and the G-200-trypsin isolation method resulted in ovomucoid which was immunologically homogenous (Figs. 4-7), in all further studies, ovomucoid was isolated from oviduct homogenates by adsorbtion on and elution from G-200-trypsin following a preliminary trichloroacetic acid precipitation step.

As there may be some non-incorporated lysine or glucosamine associated with labelled ovomucoid isolated from oviduct, an investigation was made into the effectiveness of dialysis in removing any radioactive lysine or glucosamine from ovomucoid. In Table 10 the d.p.m. associated with unlabelled ovomucoid which had [^{14}C] lysine or [^{14}C] glucosamine added to it is shown after purification steps to remove the free lysine or glucosamine were performed. It can be seen that

Table 10. The effectiveness of procedures for removing non-incorporated lysine or glucosamine from ovomucoid

Isotope	Procedure for removing isotope	d.p.m. recovered	% removed
Glucosamine	Dialysis at	82	100
Lysine	pH 4.6	90	100
Glucosamine	Dialysis against	410	99.98
Lysine	distilled water	503	99.98
Glucosamine	Passage through	10,525	99.52
Lysine	Dowex 50	2,422	99.89

To samples of ovomucoid (2mg) were added $[^{14}C]$ lysine, 1μCi or $[^{14}C]$ glucosamine, 1μCi in a total volume of 1ml. Dialysis was against 5 litres of water (or water brought to pH 4.6 with acetic acid) for 2 days with one change of solution. In one experiment the samples were brought to a volume of 40ml with HCl, pH 1.5 and passed through columns (10cm x 0.5cm) of Dowex 50, collecting the non-adsorbed material.

dialysis against water or passage over columns of Dowex 50 removed most of the free lysine or glucosamine but that dialysis against distilled water brought to pH 4.6 with acetic acid was virtually 100% effective in removing unincorporated radioactive material. As a result, when ovomucoid was isolated from oviduct after tissue incubations, the ovomucoid was dialysed over a period of two days against water brought to pH 4.6, with several changes of water.

3. 13 (3) Single Label Incorporation Studies in Two Different Incubation Media

In all the foregoing experiments, oviduct had been incubated in the medium of Hendler (1956; 1957) supplemented with antibiotics (Carey, 1966). The ovomucoid in these early studies had been isolated from the 105,000g supernatant of oviduct homogenate. It was intended to isolate ovomucoid, in these studies, from the incubation medium (S1), from the 105,000g supernatant (S2) and from a deoxycholate extract of the 105,000g pellet (S3) of oviduct homogenate. These tissue fractions have been labelled S1; "extracellular fraction", S2; "intracellular fraction", S3; "deoxycholate extracted fraction", and this is illustrated in Fig. 24. (This is similar to Fig. 1 but has been reproduced here for ease of reference).

The experiment represented by Table 9 suggested that, on tissue incubation studies, the specific radioactivity of ovomucoid in the intracellular fraction increased over about a 5 hour period when labelling with lysine. The recovery of administered radioactivity from the extracellular fraction is shown for different times of incubation in Table 11.

Fig. 24. Tissue fractionisation in single label studies

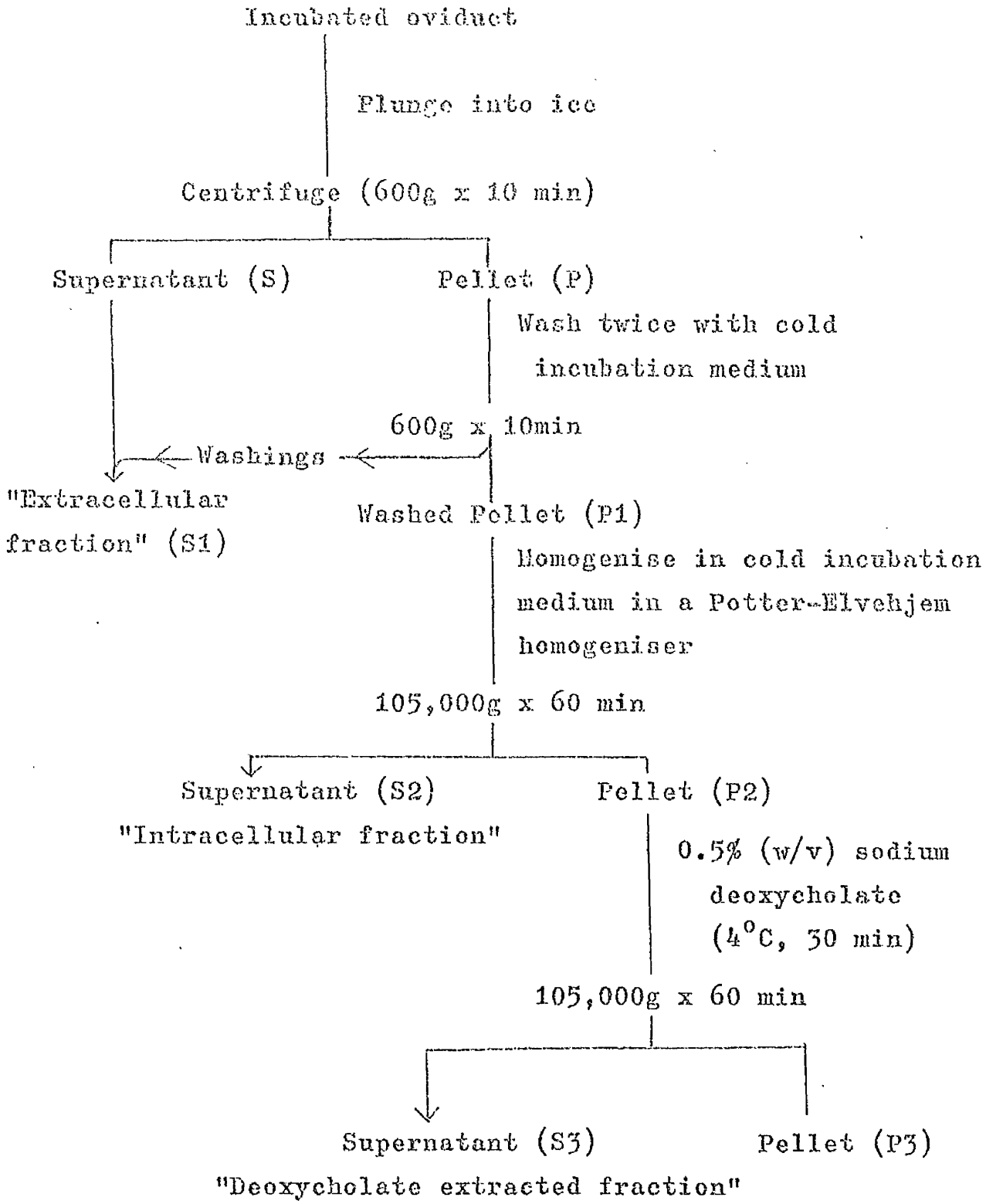


Table 11. Recovery of administered radioactivity from the incubation medium with time

Incubation time (hours)						
0	0.1	0.5	1	2	5	22
% recovery of administered radioactivity						
96	100	73.6	53.5	47.5	41.0	42.5

Oviduct portions (3g) were incubated in 7.5ml of the medium of Hendler (1956; 1957) supplemented with antibiotics (Carey, 1966) using [^{14}C] lysine (1μCi/g of oviduct) as precursor. After washing the tissue twice with fresh incubation salts (Hendler, 1956; 1957) the supernatants were combined and a portion prepared for liquid scintillation counting.

Table 12. The effectiveness of the procedure for washing the oviduct portions

Administered radioactivity	6.6×10^6 d.p.m.	% recovered
Supernatant S	5.96×10^6 d.p.m.	90.5
Wash 1	229,000 d.p.m.	3.5
Wash 2	40,000 d.p.m.	0.6
Wash 3	55 d.p.m.	0
	Total	94.6

To unincubated portion (3g) of oviduct was added 3μCi [^{14}C] lysine in a volume of 7.5ml of incubation medium (Hendler; 1956; 1957). The tissue was centrifuged (600g for 10 minutes) and the supernatant collected. The washing was repeated 3 times with fresh medium.

It can be seen that the amount of radioactivity in the extracellular fraction, S1 has decreased up to 2 hours incubation time, but that at times longer than 2 hours the amount of radioactivity in S1 has remained fairly constant. The results from Tables 9 and 11, therefore, would suggest that the time course of incorporation of lysine into ovomucoid should be followed over a period of a few hours only.

After incubation of oviduct tissue, the tissue pellet (P) was washed with fresh incubation medium prior to homogenisation. The effectiveness of the washings are illustrated in Table 12 which shows the radioactivity present in the washings of the tissue pellet (P) from an unincubated control. As recovery of administered radioactivity was quantitative, and a third wash was not washing out any additional radioactivity, all incubated samples were washed twice with cold incubation medium.

In one other zero-time sample, [^{14}C] lysine was added to the washed tissue pellet (P1) before homogenisation. After homogenisation and centrifugation, the recovery of this radioactivity was 103% from the supernatant, S2. All of this radioactivity was recovered from the supernatant after trichloroacetic acid precipitation, suggesting that no lysine had co-precipitated with the total protein fraction. There was no radioactivity associated with the ovomucoid which was isolated from the trichloroacetic acid supernatant, indicating the effectiveness of the isolation procedure in removing any free (non-incorporated) lysine which might have contributed to the counts in the ovomucoid fraction.

In Fig. 25 the time course of incorporation of lysine into intracellular ovomucoid in the medium of Hendler (1956; 1957) is shown. The

using the medium of Hendler (1956;1957)

Fig. 25a

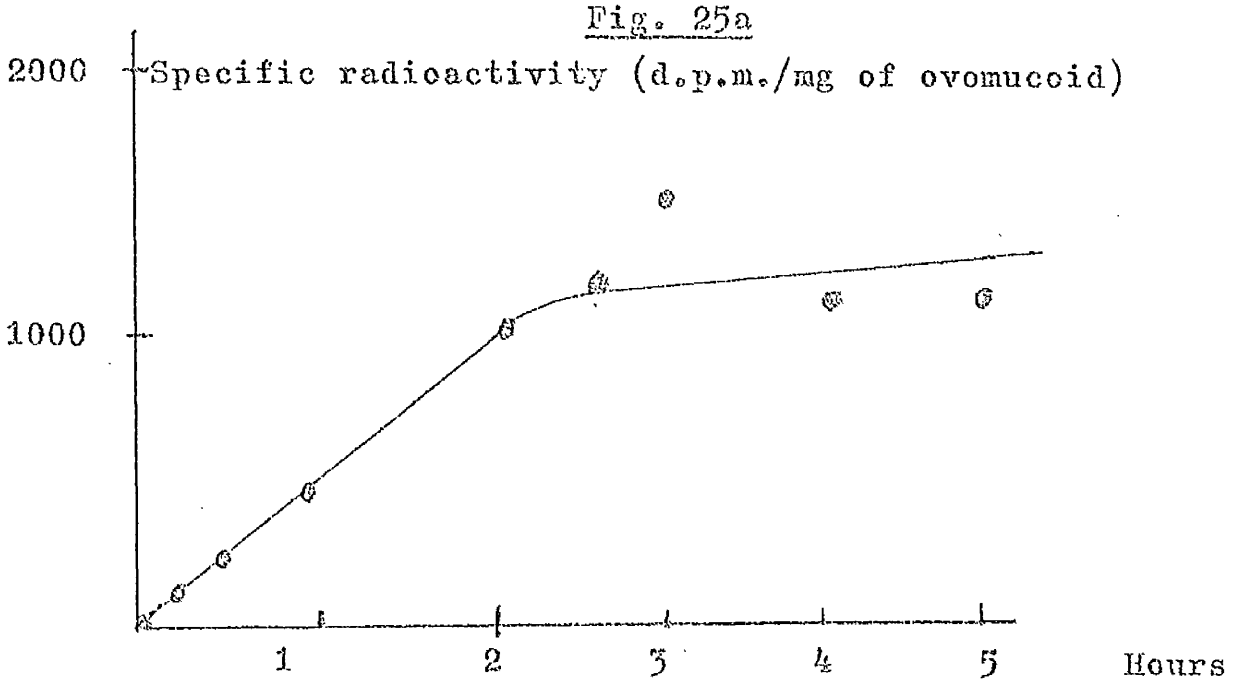
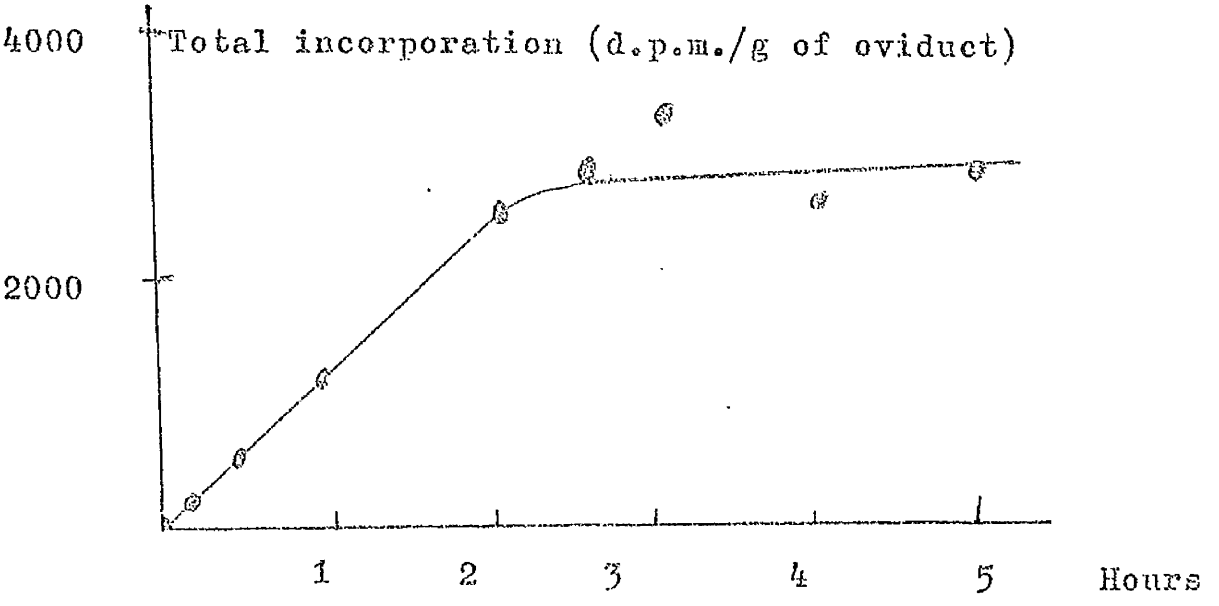
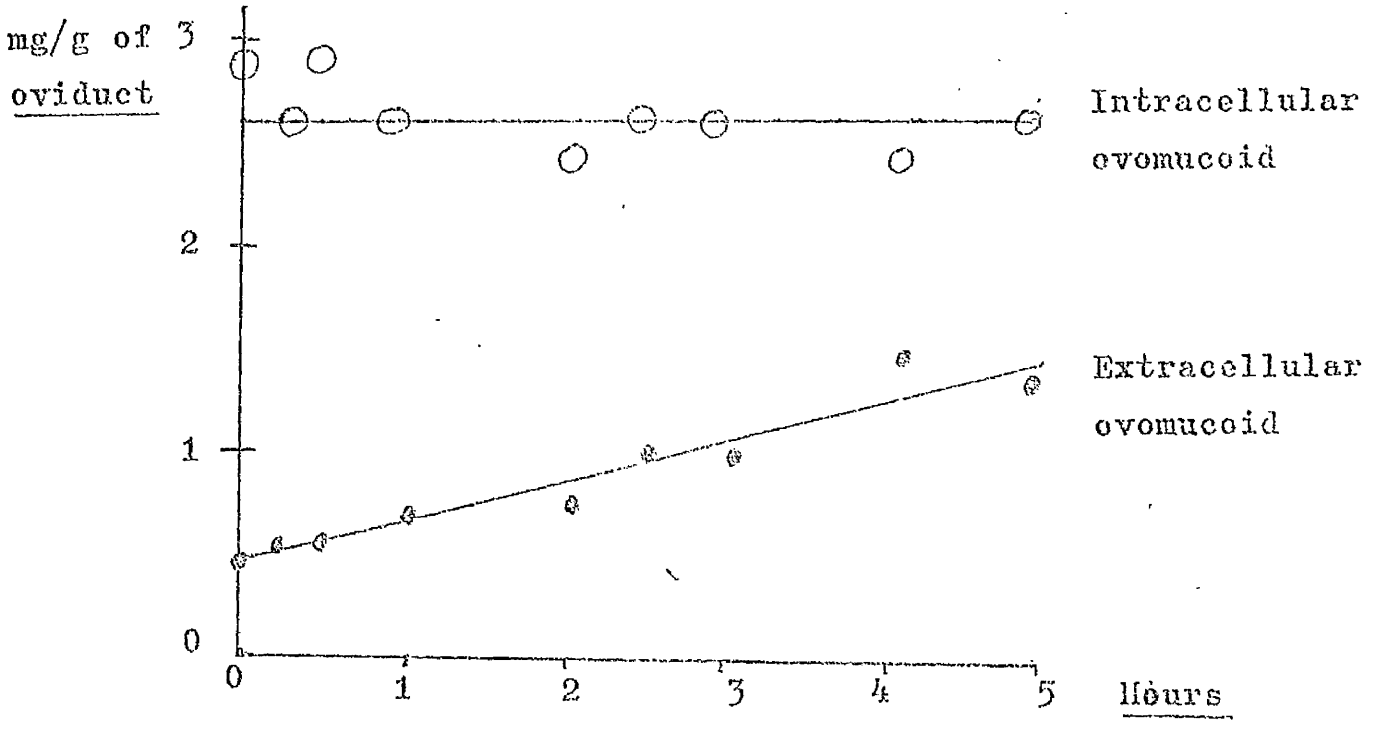


Fig. 25b



Portions of oviduct (1g) were incubated in the medium of Hendler(1956;1957) for the times shown with [^{14}C] lysine 1μCi/g as precursor. Ovomucoid was isolated from the intracellular fraction by adsorbtion on and elution from G-200-trypsin.

ular fractions



The experimental details are as given in Fig. 25. After incubation of oviduct for the stated times, ovomucoid was isolated from the tissue fractions by adsorbtion on and elution from G-200-trypsin following pH 3.5 trichloroacetic acid precipitation of other components.

specific radioactivity of ovomucoid, d.p.m./mg (Fig. 25a) and the total incorporation of lysine d.p.m./g (wet weight) tissue (Fig. 25b) increased linearly over 2 hours with no observable lag period. The amount of ovomucoid isolated from the intracellular fraction was, approximately, constant throughout the incubation period while the amount of ovomucoid isolated from the extracellular fraction increased over the time of incubation (Fig. 26). The ovomucoid isolated from the extracellular fraction represented $\frac{1}{3}$rd of the total ovomucoid isolated from the tissue after a 5 hour incubation period, but no radioactivity was associated with this ovomucoid fraction in this experiment. As the amount of ovomucoid isolated from the extracellular fraction increases with time, it may represent unlabelled ovomucoid being secreted into the medium. (The 105,000g pellet was extracted with deoxycholate, but due to mechanical failure while centrifuging, the samples were irretrievably lost and no determinations could be made on them).

An examination of the medium of Hendler (1956; 1957) shows that the potassium content and sodium content are both 72mM. This is high in potassium and low in sodium when compared to chicken serum. It was decided to repeat the above experiment with a more physiological medium. The original ringer bicarbonate of Krebs and Henseleit (1932) has a composition closely resembling the salt content of chicken serum. Rather than determine the effect of individual additions to this medium on the incorporation of precursors into ovomucoid, it was decided to supplement this physiological medium with the essential amino acids in order to supply all that the oviduct might need in synthesising glycoproteins,

without greatly complicating the medium. (For in vitro biosynthetic studies, Mandeles and Ducay (1962) supplemented the medium of Hendler (1956) with amino acids, and Palmiter, Oka and Schimke (1971) used a commercial incubation medium).

In Fig. 27b it can be seen that the total incorporation of lysine, d.p.m./g (wet weight) tissue, into intracellular ovomucoid was linear over, appxoximately 4 hours in this more physiological medium (described above). The specific radioactivity, d.p.m./mg, of deoxycholate extracted ovomucoid (Fig. 28a) was higher than that of intracellular ovomucoid (Fig. 27a). However, as the yield of ovomucoid from the deoxycholate extracted fraction was very small (Fig. 29), the total incorporation of lysine into this fraction (Fig. 28b) was much lower than the total incorporation into the intracellular fraction (Fig. 27b). The amount of ovomucoid isolated from the extracellular fraction increased with time (Fig. 29) and after 5 hours represented $\frac{1}{3}$rd of the total ovomucoid from the tissue. There was a small amount of radioactivity associated with extracellular ovomucoid in this experiment but this varied with time and it was not clear if this did represent the appearance of newly synthesised ovomucoid in the medium.

The observation that newly synthesised ovomucoid did appear in the extracellular fraction was confirmed in experiments where the incorporation of lysine into ovomucoid, intracellularly, was somewhat higher than the incorporation into intracellular ovomucoid observed in the experiment represented by Fig. 27. An illustration of the appearance of lysine labelled ovomucoid in the extracellular fraction is given in

ovomucoid in the medium of Krebs and Henseleit (1932)

Fig. 27a

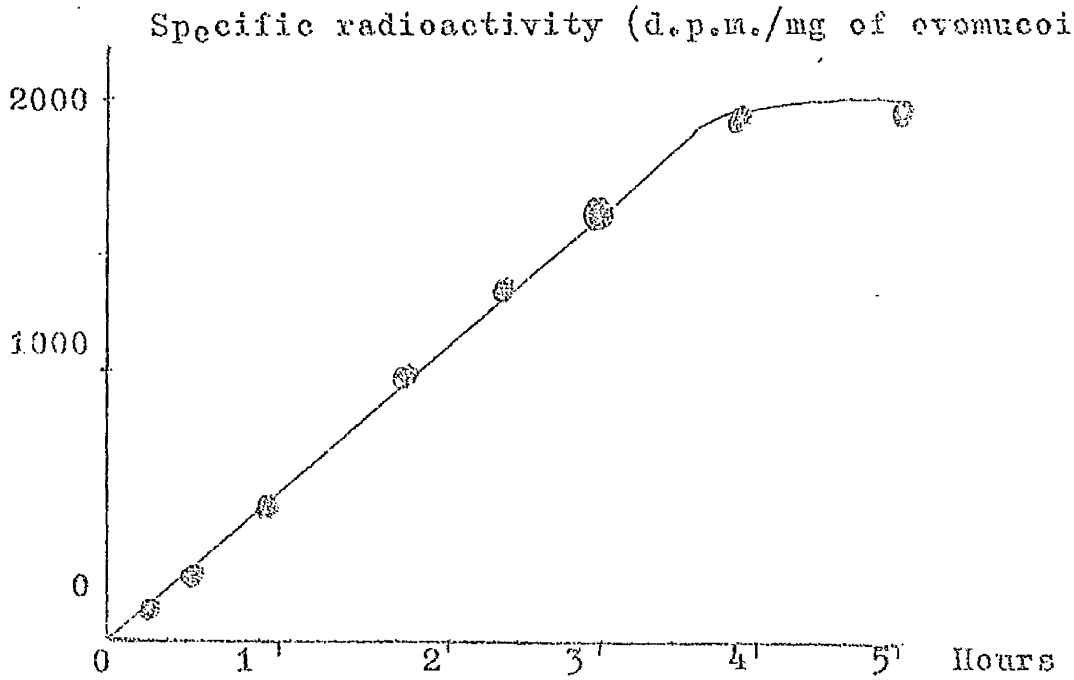
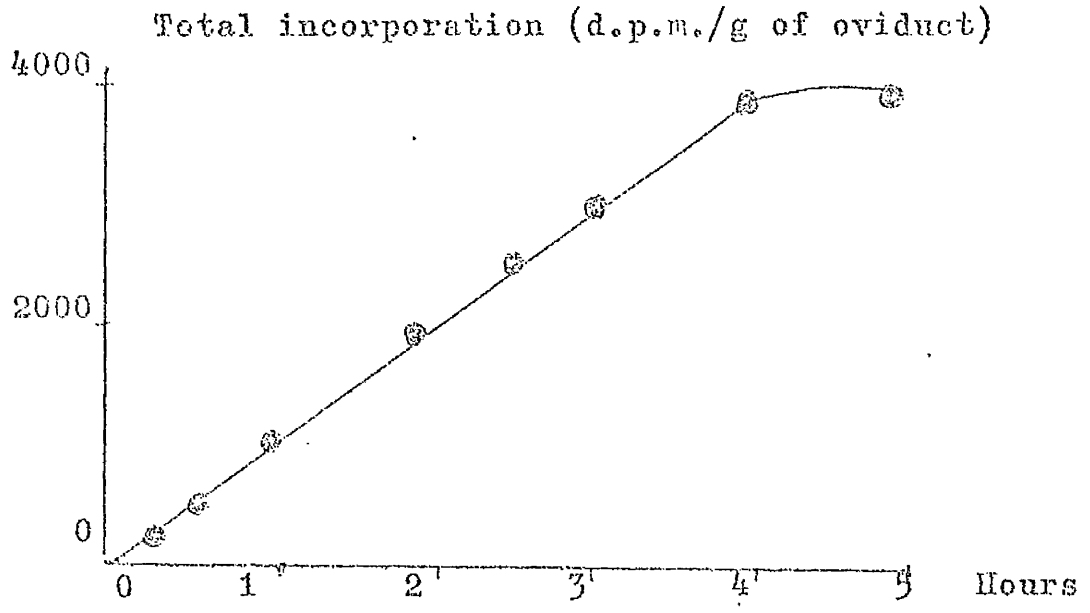


Fig. 27b



Portions of oviduct (1g) were incubated in the medium of Krebs and Henseleit (1932) for the times shown with [^{14}C]-lysine (1μCi/g of oviduct as precursor).

ovomucoid

Fig. 28a

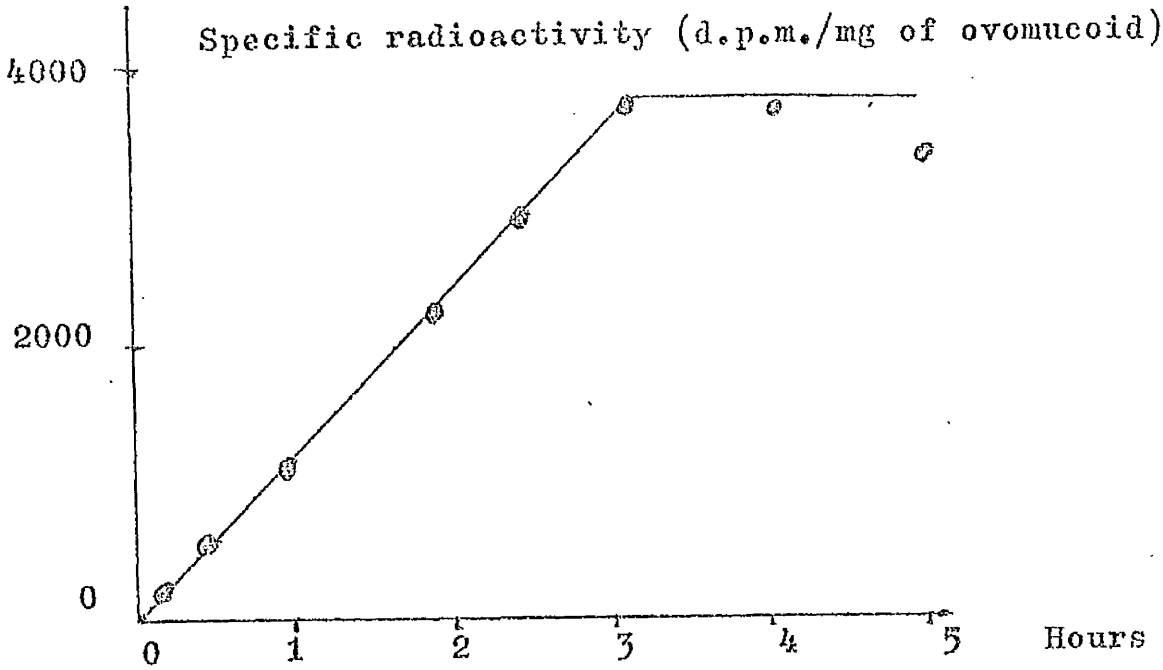
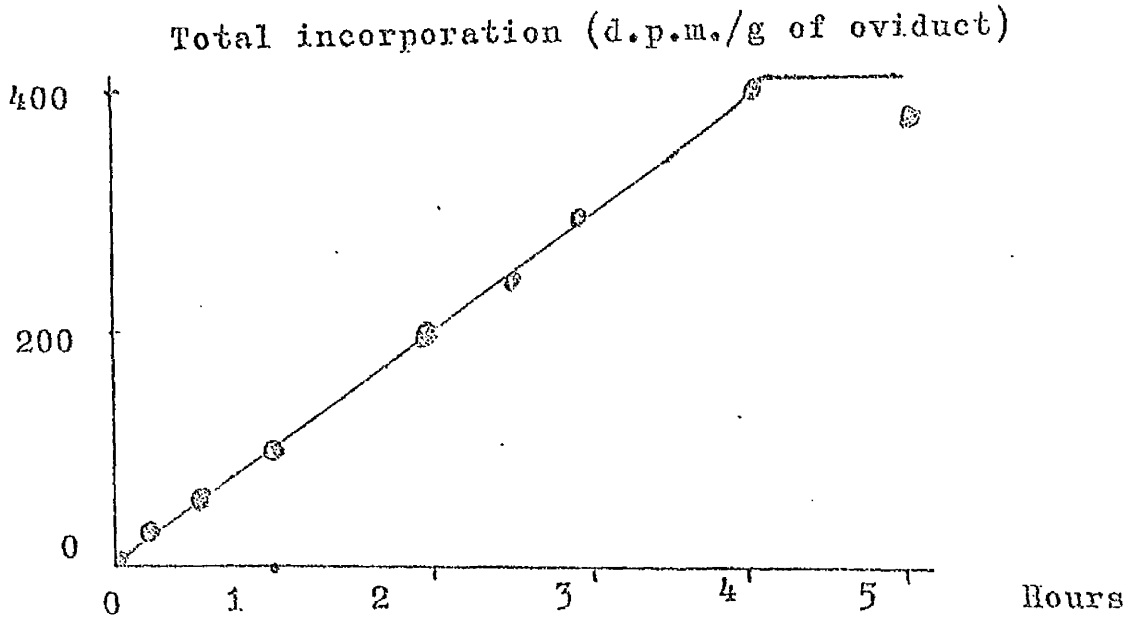
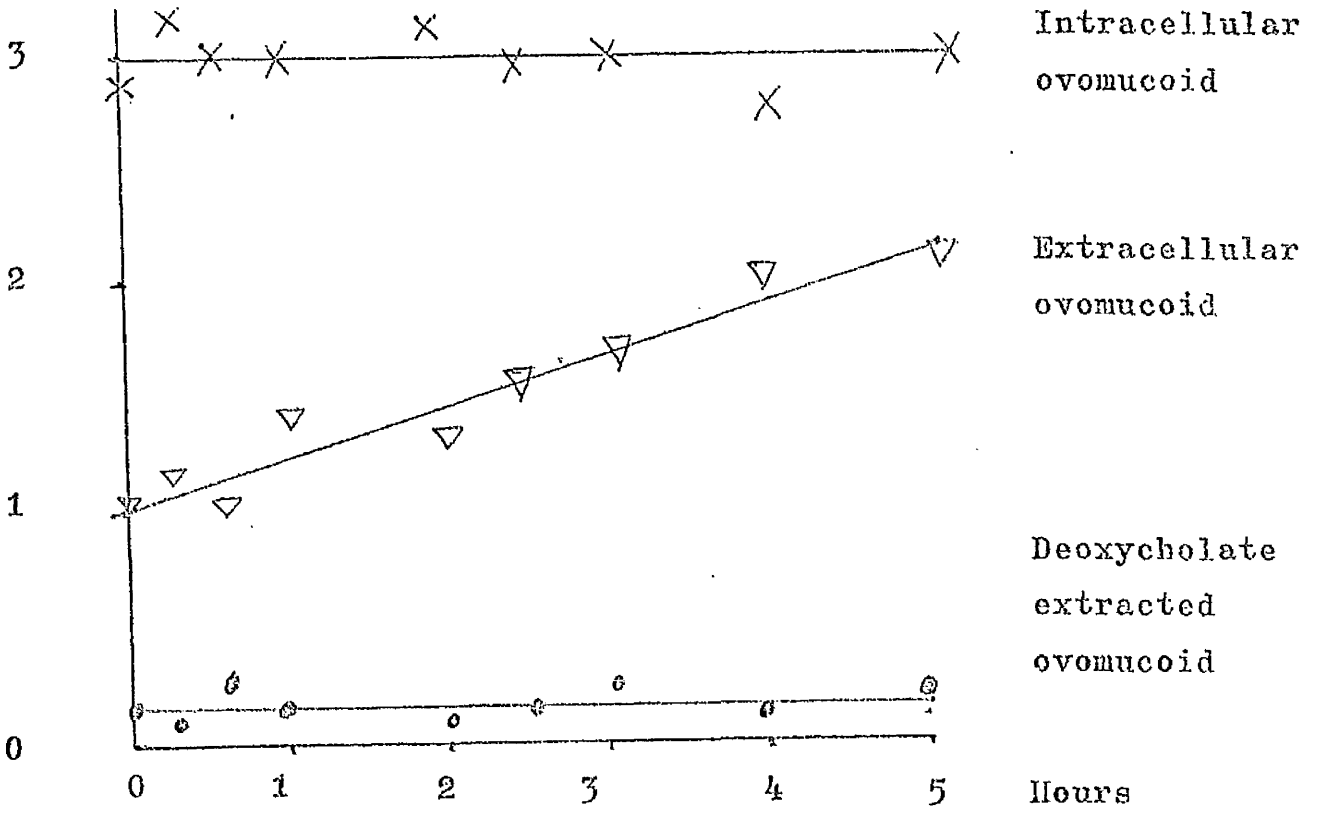


Fig. 28b



Incubation conditions were as in Fig. 27. Ovomucoid was isolated from the deoxycholate extracted fraction of oviduct homogenate by adsorbtion on and elution from G-200-trypsin following precipitation of other components with pH 3.5 trichloroacetic acid.

Fig. 27. Yields of ovomucoid from tissue fractions in incorporation studies



Incubation conditions are given in Table 27. Ovomucoid was isolated from the tissue fractions, after incubation of oviduct for the times shown, by adsorbtion on and elution from G-200-trypsin following precipitation of other components with pH 3.5 trichloroacetic acid.

Table 13. The appearance of newly synthesised ovomucoid in the incubation mediums

Incubation time (hours)	Intracellular ovomucoid d.p.m./mg of ovomucoid	Intracellular ovomucoid d.p.m./g of oviduct	Extracellular ovomucoid d.p.m./mg of ovomucoid	Extracellular ovomucoid d.p.m./g of oviduct
0	0	0	0	0
2	2000	5,800	83	35
5	6200	15,400	322	252
9	8900	20,200	874	695

Oviduct portions (2g) were incubated in 5ml of the medium of Krebs and Henseleit (1932) supplemented with amino acids and antibiotics using [^{14}C] lysine (1μCi/g of oviduct) as precursor. The tissue portions were homogenised in a Potter-Elvehjem homogeniser in fresh incubation medium (Krebs & Henseleit, 1932).

Table 13. It can be seen that while both the specific radioactivity and total incorporation of lysine into extracellular ovomucoid are fairly low, they both do increase with time, representing the appearance of newly synthesised ovomucoid extracellularly.

In view of the more physiological nature of the original ringer bicarbonate (Krebs & Henseleit, 1932) and the observation that it was capable of supporting the incorporation of lysine into ovomucoid, this was the medium chosen for all further *in vitro* studies.

3. 13 (4) Methods of Homogenising Oviduct Tissue Prior to Isolation of Ovomucoid from the Intracellular and Deoxycholate Extracted Fractions

In one of the experiments on radiological purity of ovomucoid represented by Fig. 22, the oviduct had been homogenised with a Potter-Elvehjem homogeniser (Fig. 22a), while in the other a Dounce homogeniser was used (Fig. 22b). When using the Potter-Elvehjem homogeniser, the tissue had been homogenised in incubation medium, while with the Dounce homogeniser, buffered sucrose was used. An investigation was made on the specific radioactivities of intracellular and deoxycholate extracted ovomucoid isolated after homogenisation of comparable tissue samples from a single oviduct by these two types of homogenisers.

After incubating tissue portions with lysine for 2 hours, ovomucoid was isolated from the intracellular fraction. The specific radioactivities of intracellular ovomucoid isolated after homogenisation of the tissue by various methods are shown in Table 14. It can be seen that the specific

Table 14. The specific radioactivity of intracellular ovomucoid isolated after homogenisation of the tissue by various methods

Homogenisation method	Specific radioactivity d.p.m./mg of ovomucoid
Potter-Elvehjem in incubation medium	1999
Dounce in incubation medium	1980
Potter-Elvehjem in 0.44M sucrose-TKM, pH 7.5	2060
Dounce in 0.44M sucrose-TKM, pH 7.5	2150

Oviduct portions (4g) were incubated in 10ml of the medium of Krebs and Henseleit (1932) supplemented with amino acids and antibiotics. Incubation was for 2 hours with [^{14}C] lysine (1μCi/g of oviduct) as precursor. After incubation the tissue pieces were split into 4 x 1g (approximately) portions which were then homogenised by the above methods.

radioactivities of intracellular ovomucoid were virtually identical, no matter whether the ovomucoid was isolated after homogenisation of the tissue with a Dounce or Potter-Elvehjem homogeniser in incubation medium or in buffered sucrose.

In earlier experiments it was found that the amount of ovomucoid isolated by deoxycholate extraction of the 105,000g pellet was very small (Fig. 29). This fraction might contain some precursor material of ovomucoid which has an incompleted carbohydrate moiety and perhaps, therefore differing in solubility properties from ovomucoid secreted into egg white. As well as comparing the homogenisation techniques, therefore, ovomucoid was isolated from the deoxycholate extracted fraction by use of G-200-trypsin both with and without a preliminary trichloroacetic acid precipitation stage.

It can be seen that the yields of deoxycholate extracted ovomucoid were greatly reduced if the preliminary trichloroacetic acid precipitation was included in the isolation method (Table 15). Although this to a certain extent might have reflected a difficulty in isolating very small quantities of ovomucoid from the deoxycholate extracted fraction, it may have represented a chemical difference between deoxycholate extracted ovomucoid and intracellular ovomucoid.

When the trichloroacetic acid precipitation stage was omitted from the method of isolation of deoxycholate extracted ovomucoid, the specific radioactivities of ovomucoid, isolated after homogenisation of the tissue by the differing techniques, were virtually indistinguishable (Table 15). If the trichloroacetic acid precipitation was included, then the specific

Table 15. The isolation of ovomucoid from the deoxycholate extracted fraction of oviduct homogenate

Homogenisation method	Precipitation with trichloro-acetic acid included (+) or (-)	mg of ovomucoid recovered	d.p.m. recovered	Specific radioactivity d.p.m./mg of ovomucoid
Potter-Elvehjem,	(+)	0	0	-
salts medium	(-)	0.24	720	3000
Dounce,	(+)	0.05	142	2840
salts medium	(-)	0.18	598	3320
Potter-Elvehjem,	(+)	0.03	86	2840
0.44M sucrose TKM	(-)	0.30	916	3050
Dounce,	(+)	0.05	140	2800
0.44M sucrose TKM	(-)	0.21	652	3100

Incubation conditions were as in Table 14. The portions (equivalent to 1g originally of oviduct) were extracted with deoxycholate. The deoxycholate extracted supernatant was split into two equal portions and ovomucoid isolated using G-200-trypsin with or without including the preliminary precipitation step with trichloroacetic acid.

radioactivity of ovomucoid was similar to that of ovomucoid isolated without inclusion of this preliminary precipitation step (Table 15). The small amount of ovomucoid isolated when trichloroacetic acid precipitation is included in the isolation of deoxycholate extracted ovomucoid and the low d.p.m. values (Table 15), make the calculation of specific radioactivities very difficult to determine with any degree of certainty.

Although ovomucoid isolated by use of G-200-trypsin without a preliminary trichloroacetic acid precipitation stage may contain ovoinhibitor, this is a minor component of egg white (0.1% by weight). Also, it has been shown that the specific radioactivities of intracellular ovomucoid isolated by use of G-200-trypsin with or without a preliminary trichloroacetic acid precipitation stage were virtually the same, and that the specific radioactivity of ovomucoid was essentially unaltered by passage over G-200-chymotrypsin (Fig. 22). In view of these observations, in all subsequent studies where ovomucoid was isolated from the deoxycholate extracted fraction by use of G-200-trypsin, the preliminary trichloroacetic acid precipitation stage was omitted.

As the specific radioactivities of intracellular ovomucoid isolated from tissue homogenised by different methods closely resembled one another (Table 14), and the specific radioactivities of deoxycholate extracted ovomucoid from tissue homogenised by these methods were likewise similar (Table 15), there seemed to be little to choose between the homogenisation methods. Hendler (1956;1957) used a Potter-Elvehjem

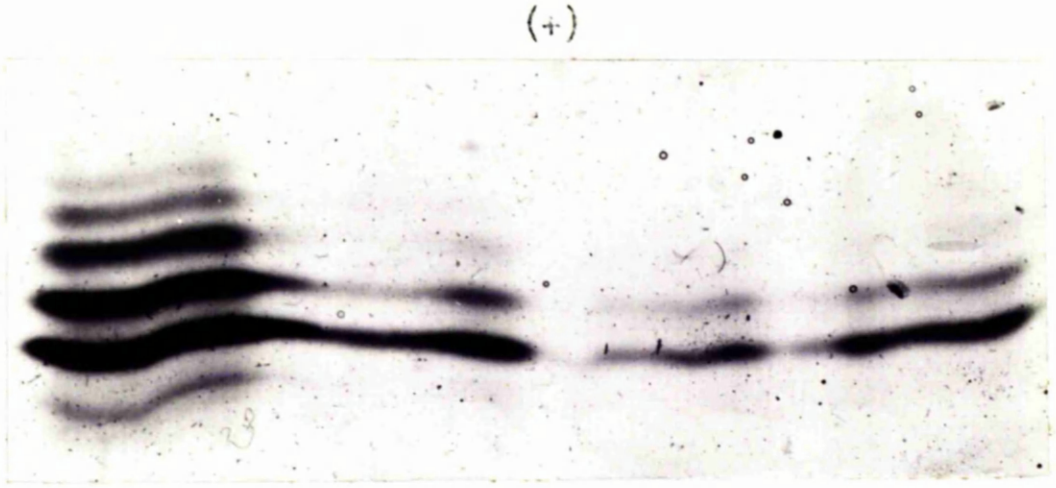
homogeniser while Carey (1966) used a Dounce homogeniser to homogenise oviduct portions. Carey (1966) suggested that the use of 0.44M sucrose when fractionating oviduct homogenate in the centrifuge might have helped to prevent microsome-like material from sedimenting at the low centrifugal fields found by Hendler (1956;1957), and Anderson (1956) suggested that a Dounce homogeniser was less likely to damage cell particles. In further studies it was decided to homogenise portions of oviduct in 0.44M sucrose-TKM buffer, pH 7.5, with a Dounce-type homogeniser.

3. 13 (5) Characterisation of Labelled Ovomucoid from Tissue Incubation Studies

On isoelectric focusing of ovomucoid isolated from the deoxycholate extracted fraction, it can be seen (Fig. 30) that the major ovomucoid variants O_1, O_2 and a trace of O_3 were present both in an unincubated sample (Fig. 30d) and in samples which had been incubated for 2 or 7 hours (Figs. 30b and 30c). No trace of any components with an isoelectric point higher than that of the most basic ovomucoid variant, O_1, was found. Due to the very small amount of material which was present in this fraction, it cannot be said with certainty that the more acidic ovomucoid species, O_4 and O_5, were not present, as these are minor components which do not stain readily unless there is sufficient ovomucoid applied to the gel.

In Fig. 31 it can be seen that the isoelectric focusing band patterns of intracellular ovomucoid isolated from the tissue after 2 hours or 7 hours (Figs. 31b and 31c) resembled that of intracellular ovomucoid from an unincubated portion of oviduct (Fig. 31a). Ovomucoid isolated

Fig. 30. Isoelectric focusing of deoxycholate extracted ovomucoid from tissue incubation studies



(a) Ovomucoid isolated from egg white by the method of Fredericq and Deutsch (1949). (b) Ovomucoid isolated from the deoxycholate extracted fraction of oviduct homogenate by adsorbtion on and elution from G-200-trypsin after a 2 hour incubation period *in vitro*. The preliminary trichloroacetic acid precipitation stage was omitted. (c) Ovomucoid isolated similarly after a 7 hour incubation period. (d) Ovomucoid isolated by the same method from an unincubated piece of tissue.

Fig. 31. Isoelectric focusing of intracellular and extracellular ovomucoid from tissue incubations

(+)

(a) (b) (c) (d)

(-)

(a) Ovomucoid isolated from the intracellular fraction of an unincubated oviduct homogenate by adsorbtion on and elution from G-200-trypsin following a preliminary precipitation of other components with pH 3.5 trichloroacetic acid. (b) Intracellular ovomucoid isolated similarly after a 2 hour incubation period. (c) Intracellular ovomucoid isolated by the same method after a 7 hour incubation period. (d) Ovomucoid isolated by the same method from the extracellular fraction after a 2 hour incubation period in vitro.

from the extracellular fraction after a 2 hour incubation period (Fig. 31d) also resembled intracellular ovomucoid from an unincubated sample of oviduct (Fig. 31a) on isoelectric focusing. The same band pattern was found for ovomucoid isolated from the extracellular fraction after a 7 hour incubation period and for extracellular ovomucoid from an unincubated control (not shown).

The results would suggest that isoelectric focusing has not detected any obvious differences between ovomucoid isolated from tissue fractions after a 7 hour incubation period and ovomucoid from an unincubated sample of oviduct. As has been implied elsewhere in the text, oviduct ovomucoid (Fig. 31a) resembled egg white ovomucoid (Fig. 30a) on isoelectric focusing, and use of G-200-trypsin in the isolation of ovomucoid (Fig. 31a) has removed the component with an isoelectric point higher than that of ovomucoid O_1 which was sometimes present (Fig.30a) in ovomucoid prepared by the method of Fredericq and Deutsch (1949).

The carbohydrate content of intracellular ovomucoid isolated after incubation of oviduct for various periods of time is shown in Table 16. It can be seen that the hexosamine content of ovomucoid was essentially unaltered in incubation studies up to 7 hours and that the hexose content of ovomucoid isolated from the tissue after 1.5 hours incubation resembled that of ovomucoid from an unincubated portion of tissue. The sialic acid content has fluctuated somewhat over the incubation period (0.25 - 0.50%) but this is within the range of values normally found for the sialic acid content of egg white ovomucoid.

The lysine and histidine content of intracellular ovomucoid isolated

Table 16. The carbohydrate content of intracellular ovomucoid from tissue incubation studies

(N. D. implies determination not carried out).

Incubation time (hours)	Hexose g/100g	Hexosamine g/100g	Sialic acid g/100g
Unincubated control	8.75	14.1	0.50
1.5	8.4	14.0	0.25
5	N.D.	13.4	0.49
7	N.D.	13.1	0.29

Table 17. The lysine and histidine content of intracellular ovomucoid from tissue incubation studies

	Moles/10,000g		Moles/27,000g	
Incubation time	3.5 hour	7 hour	3.5 hour	7 hour
Lysine	4.78	4.90	12.9	13.2
Histidine	1.31	1.47	3.55	3.98

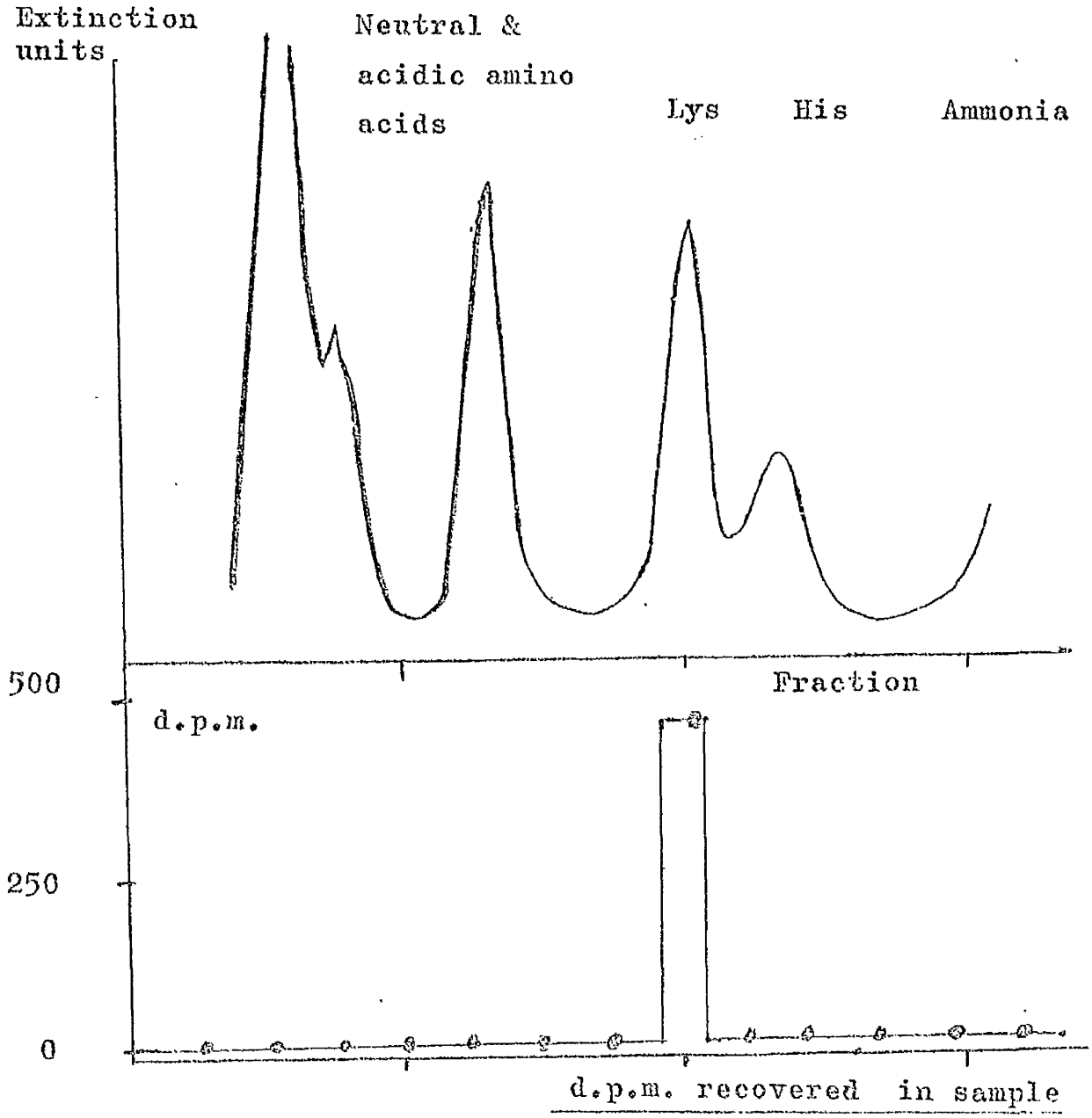
from the tissue after a 3.5 hour and a 7 hour incubation period are given in Table 17. It can be seen that the lysine content agreed well at both times as did the histidine content. On assuming a molecular weight of 27,000 for ovomucoid, the residues of lysine and histidine per mole ovomucoid have been calculated (Table 17) and these values agree well with the published values on lysine and histidine content of egg white ovomucoid (Davis et al, 1971; Beeley, 1971a).

These results suggested that ovomucoid isolated from oviduct after periods of incubation up to 7 hours resembled egg white ovomucoid or ovomucoid isolated from an unincubated piece of tissue.

3. 13 (6) Suitability of Lysine and Glucosamine as Precursors in Incubation Studies

By hydrolysis of labelled ovomucoid, isolated after incubation of oviduct with [^{3}H] lysine for 3.5 hours and 7 hours, and separation of the amino acids, it was found that all the [^{3}H] radioactivity was recovered in the fraction corresponding to lysine (Fig. 32). The recovery of incorporated radioactivity was 101% in the lysine fraction isolated after a 3.5 hour incubation and 102% after a 7 hour incubation (Table 18). Lysine was originally chosen as it was known to be an essential amino acid for the hen, and this has demonstrated its suitability as a labelled precursor for incubation studies.

When [^{14}C] glucosamine was used as labelled precursor in tissue incubation studies, then the incorporated radioactivity was associated with both glucosamine and N-acetylneuraminic acid from ovomucoid (Table 19). Most of the incorporated radioactivity from the labelled



Labelled ovomucoid was hydrolysed with HCl and the amino acids fractionated on an amino acid analyser as described. Radioactivity was recovered from the lysine fraction only. Recovery of radioactivity was 100% in lysine from ovomucoid isolated from the tissue after a 7 hour or a 3.5 hour incubation period in vitro (Table 18).

Table 18. Recovery of $[^{3}H]$ radioactivity in intracellular ovomucoid with $[^{3}H]$ lysine as precursor in tissue incubation studies

Incubation time (hours)	d.p.m. in ovomucoid	d.p.m. recovered in lysine	% recovery
3.5	5700	5750	101
7	4650	4760	102

Table 19. Recovery of $[^{14}C]$ radioactivity in intracellular ovomucoid with $[^{14}C]$ glucosamine as precursor in tissue incubation studies

(N.D. implies no determination was carried out)

Incubation time hours	d.p.m. in ovomucoid (100%)	d.p.m. recovered in sialic acid	d.p.m. recovered in glucosamine	% recovery of radio-activity
2	820	150 (18%)	N.D.	-
2	360	64 (18%)	340 (94%)	112%
5	2700	N.D.	2260 (84%)	-
5	1200	350 (29%)	1000 (83%)	112%
7	2200	490 (22%)	1980 (90%)	112%

glucosamine has been recovered as glucosamine, although 18-29% was metabolised to N-acetylneuraminic acid. The recovery of incorporated radioactivity was greater than 100% at all times of incubation. As about 24% of the labelled glucosamine was metabolised to N-acetylneuraminic acid the total d.p.m. in the N-acetylneuraminic acid fraction was much less than the d.p.m. associated with glucosamine (Table 19) and would be more likely to be in error.

The observation that the radioactivity of administered glucosamine was recovered in both glucosamine and sialic acid would confirm that the normal route of synthesis of the sialic acid nucleotides (Kornfield et al, 1964) was occurring in the tissue during the incubation period, and that glucosamine was a suitable choice of precursor for biosynthetic studies.

When the amount of [^{14}C] glucosamine added to the incubation medium was increased from 1μCi/g oviduct to 5μCi/g oviduct, the specific radioactivity of intracellular ovomucoid, isolated after an incubation period of 9 hours, was increased approximately 5 fold (13,000 - 57,000 d.p.m./mg ovomucoid). In view of this, in dual label studies it was decided to increase the amount of labelled precursor to 4μCi [^{14}C] glucosamine/g (wet weight) oviduct and to use 40μCi [^{3}H] lysine/g (wet weight) oviduct. It was also decided to use 5ml incubation medium/g tissue rather than 2.5ml/g tissue to counteract any volume losses during the incubation.

A summary of the conditions used for dual label incubation studies is given below. A list of relevant points is given, the reason for

choosing a particular method has occurred in the above sections and is not reproduced here.

(1) The tissue was finely chopped with scissors.

(2) Portions of oviduct (1g) were incubated in the original ringer bicarbonate (5ml) of Krebs and Henseleit (1932) supplemented with essential amino acids, glucose, penicillin and streptomycin.

(3) Ovomucoid was isolated from the intracellular and extracellular fractions by adsorbtion on and elution from G-200-trypsin following a preliminary trichloroacetic acid precipitation step.

(4) Ovomucoid was isolated from the deoxycholate extracted fraction by use of G-200-trypsin without including the preliminary trichloroacetic acid precipitation step.

(5) Homogenisation of the tissue was carried out in 0.44M sucrose-TKM buffer, pH 7.5, using a Dounce homogeniser.

(6) Labelled precursors were [^{3}H] lysine (40μCi/g oviduct) and [^{14}C] glucosamine (4μCi/g oviduct).

Palmiter, Oka and Schimke (1971) found that insulin was necessary to maintain incorporation of isotopes into protein in long term in vitro studies using a commercial incubation medium. The incorporation of precursors into protein in the absence of insulin was the same as that in the presence of added insulin up to 6 hours. As the in vitro studies on ovomucoid biosynthesis to be described were of the order of 6 hours, no insulin was added to the medium.

3. 13 (7) The Incorporation of [^{3}H] Lysine and [^{14}C] Glucosamine into Intracellular and Extracellular Ovomucoid, and into Intracellular and Extracellular Total Protein

From Fig. 33 it can be seen that the incorporation (d.p.m./g of tissue) of [^{3}H] lysine into intracellular ovomucoid was linear over about a 6 hour period. Newly synthesised ovomucoid (labelled with lysine) did not appear in the medium until after, approximately, 1 hour. The incorporation (d.p.m./g of tissue) of [^{14}C] glucosamine into intracellular ovomucoid was also linear over the 6 hour period, although there was an initial lag period observed (Fig. 34). This initial lag in the incorporation of glucosamine into intracellular ovomucoid probably reflected the slower build up of the intracellular glucosamine pool compared to the build up of the lysine pool. Similar to the observations with lysine labelling (Fig. 33), the incorporation of glucosamine into extracellular ovomucoid was very much lower than the incorporation of glucosamine into intracellular ovomucoid (Fig. 34). The yield of ovomucoid from the tissue fractions is given in Fig. 35, and it can be seen that the proportion of ovomucoid in the extracellular fraction increased from 10% of the total ovomucoid isolated at zero time up to 35% of the total at 6 hours. It is obvious therefore, that as the total incorporation of radioactive precursors into extracellular ovomucoid was much smaller than their incorporation into intracellular ovomucoid (Figs. 33 and 34), only a small proportion of the ovomucoid isolated from the extracellular fraction was newly synthesised material.

For a comparison, the results from an experiment performed with

Fig. 33. The incorporation of [^{3}H] lysine into intracellular and extracellular ovomucoid. (Experiment 1).

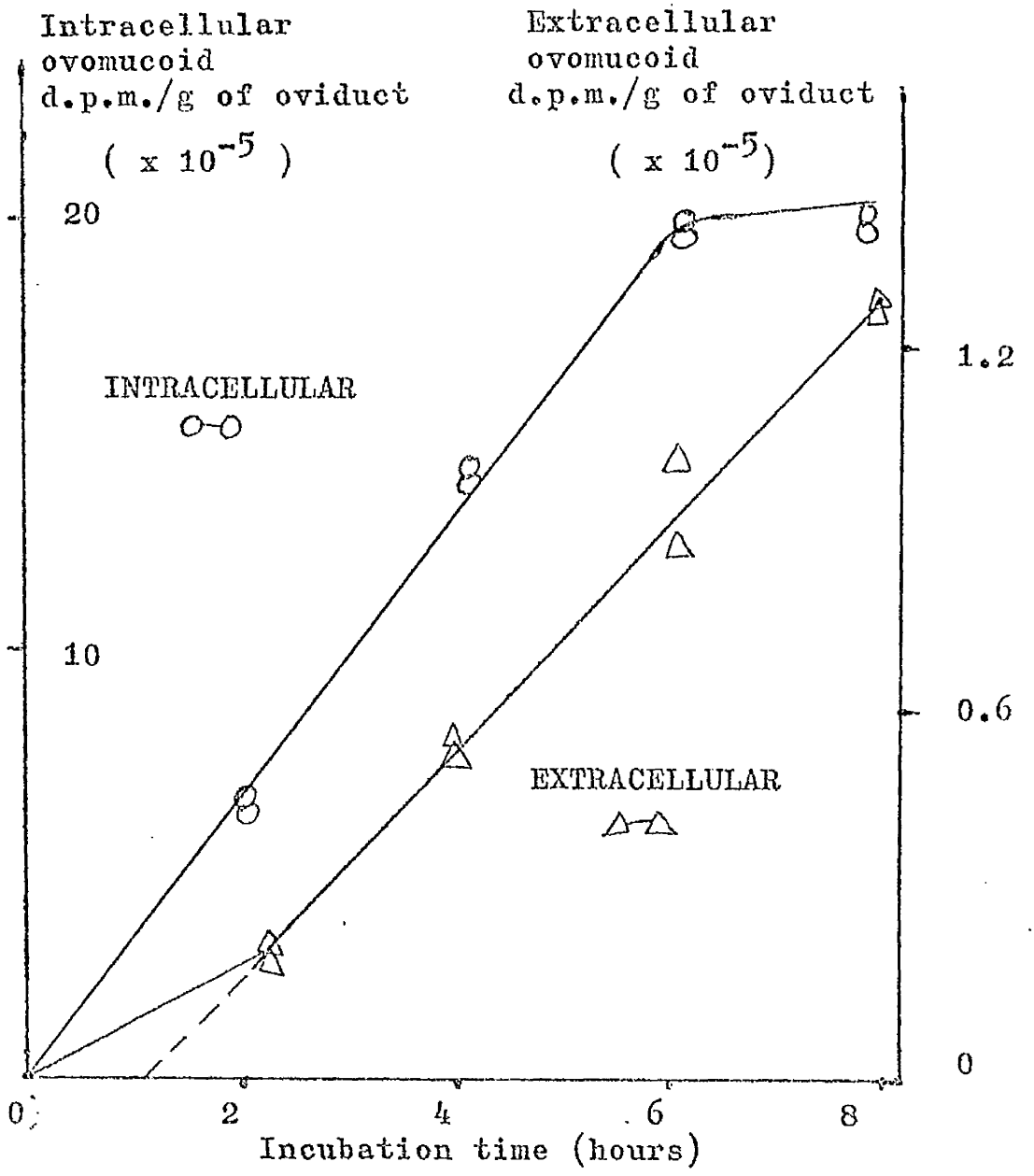


Fig. 34. The incorporation of [^{14}C] glucosamine into intracellular and extracellular ovomucoid. (Experiment 1).

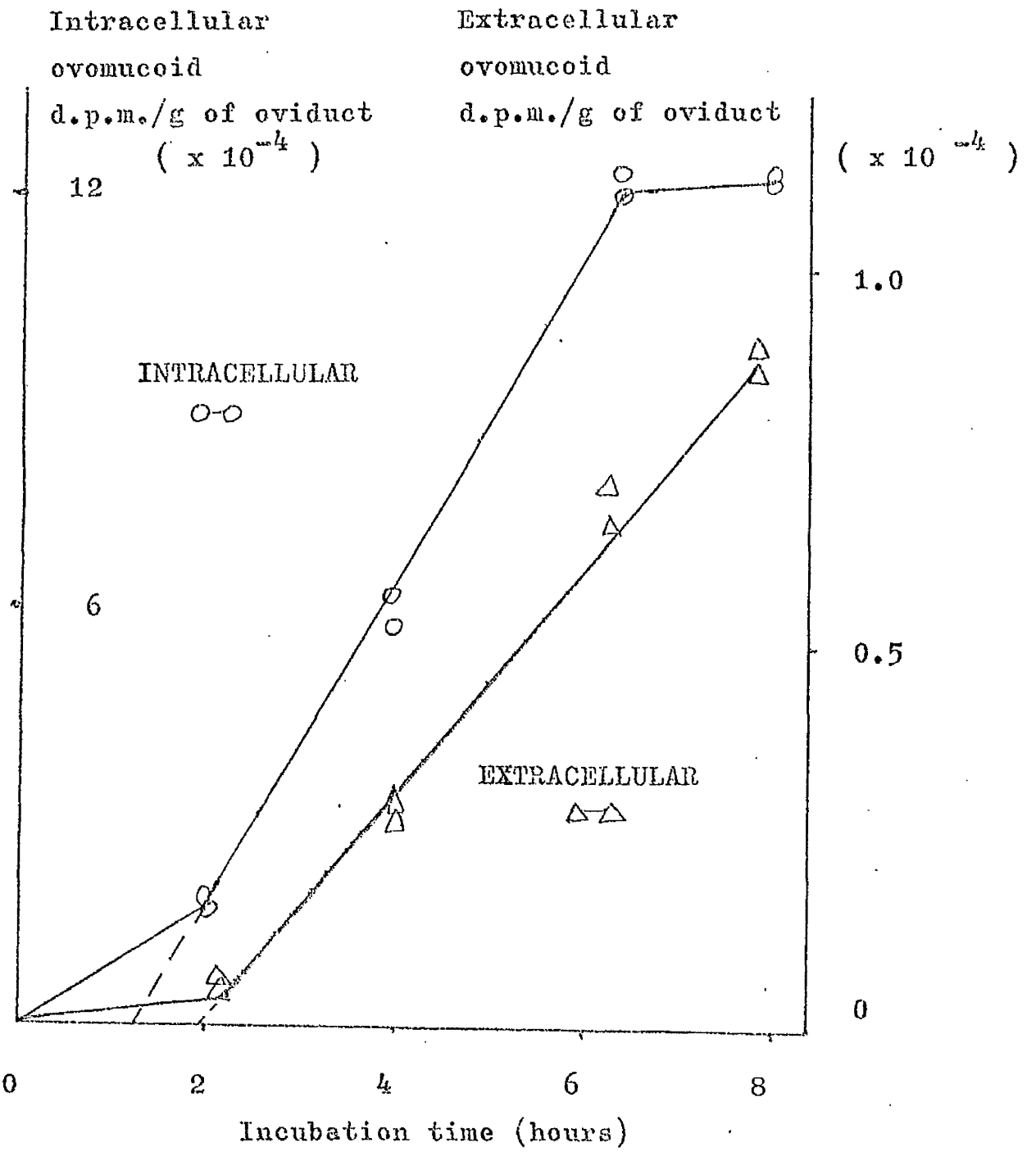
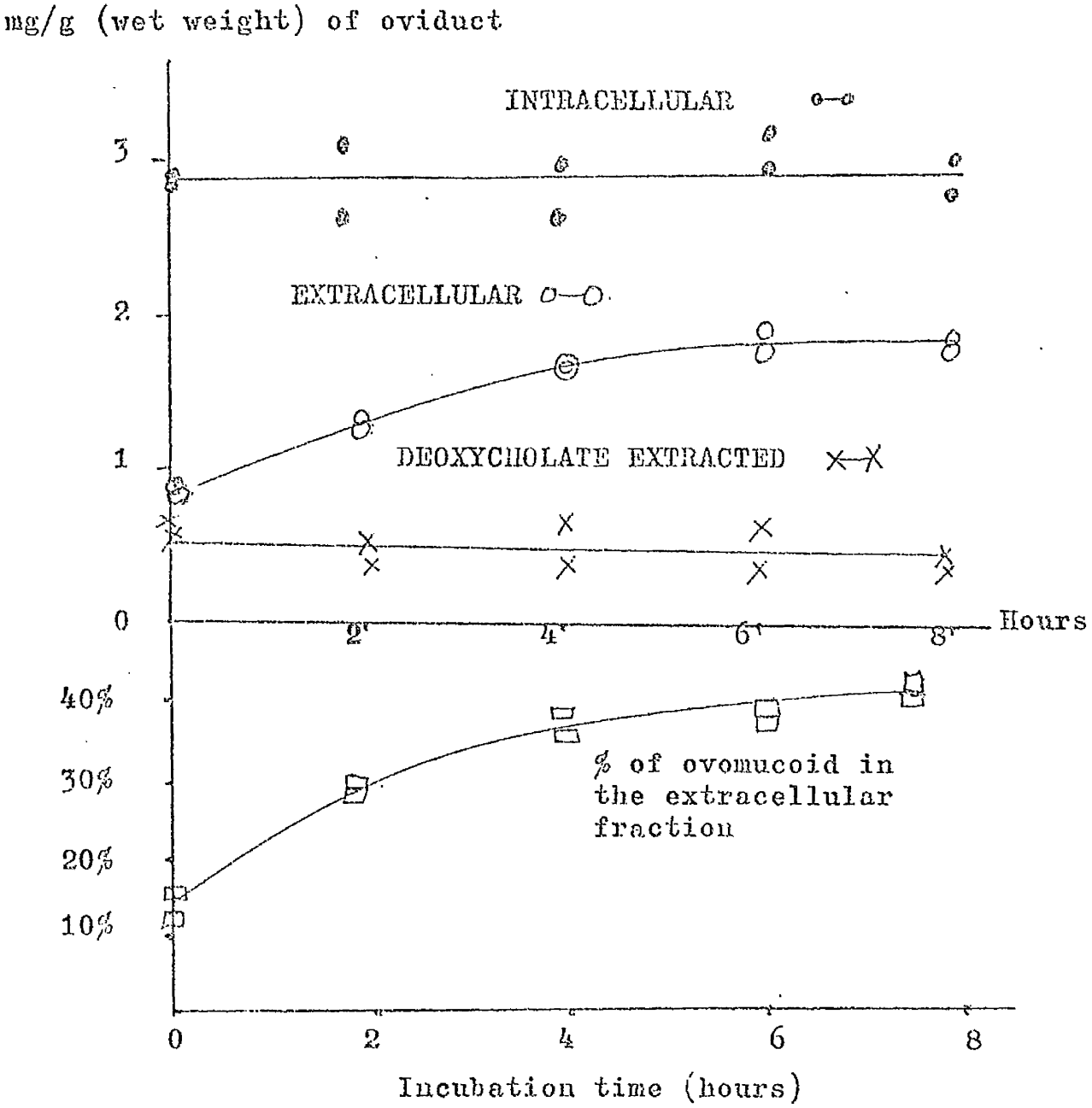


Fig. 35. Yields of ovomucoid from the tissue fractions in radioisotope studies. (Experiment 1)



another oviduct are also presented. In Fig. 36 it is seen that the incorporation of lysine into intracellular ovomucoid was linear over, approximately a 5 hour period in this experiment. After a lag period of about an hour, a small amount of newly synthesised material appeared in the extracellular fraction. The incorporation of glucosamine into intracellular ovomucoid (Fig. 37) again indicated the presence of an initial lag period. After this lag period (approximately 1 hour) the incorporation of glucosamine into intracellular ovomucoid was linear over the 7 hour period examined. In contrast to the experiment represented by Fig. 34, there was no observable lag period detected in the incorporation of glucosamine into extracellular ovomucoid in the experiment represented by Fig. 37. This may have reflected the availability of ovomucoid precursors to which carbohydrate could be added. While the amount of ovomucoid isolated from the intracellular fraction was fairly constant over the incubation period, the amount of ovomucoid isolated from the extracellular fraction increased from 10% of the total ovomucoid isolated at zero time up to 30% of the total after 7 hours (Fig. 38). From the total incorporation of lysine and glucosamine into extracellular ovomucoid compared to their incorporation into intracellular ovomucoid (Figs. 36 and 37), it was again indicated that only a small proportion of extracellular ovomucoid was newly synthesised material.

The $[^{3}H] : [^{14}C]$ ratio has decreased during the period 2 hours to 8 hours in both intracellular and extracellular ovomucoid (Fig. 39) in the experiment represented by Figs. 33-35. This observation was confirmed

Fig. 36. The incorporation of [^{3}H]lysine into intracellular and extracellular ovomucoid. (Experiment 2)

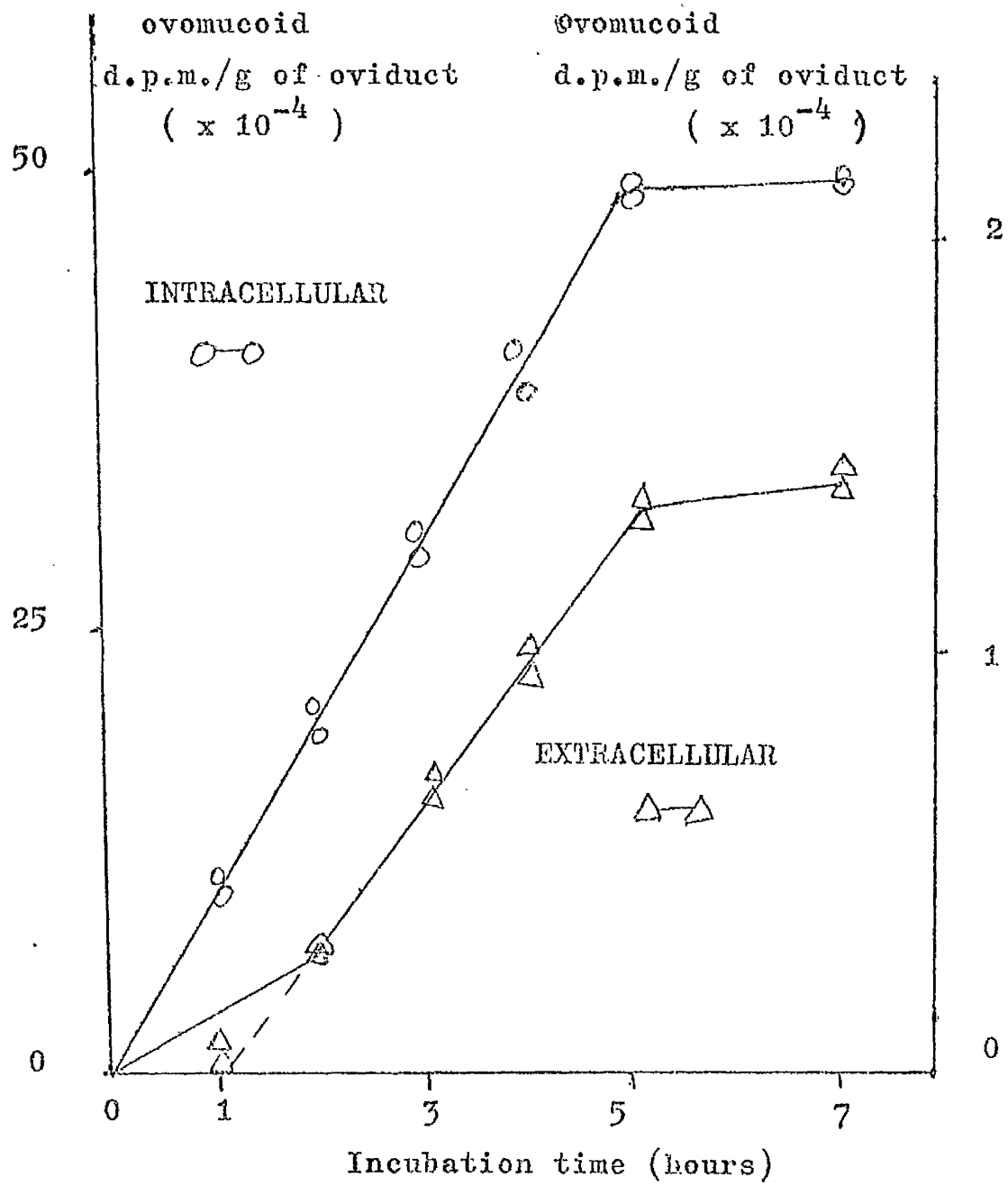
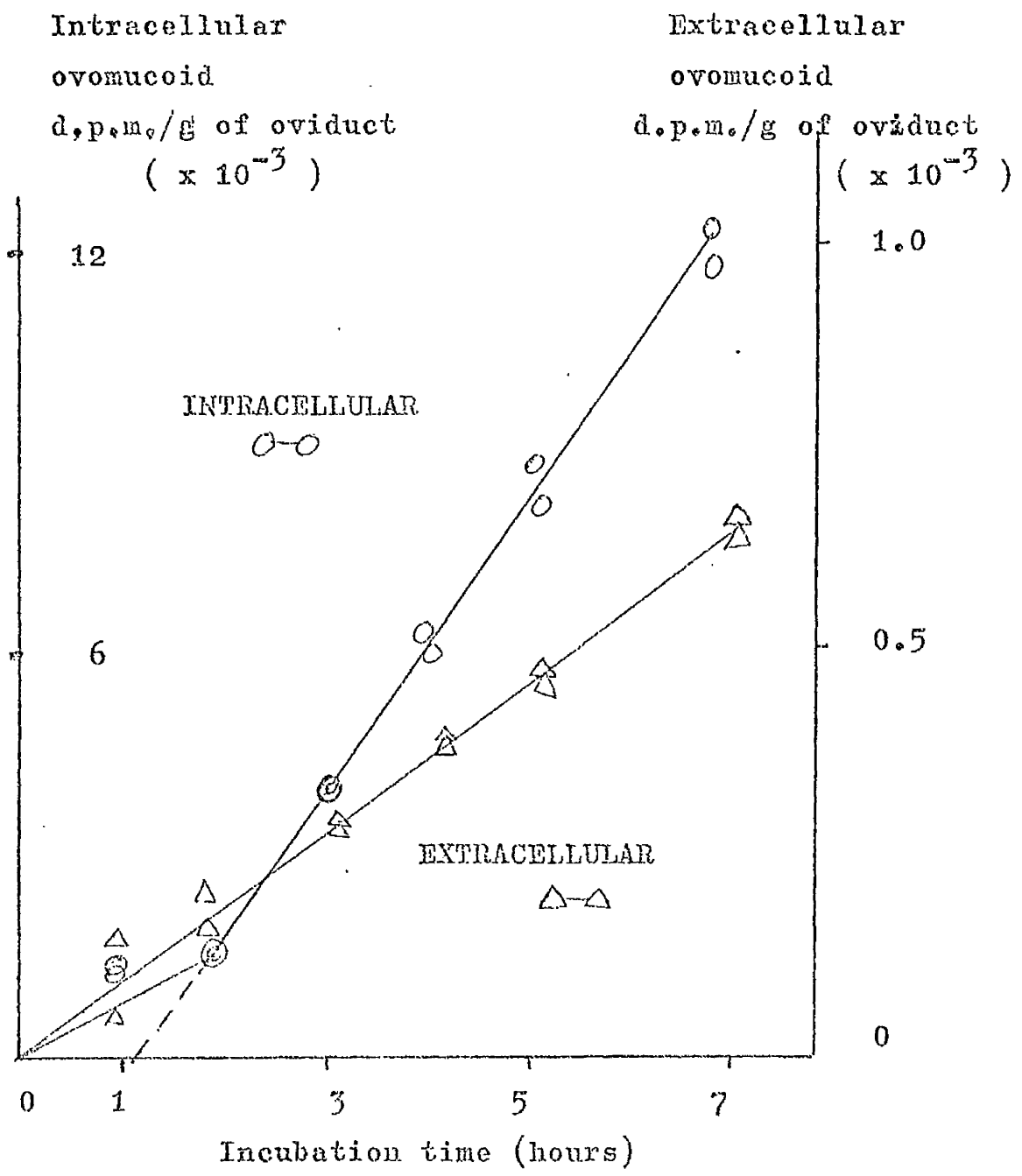
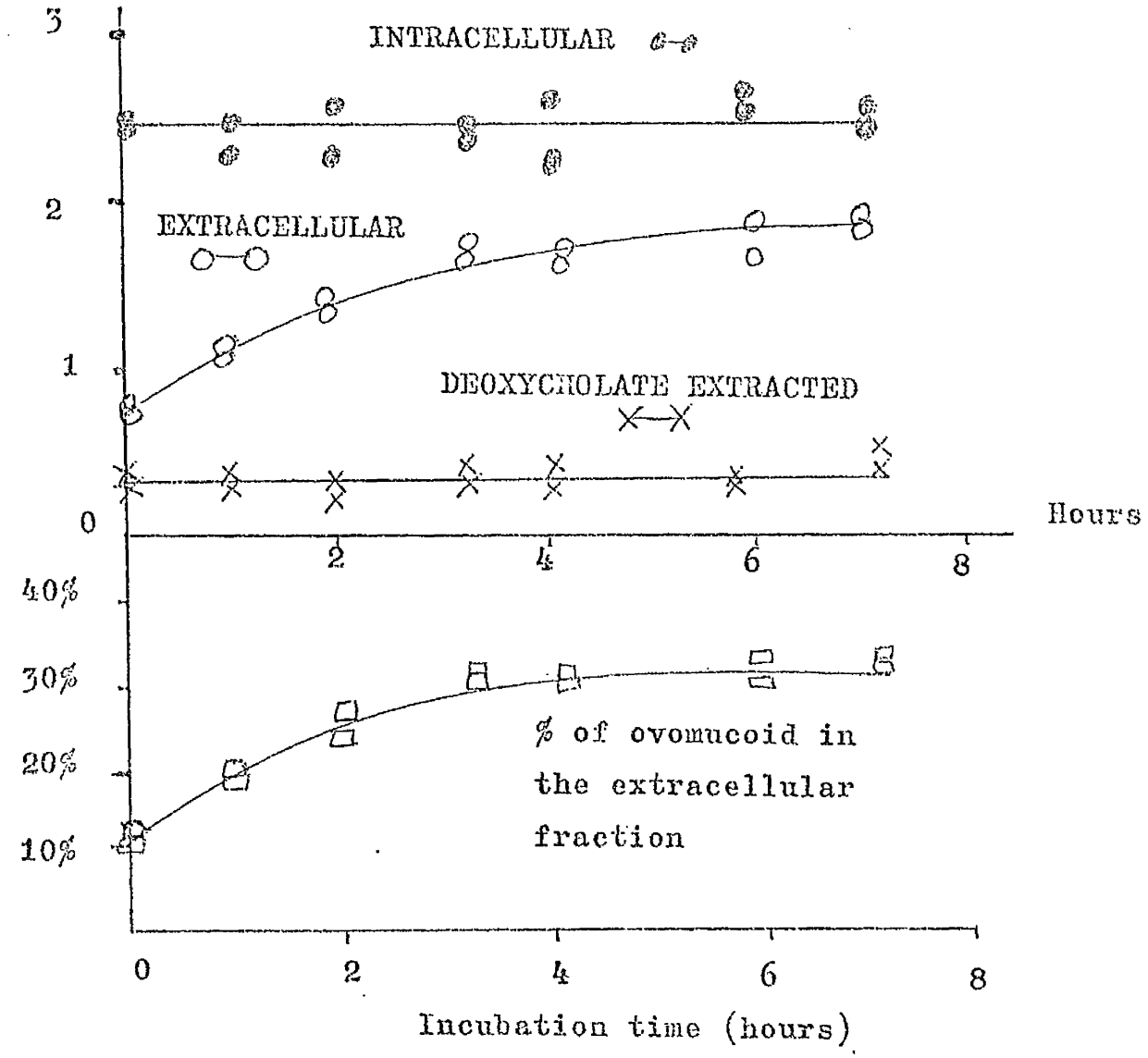


Fig. 37. The incorporation of [^{14}C]glucosamine into intracellular and extracellular ovomucoid. (Experiment 2).



Separation of ovomucoid from the tissue fractions in radioisotope studies. (Experiment 2).



Figs. 39 & 40. Ratios of $[^{3}H]$ lysine: $[^{14}C]$ glucosamine in intracellular and extracellular ovomucoid with time

Fig. 39. $[^{3}H]$: $[^{14}C]$ ratios (Experiment 1)

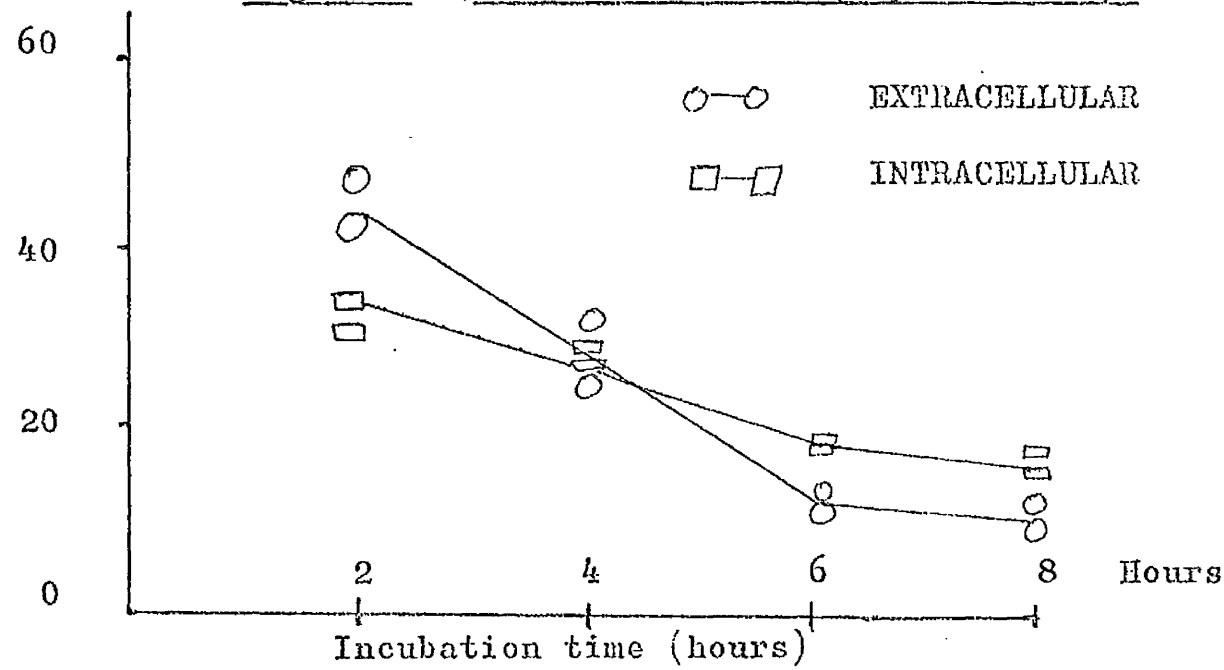
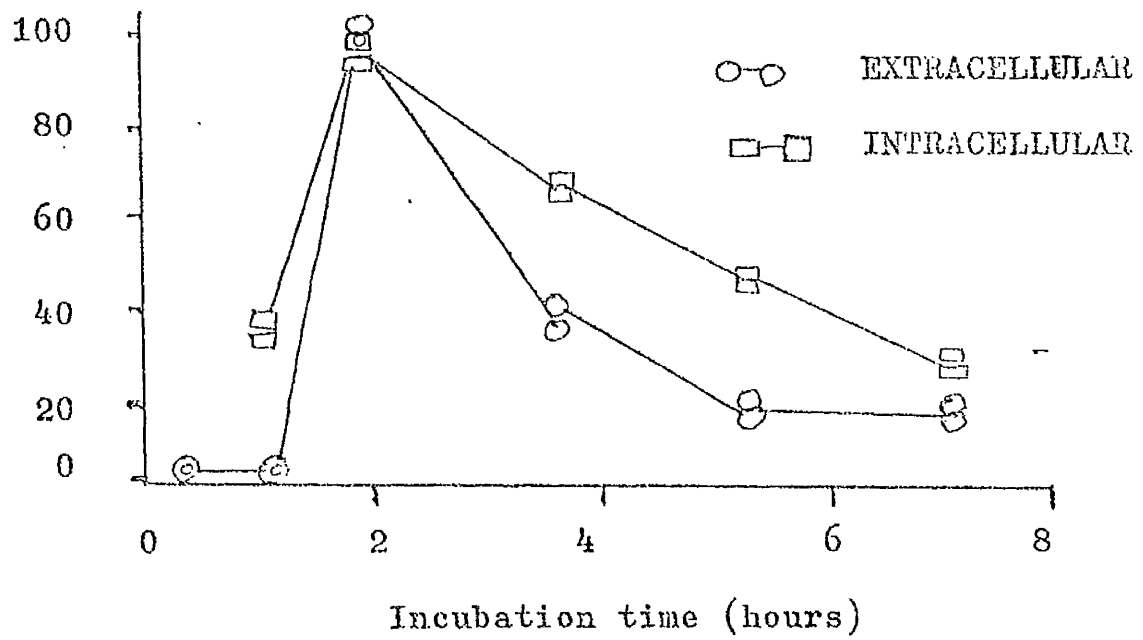


Fig. 40. $[^{3}H]$: $[^{14}C]$ ratios (Experiment 2)

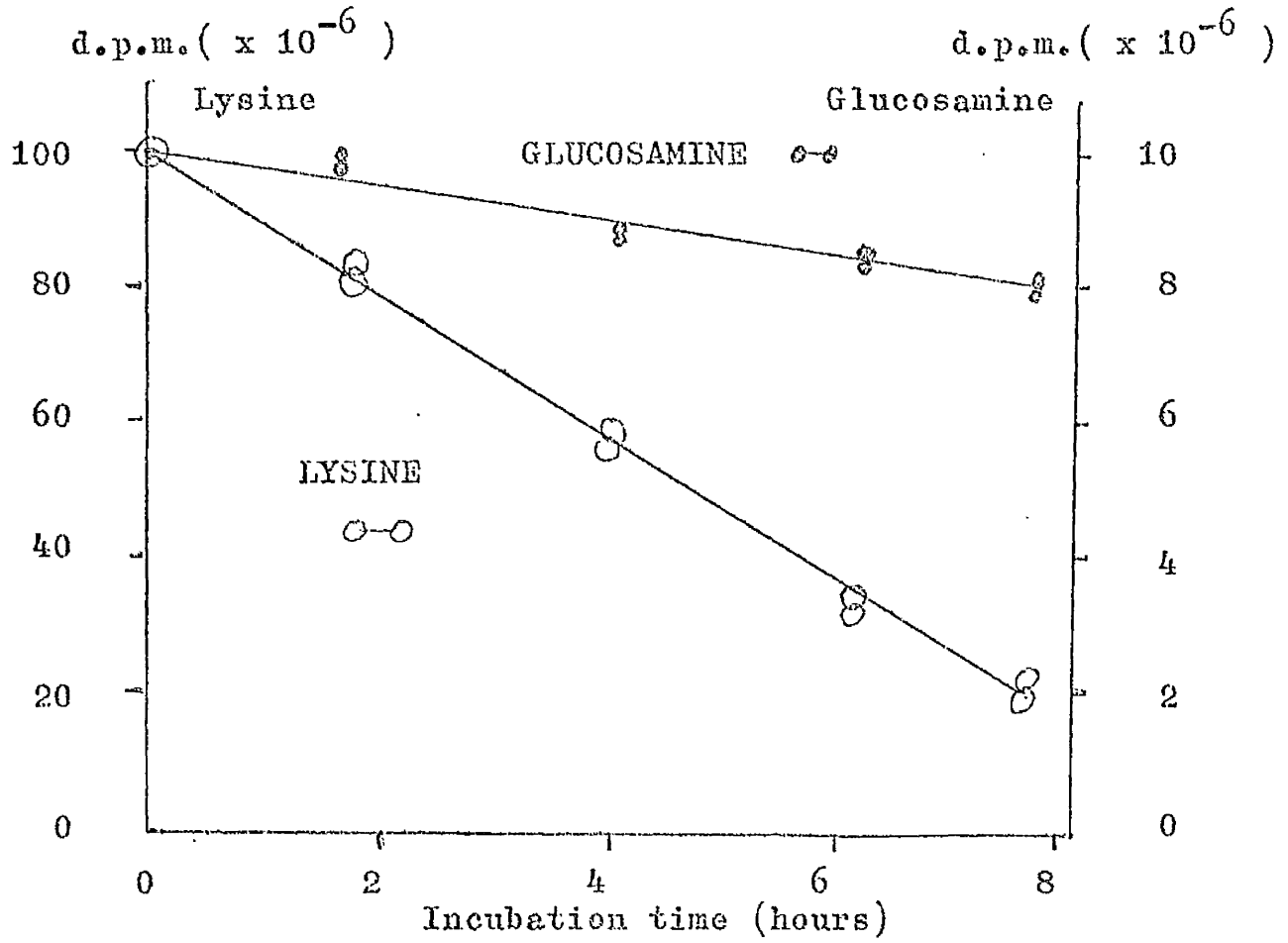


in Fig. 40 for the experiment represented by Figs. 36-38. However in Fig. 40 it can be seen that at times less than 2 hours, the $[^3H]$: $[^{14}C]$ ratio was much lower than it was at 2 hours. This may have represented the addition of $[^{14}C]$ labelled carbohydrate to ovomucoid precursors which had completed peptide portions but incompleted carbohydrate moieties.

After trichloroacetic acid precipitation of the incubation medium, the amounts of $[^3H]$ radioactivity and $[^{14}C]$ radioactivity remaining in the supernatant were calculated as indications of the amounts of free $[^3H]$ lysine and $[^{14}C]$ glucosamine remaining in the extracellular fraction with time. Both lysine and glucosamine were taken up linearly by the tissue, but lysine was taken up more rapidly than glucosamine (Fig. 41). After 6 hours, 80% of the added $[^3H]$ lysine had been taken up by the tissue while only 20% of the initial $[^{14}C]$ glucosamine had been taken up into the intracellular spaces of the tissue.

In the isolation of ovomucoid from the intracellular and extracellular fractions of the tissue, a preliminary precipitation with 5% (w/v) trichloroacetic acid at pH 3.5 was included. The precipitate from this step was washed twice on the centrifuge with 5% (w/v) trichloroacetic acid and the washed precipitate used as an indication of the total protein (minus ovomucoid) fraction of the tissue. It was more convenient to use the precipitate from 5% (w/v) trichloroacetic acid, pH 3.5, than to remove aliquots for a separate precipitation with 5% (w/v) trichloroacetic acid (not brought to pH 3.5). Both precipitation methods gave the same results. After washing the precipitate twice (a third wash was not effective in washing out any additional radioactivity) it was dissolved

Fig. 41. Uptake of isotope by the tissue



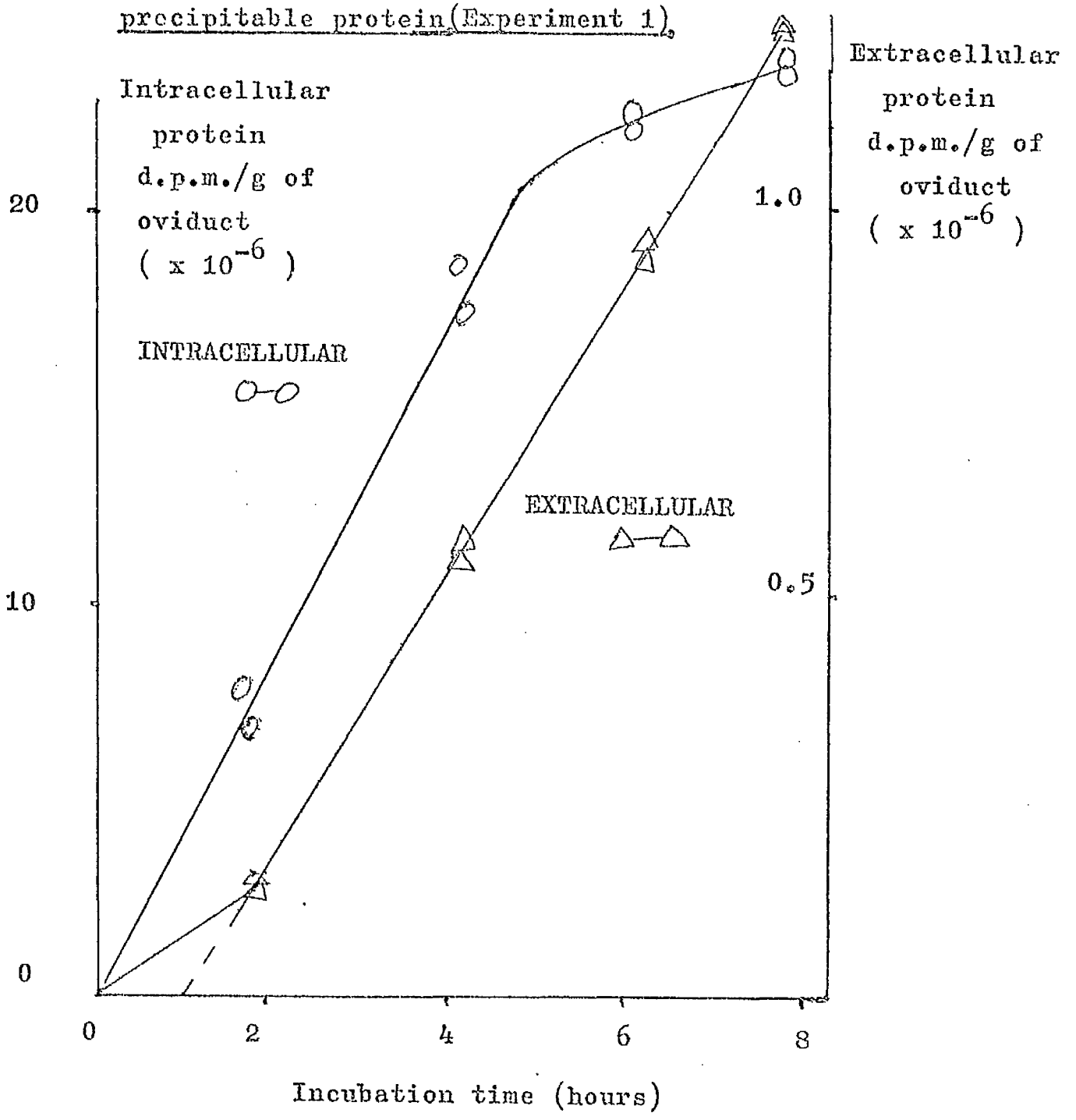
Data refer to Experiment 1. After trichloroacetic acid precipitation of the extracellular fraction an aliquot of the supernatant was prepared for liquid scintillation counting. The amount of radioactivity remaining in the medium with time is illustrated above.

in 0.5N NaOH for determination of protein content by the method of Lowry et al (1951) and radioactivity by liquid scintillation counting (Methods section).

The incorporation of lysine into the intracellular and extracellular total protein fractions in the experiment corresponding to Fig. 33 is given in Fig. 42. The incorporation of lysine into intracellular total protein was linear over, approximately, a 5 hour period with no detectable lag period. After a lag of about an hour, lysine labelled material began to appear in the incubation medium. The incorporation of glucosamine into total protein for the experiment corresponding to Fig. 34 is given in Fig. 43. There may have been a small lag period before glucosamine was incorporated into extracellular total protein. There was, however, a lag period of about an hour before glucosamine was incorporated into intracellular total protein. After this lag period, the incorporation of glucosamine into intracellular and extracellular total protein was linear over the time course (8 hours) examined.

In Fig. 44 it can be seen that the incorporation of lysine into intracellular total protein, in the experiment corresponding to Fig. 36, was also linear over 5 hours with no detectable lag period. There was a lag period of approximately 1 hour before the incorporation of lysine into extracellular total protein. It can be seen in Fig. 45, for the experiment corresponding to Fig. 37, that there was no detectable lag period in the incorporation of glucosamine into extracellular total protein. The incorporation of glucosamine into intracellular total

precipitable protein (Experiment 1).



acetic acid precipitable protein. (Experiment 1)

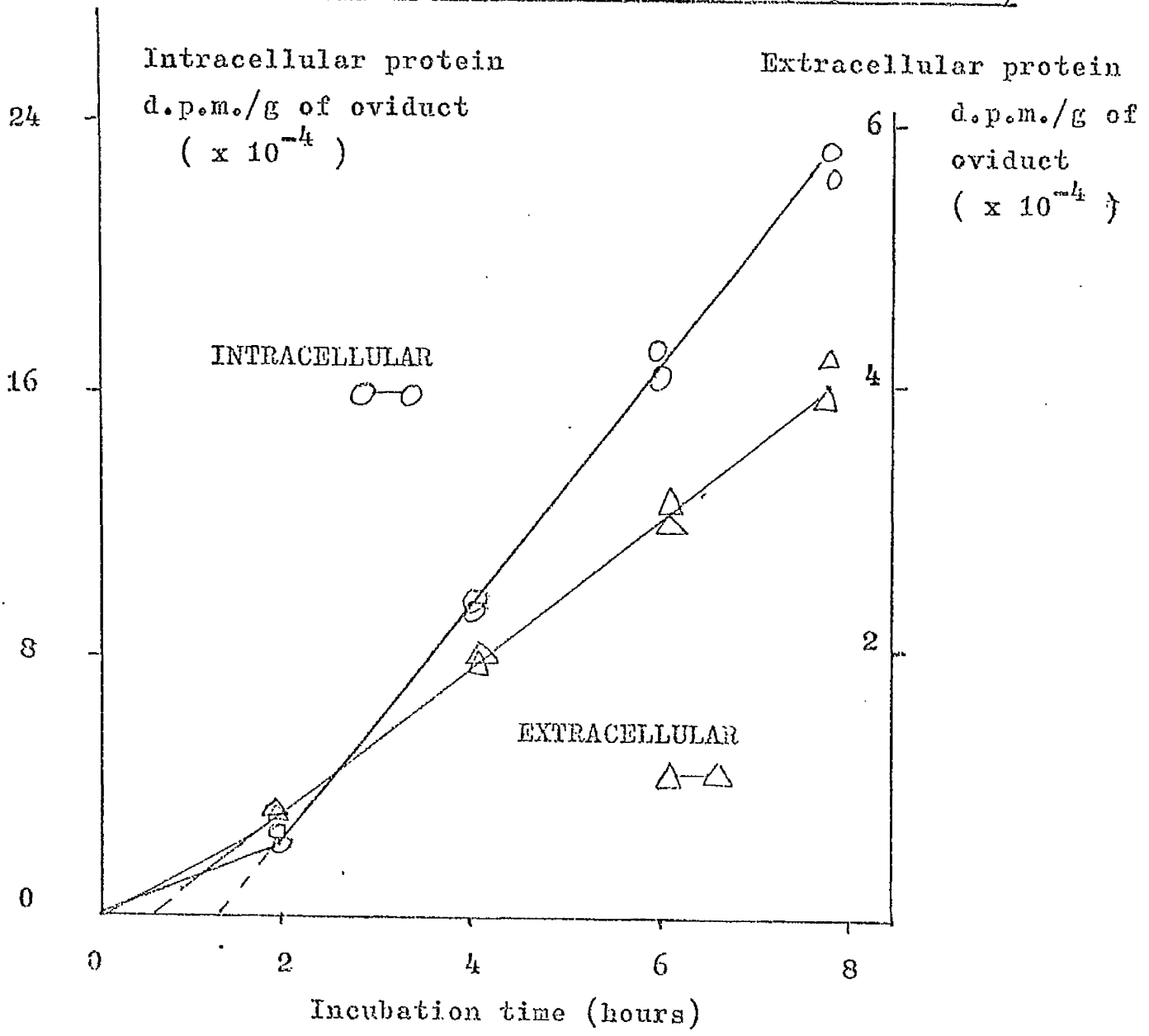


Fig. 44. Incorporation of lysine into the total trichloroacetic acid precipitable protein. (Experiment 2).

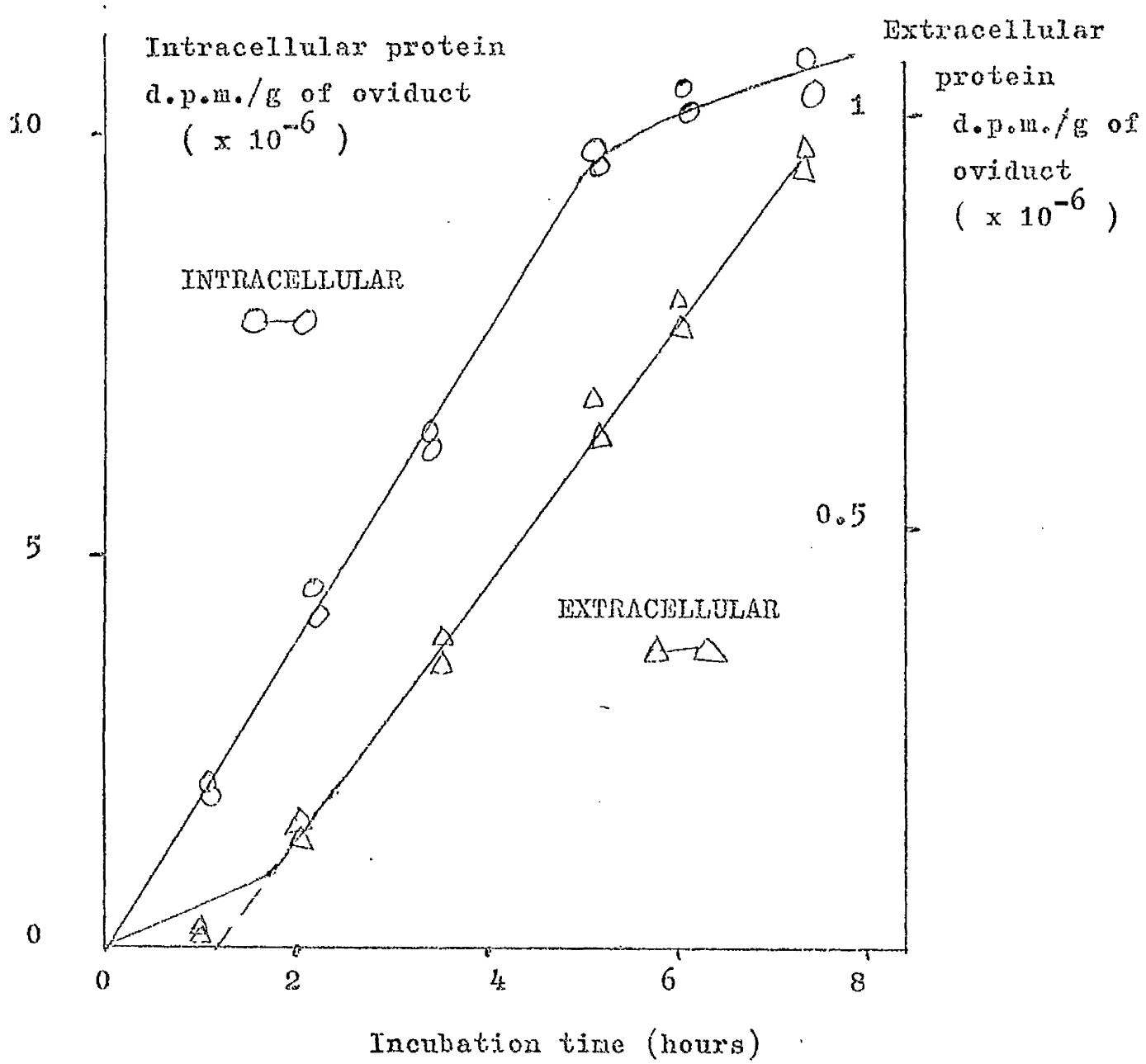
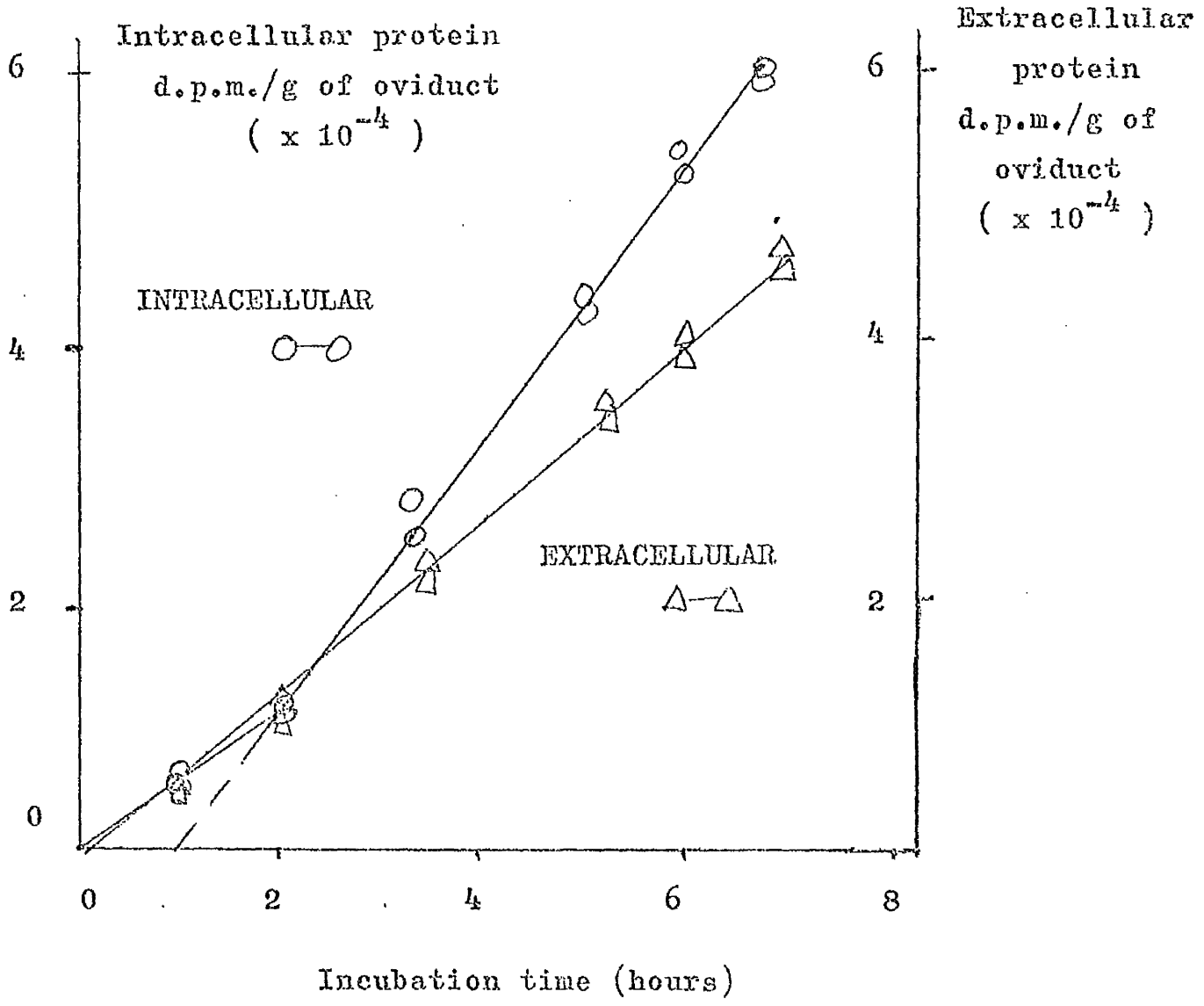


Fig. 42. Incorporation of glucosamine into the total trichloroacetic acid precipitable protein. (Experiment 2).



protein was linear over the time course (7 hours) examined, after an initial short lag period.

Apart from the absolute values obtained, the overall pattern of results from the two separate experiments depicted (Figs. 33, 34, 42 and 43 and Figs. 36, 37, 44 and 45) were similar. It was calculated that after 6 hours, in one experiment (Fig. 42) 22% of the added lysine was in intracellular total protein while 1% was in extracellular total protein. For the same experiment, 2% of the initial lysine was in intracellular ovomucoid, while 0.09% was in extracellular ovomucoid after this time (Fig. 33). In the other experiment (Fig. 44) 12% of the added lysine was in intracellular total protein while 1% was in extracellular total protein after 6 hours. In this experiment, 0.45% of the initial lysine was in intracellular ovomucoid and 0.012% in extracellular ovomucoid after this time (Fig. 36). From these figures it was calculated that after 6 hours in one experiment (Figs. 33 and 42) 8.4% of the lysine incorporated into all intracellular protein was in intracellular ovomucoid, while 8.3% of the lysine incorporated into all extracellular protein was in extracellular ovomucoid. In the other experiment after this time, 3.6% of the lysine incorporated into all intracellular protein was in intracellular ovomucoid, while 1.2% of the lysine incorporated into all extracellular protein was in extracellular ovomucoid (Figs. 36 and 44). Similar calculations showed that after 6 hours in one experiment (Fig. 43), 2% of the added glucosamine was in intracellular total protein while 0.25% was in extracellular total protein. After this time, 1.2% of the initial glucosamine was in

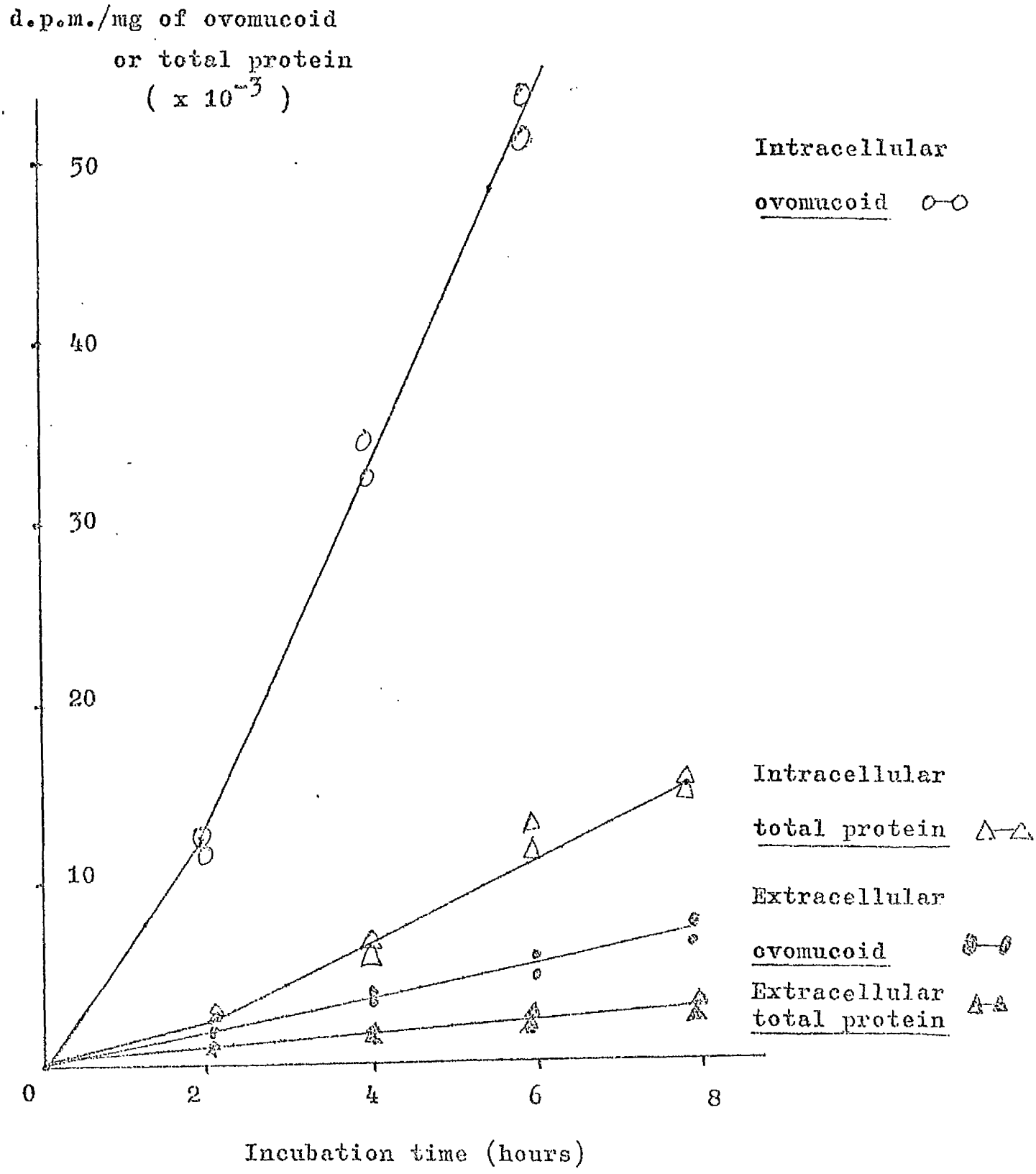
intracellular ovomucoid and 0.05% in extracellular ovomucoid (Fig. 34). The figures for the other experiment were that after 6 hours 0.55% of the initial glucosamine was in intracellular total protein while 0.4% was in extracellular total protein (Fig. 45). After this time, 0.11% of the added glucosamine was in intracellular ovomucoid while 0.005% was in extracellular ovomucoid (Fig. 37). From these figures it was calculated that after 6 hours in one experiment (Figs. 34 and 43) 37% of the glucosamine incorporated into all intracellular protein was in intracellular ovomucoid and 17% of all the glucosamine incorporated into extracellular protein was in extracellular ovomucoid. In the other experiment after this time (Figs. 37 and 45), 16.6% of the glucosamine incorporated into all intracellular protein was in intracellular ovomucoid while 1.2% of the glucosamine incorporated into all extracellular protein was in extracellular ovomucoid.

The incorporation of lysine into intracellular ovomucoid was much larger than the incorporation of lysine into extracellular ovomucoid (Fig. 33 and Fig. 36). There was a similar difference between the incorporation of glucosamine into intracellular and extracellular ovomucoid (Fig. 34 and Fig. 37). However, although the incorporation of lysine into intracellular total protein was greater than the incorporation of lysine into extracellular total protein (Fig. 42 and Fig. 44), the difference between the incorporation of glucosamine into intracellular and extracellular total protein was not so marked (Fig. 43 and Fig. 45). There may be some intracellular proteins synthesised which are not for secretion, and the smaller difference in incorporation of glucosamine

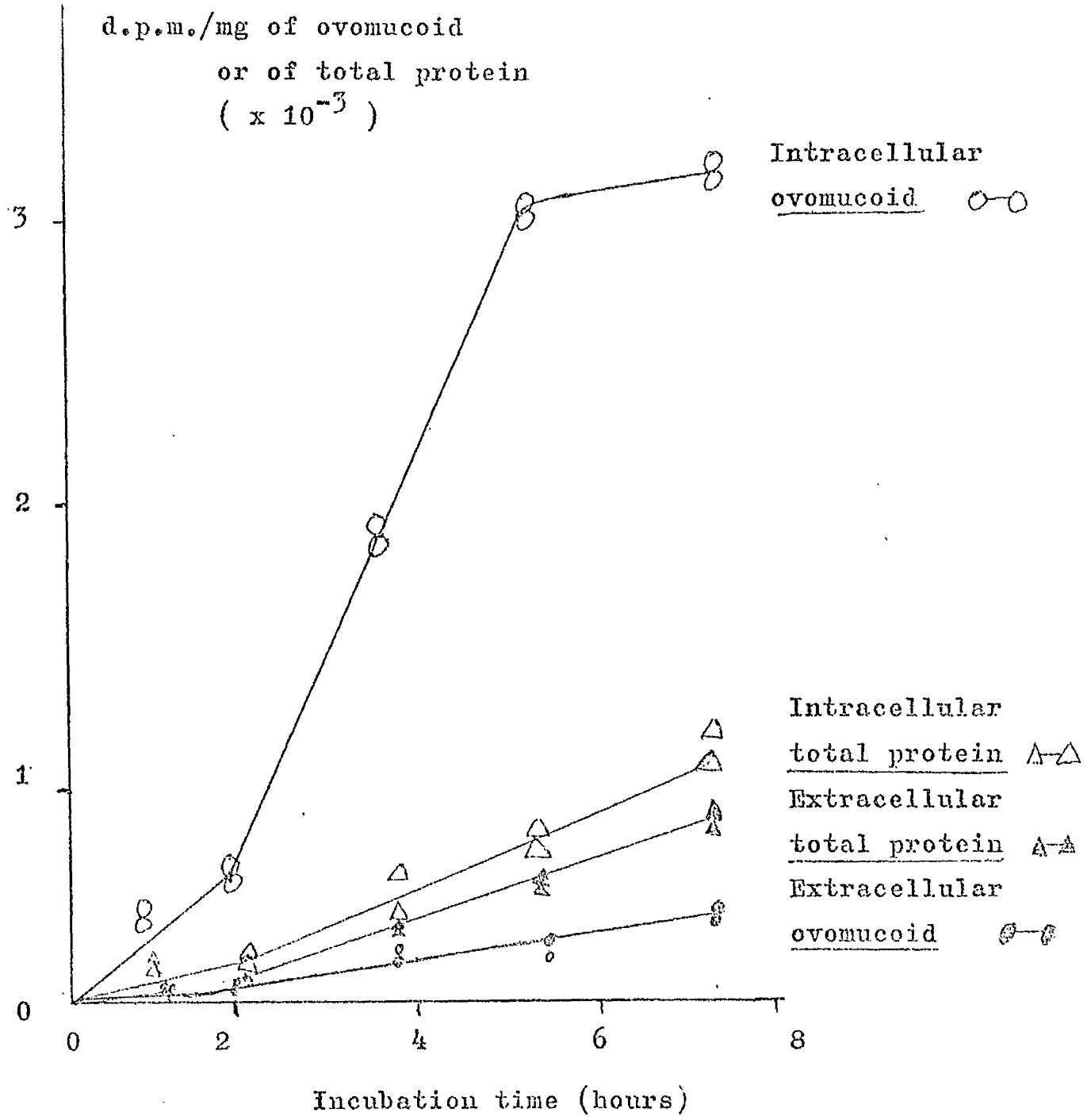
into intracellular and extracellular total protein when compared to the difference between the incorporation of lysine into intracellular and extracellular total protein (Fig. 43 compared to Fig. 42 and Fig. 45 compared to Fig. 44) may be a reflection of the large proportion of glycosylated proteins in oviduct secretions. It can be seen in Fig. 46, for the experiment corresponding to Figs. 34 and 43, that the specific radioactivity (d.p.m./mg of ovomucoid) of intracellular glucosamine labelled ovomucoid was much higher at all times than the specific radioactivity (d.p.m./mg of protein) of glucosamine labelled intracellular total protein. Although the specific radioactivity of extracellular glucosamine labelled ovomucoid was higher than the specific radioactivity of glucosamine labelled extracellular total protein, these values were nearer to each other than were the values for intracellular total protein and ovomucoid. In Fig. 47, for the experiment represented by Figs. 37 and 45, it was confirmed that the specific radioactivity of intracellular glucosamine labelled ovomucoid was much higher than the specific radioactivity of intracellular glucosamine labelled total protein at all times. This time, the specific radioactivity of glucosamine labelled extracellular total protein was higher than the specific radioactivity of glucosamine labelled extracellular ovomucoid, but once again these values were much closer together than the specific radioactivities of intracellular ovomucoid and total protein.

In Fig. 48, for the experiment corresponding to Figs. 33 and 42, it can be seen that the specific radioactivity of lysine labelled intracellular ovomucoid was only marginally lower at all times than the

labelled ovomucoid and total protein. (Experiment 1).



glucosamine labelled ovomucoid and total protein. (Experiment 2).



labelled ovomucoid and total protein. (Experiment 1).

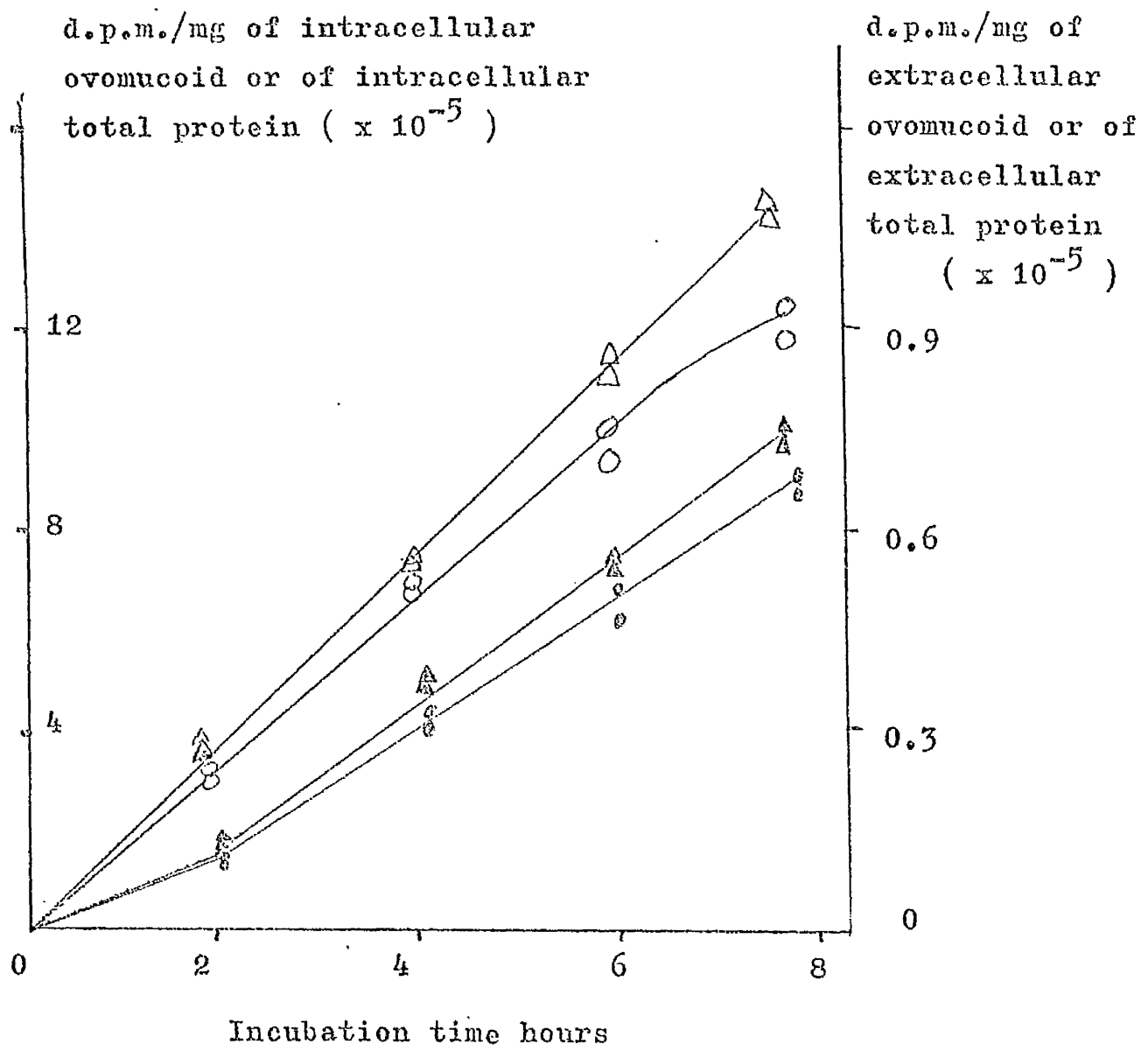
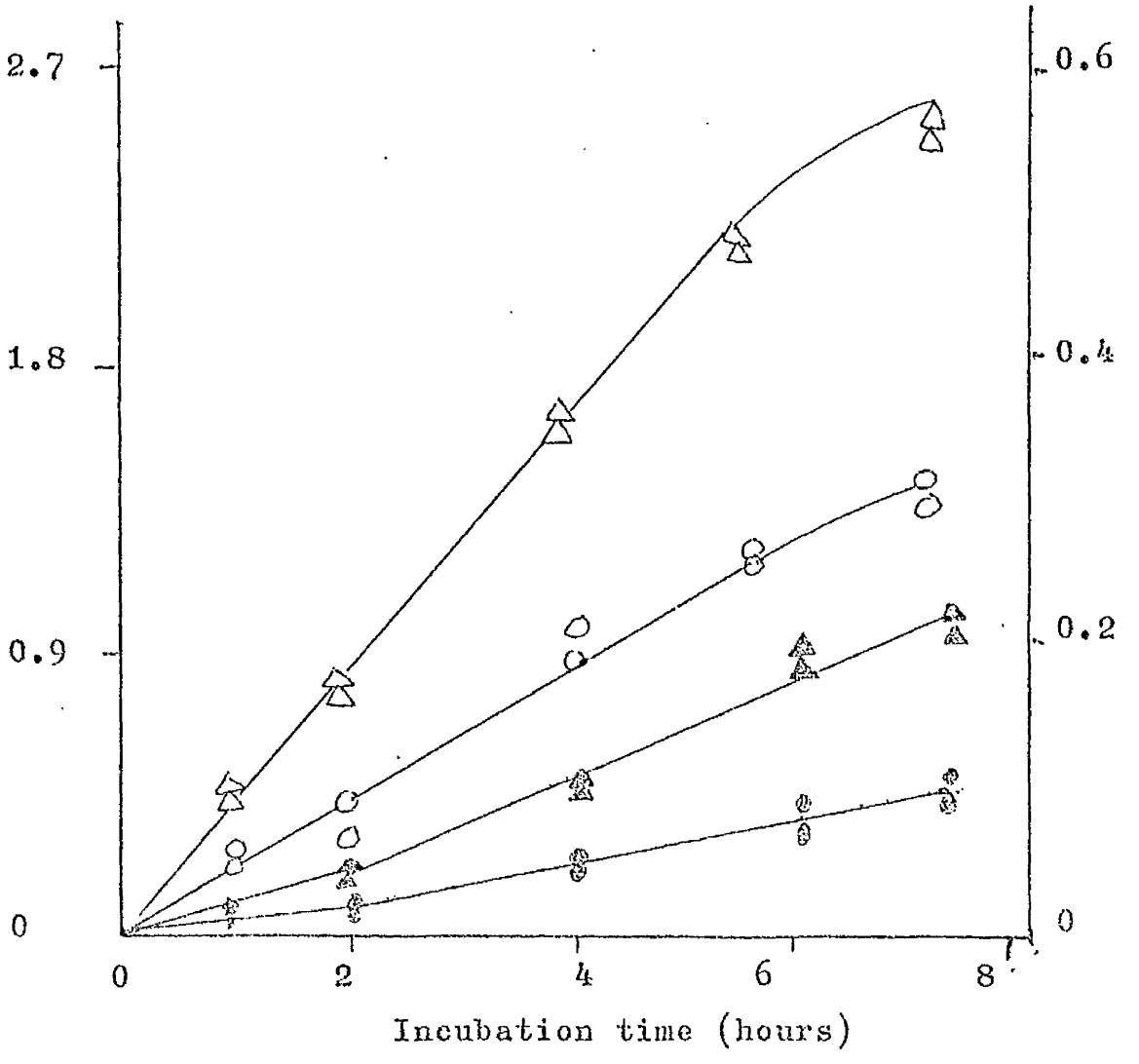


Fig. 49. Comparison of the specific radioactivities of lysine labelled ovomucoid and total protein. (Experiment 2)



specific radioactivity of lysine labelled intracellular total protein. The specific radioactivities of lysine labelled extracellular ovomucoid and total protein were likewise similar. In Fig. 49, for the experiment corresponding to Figs. 36 and 44, however, it can be seen that the specific radioactivity of intracellular lysine labelled ovomucoid was much lower than (approximately $\frac{1}{2}$) that of intracellular lysine labelled total protein over the time course examined (7 hours). Similarly, the specific radioactivity of extracellular lysine labelled ovomucoid was lower than that of extracellular lysine labelled total protein over the time course. As the specific radioactivity of ovomucoid was variable when compared to that of the total acid precipitable protein fraction in experiments with different oviducts, it may be that ovomucoid can be synthesised at a different though varying rate from total protein (minus ovomucoid) synthesis. Egg white proteins may, of course, be synthesised at cytologically exclusive sites (Wyburn et al, 1970; Kohler et al, 1968; O'Malley et al, 1967; Korenman & O'Malley, 1968).

In the experiment represented by Figs. 33 and 34, and 42 and 43, when the oviduct was excised, an egg was just moving from the protein secreting magnum portion of the oviduct into the shell gland region for the laying down of the shell. In the experiment represented by Figs. 36 and 37, and 44 and 45, the shell had been completed and the egg was about to be laid. This may explain the variation in absolute values between the results from experiments performed on separate oviducts, if there was a difference in the rate of synthesis of egg white proteins at different times in the egg laying cycle. In terms of lysine

labelling, the variation in the specific radioactivity of ovomucoid when compared to the specific radioactivity of total protein (Figs. 48 and 49) was similar to that found by Mandeles and Ducay (1962). They found that when oviducts were excised at varying times post-oviposition, the specific radioactivity of glycine labelled ovomucoid was sometimes higher and sometimes lower than the specific radioactivity of ovalbumin after a 3 hour incubation period of oviduct *in vitro* with [^{14}C] glycine. As the variable rate of ovomucoid synthesis when compared to ovalbumin or total protein synthesis seems to depend on the stage of the egg laying cycle, it is possible that this is a reflection of the hormone balance of the hen.

3. 13 (8) The Possible Effects of Hormones on the Synthesis or Secretion of Ovomucoid

To examine the possibility that ovomucoid synthesis or secretion might be stimulated *in vitro* by hormones, tissue incubation studies were carried out in the presence of progesterone or estrogen. In Table 20 it can be seen that 0.01μM progesterone had virtually no effect on the specific radioactivity of intracellular ovomucoid, in terms of lysine labelling or glucosamine labelling, isolated after a 5 hour incubation period. The specific radioactivities of both lysine labelled and glucosamine labelled intracellular ovomucoid were reduced when tissue incubations were carried out in the presence of 1μM progesterone. Progesterone, 0.01μM or 1μM had little effect on the specific radioactivity of extracellular ovomucoid, isolated from the tissue after a 5 hour incubation period. While incubation for 5 hours in the presence of

Table 20. The possible effects of hormones on the synthesis or secretion of ovomucoid

Fraction	Intracellular ovomucoid		Extracellular ovomucoid	
	d.p.m./mg of ovomucoid		d.p.m./mg of ovomucoid	
Isotope	Lysine	Glucosamine	Lysine	Glucosamine
Control	38,900	1420	3520	185
+Progesterone (0.01μM)	39,600	1590	4270	270
+Progesterone (1μM)	22,400	525	4350	144
+Estrogen (0.01μM)	24,100	885	3240	151
+Estrogen (1μM)	37,700	1310	4740	240
+Progesterone (0.01μM) & estrogen (0.01μM)	26,600	1030	5600	290
+Progesterone (1μM) & estrogen (1μM)	27,200	960	3500	203

Incubation was for a 5 hour period with $[^{3}H]$ lysine (40 μCi/g of oviduct) and $[^{14}C]$ glucosamine (4μCi/g of oviduct).

0.01μM estradiol has caused some reduction in the specific radioactivities of lysine labelled and glucosamine labelled intracellular ovomucoid, tissue incubations in the presence of 1μM estradiol resulted in intracellular ovomucoid which had a similar specific radioactivity to the control. Estradiol, 0.01μM or 1μM had little effect on the specific radioactivity of extracellular ovomucoid isolated from the tissue after a 5 hour incubation period. When a combination of progesterone and estradiol was used, there was no increase in the specific radioactivity of lysine labelled or glucosamine labelled intracellular ovomucoid compared to the control. The combination of hormones, progesterone 0.01μM and estradiol 0.01μM has increased, to an extent, the specific radioactivity of extracellular ovomucoid isolated after a 5 hour incubation. This increase was not large, however, and when tissue incubations were carried out in the presence of both hormones at a concentration of 1μM, the extracellular ovomucoid isolated after 5 hours had a similar specific radioactivity to that of the control, in terms of both lysine and glucosamine labelling.

It is possible that in these studies neither the right concentration of either hormone nor the correct balance of the hormones was chosen. However, under the conditions tested, there was no combination of progesterone and estrogen which would stimulate ovomucoid synthesis or secretion in vitro. In view of this lack of stimulation, no further examinations were made on the possible effects of hormones on the synthesis or secretion of ovomucoid.

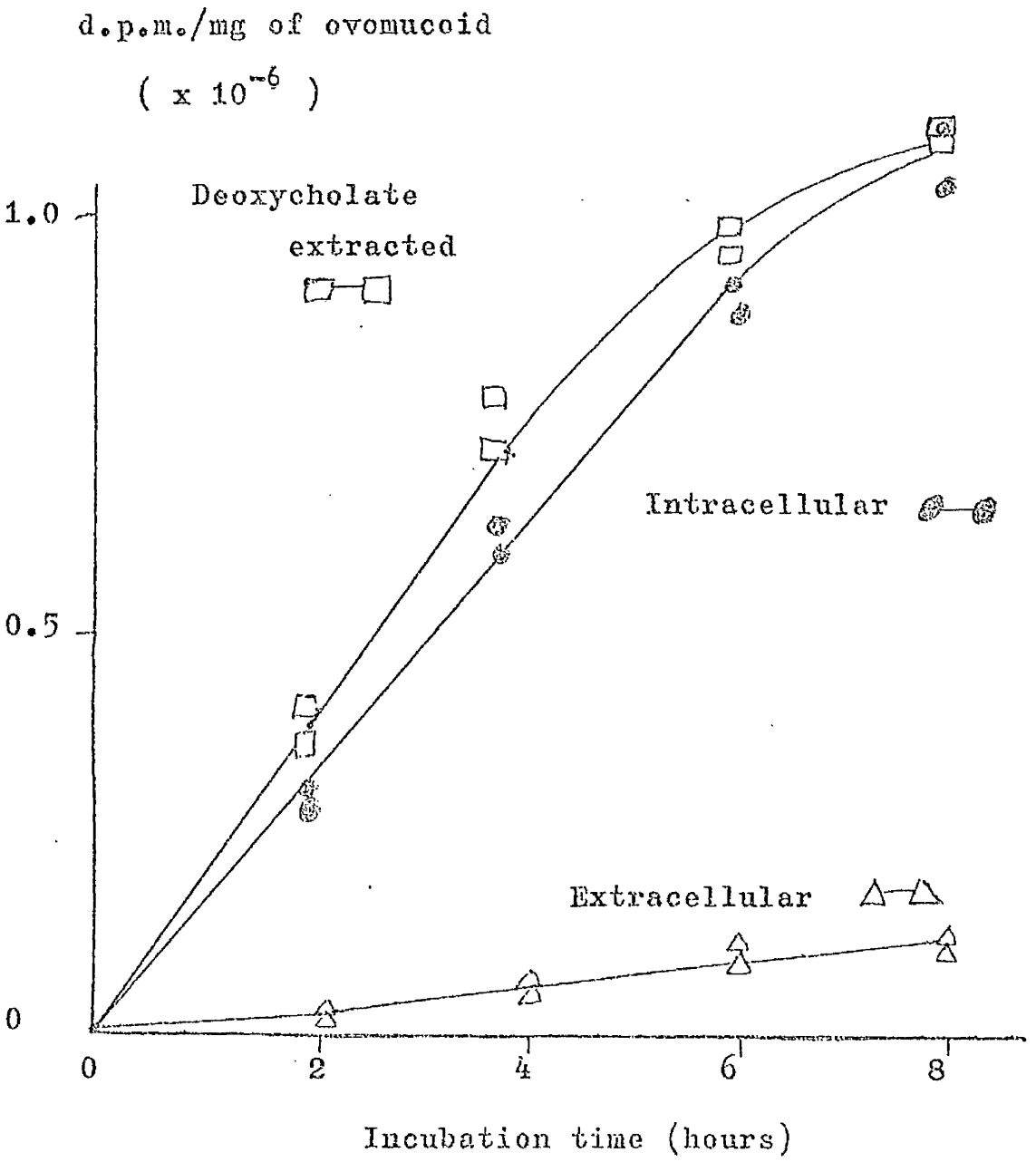
3. 13 (9) The Incorporation of Precursors into Deoxycholate Extracted Ovomucoid

The general pattern of glycoprotein biosynthesis is that the growing glycoprotein moves from the rough membranes of the endoplasmic reticulum through the smooth membranes of the endoplasmic reticulum to the Golgi apparatus (Rambourg et al, 1969; Choi et al, 1971; Melchers, 1971). Particle bound glycoprotein precursors which could be released by deoxycholate have been found in tissues (Spiro & Spiro, 1966). In view of these findings, the specific radioactivity of the ovomucoid which washed out of the 105,000g pellet from oviduct homogenate by use of deoxycholate was determined in radioisotope studies.

With [^{3}H] lysine as precursor, the specific radioactivity (d.p.m./mg) of deoxycholate extracted ovomucoid was slightly higher than that of intracellular ovomucoid in the experiment corresponding to Fig. 33 (Fig. 50). The specific radioactivity of extracellular ovomucoid was much less than these values (Fig. 50). With [^{14}C] glucosamine as precursor, the specific radioactivity of deoxycholate extracted ovomucoid was indistinguishable from that of intracellular ovomucoid, while the specific radioactivity of extracellular ovomucoid was much lower than both of these (Fig. 51). Due to the low yield of ovomucoid, 0.3mg/g of oviduct in this fraction (Fig. 35), the total incorporation (d.p.m./g of oviduct) of [^{3}H] lysine (Fig. 52a) and [^{14}C] glucosamine (Fig. 52b) into deoxycholate extracted ovomucoid were very low.

In the experiment represented by Fig. 36, the specific radioactivity of lysine labelled ovomucoid from the deoxycholate extracted fraction

Fig. 50. The specific radioactivity of lysine labelled deoxycholate extracted ovomucoid. (Experiment 1)



deoxycholate extracted ovomucoid. (Experiment 1)

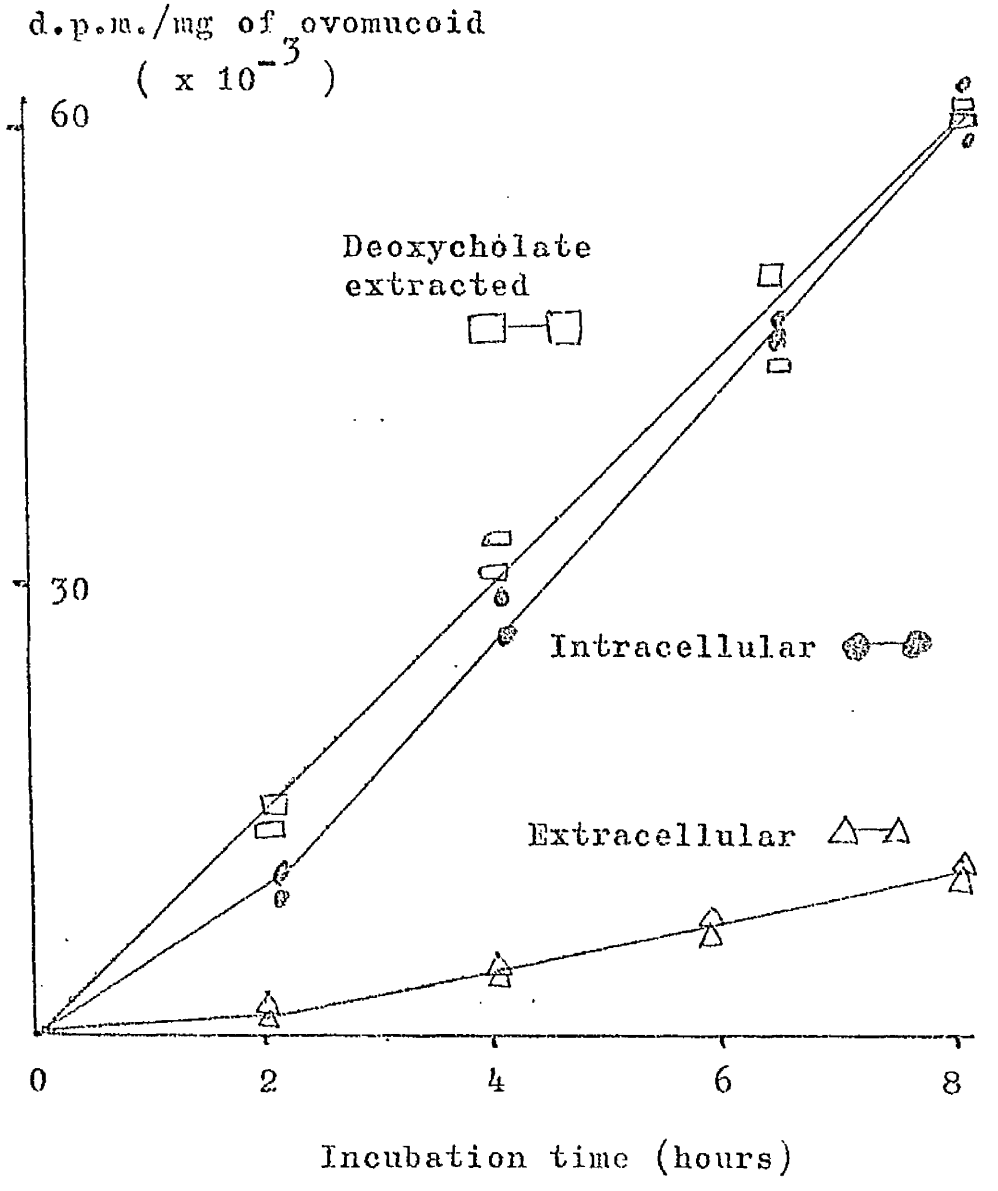


Fig. 52. Total incorporation of isotope into deoxycholate extracted ovomucoid. (Experiment 1)

Fig. 52a. Lysine incorporation

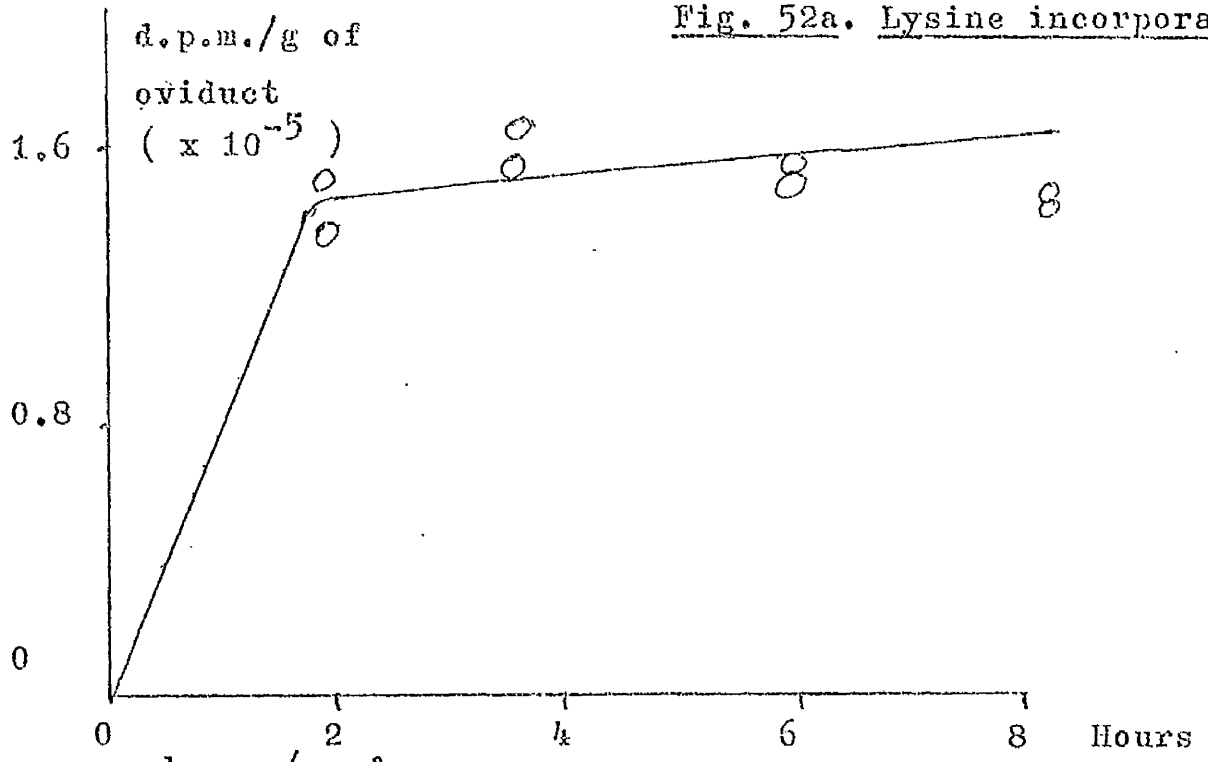
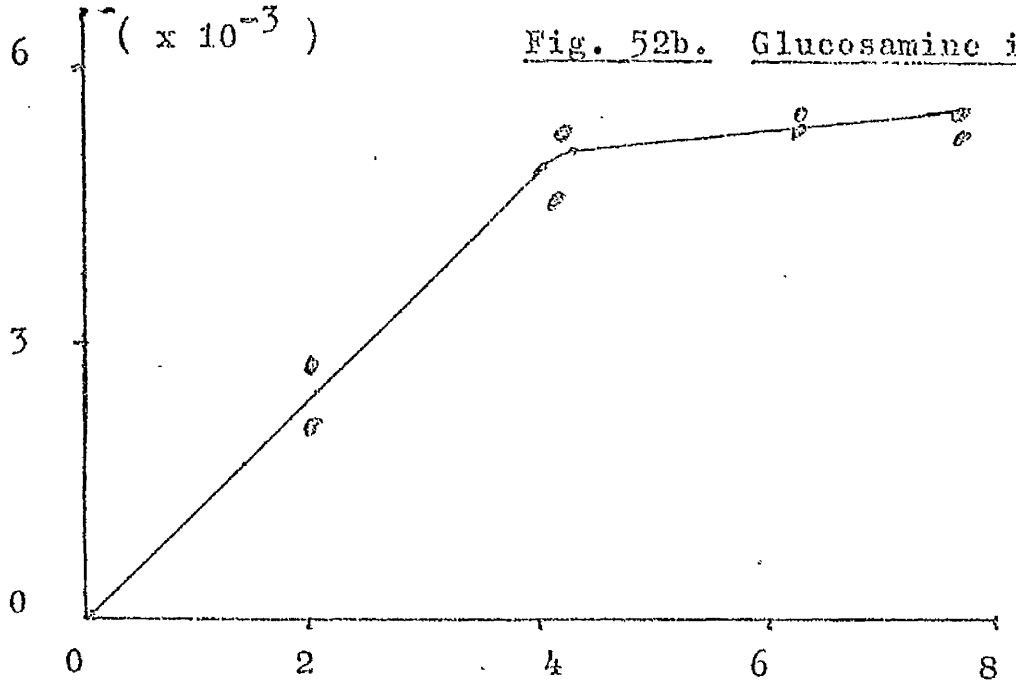


Fig. 52b. Glucosamine incorporation



(Fig. 53) was higher than that of intracellular lysine labelled ovomucoid. The specific radioactivity of deoxycholate extracted glucosamine labelled ovomucoid was higher than that of intracellular glucosamine labelled ovomucoid (Fig. 54). In terms of both lysine labelling (Fig. 53) and glucosamine labelling (Fig. 54), the specific radioactivities of extracellular ovomucoid were lower than those of intracellular or deoxycholate extracted ovomucoid. The low yield of deoxycholate extracted ovomucoid, 0.3mg/g of oviduct (Fig. 38) meant that the total incorporation of labelled lysine (Fig. 55a) into ovomucoid from this fraction was lower than the total incorporation into intracellular ovomucoid (Fig. 36). The total incorporation of glucosamine into deoxycholate extracted ovomucoid (Fig. 55b) was likewise lower than the incorporation of glucosamine into intracellular ovomucoid (Fig. 37).

In the experiment represented by Figs. 50-52, the ratio of $[^{3}H]$ lysine : $[^{14}C]$ glucosamine in deoxycholate extracted ovomucoid fell during the period 2-8 hours (Fig. 56). Similar ratios of amino acid : carbohydrate were found in intracellular and extracellular ovomucoid (Fig. 39). These observations were confirmed for the period 2-7 hours in Fig. 57 for the experiment represented by Figs. 53-55. However, at times less than 2 hours (Fig. 57), the ratio of $[^{3}H]$ lysine : $[^{14}C]$ glucosamine was very low and may have represented addition of carbohydrate to ovomucoid precursors which had a completed peptide chain but incompleted carbohydrate moiety. The ratio of amino acid : carbohydrate was particularly low in extracellular ovomucoid at times less than 2 hours (Fig. 40). As glucosamine may be metabolised to sialic acid, this may

Fig. 53. The specific radioactivity of lysine labelled deoxycholate extracted ovomucoid. (Experiment 2).

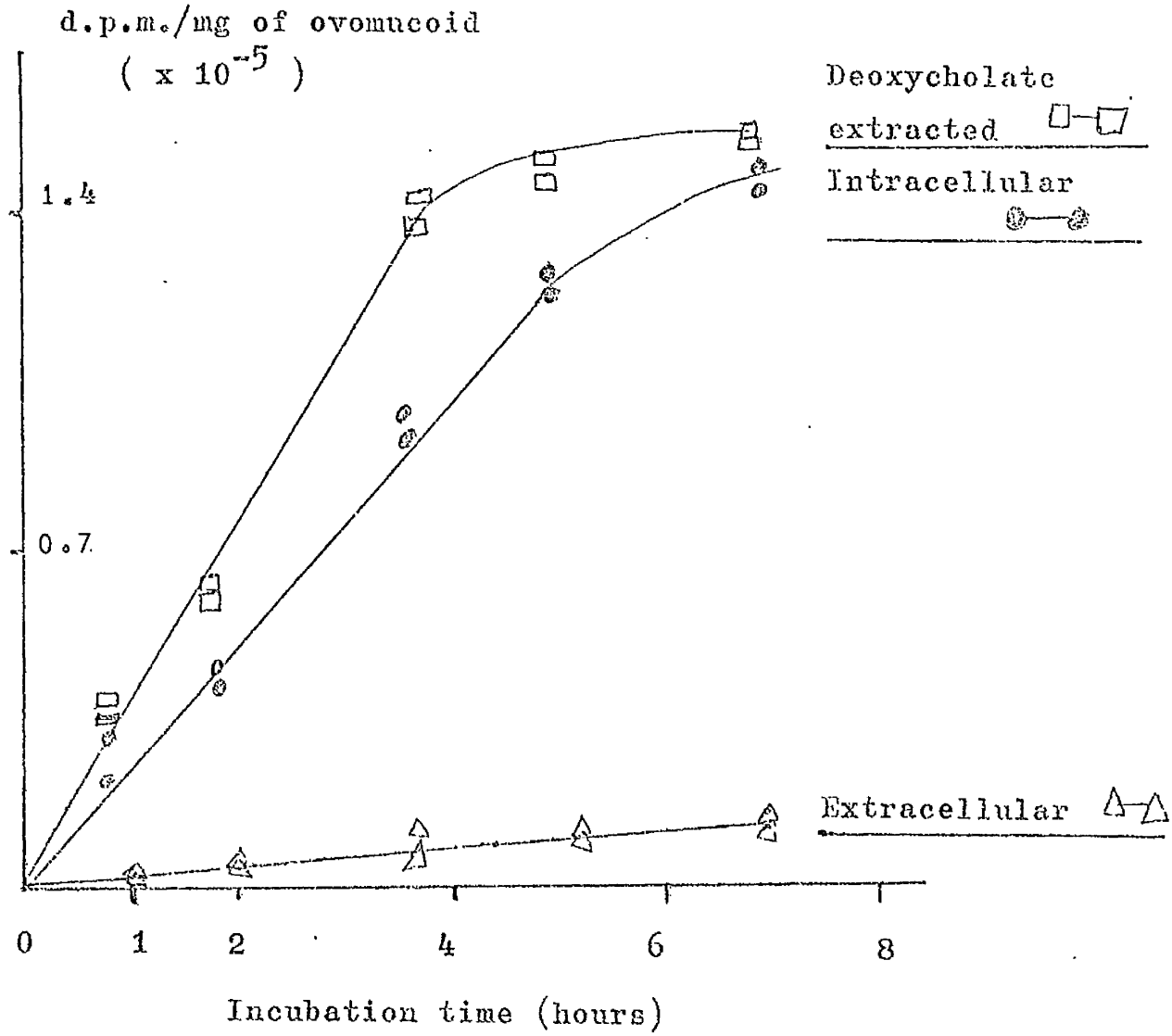


Fig. 54. The specific radioactivity of glucosamine labelled deoxycholate extracted ovomucoid . (Experiment2)

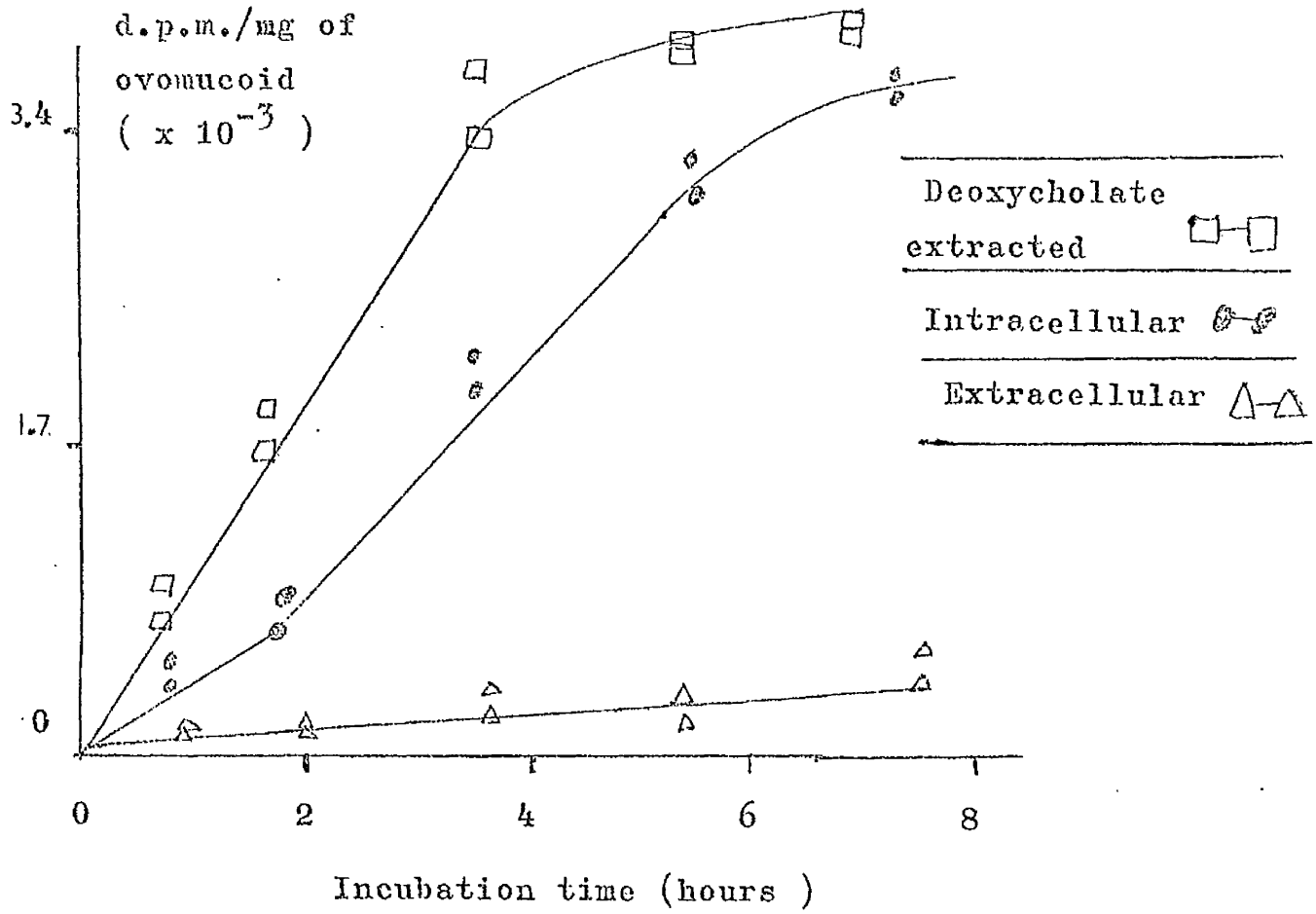


Fig. 55. Total incorporation of isotope into deoxycholate extracted ovomucoid. (Experiment 2).

Fig. 55a. Lysine incorporation

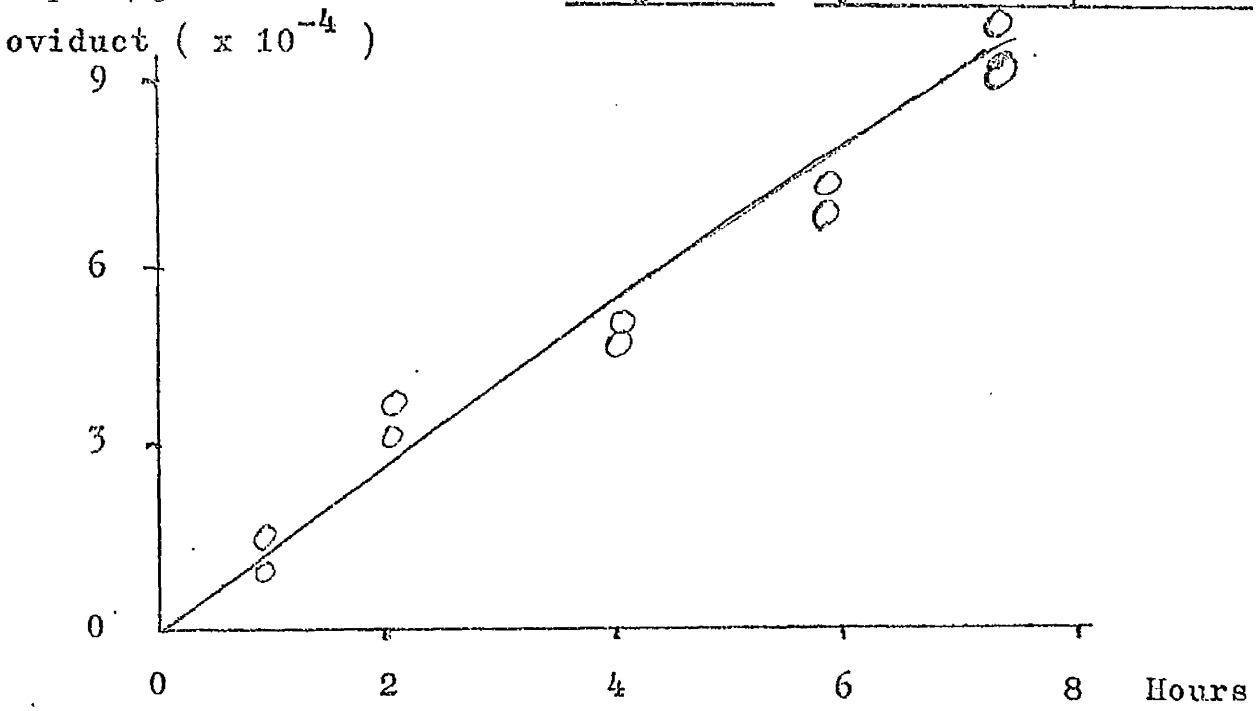
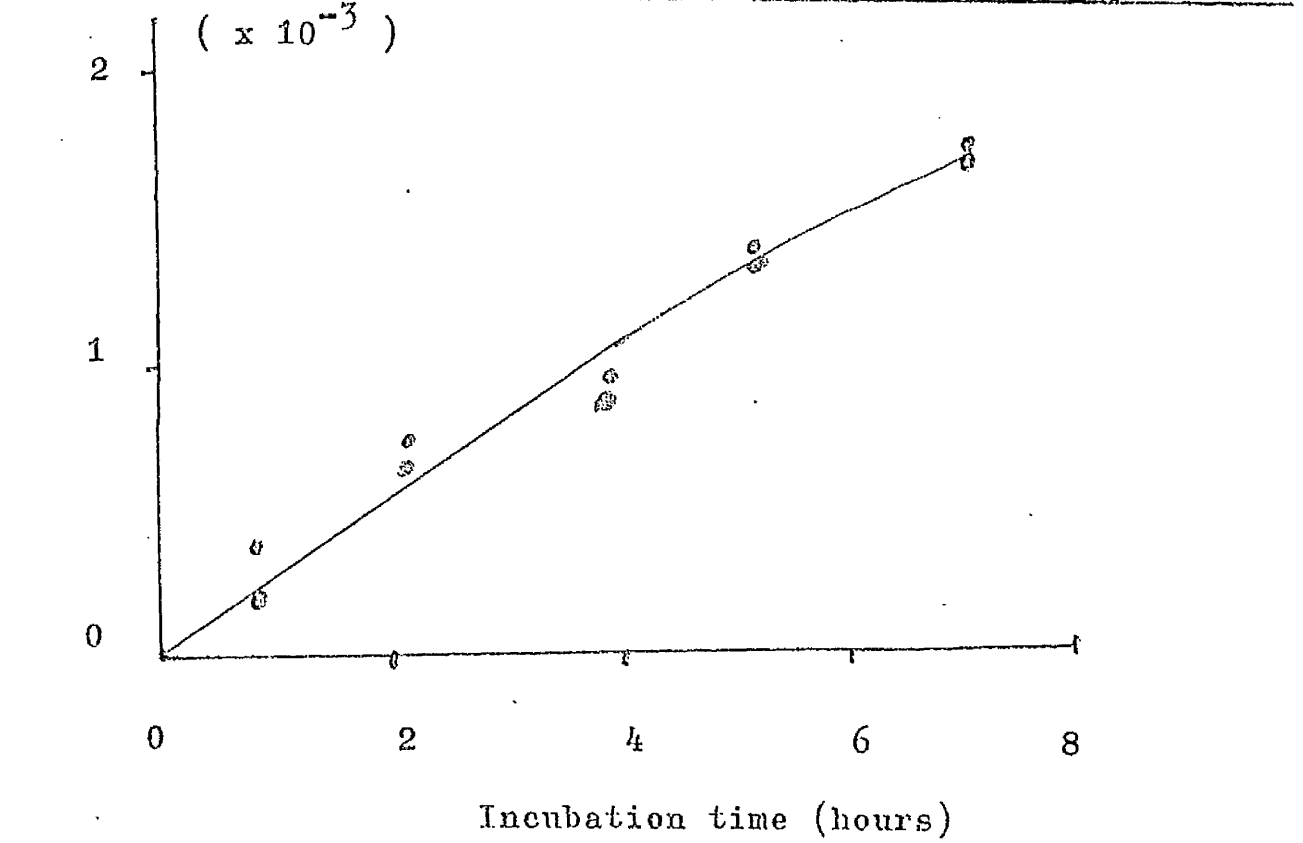


Fig. 55b. Glucosamine incorporation



Figs. 56 & 57. Ratios of [^{3}H] lysine : [^{14}C] glucosamine in deoxycholate extracted ovomucoid.

Fig. 56. [^{3}H] : [^{14}C] ratios (Experiment 1)

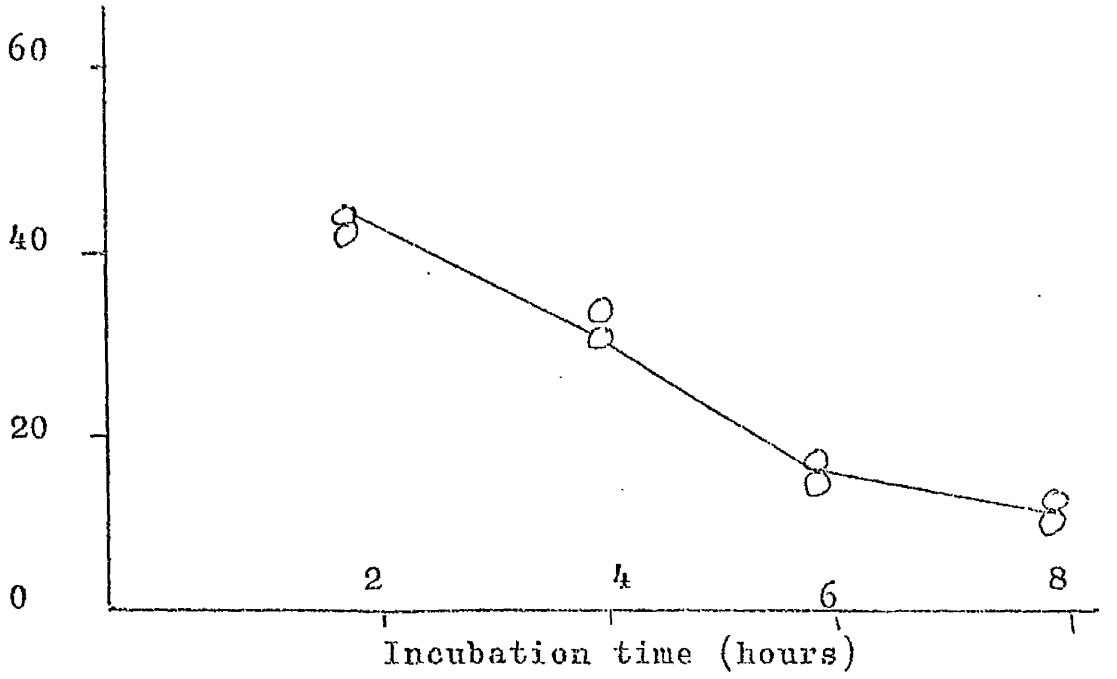
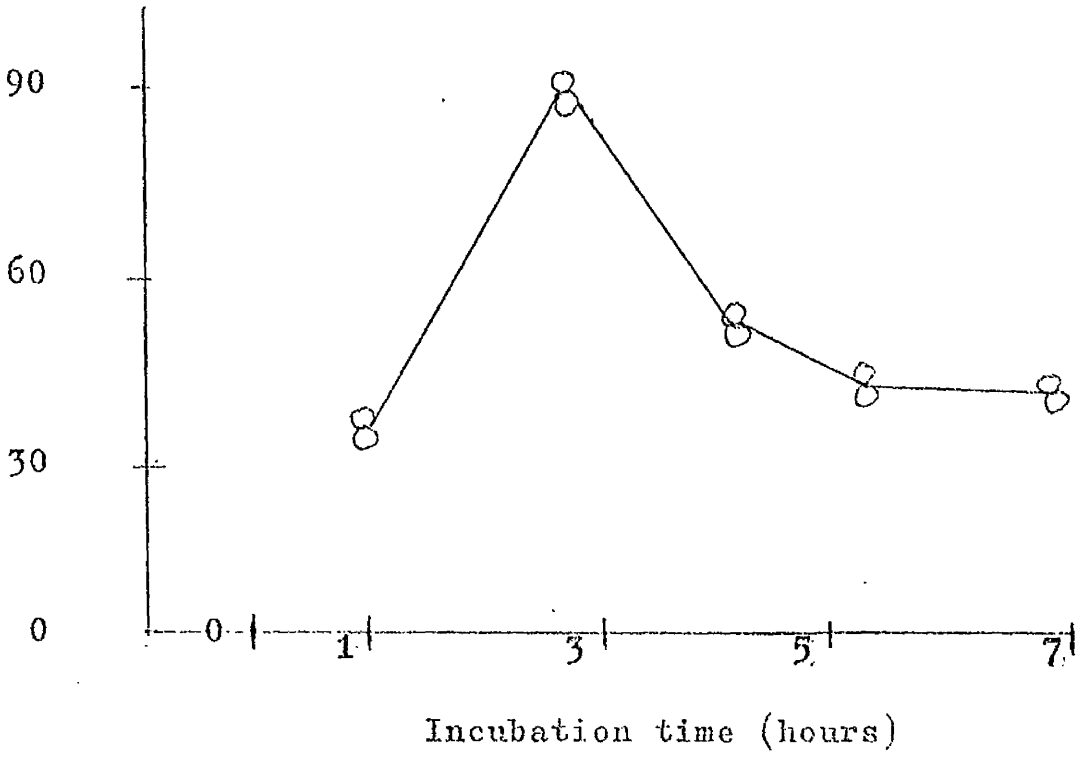


Fig. 57. [^{3}H] : [^{14}C] ratios (Experiment 2)



have represented the addition of either glucosamine or sialic acid to ovomucoid shortly before secretion.

As the specific radioactivity of deoxycholate extracted ovomucoid was slightly higher over a 5 hour incubation period than the specific radioactivity of intracellular ovomucoid, it may have represented a precursor of soluble intracellular ovomucoid. The closeness of the specific radioactivities of intracellular and deoxycholate extracted ovomucoid, however, might have meant that any particle bound ovomucoid precursor in the deoxycholate extracted fraction was "contaminated" by ovomucoid of a lower specific radioactivity. The low yield of ovomucoid from the deoxycholate extracted fraction meant that it was only a small proportion of the total ovomucoid isolated from the tissue (approximately 7%).

3. 13 (10) Preliminary Investigation on the Incorporation of Neutral Sugars into Ovomucoid

The incorporation of [^{14}C] mannose and [^{3}H] lysine into ovomucoid is represented by Table 21a. With [^{3}H] lysine as precursor, the specific radioactivities (d.p.m./mg) of ovomucoid in the intracellular and extracellular fractions have increased over a 5 hour period. The specific radioactivity of deoxycholate extracted ovomucoid was similar to that of intracellular ovomucoid at 2 hours and 5 hours, although it was lower at 8 hours. With [^{14}C] mannose as precursor, the specific radioactivity of extracellular ovomucoid increased over the 8 hour incubation period while the specific radioactivity of intracellular ovomucoid increased over, approximately, 5 hours. The specific radioactivity of deoxycholate

Table 21. Incorporation of neutral sugars into ovomucoid

Table 21a: Incorporation of mannose

Isotope	[^{14}C] mannose			[^{3}H] lysine		
Fraction	S1	S2	S3	S1	S2	S3
	d.p.m./mg of ovomucoid			d.p.m./mg of ovomucoid		
Incubation time (hours)						
0	0	0	0	0	0	0
2	100	5000	8000	7000	0.4×10^{6}	0.4×10^{6}
5	600	25000	22000	28000	1×10^{6}	0.9×10^{6}
8	1100	32000	15000	30000	1×10^{6}	0.4×10^{6}

Table 21b: Incorporation of galactose

Isotope	[^{14}C] galactose			[^{3}H] lysine		
Fraction	S1	S2	S3	S1	S2	S3
	d.p.m./mg of ovomuccid			d.p.m./mg of ovomucoid		
Incubation time (hours)						
0	0	0	0	0	0	0
2	220	9000	8400	12000	0.7×10^{6}	0.65×10^{6}
5	600	18000	16800	32000	0.7×10^{6}	0.85×10^{6}
8	1200	30000	8000	36000	0.75×10^{6}	0.4×10^{6}

Labelled precursors were [^{3}H] lysine (40μCi/g of oviduct) and either [^{14}C] mannose (4μCi/g of oviduct), Table 21a, or [^{14}C] galactose (4μCi/g of oviduct), Table 21b. (S1; extracellular, S2; intracellular, S3; deoxycholate extracted fraction).

extracted ovomucoid was similar at 2 hours and 5 hours to that of intracellular ovomucoid. The specific radioactivity of extracellular ovomucoid was much lower at all times than the specific radioactivity of intracellular ovomucoid.

The incorporation of [^{14}C] galactose and [^{3}H] lysine into ovomucoid in a separate incorporation experiment is represented by Table 21b. In this case with [^{3}H] lysine as precursor, the specific radioactivity of ovomucoid increased rapidly over 2 hours but was no higher at longer times. The specific radioactivity of deoxycholate extracted ovomucoid at 2 hours and 5 hours was similar to that of intracellular ovomucoid at the same time. The specific radioactivity of extracellular ovomucoid increased over 5 hours, but was always lower than that of intracellular ovomucoid at the same time. With [^{14}C] galactose as precursor, the specific radioactivities of ovomucoid in the intracellular and extracellular fractions increased over the 8 hour incubation period, although at each time the specific radioactivity of extracellular ovomucoid was lower than that of intracellular ovomucoid. At 2 hours and at 5 hours the specific radioactivity of deoxycholate extracted ovomucoid was similar to that of intracellular ovomucoid.

The results suggested that when oviduct was incubated <u>in vitro</u> with [^{14}C] mannose or [^{14}C] galactose, that radioactivity was associated with ovomucoid in all three tissue compartments. The specific radioactivity of extracellular ovomucoid was always lower than that of intracellular ovomucoid in the time course examined (8 hours). The specific radioactivities of intracellular and deoxycholate extracted

ovomucoid were similar over a 5 hour incubation period.

3. 13 (11) Fractionation of Oviduct Homogenates by Differential Centrifugation and by Centrifugation on Sucrose Density Gradients

A portion of hen oviduct was homogenised in 0.44M sucrose with a Dounce homogeniser and fractionated by the differential centrifugation scheme illustrated in Fig. 58 (Carey, 1966). The distribution of DNA, RNA and protein between oviduct cell fractions P1, P2, P3 and S3 (Fig. 58) is shown in Table 22a. By examining the DNA/protein and the RNA/protein ratios it is clear that the 600g pellet (P1) was enriched in DNA and the 105,000g pellet (P3) was enriched in RNA (Table 22b). However, the percentage recoveries (Table 22a) show that there was a substantial amount of RNA in all fractions and that fraction P1 contained only about 63% of the total DNA. The supernatant fraction S3 contained most of the protein of the homogenate.

This fractionation scheme did not seem to produce a particularly clear separation of subcellular components as determined by the chemical assays (Table 22). Attempts were made, therefore, to fractionate oviduct homogenates by sucrose density gradient centrifugation. Such a fractionation would be necessary to follow the movement of ovomucoid through the subcellular components of the tissue.

Oviduct homogenates were centrifuged at 80,000g for 10 hours on stepwise sucrose density gradients. The optical density profiles of fractionated oviduct across the gradients at 260nm (E260) and 280nm (E280) are given in Fig. 59. There was a large amount of ultraviolet

Figure 58. Fractionisation of oviduct homogenates by differential centrifugation

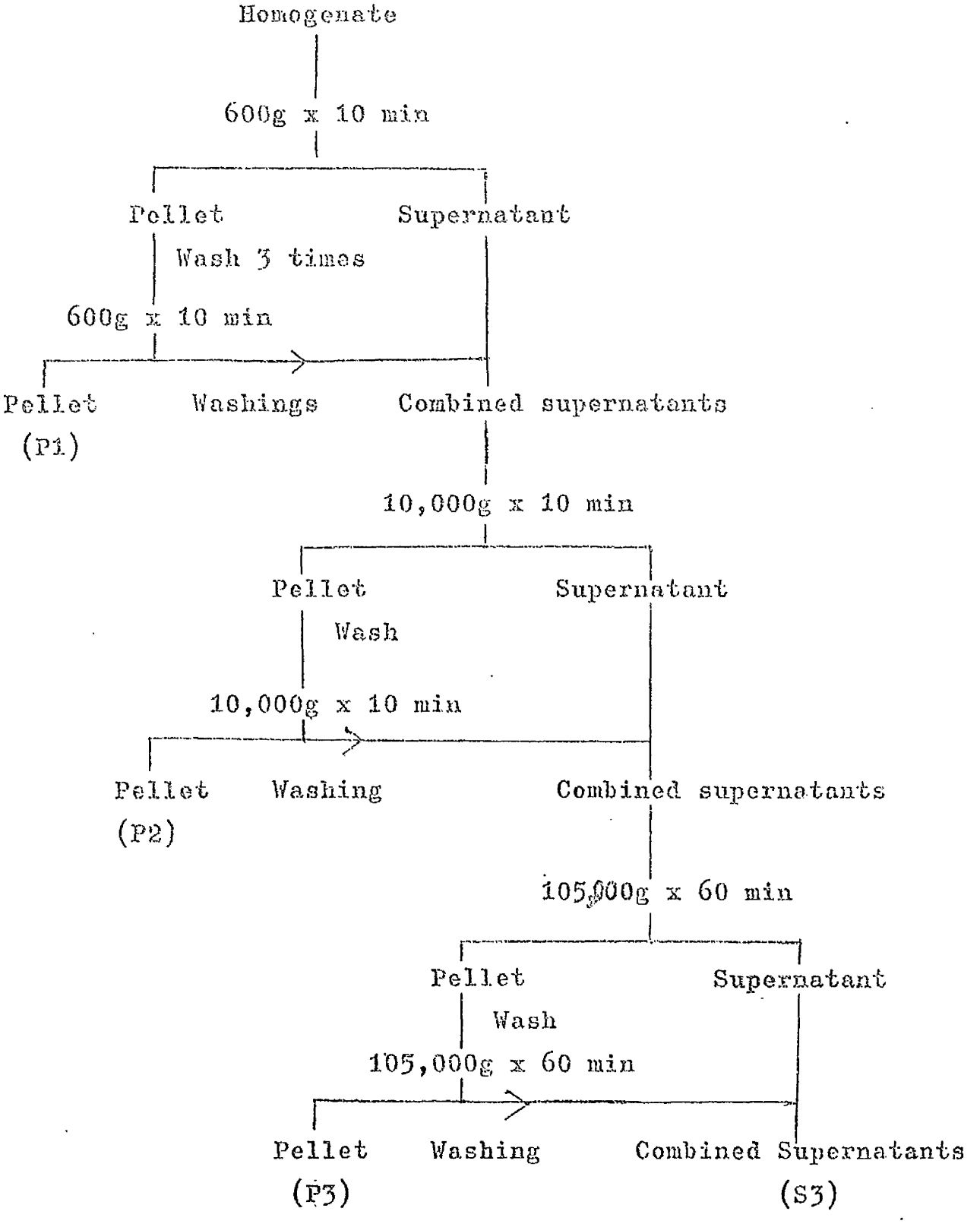


Table 22. Distribution of DNA, RNA and protein between oviduct cell fractions

For nomenclature of fractions see Fig. 58. In column (1) chemical fractionation of nucleic acids and protein was by the method of Fleck and Munro (1962). In column (2) chemical fractionation (Carey, 1966) was by cold trichloroacetic acid (protein) and hot trichloroacetic acid (nucleic acids). In Table 22a the yield of the cell fractions is expressed as a percentage of the yield from the unfractionated homogenate. The yield in the unfractionated homogenate is expressed in mg/g (wet weight) of tissue.

Table 22a

Cell Fraction	DNA (1) %	DNA (2) %	RNA (1) %	RNA (2) %	Protein (1) %	Protein (2) %
mg/g	0.73	0.82	2.10	2.16	44.3	52.1
Homogenate	(100%)	(100%)	(100%)	(100%)	(100%)	(100%)
P1	62.6	64.8	15.7	10.4	23.1	20.0
P2	5.0	15.4	17.5	30.0	13.6	10.8
P3	10.7	11.3	38.9	38.5	9.1	6.8
S3	21.7	8.5	27.9	21.1	54.2	62.4

Table 22b

Cell Fraction	DNA/Protein (mg/mg) (1)	DNA/Protein (mg/mg) (2)	RNA/Protein (mg/mg) (1)	RNA/Protein (mg/mg) (2)	RNA/DNA (mg/mg) (1)	RNA/DNA (mg/mg) (2)
Homogenate	0.0164	0.0158	0.0475	0.0415	2.9	2.6
P1	0.0445	0.0510	0.0323	0.0216	0.7	0.4
P2	0.0063	0.0224	0.0610	0.1150	10.1	5.1
P3	0.0195	0.0264	0.2040	0.2360	10.4	8.9
S3	0.0065	0.0021	0.0244	0.0140	3.7	6.5

absorbing material at the interface between 2M and saturated sucrose and at the top (low density sucrose) of the gradient. The profiles of the gradients of oviduct homogenised with a Potter-Elvehjem homogenizer (Fig. 59a) and with a Dounce homogeniser (Fig. 59b) were similar. Most of the DNA and RNA were at the interface between 2M and saturated sucrose, while most of the protein was near the top of the gradient (Fig. 60). In samples of oviduct which were incubated for 5 hours with [^{3}H] lysine, a small amount of radioactivity was associated with the protein at the interface between 2M and saturated sucrose while most of the trichloroacetic acid precipitable radioactivity was near the top of the gradient (Fig. 61). A preliminary centrifugation at 600g for 10 minutes before application of oviduct homogenates to gradients, only removed some of the ultraviolet absorbing material which was found at the interface between 2M and saturated sucrose when the preliminary centrifugation was omitted.

As most of the DNA and most of the RNA of the homogenate were both recovered at the interface between 2M and saturated sucrose, there seemed to be no clear fractionation of subcellular components of oviduct. Centrifugation of oviduct homogenates at 20,000g for 16 hours or at 5,000g for 16 hours on linear (0.5-2.5M) sucrose density gradients did not improve the fractionation.

In Fig. 62 the optical density profiles (E260 and E280) of an oviduct homogenate centrifuged on a linear sucrose density gradient at 2,000g for 16 hours is shown. There were two peaks of ultraviolet absorbing material, fractions I and II (fraction I at denser sucrose than fraction II). In Fig. 63 it can be seen that there was RNA and DNA associated

Fig. 59. Fractionation of oviduct homogenate by discontinuous sucrose gradient centrifugation

The gradients were prepared as described in the Methods section. The extinction profiles of the gradients at 260nm and 280nm are illustrated. In Fig. 59a the gradient was from a portion of oviduct (0.4g) which was homegenised in a Potter-Elvehjem homogeniser. In Fig. 59b the oviduct was homogenised in a Dounce homogeniser. Samples were applied to the gradients in 0.25M sucrose TKM, pH 7.5. The gradient is illustrated below these Figs. Centrifugation was at 80,000g for 10 hours.

Fig. 59a.

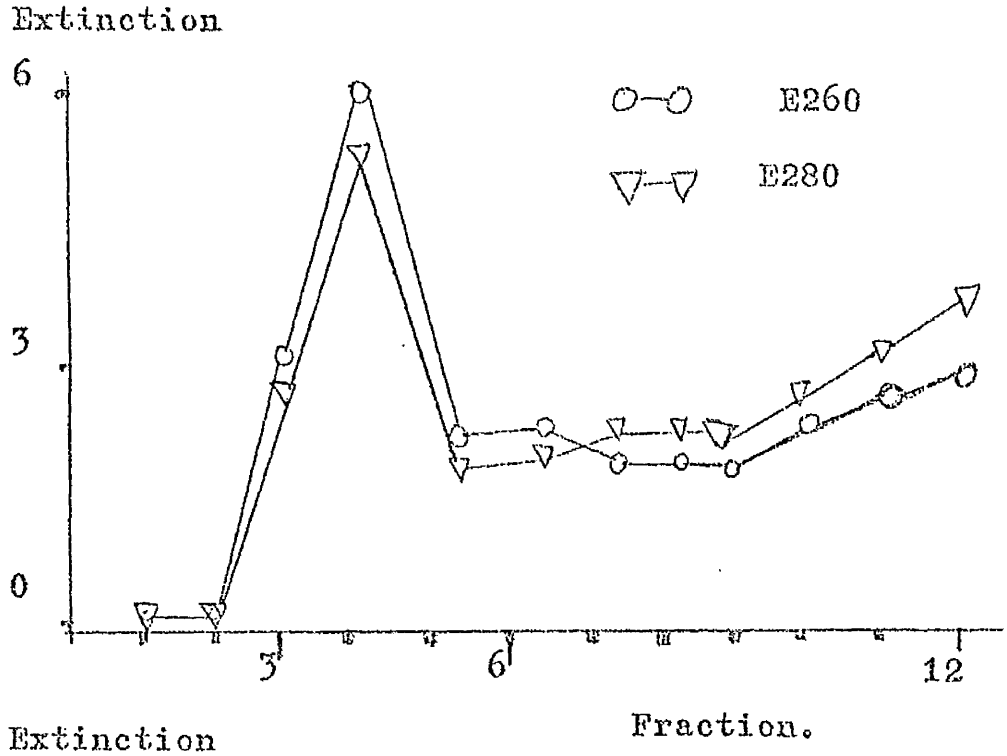


Fig. 59b.

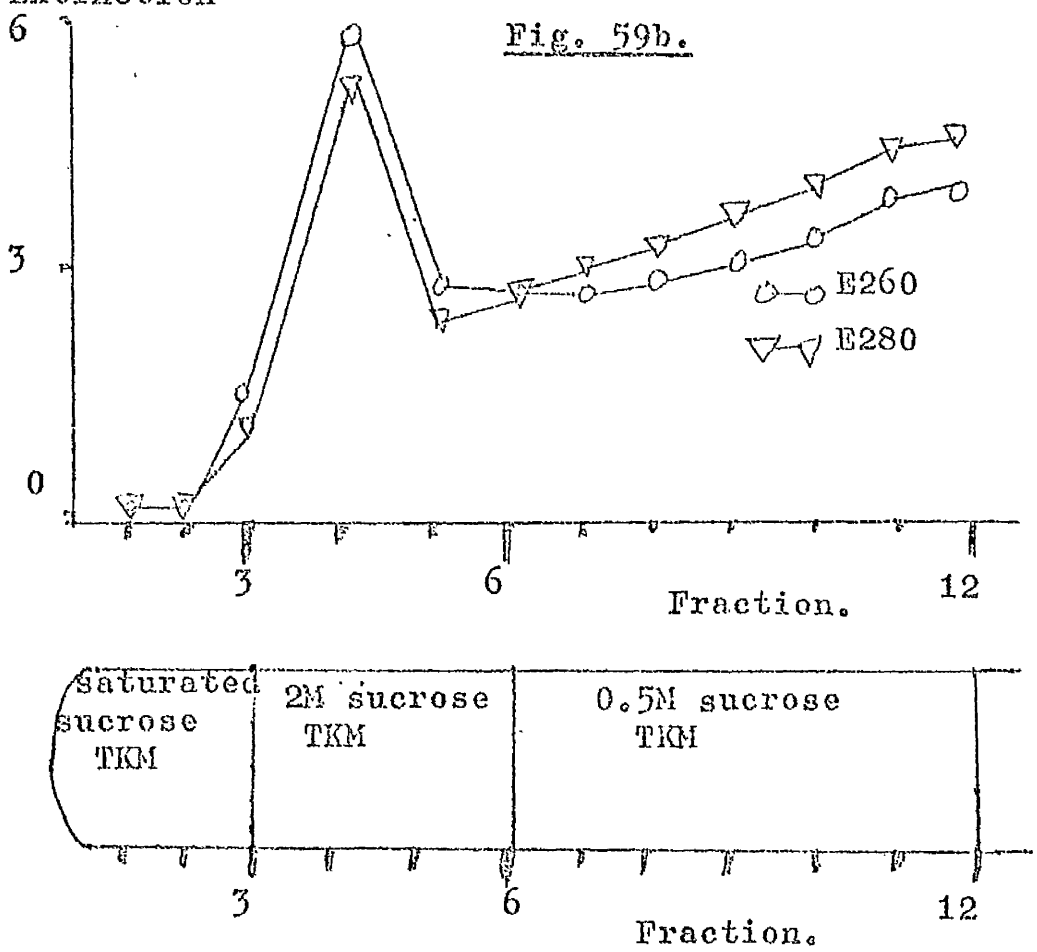


Fig. 60. DNA, RNA and protein content of oviduct fractions

Fig. 60a.

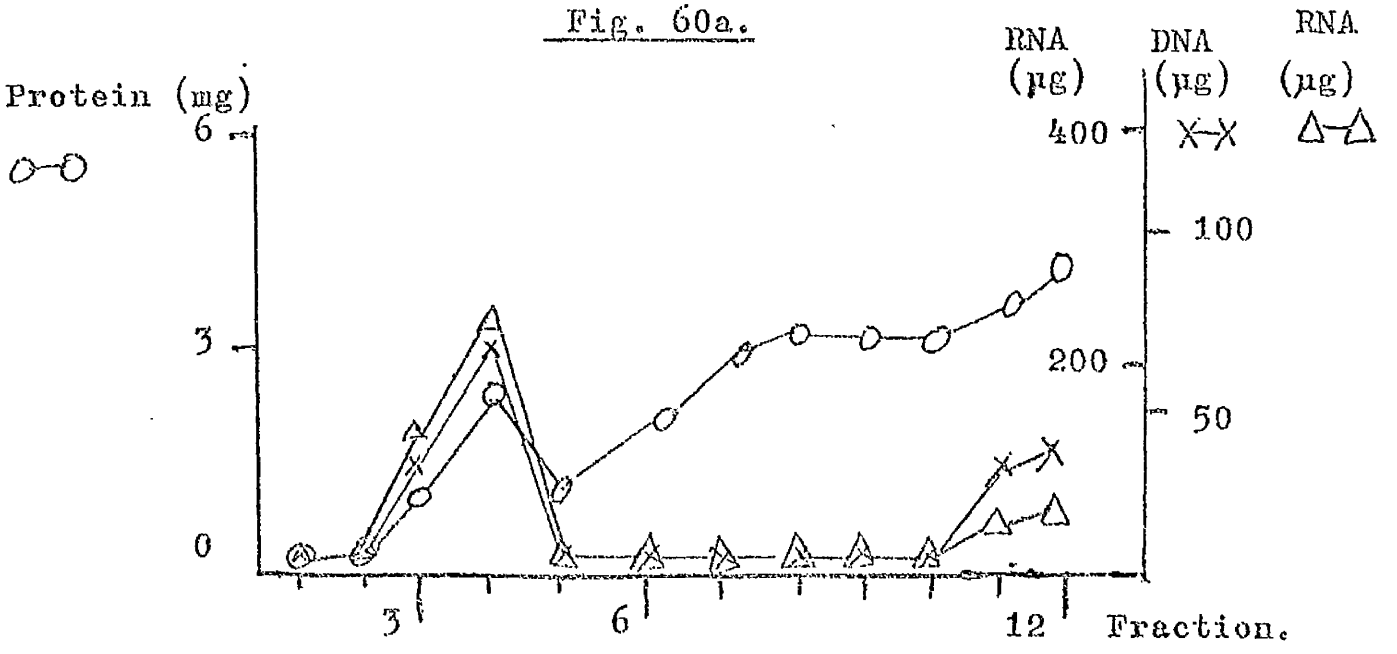
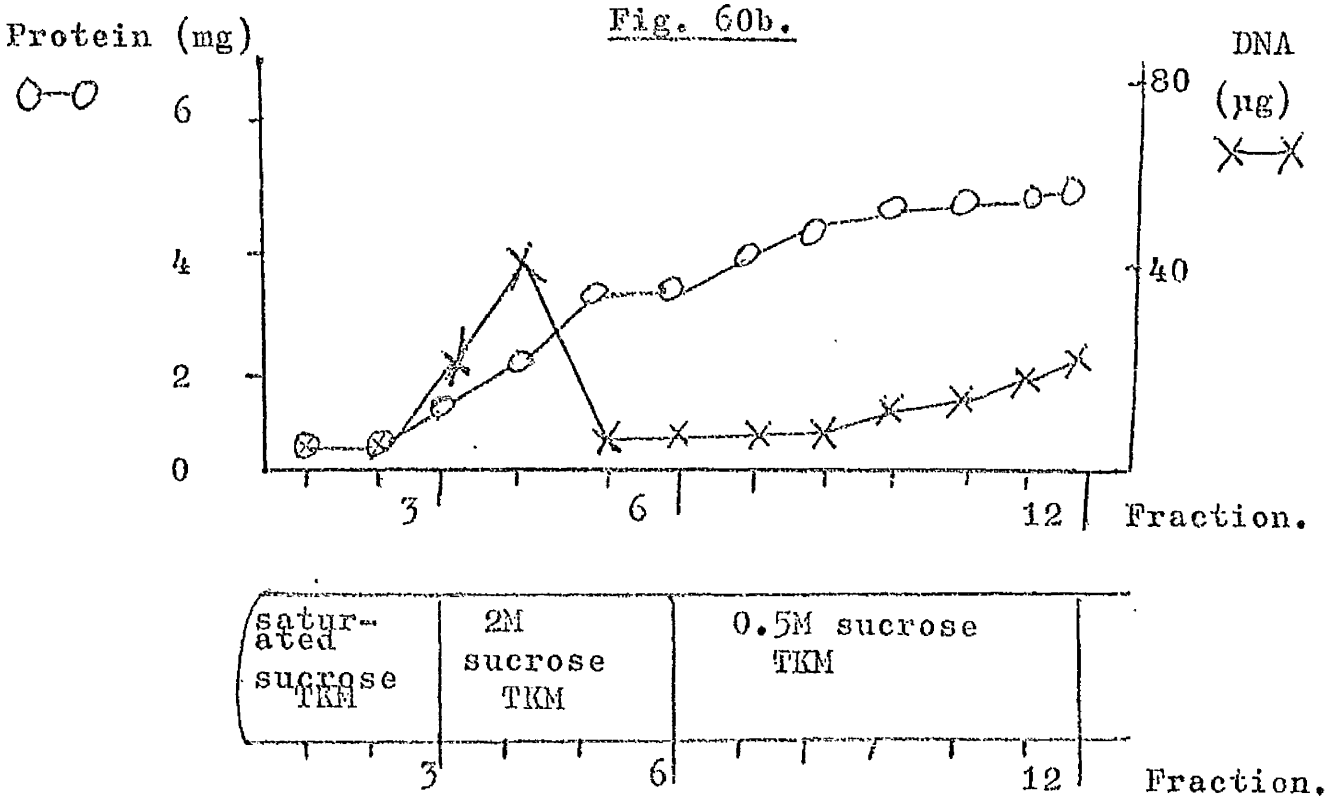


Fig. 60b.



Conditions are described in Fig. 59. Fig. 60a corresponds to Fig. 59a and Fig. 60b corresponds to Fig. 59b.

Fig. 61. Recovery of radioactivity in oviduct fractions

Fig. 61a.

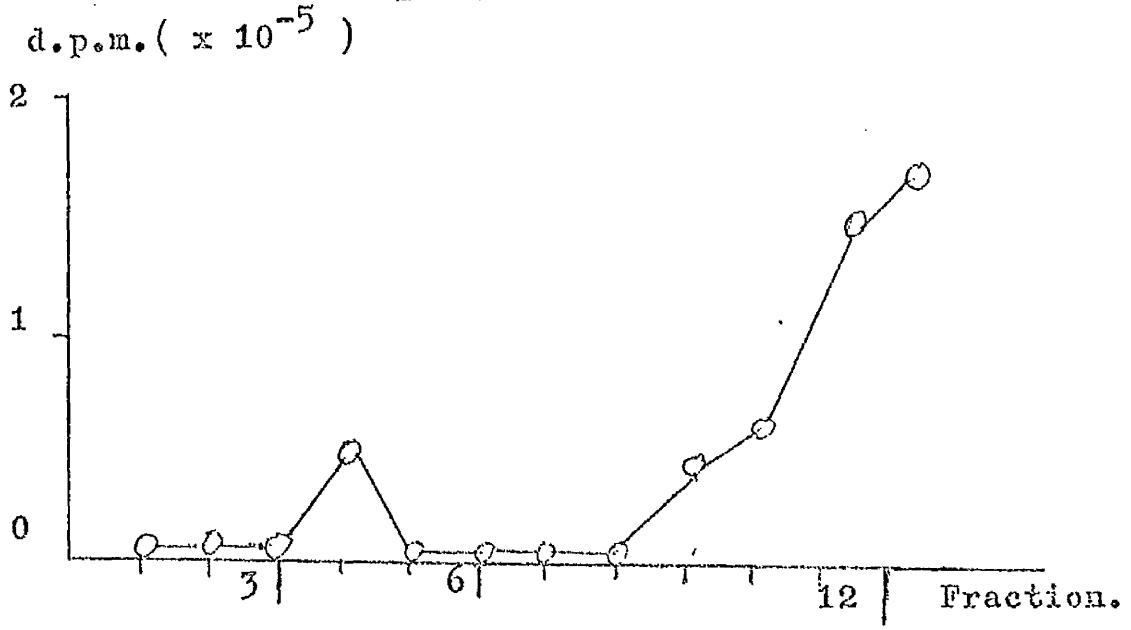
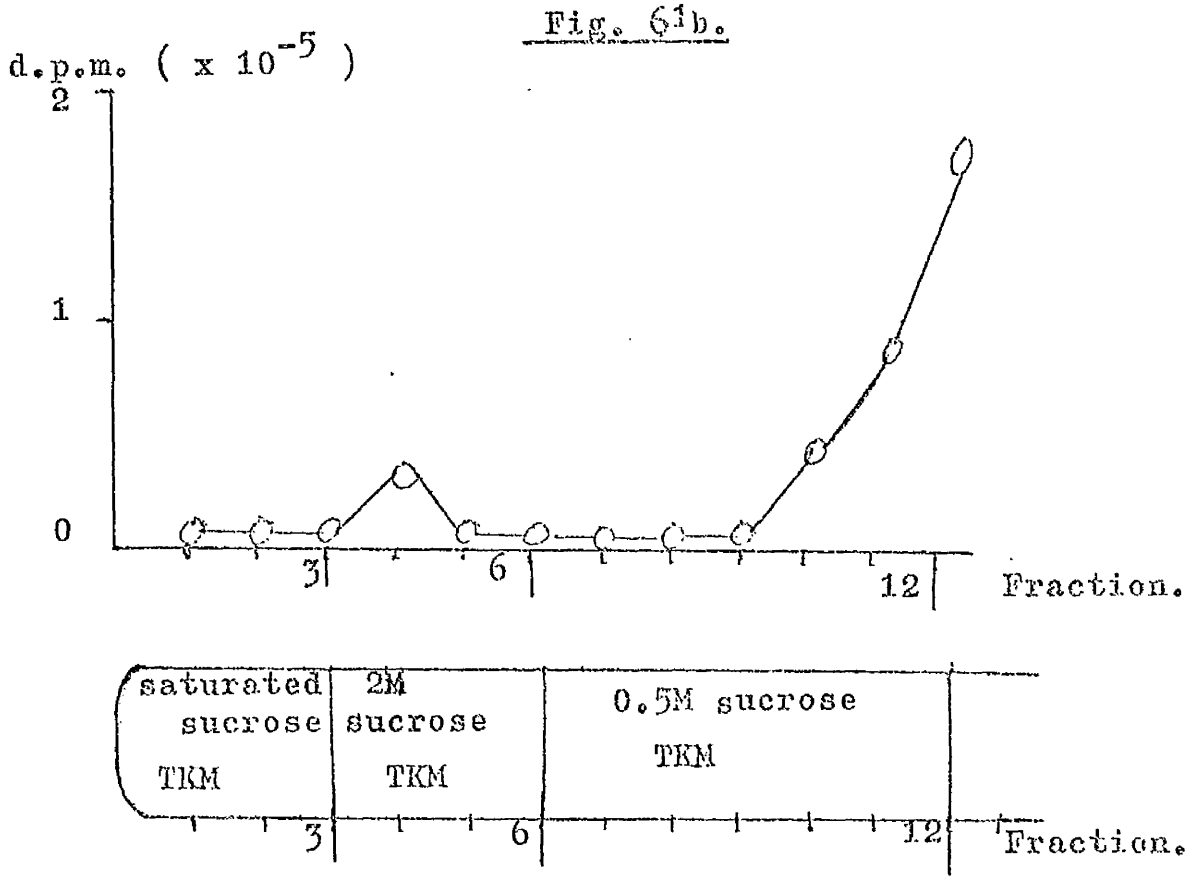


Fig. 61b.



Conditions were as in Fig. 59. Fig. 61a corresponds to Fig. 59a. and Fig. 61b to Fig. 59b. Incubation was for 5 hours with [3-H]lysine as precursor. The d.p.m. values are those recovered from trichloroacetic acid precipitation of the fractions.

Fig. 62. Fractionation of oviduct homogenate on linear (0.5M to 2.5M) sucrose gradients.

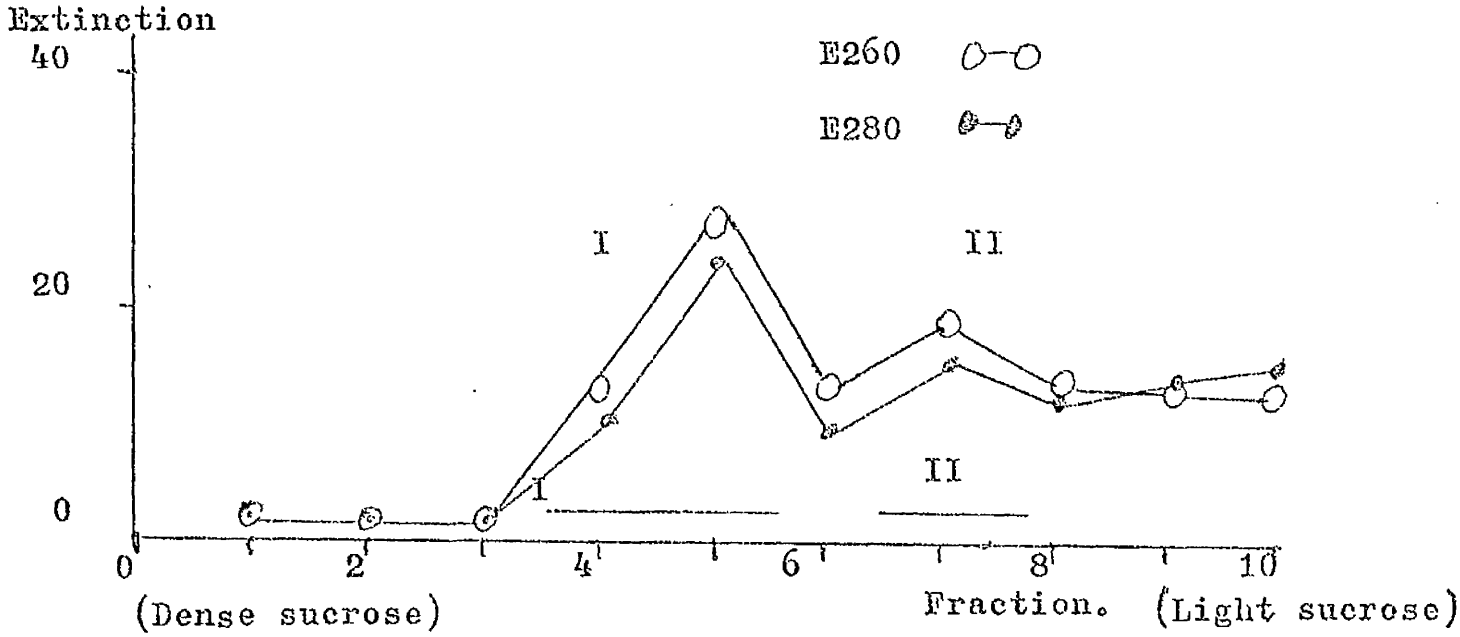
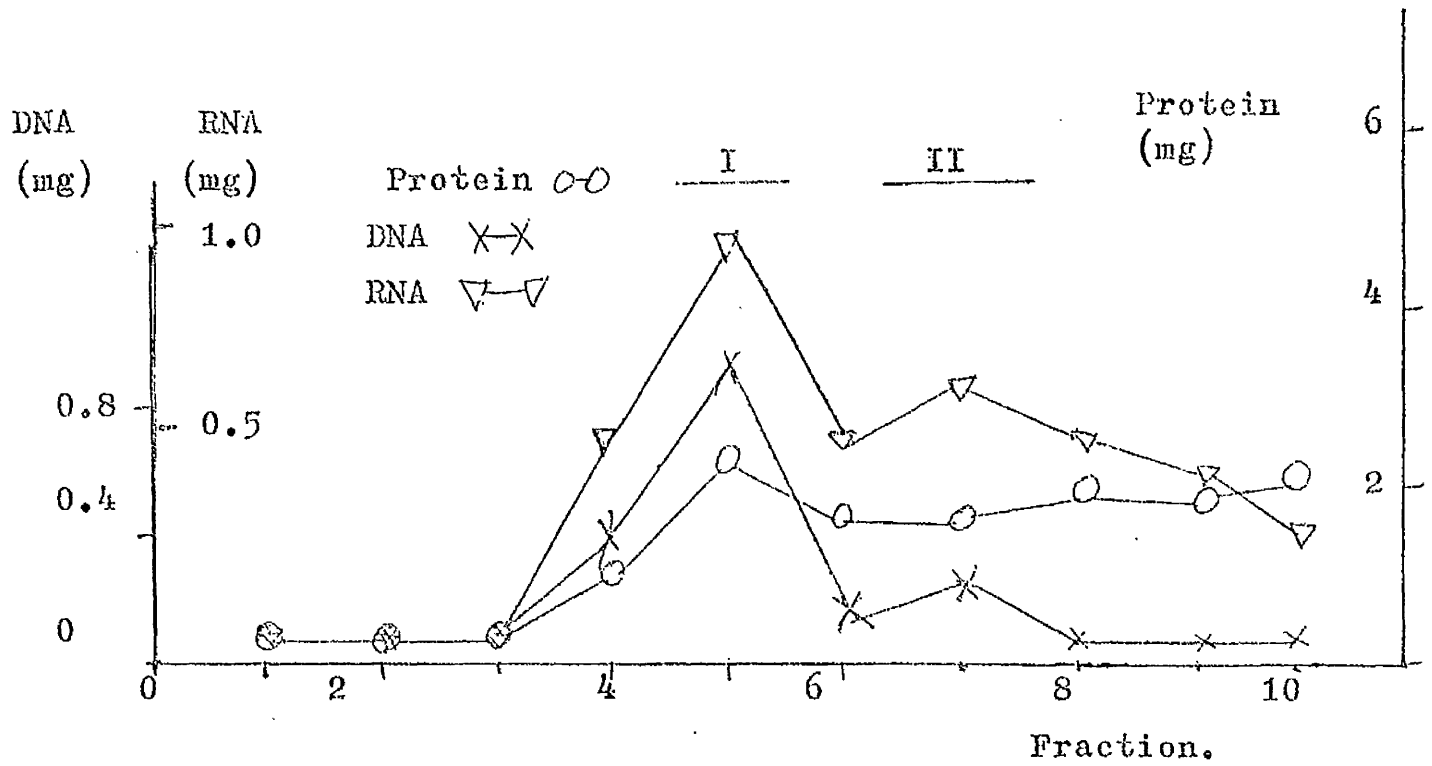
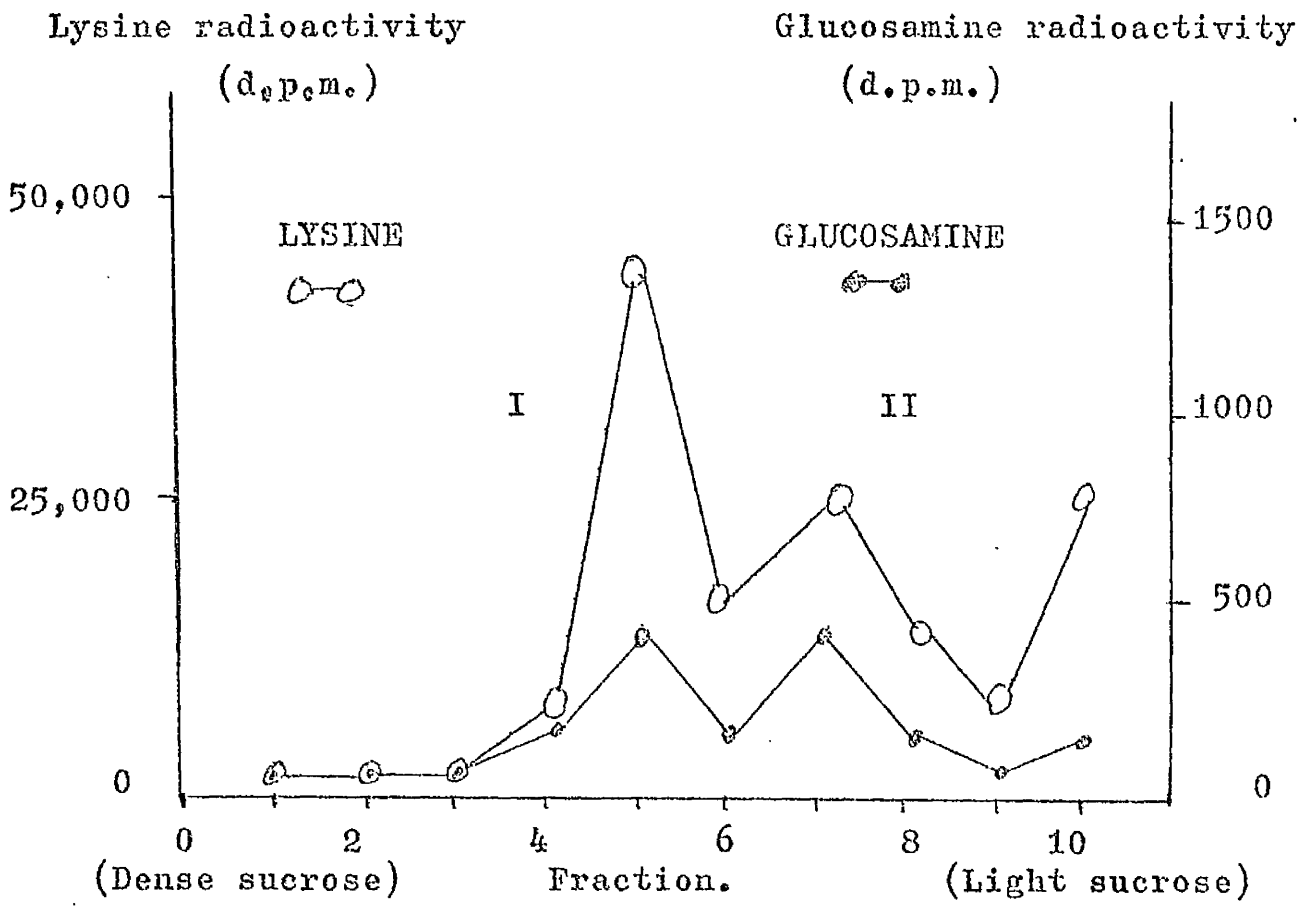


Fig. 63. Chemical determinations on oviduct fractions



Oviduct homogenate was centrifuged on a linear sucrose gradient at 2,000g for 16 hours as described in the Methods section.

Fig. 64. Recovery of radioactivity in oviduct fractions resulting from linear sucrose gradient centrifugation



Experimental conditions were as in Fig. 62. The oviduct in this study (Fig. 64) had been incubated for 2 hours with [^{3}H] lysine (40μCi/g of tissue) and [^{14}C] glucosamine (4μCi/g of oviduct). The radioactivity recovered is that in trichloroacetic acid precipitates of the fractions.

with both fractions. There was [^{3}H] lysine and [^{14}C] glucosamine radioactivity present in trichloroacetic acid precipitates of fractions I and II (Fig. 64) from an oviduct which had been incubated for 2 hours with [^{3}H] lysine and [^{14}C] glucosamine. The ratio of [^{3}H] radioactivity in terms of specific radioactivity (d.p.m./mg of protein) of the protein components of fractions I and II was 1.2 : 1 (fraction I : II). The ratio of the specific radioactivities of fraction I protein to fraction II protein in terms of [^{14}C] glucosamine labelling was 0.6 : 1 (fraction I : II). Fraction I has proportionately more of the [^{3}H] lysine radioactivity and less of the [^{14}C] glucosamine radioactivity than fraction II. The RNA/protein ratio in fraction I was 0.36, while the RNA/protein ratio in fraction II was 0.16. These values are similar to the values found for rough membranes (fraction I) and smooth membranes (fraction II) of other tissues on fractionation (Jamieson & Palade, 1967; Choi et al, 1971). However, as both fraction I and II contained DNA, this would suggest nuclear contamination of the fractions.

This inability to fractionate oviduct homogenates by differential or sucrose density gradient centrifugation meant that it was not possible to follow the movement of labelled ovomucoid through identifiable sub-cellular components of the tissue.

3. 13 (12) The Effect of Inhibitors of Protein Synthesis on the Incorporation of Precursors into Ovomucoid

In order to investigate the temporal relationship between the synthesis of the peptide and carbohydrate portions of ovomucoid, tissue incubation studies were carried out in the presence of puromycin or

cycloheximide. In Table 23 the specific radioactivities of ovomucoid in control samples isolated from the three tissue fractions (intracellular, extracellular and deoxycholate extracted) after a 2 hour and 7 hour incubation period are shown. The specific radioactivities of ovomucoid isolated from tissue fractions in incorporation studies in the presence of puromycin or cycloheximide are expressed as percentages of these values.

When tissue incubations were carried out in the presence of puromycin (50µg/ml) the specific radioactivity of [^{3}H] lysine labelled ovomucoid was reduced to 15-22% of the control value in all tissue fractions at both times (Table 23). With incubation in the presence of puromycin at a concentration of 100µg/ml, the specific radioactivity of lysine labelled ovomucoid was 8-15% of that of a control at the same time. The specific radioactivity of [^{14}C] glucosamine labelled ovomucoid was 90-100% of that of a control when tissue incubations were carried out in the presence of puromycin (50µg/ml or 100µg/ml). Puromycin has therefore caused some inhibition of the incorporation of lysine into ovomucoid, while the incorporation of glucosamine was virtually unaltered. The effects of cycloheximide (50µg/ml) on the incorporation of precursors into ovomucoid were similar to those described for puromycin. Cycloheximide at a concentration of 100µg/ml has virtually abolished the incorporation of lysine into ovomucoid, while the incorporation of glucosamine was essentially unaltered at both 2 hours and 7 hours, in all three tissue fractions (Table 23).

These results would suggest that oviduct contained a pool of ovomucoid precursors with completed peptide chains but incompleted carbohydrate moieties to which carbohydrate could be added. It is also implied that

Table 23. The effect of inhibitors of protein synthesis on the incorporation of precursors into ovomucoid

Values are expressed as percent activity (d.p.m./mg of ovomucoid) of a control sample. The specific radioactivity of the control is given in terms of d.p.m./mg of ovomucoid. Incubations were for 2 hours or 7 hours with $[^{3}H]$lysine (40μCi/g of oviduct) and $[^{14}C]$ glucosamine (4μCi/g of oviduct).

Fraction	Extracellular ovomucoid		Intracellular ovomucoid		Deoxycholate extracted ovomucoid	
Isotope	Lys	$GlcNH_2$	Lys	$GlcNH_2$	Lys	$GlcNH_2$
Control (2 hours)	N.D.		13846 (100%)	320 (100%)	51991 (100%)	815 (100%)
+Puromycin (50µg/ml)	N.D.		20%	100%	22%	95%
+Puromycin (100µg/ml)	N.D.		9%	90%	8%	90%
+Cycloheximide (50µg/ml)	N.D.		5%	94%	2%	99%
+Cycloheximide (100µg/ml)	N.D.		0%	90%	2%	95%
Control (7 hours)	5774 (100%)	158 (100%)	43805 (100%)	1610 (100%)	91909 (100%)	3000 (100%)
+Puromycin (50µg/ml)	15%	100%	16%	94%	17%	97%
+Puromycin (100µg/ml)	15%	100%	15%	97%	15%	95%
+Cycloheximide (50µg/ml)	15%	102%	6%	97%	5%	98%
+Cycloheximide (100µg/ml)	0%	104%	0%	98%	7%	94%

(N.D. implies that no determination was carried out).

carbohydrate addition follows peptide synthesis and could go on for several hours in the absence of protein synthesis.

Although it is known that puromycin or cycloheximide can inhibit the synthesis of the peptide portion of glycoproteins no such well documented inhibitors of the synthesis of the carbohydrate portion of glycoproteins have been recorded. In an attempt to inhibit, or block, the synthesis of the carbohydrate portion of ovomucoid, tissue incubations were carried out in the presence of 2-deoxyglucose. If this substrate was incorporated in error into the growing glycoprotein, it might result in an accumulation of ovomucoid precursors with incompleted carbohydrate moieties.

In one experiment (Table 24) when tissue incubations were carried out for 7 hours in the presence of 2-deoxyglucose, 0.1mM or 10mM, it can be seen that the specific radioactivity of extracellular ovomucoid in terms of lysine or glucosamine labelling was essentially unaltered. The specific radioactivity of intracellular ovomucoid in terms of lysine and glucosamine labelling was increased. The specific radioactivity of deoxycholate extracted ovomucoid in terms of glucosamine labelling was essentially unaltered. Deoxyglucose (0.1mM) had essentially no effect on the specific radioactivity of deoxycholate extracted lysine labelled ovomucoid, while incubation in the presence of deoxyglucose (10mM) increased the specific radioactivity of lysine labelled deoxycholate extracted ovomucoid.

In a separate experiment (Table 25) it was found, after a 5 hour incubation period in the presence of deoxyglucose, 0.1mM or 10mM, that the specific radioactivity of intracellular ovomucoid in terms of lysine

Tables 24, 25. The possible effects of deoxyglucose on the incorporation of precursors into ovomucoid

7 hour incubation period Table 24

Fraction	Extracellular ovomucoid d.p.m./mg of ovomucoid		Intracellular ovomucoid d.p.m./mg of ovomucoid		Deoxycholate extracted ovomucoid d.p.m./mg of ovomucoid	
Isotope	$GlcNH_2$	Lys	$GlcNH_2$	Lys	$GlcNH_2$	Lys
Control	160	5800	1610	43,805	3000	91,909
+Deoxyglucose (0.1mM)	220	5780	2720	70,000	3340	103,000
+Deoxyglucose (10mM)	260	6440	2520	51,900	2220	153,000

5 hour incubation period Table 25

Fraction	Extracellular ovomucoid d.p.m./mg of ovomucoid		Intracellular ovomucoid d.p.m./mg of ovomucoid	
Isotope	Glucosamine	Lysine	Glucosamine	Lysine
Control	185	3520	1420	38,900
+Deoxyglucose (0.1mM)	165	3200	825	23,100
+Deoxyglucose (10mM)	125	3340	930	26,700
+Deoxyglucose (10mM) & mannose (10mM)	184	3840	940	32,500

and glucosamine labelling was reduced. The specific radioactivity of lysine labelled extracellular ovomucoid was essentially unaltered, and the specific radioactivity of glucosamine labelled extracellular ovomucoid was similar to that of the control. The slight reductions in the specific radioactivities of ovomucoid observed when tissue incubations were carried out in the presence of 10mM deoxyglucose were to an extent, relieved by the addition of mannose to the incubation medium (Table 25).

In view of the lack of any large change in the specific radioactivity of lysine or glucosamine labelled ovomucoid when tissue incubations were carried out in the presence of 2-deoxyglucose, and the variable effects it seemed to produce on the specific radioactivity of ovomucoid, this was not an effective inhibitor of the synthesis of the carbohydrate portion of ovomucoid.

Chapter 14 Fractionation of Labelled Ovomucoid

It was intended to fractionate ovomucoid so that the specific radioactivities of the ovomucoid variants could be compared in order to determine if the species were all synthesised at the same time or whether they bore a precursor-product relationship to one another. The relationship of the observations to the microheterogeneity of ovomucoid is discussed.

Ovomucoid could be completely separated from Ampholine by gel filtration (Methods section). After isoelectric focusing of labelled ovomucoid, the ovomucoid variants were eluted from the gel and separated from any ampholytes present. However, the small amount of protein present in each variant after focusing meant that the determination of protein content was very difficult to estimate accurately. This was particularly true of the more acidic species which were present in lower proportions. It was possible to detect that ovomucoid O_1 (the most basic variant) had a similar specific radioactivity to that of unfractionated ovomucoid, but the specific radioactivities of all the species could not be compared to each other. In view of this, ovomucoid was fractionated by sulphoethyl (SE)-Sephadex C-50 chromatography into two components, one (fraction I) corresponding to the sialic acid free ovomucoid variant O_1, and the other (fraction II) to the sialic acid containing variants O_2 - O_5 (described in Fig. 15).

3. 14 (1) Sulphoethyl-Sephadex Chromatography

In preliminary single label studies with $[^{14}C]$ lysine as precursor, it was indicated that intracellular ovomucoid O_1 had a higher specific

radioactivity than ovomucoids O_2 - O_5, after a 2 hour incubation period (Fig. 65). These studies were repeated with [^{3}H] lysine and [^{14}C] glucosamine as precursors in dual label studies.

The elution profiles of ovomucoid isolated from the intracellular fraction after incubation for 2, 4, 6 and 8 hours are shown in Fig. 66. In terms of lysine labelling it can be seen (Fig. 66a) that the sialic acid free ovomucoid has a higher specific radioactivity than the sialic acid containing variants after 2 hours. At 4 hours the ovomucoid species have approximately the same specific radioactivity (Fig. 66b). The same was true at 6 and 8 hours (Figs. 66c and 66d). As the lysine content of the species is virtually identical (Beeley, 1971a) these results would suggest that the sialic acid free ovomucoid variant is synthesised first. At times longer than 2 hours the specific radioactivities of all the species were approximately the same, and this may have reflected the addition of carbohydrate to ovomucoid precursors which were already labelled with lysine. At 2 hours there would presumably be a smaller proportion of lysine labelled precursors to which carbohydrate could be added resulting in a lower specific radioactivity in the more acidic species.

At all times the specific radioactivities of the intracellular species, in terms of glucosamine labelling, were similar to each other (Fig. 66). As carbohydrate is added to completed peptide chains, this is probably a reflection of the late build up of the carbohydrate moiety of ovomucoid. Clearly at 2 hours (Fig. 66a) the ratio of [^{14}C] glucosamine : [^{3}H] lysine is higher in the more acidic ovomucoid variants than in ovomucoid O_1. This would be consistent with the addition of carbohydrate to completed

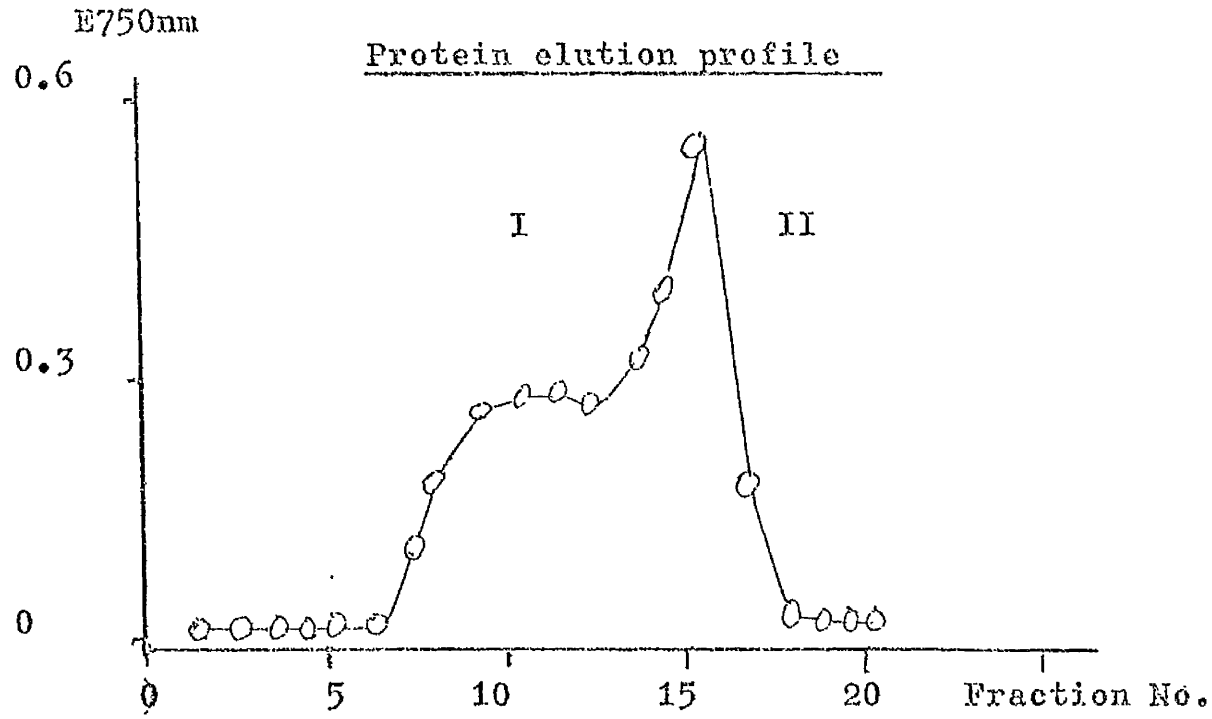
Fig. 65. The specific radioactivity of ovomucoid species fractionated by SE-Sephadex chromatography

After incubation of oviduct with [^{14}C]lysine (1μCi/g of oviduct) a portion of intracellular ovomucoid was fractionated by chromatography on columns (60cm x 1cm) of SE-Sephadex. The flow rate was 4ml/hour and 1.6ml fractions collected. Eluant was 0.014M sodium acetate containing 1mM sodium azide, pH 4.90.

The upper graph represents the elution profile of ovomucoid isolated from the intracellular fraction after a 2 hour incubation period as determined by the method of Lowry et al (1951).

The elution profile is similar to that of egg white ovomucoid (Fig. 15).

The lower graph represents the specific radioactivities (d.p.m./mg of ovomucoid) of the fractions.


E750nm
Protein elution profile
0.6
0.3
0
I
II
0
5
10
15
20
Fraction No.

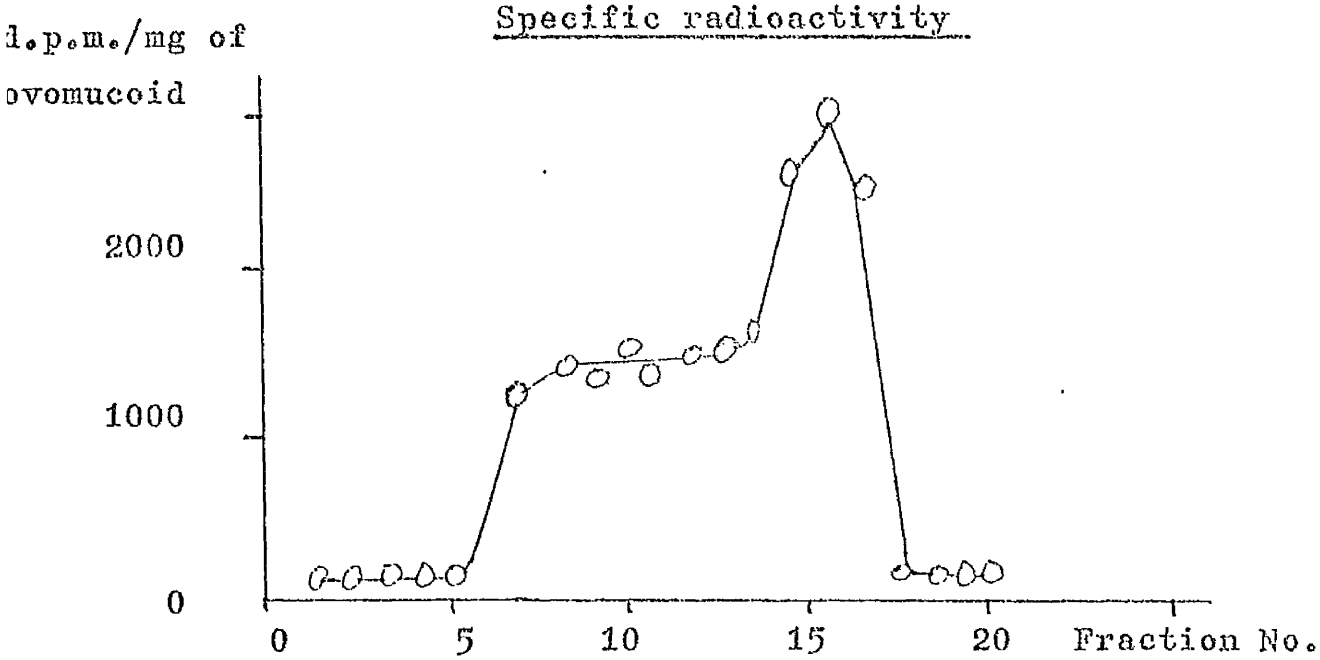
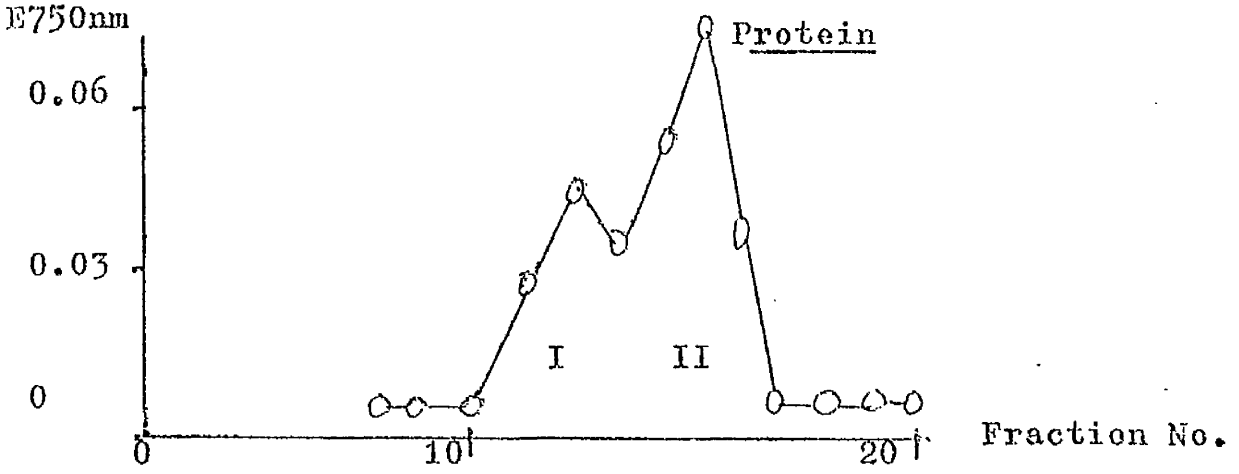
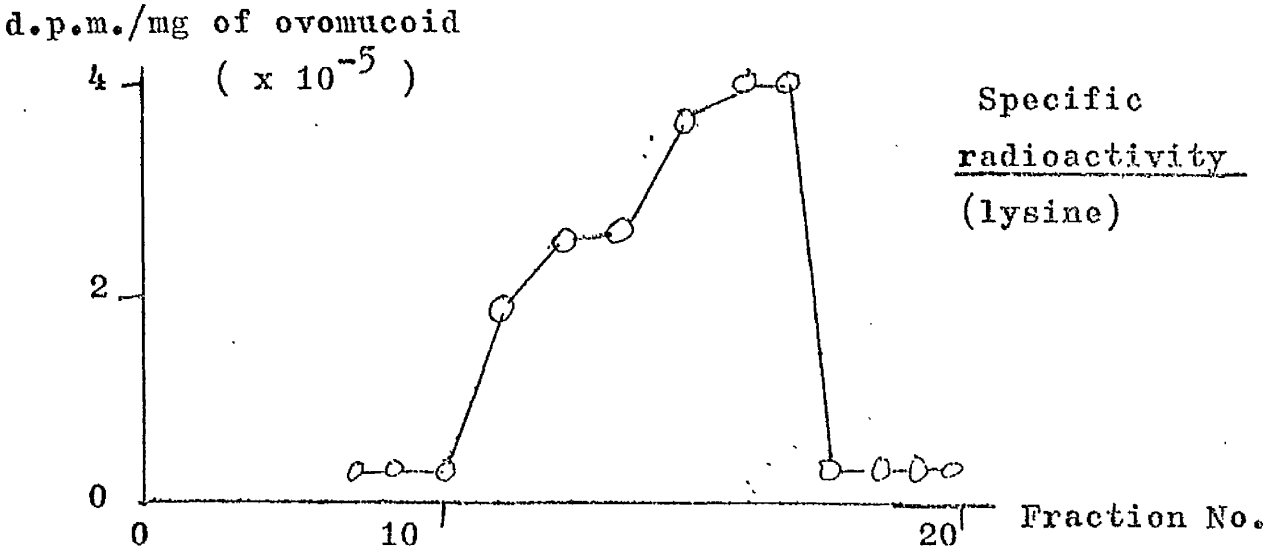

d.p.m./mg of ovomucoid
Specific radioactivity
2000
1000
0
0
5
10
15
20
Fraction No.

Fig. 66. SE-Sephadex chromatography of dual labelled ovomucoid

Oviduct was incubated with [^{3}H]lysine (40μCi/g of oviduct) and [^{14}C] glucosamine (4μCi/g of oviduct). Ovomucoid was isolated from the intracellular fraction of oviduct at various incubation times and fractionated by SE-Sephadex chromatography as in Fig. 65. The protein elution profile and the specific radioactivities of ovomucoid, in terms of lysine labelling and glucosamine labelling, are shown after 2 hour (Fig. 66a), 4 hour (Fig. 66b), 6 hour (Fig.66c) and 8 hour (Fig. 66d)incubations.


E750nm
0.06
0.03
0
Protein
I
II
0
10
20
Fraction No.


d.p.m./mg of ovomucoid
(x 10⁻⁵)
4
2
0
Specific
radioactivity
(lysine)
0
10
20
Fraction No.

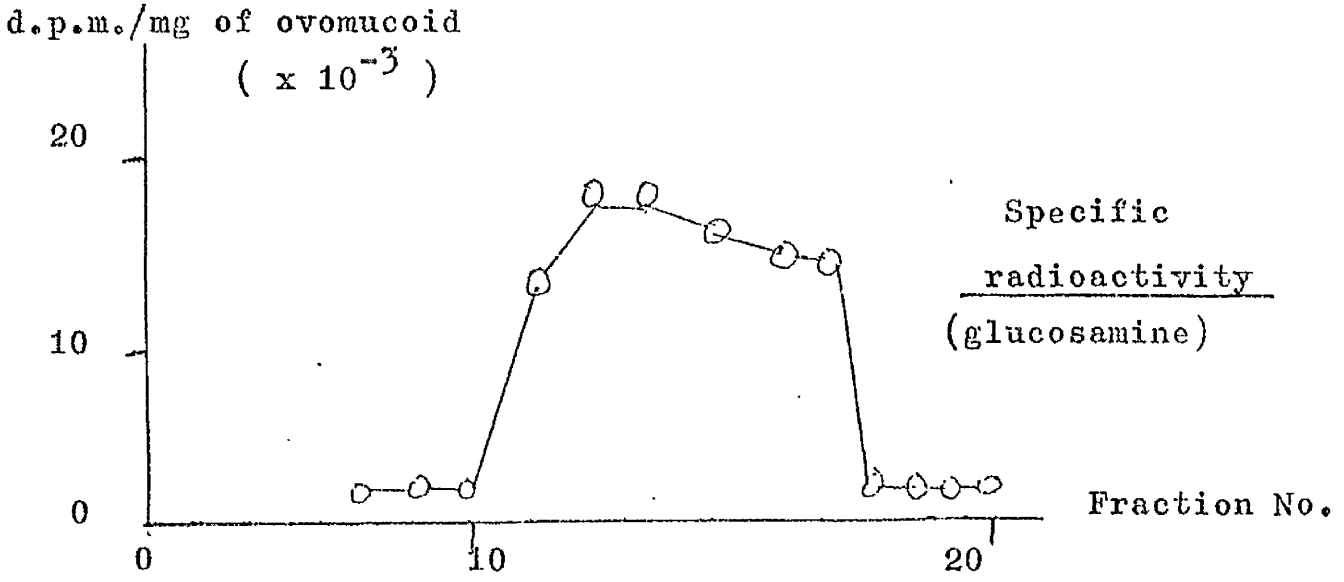
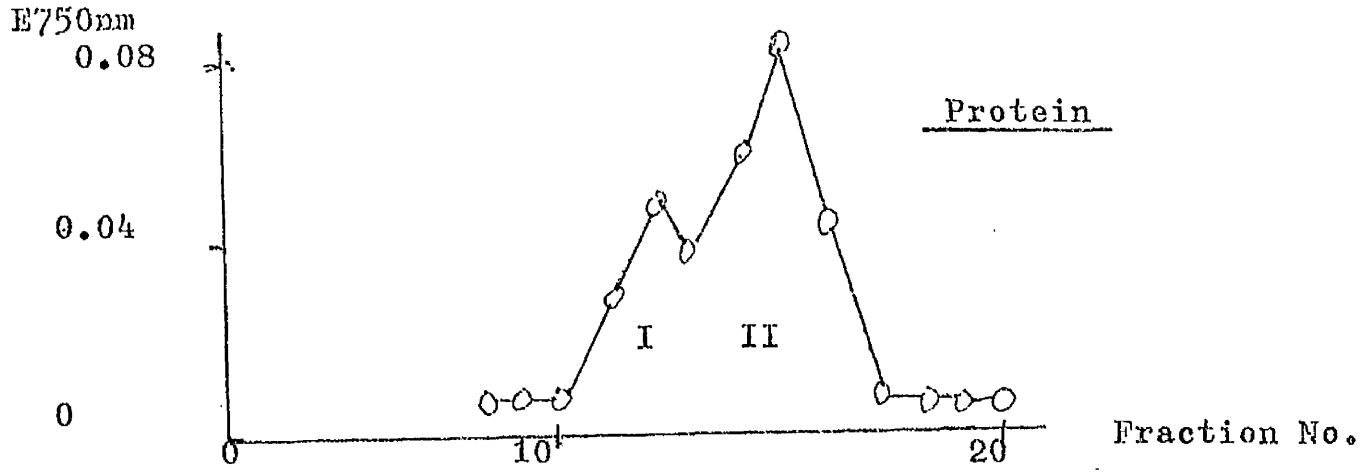
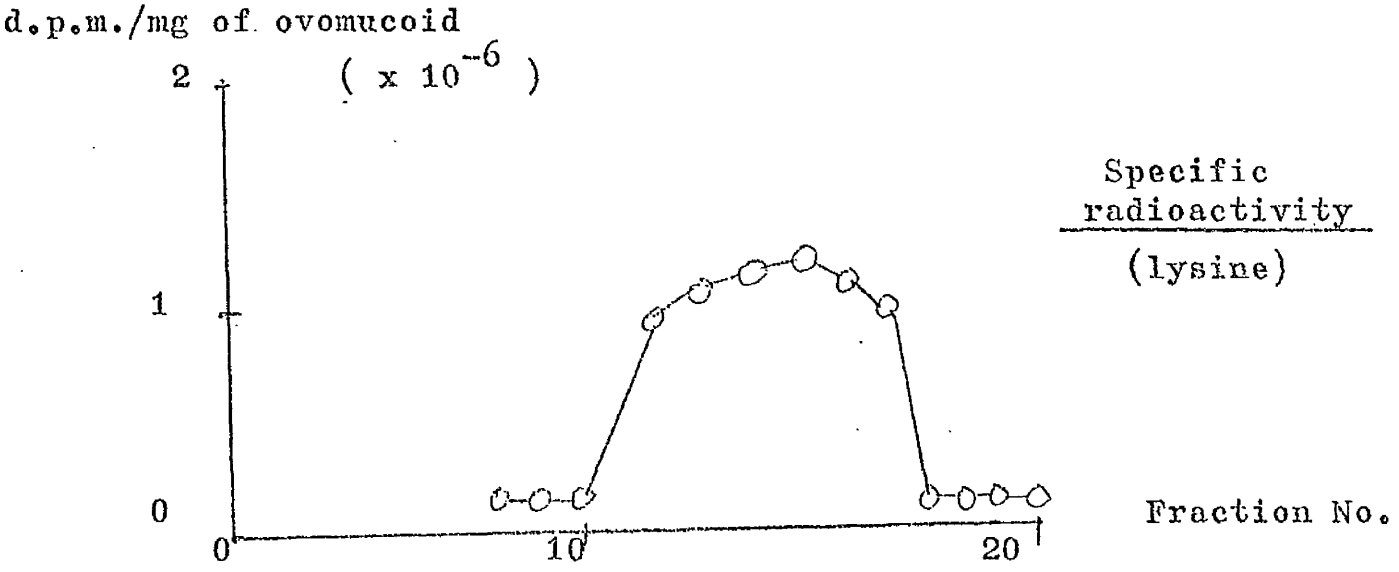

d.p.m./mg of ovomucoid
(x 10⁻³)
20
10
0
Specific
radioactivity
(glucosamine)
0
10
20
Fraction No.

Fig. 3b. (Intracellular ovomucoid; 4 hour incubation)





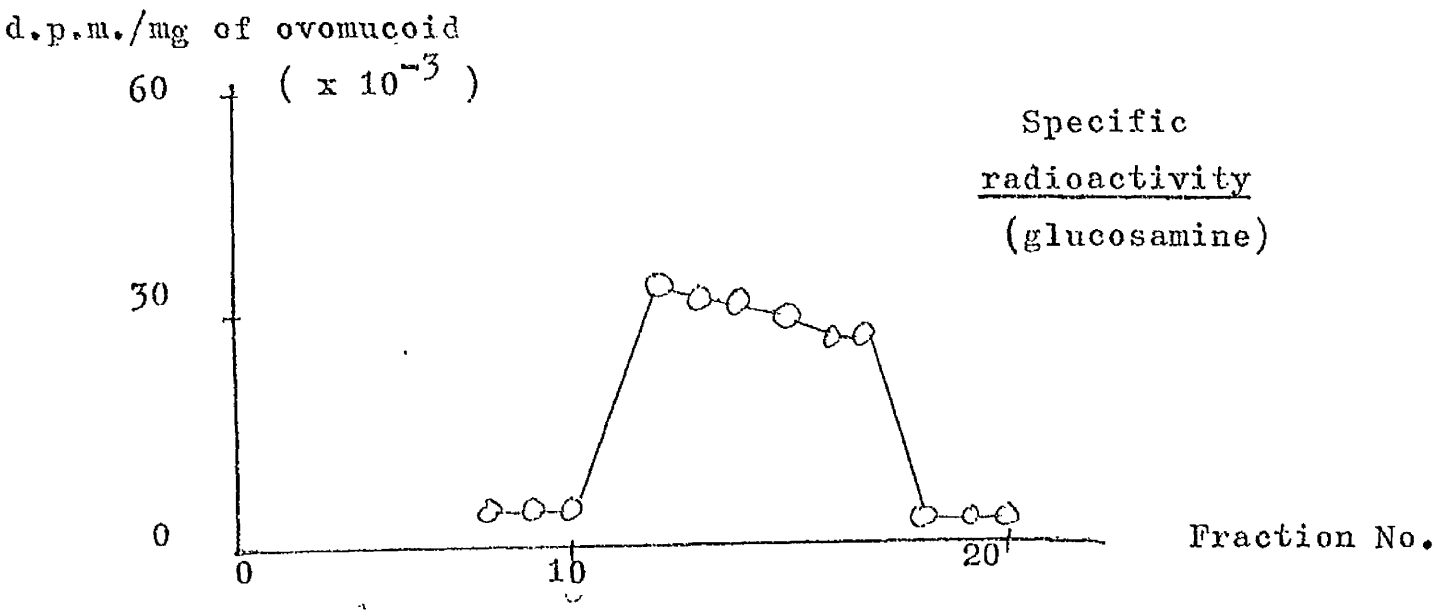
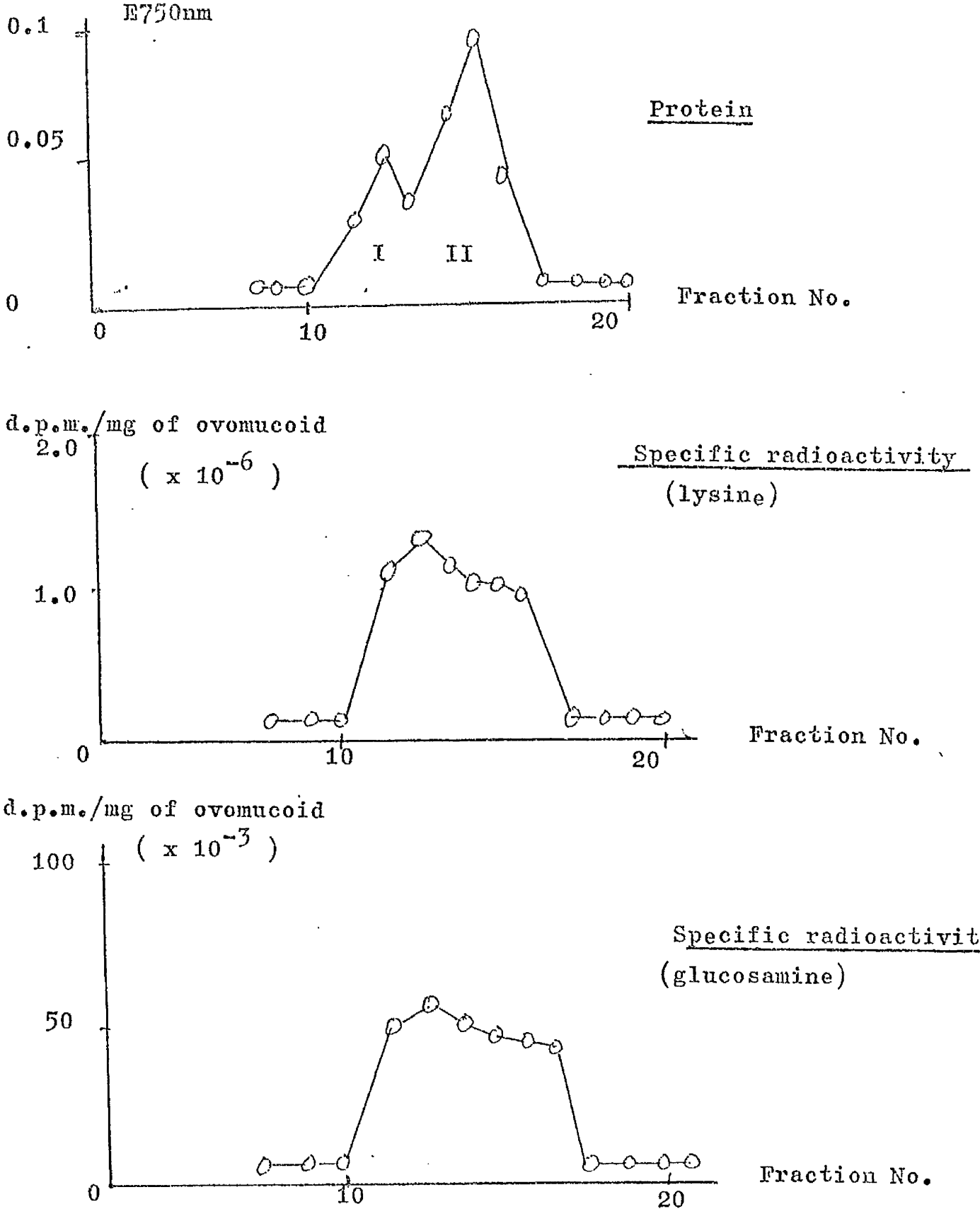
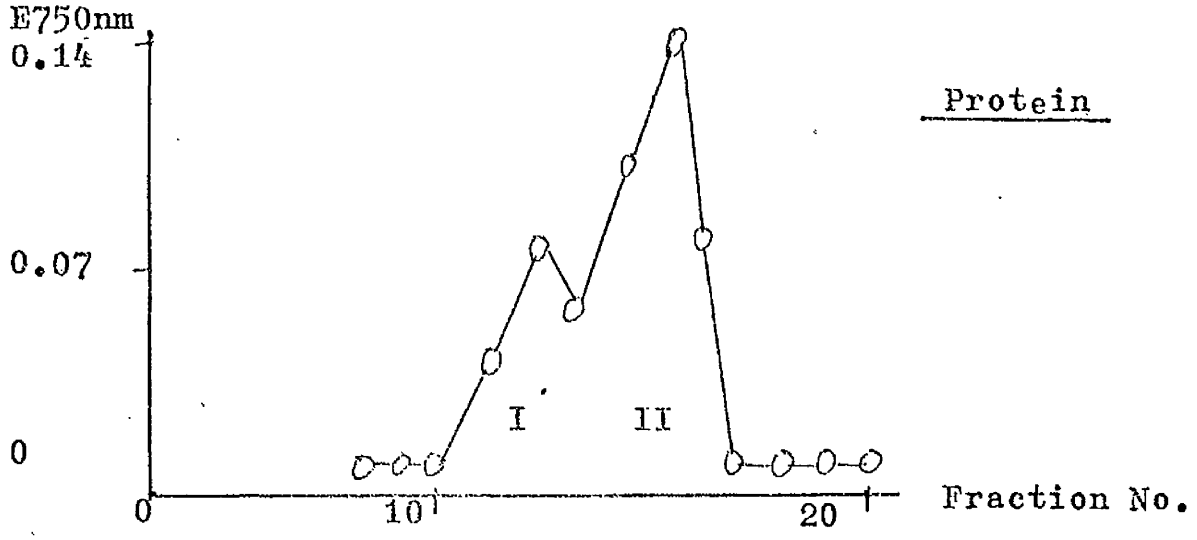
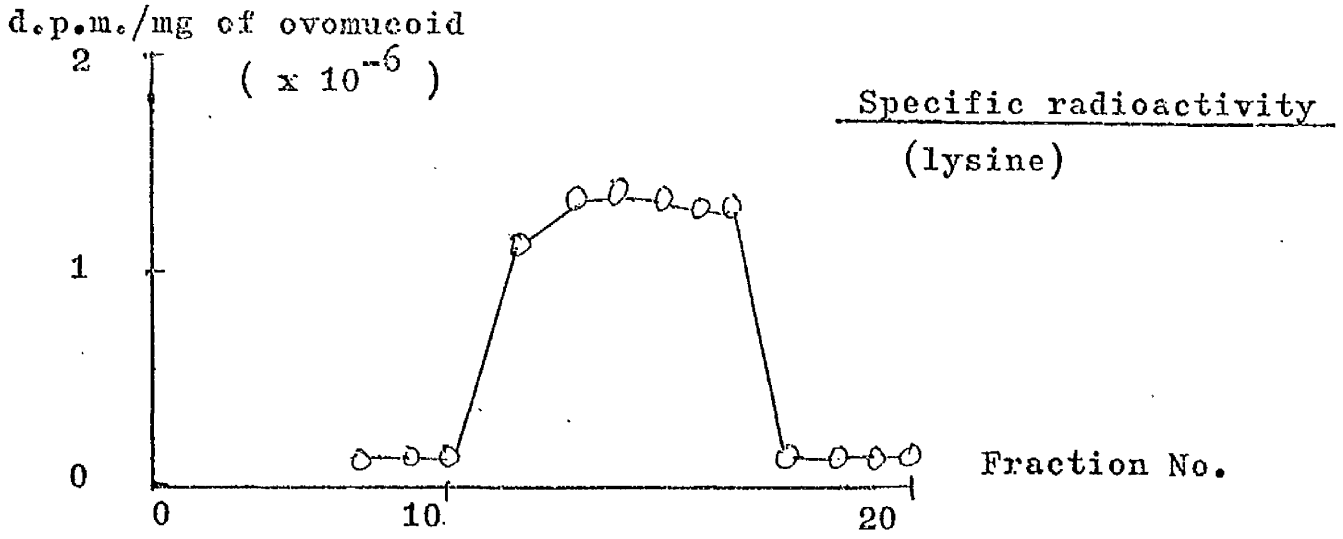
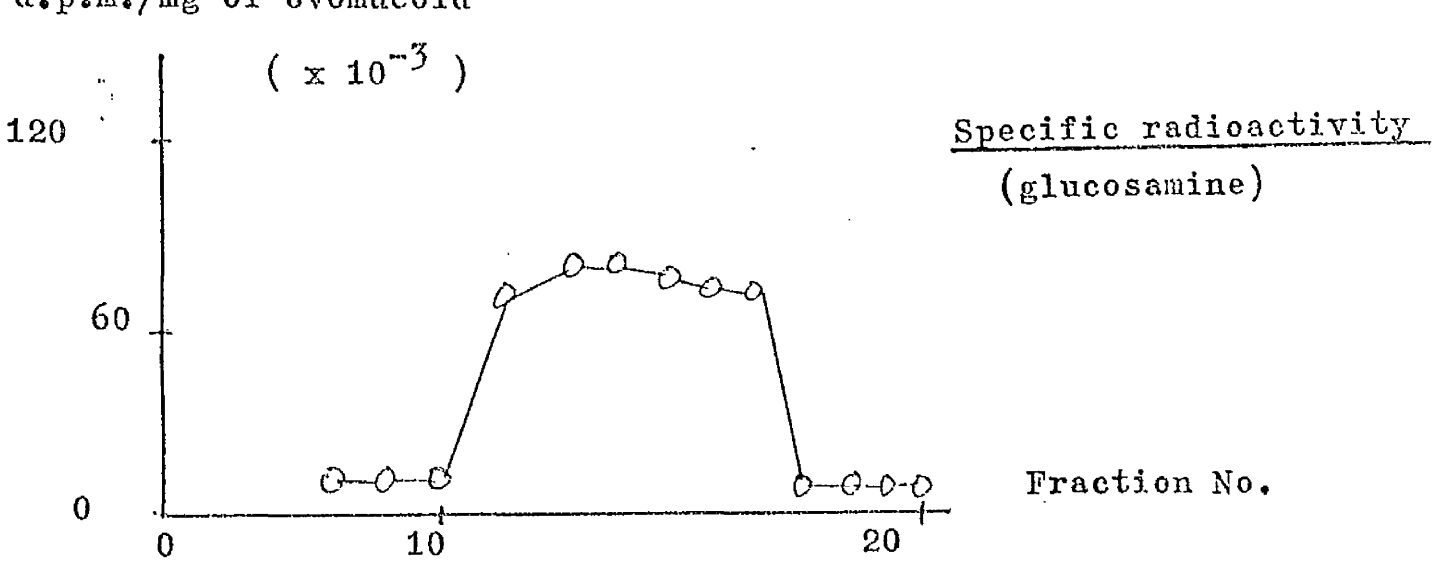


Fig. 5-6 (Intracellular ovomucoid; 6 hour incubation)




E750nm
0.14
0.07
0
I
II
0
10
20
Fraction No.
Protein


d.p.m./mg of ovomucoid
(x 10-6)
2
1
0
0
10
20
Fraction No.
Specific radioactivity
(lysine)


d.p.m./mg of ovomucoid
(x 10-3)
120
60
0
0
10
20
Fraction No.
Specific radioactivity
(glucosamine)

peptide chains, and that ovomucoid 0_1 was the first to be labelled with lysine.

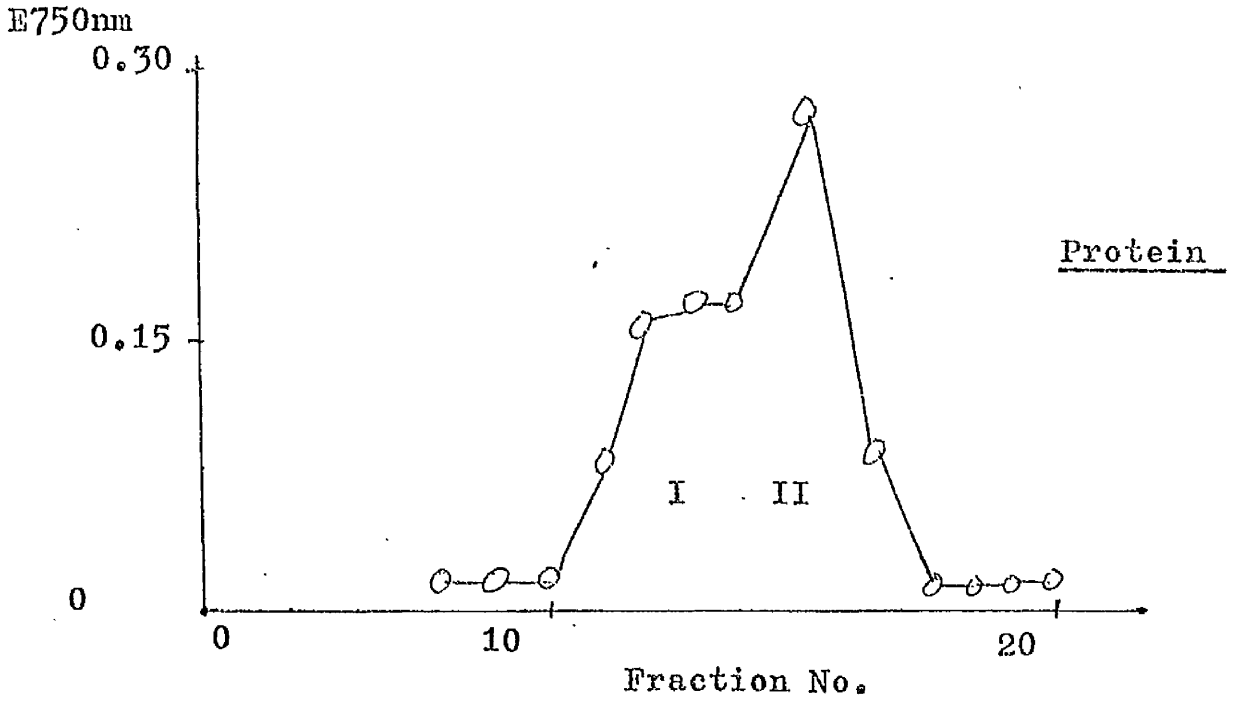
These results suggest that ovomucoid 0_1 may be the precursor to the more acidic species, intracellularly, but that the more acidic species could not be the precursor of ovomucoid 0_1. It is still possible that in the secreted product the multiplicity of species could result from the removal of sialic acid from the most completed species to a varying extent. To test this possibility, the above studies were repeated with extracellular ovomucoid.

After a 4 hour incubation period with $[^3H]$ lysine as precursor, all the extracellular ovomucoid species had a similar specific radioactivity (Fig. 67b). The same was true after 6 hours (Fig. 67c). With $[^{14}C]$ glucosamine as precursor, the specific radioactivities of the variants were similar to each other both at 4 and 6 hours (Figs. 67b and 67c). At 2 hours the specific radioactivities of the $[^{14}C]$ glucosamine labelled ovomucoid species could not be determined accurately due to the low specific radioactivity of the starting material (400d.p.m./mg of ovomucoid). However it can be seen (Fig. 67a) that the specific radioactivity of extracellular 0_1 was higher than the specific radioactivities of the other variants in terms of lysine labelling after 2 hours. This would suggest that the most basic ovomucoid variant (0_1) does not result from the removal of sialic acid from a more acidic species prior to secretion. It would also imply that the sialic acid free ovomucoid may be secreted without having had sialic acid added.

Fig. 67. The specific radioactivities of the extracellular ovomucoid species fractionated by SE-Sephadex chromatography

From the same experiment indicated in Fig. 66 ovomucoid was isolated from the extracellular fraction of oviduct and fractionated by SE-Sephadex chromatography. The protein elution profile and specific radioactivities of ovomucoid are shown at 2 hour (Fig. 67a), 4 hour(Fig. 67b) and 6 hour (Fig. 67c) incubation periods.

Fig. 67a (Extracellular ovomucoid ; 2 hour incubation)



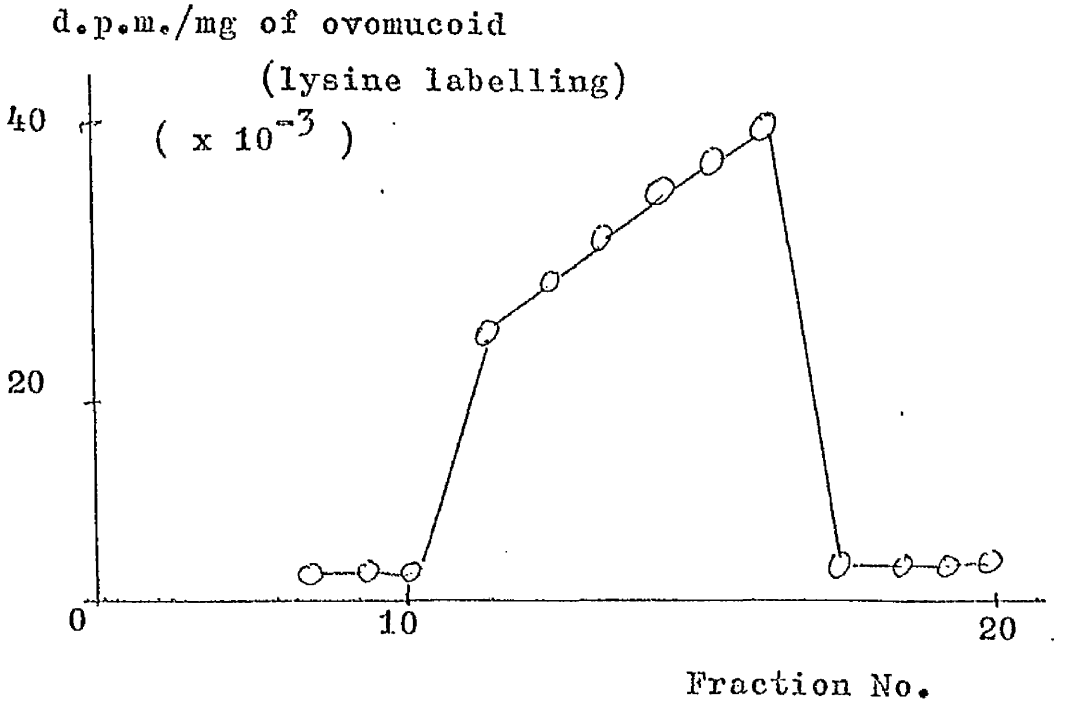
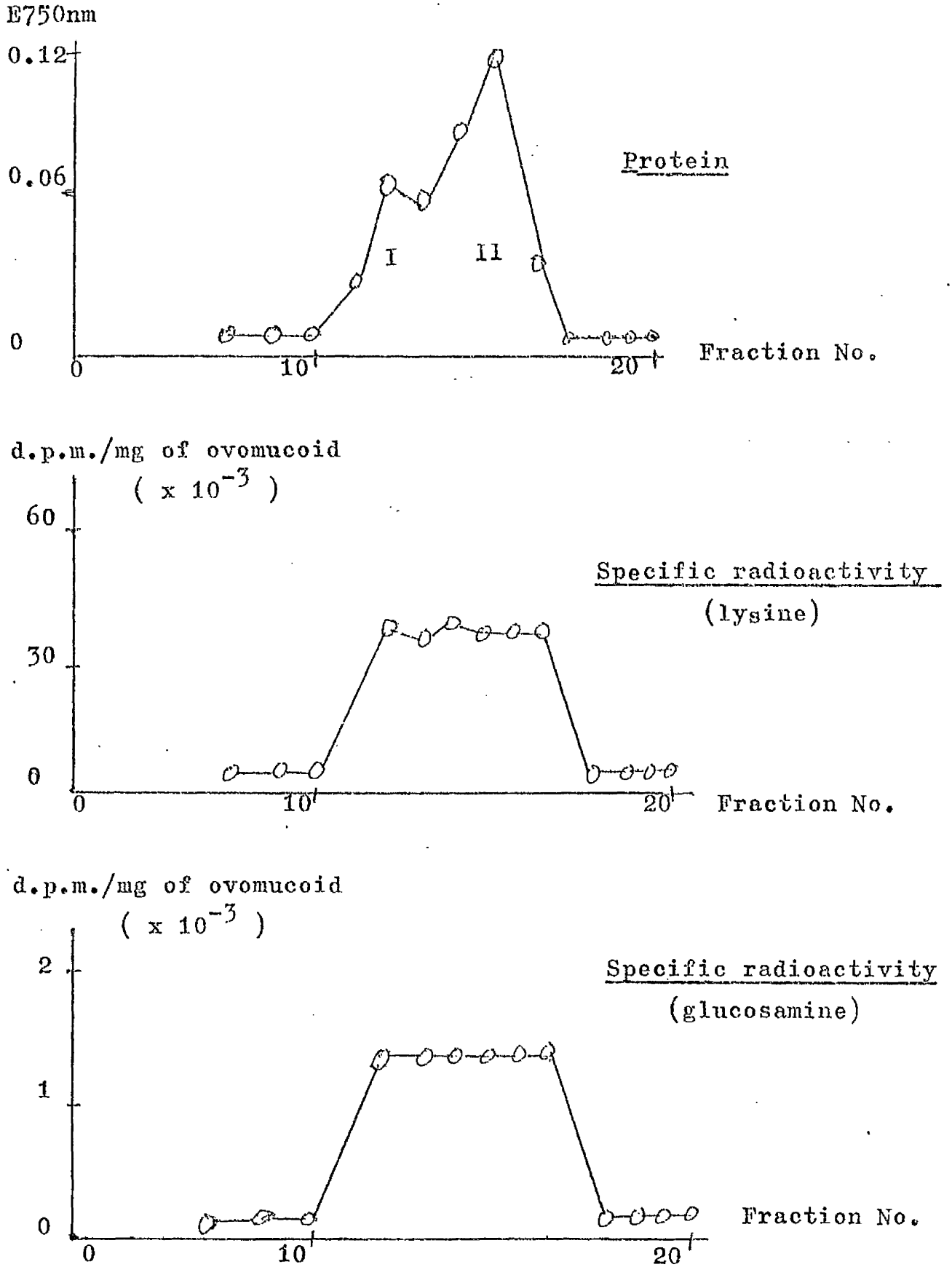
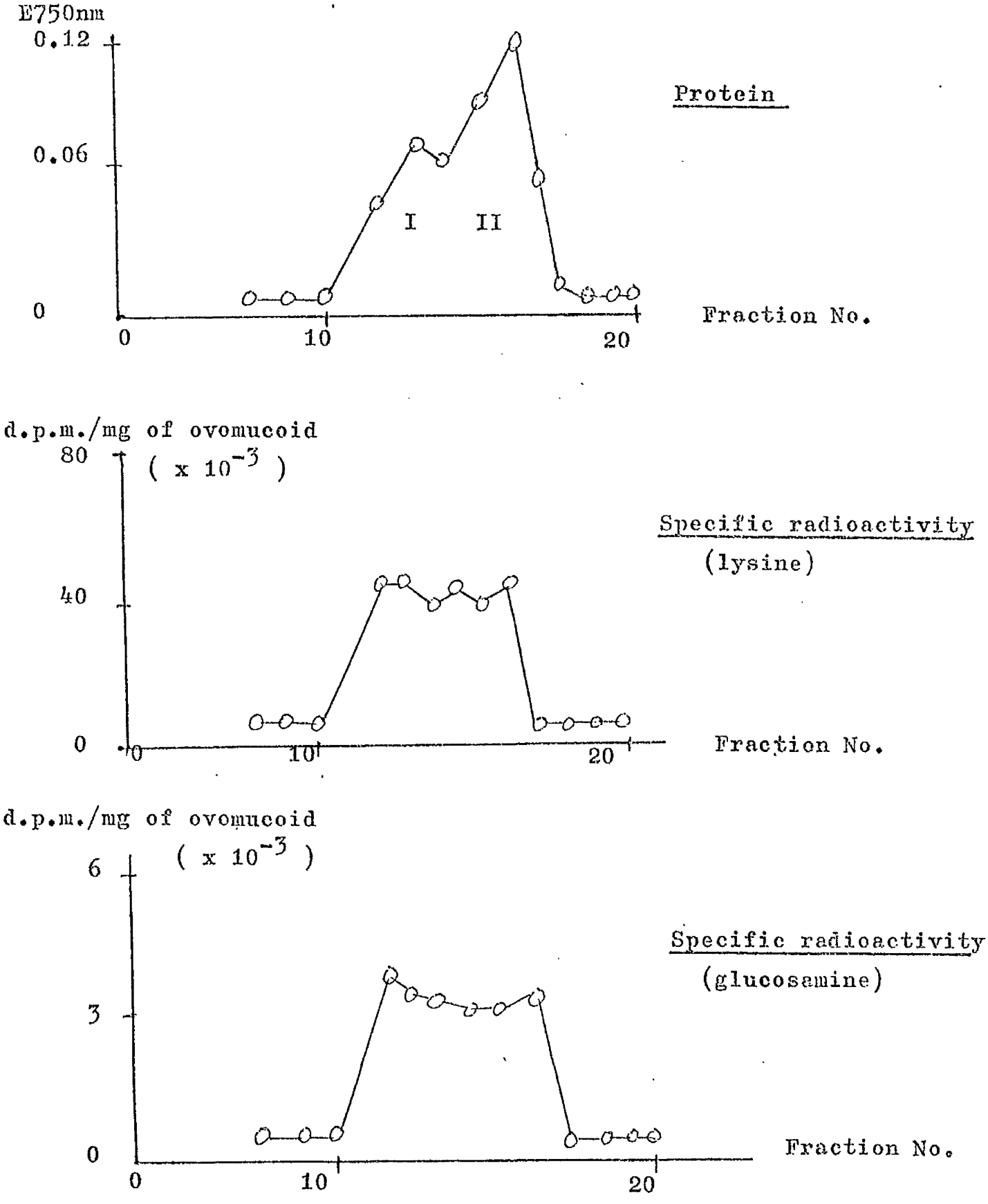


Fig. 67b. (Extracellular ovomucoid; 4 hour incubation)



ig. 67c. (Extracellular ovomucoid; 6 hour incubation)



3. 14 (2) Heterogeneity of Ovomucoid Examined by Isoelectric Focusing

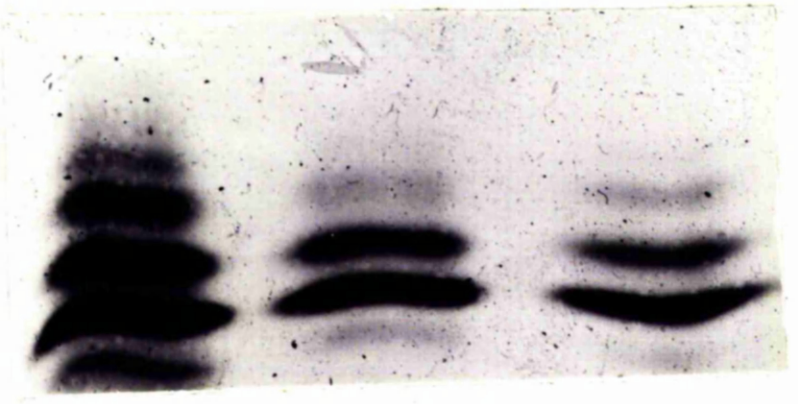
The above results have indicated that heterogeneity of ovomucoid could partly be explained by the secretion of ovomucoid variants without a "completed" carbohydrate moiety. Beeley (1971b) and Melamed (1967) showed that after neuraminidase treatment of egg white ovomucoid there were still two species remaining. In egg white, this may be partly due to effects of storage e.g. deamidation of asparagine residues. The heterogeneity of oviduct intracellular ovomucoid was therefore examined by isoelectric focusing before and after neuraminidase treatment.

In Fig. 68c the three major ovomucoid variants can be seen in a sample of oviduct ovomucoid. After neuraminidase treatment of this sample (Fig. 68b), the three bands still remain. Fig. 68a represents a crude preparation of ovomucoid to show the location of the species. In Fig. 69a the band pattern of a crude preparation of ovomucoid from egg white is shown. Fig. 69d represents this preparation after adsorbtion on and elution from G-200-trypsin. It can be seen that the patterns were very similar except that the use of G-200-trypsin has removed the contaminant which is more basic than O_1. After neuraminidase treatment of both these samples, the number of ovomucoid species has been reduced to 3 (Figs. 69b and 69c). Although the effectiveness of the removal of sialic acid was not checked in these studies, there is a residual charge heterogeneity remaining after neuraminidase treatment of ovomucoid.

Clearly then, part of the charge heterogeneity of ovomucoid is due to sialic acid. The residual heterogeneity remaining after neuraminidase treatment of ovomucoid may be due to carboxyl side chains with abnormal

Fig. 68. Isoelectric focusing of oviduct intracellular ovomucoid after neuraminidase treatment

(+)

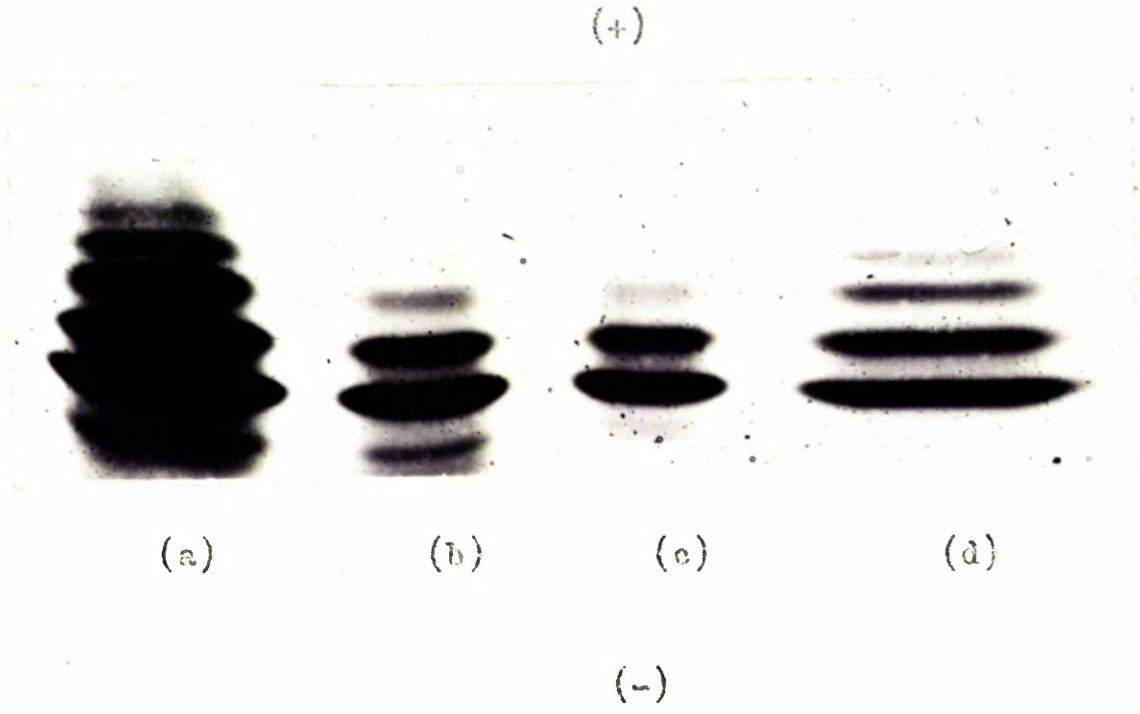


(a) (b) (c)

(-)

(a) A crude preparation of ovomucoid from egg white prepared by the ethanol precipitation method of Fredericq and Deutsch (1949). (b) Intracellular ovomucoid from oviduct homogenate after neuraminidase treatment. (c) Sample (b) before neuraminidase treatment. Samples (b) & (c) were isolated from oviduct by adsorbtion on and elution from G-200-trypsin following a preliminary precipitation of other components with pH 3.5 trichloroacetic acid.

Fig. 69. Isoelectric focusing of egg white ovomucoid after neuraminidase treatment



(a) A crude preparation of ovomucoid isolated from egg white by the ethanol precipitation method of Fredericq and Deutsch (1949). (b) Sample (a) after neuraminidase treatment. (c) Ovomucoid isolated from egg white by adsorbtion on and elution from G-200-trypsin following a preliminary precipitation of other components with pH 3.5 trichloroacetic acid and treated with neuraminidase. (d) Sample (c) prior to neuraminidase treatment.

pK values (Melamed, 1967; Donovan, 1967) or to a difference in amino acid content of the species (Melamed, 1967). However any differences in amino acid content would have to be minor as the amino acid analyses of the variants are similar and immunodiffusion can detect no differences between the species (Beeley, 1971a).

PART 4

DISCUSSION

By using the highly associated but reversible binding of ovomucoid to trypsin it has been possible to isolate ovomucoid from egg white or oviduct homogenates with a high degree of purity.

As opposed to electrophoretic or chromatographic isolation procedures which depend on size and charge, affinity chromatography is selective for molecules which react in the system chosen. In this case an insolubilised derivative of trypsin was employed which would have the capacity to isolate any molecules which bind to trypsin. The isolation method was shown to be selective for ovomucoid if a preliminary precipitation stage with trichloroacetic acid was included (Fig. 3). If ovomucoid was isolated directly from blended egg white or oviduct homogenate then a more basic group of components was also observed on isoelectric focusing (Fig. 3). This presumably would represent ovoinhibitor (Matsushima, 1958), an egg white protein which inhibits both trypsin and chymotrypsin (Feeney, Stevens & Osuga, 1963). After trichloroacetic acid precipitation no material remained in the supernatant which would bind to G-200-chymotrypsin. As ovomucoid isolated by adsorbtion on and elution from G-200-trypsin following a preliminary precipitation with trichloroacetic acid showed no evidence of chymotrypsin inhibiting activity, this would suggest the absence of ovo-inhibitor.

Ovomucoid isolated by adsorbtion on and elution from G-200-trypsin had the same trypsin inhibiting activity as ovomucoid isolated by the method of Fredericq and Deutsch (1949). It was found that 1µg of ovomucoid inhibited 1µg of trypsin and due to the similarity of molecular weights of

ovomucoid (27,000 - 30,000) and trypsin (23,300) this may have meant that inhibition was in a 1 : 1 molar basis if all the trypsin was enzymatically active. Green (1953) showed that the inhibition of trypsin by ovomucoid was competitive.

The hexose, hexosamine and sialic acid content of ovomucoids isolated by use of G-200-trypsin and by the method of Fredericq and Deutsch (1949) were virtually indistinguishable (Tables 5 and 6). These analytical values found for egg white and oviduct ovomucoids were similar to the carbohydrate assays on egg white ovomucoid reported by other workers (Beeley, 1971a; Chatterjee & Montgomery, 1962; Montgomery & Wu, 1963). As the molecular weight of ovomucoid is reported to be in the range 27,000 - 30,000 (Melamed, 1966), the carbohydrate content of ovomucoid (Tables 5 and 6) in terms of residues per mole of protein would be hexose 14 - 15, glucosamine 21 - 24 and sialic acid approximately 0.4 residue per mole of ovomucoid.

When the molecular weight of ovomucoid was determined by gel electrophoresis in the presence of sodium dodecyl sulphate (SDS) the value of 30,000 was found. Weber and Osborn (1969) demonstrated that SDS gel electrophoresis gave remarkably accurate molecular weight determinations for some 40 polypeptide chains of varying size. They did not, however, use any proteins with an extensive carbohydrate moiety. Reynolds and Tanford (1970) illustrated that proteins bound identical amounts of SDS on a gram to gram basis and that the complexes formed had identical hydrodynamic shapes. As the hydrodynamic properties of protein-SDS complexes were a function of the polypeptide chain length and their charge per unit mass was approximately constant, this formed the basis

of the molecular weight estimation. However, Nelson (1971) found that some proteins were resistant to SDS denaturation and that the charge of the protein could affect the amount of SDS bound. Segrest, Jackson, Andrews and Marchesi (1971) showed that the molecular weight of human erythrocyte membrane glycoprotein determined by SDS gel electrophoresis was dependent on the acrylamide concentration. They found that the membrane glycoprotein bound less SDS per g of protein than standard polypeptides resulting in a lower charge : mass ratio than expected. The effect of this on the mobility of the glycoprotein was greatest at low gel concentrations where the sieving effect was least (Segrest et al, 1971). They also found that their estimation of the molecular weights of the dimorphic species of porcine ribonuclease 24,000 and 20,000 were higher than those of Reinhold et al (1968), 21,000 and 17,000. On molecular weight estimations by gel filtration Andrews (1964) found that the glycoproteins ovomucoid and fetuin had elution volumes smaller than expected which he attributed to the large carbohydrate content and shape of these molecules. As ovomucoid contains 20 - 25% carbohydrate the molecular weight (30,000) determined in the studies here may err on the high side. Davis et al (1971) from sedimentation studies and from the amino acid content determined the molecular weight to be 27,300. The observation that no other molecular component was present in ovomucoid prepared by adsorbtion on and elution from G-200-trypsin in the SDS gels was an indication of the purity of the preparation.

The isoelectric focusing band pattern of ovomucoid isolated by use of G-200-trypsin was similar to that of ovomucoid prepared by the method

of Fredericq and Deutsch (1949). Furthermore, comparison of ovomucoid isolated from egg white with that extracted from a homogenate of washed oviduct tissue (Figs. 3, 30, 31, 68 and 69) indicated similarity in the charged species present. The ovomucoid isolated from oviduct homogenate was presumably newly secreted material trapped in the secretory tubules of the tissue or glycoprotein which was in the process of preparation for secretion. If this was the case, the similarity of charged species in egg white and oviduct would make it unlikely that the multiplicity of species arose from degradation following secretion. The observation that each variant refocused in the same position as it did previously (Fig. 20) suggested that the multiplicity of species was not an artifact of the focusing procedure. The isoelectric points of the variants 0_1, 4.45; 0_2, 4.30; 0_3, 4.15; 0_4, 4.0 (and 0_5 less than 4) may correspond to the five molecular species with isoelectric points, 4.41, 4.28, 4.17, 4.01 and 3.83 separated by zone electrophoresis (Bier et al, 1953).

The above results have indicated that ovomucoid isolated by adsorbtion on and elution from G-200-trypsin had similar properties to ovomucoid isolated by the method of Fredericq and Deutsch (1949). Further, ovomucoid isolated from oviduct closely resembled ovomucoid from egg white by the above criteria. However, using the sensitive techniques of immunochemistry it was shown (Figs. 4, 5, 6 and 7) that ovomucoid isolated by adsorbtion on and elution from G-200-trypsin was immunologically homogenous under all the conditions tested while ovomucoid prepared by the ethanol precipitation method of Fredericq and Deutsch (1949) contained

more than one antigenic species. From the similar mobility of the contaminating species on electrophoresis it seemed probable that the major contaminant of crude ovomucoid prepared by ethanol precipitation was ovoglycoprotein (Ketterer, 1965) although a trace of ovalbumin could sometimes be detected.

Ovomucoid isolated from oviduct by use of insolubilised trypsin after 7 hours of incubation in radioisotope studies resembled egg white ovomucoid on isoelectric focusing (Figs. 30 and 31). The carbohydrate content of ovomucoid isolated from the tissue after incubation studies up to 7 hours was also similar to the carbohydrate content of egg white ovomucoid or ovomucoid from an unincubated piece of tissue (Table 16). The lysine and histidine content of ovomucoid from tissue incubation studies (Table 17) were similar to the published values of lysine and histidine content of egg white ovomucoid (Beeley, 1971a; Davis et al, 1971). These observations would suggest that the G-200-trypsin method was suitable for isolating labelled ovomucoid from tissue incubation studies. Radiological purity was indicated by the lack of change in specific radioactivity (d.p.m./mg of ovomucoid) when additional purification steps (e.g. ethanol precipitation or gel filtration) were included in the isolation of ovomucoid from labelled homogenates (Figs. 22 and 23).

When [^{14}C] glucosamine was used as precursor in incorporation studies there was radioactivity associated with both glucosamine and N-acetylneuraminic acid from ovomucoid (Table 19). This may have been expected from the route of synthesis of the sialic acid nucleotides (Kornfield et al, 1964), and indicated the suitability of glucosamine as

a precursor material. With [^{3}H] lysine as precursor all of the radioactivity incorporated into ovomucoid was recovered in lysine (Table 18 and Fig. 32) and as lysine is an essential amino acid, this demonstrated the suitability of its choice as a precursor for in vitro studies.

Both lysine and glucosamine were taken up linearly by the tissue although lysine was taken up more rapidly than glucosamine (Fig. 41). The slower uptake of glucosamine may to an extent have reflected the large amounts of glucose in the medium as energy source, but there was obviously a slower build up of the radioactive intracellular glucosamine pool compared to lysine.

Lysine was incorporated linearly into intracellular ovomucoid over approximately a 5 hour period (Figs. 33 and 36), while there was a lag of about an hour before glucosamine was incorporated linearly into intracellular ovomucoid (Figs. 34 and 37). Schubert (1970) observed a linear uptake of leucine and glucosamine into mouse myeloma protein intracellularly with no detectable lag period. However Parkhouse and Melchers (1971) did observe a lag in incorporation of hexosamine (derived from mannose) into intracellular myeloma immunoglobulin IgM. In radioisotope studies with thyroid slices Spiro and Spiro (1966) found that there was a lag in the incorporation of glucosamine (derived from glucose) into intracellular thyroglobulin. It would seem likely that a lag of incorporation of glucosamine into intracellular ovomucoid would be a reflection of the slower build up of the intracellular radioactive glucosamine pool and perhaps also the relatively larger number of reactions or intermediate pools that the glucosamine may have to go through before

incorporation into the protein compared to lysine.

It was difficult to determine if there was a lag in the incorporation of glucosamine into extracellular ovomucoid (Figs. 34 and 37). The total incorporation of glucosamine into extracellular ovomucoid was lower at all times (up to 8 hours) than the incorporation into intracellular ovomucoid. This would be in agreement with the results of Schubert (1970) with myeloma immunoglobulin up to 2 hours of incubation. Spiro and Spiro (1966) demonstrated that thyroglobulin recovered from the incubation medium after a 3 hour incubation period had a lower specific radioactivity (in terms of sialic acid, galactose, glucosamine and mannose) than intracellular thyroglobulin. Parkhouse and Melchers (1971) demonstrated that mannose and glucosamine were incorporated early into immunoglobulin IgM when compared to fucose. Fucose labelled IgM was secreted without a lag, while there was a lag of about 30 minutes before the secretion of mannose labelled material. The curve for the incorporation of mannose into extracellular IgM crossed the curve for the incorporation of mannose into intracellular IgM at 8 hours. Schubert (1970) found a lag of about 20 minutes before secretion of glucosamine labelled immunoglobulin. As the incorporation of glucosamine into extracellular ovomucoid was lower than the incorporation into intracellular ovomucoid there seems to be broad agreement between the results presented here and the results from the above studies. Glucosamine is considered to belong to the core portion of glycoproteins while sialic acid or fucose are terminal residues (Dunn & Spiro, 1967; Kabasawa & Hirs, 1972; Chatterjee & Montgomery, 1962; Watkins, 1966). It has been shown that glucosamine may be

metabolised to sialic acid and it is possible that sialic acid could be added to ovomucoid shortly before secretion. The availability of ovomucoid precursors would determine the extent of addition of sialic acid to ovomucoid being prepared for secretion.

Parkhouse and Melchers (1971) showed that there was a lag in the incorporation of leucine into extracellular IgM and in agreement with their studies with mannose as precursor that the curves for incorporation of leucine into intracellular and extracellular IgM crossed at 6 hours. In the radioisotope studies with oviduct tissue presented here it was shown that there was a lag period of about an hour (Figs. 33 and 36) before the linear incorporation of lysine into extracellular ovomucoid. In a similar case to glucosamine labelling the incorporation of lysine into extracellular ovomucoid was lower over the 8 hour incubation period than its incorporation into intracellular ovomucoid. Schubert (1970) showed that there was a lag of 20 minutes before the secretion of leucine labelled myeloma immunoglobulin and that the incorporation of leucine into extracellular protein was lower than the incorporation into intracellular protein. Spiro and Spiro (1966) showed that the specific radioactivity of leucine labelled thyroglobulin from the incubation medium was much lower at 8 hours than the activity of the intracellular protein after incubation of thyroid slices. In all these studies, including the work presented here, it seems to be a common factor that there is a lag in the incorporation of radioactive amino acids into extracellular glycoprotein and that the incorporation of a labelled amino acid into extracellular protein is lower than the incorporation into

intracellular protein during the linear period of incorporation of precursor into intracellular protein.

Newly synthesised ovomucoid, labelled with lysine, appeared in the incubation medium after 1 hour. After 6 hours about 30% of the total ovomucoid isolated from the tissue was recovered from the extracellular fraction (Figs. 35 and 38) although only a small proportion of this was labelled (Figs. 33, 34, 36 and 37). It seems likely that there was a mixing of newly synthesised and preformed ovomucoid in the medium. As Hendler (1957) showed that oviduct lost its ability to incorporate precursors into protein on homogenisation, it is very unlikely that the newly synthesised ovomucoid came from cell free synthesis in the medium and it is more probable that it originated from intracellular ovomucoid from the tissue. However, the bulk of the newly synthesised ovomucoid seems to be retained by the tissue in the 8 hour incubation period examined here.

Palmiter, Oka and Schimke (1971) demonstrated, in tissue culture studies using explants of oviduct from immature chicks pretreated in vivo with estrogen, that after 33 hours 26% of the newly synthesised soluble proteins were in the medium while only 5% of proteins made during a 2 hour pulse were secreted. They do not quote figures for the total protein isolated from the medium (i.e. labelled and unlabelled) but give c.p.m. recovered only. However there seems to be broad agreement with the results presented here that incorporation of amino acids into protein from the extracellular fraction was lower than incorporation into intracellular protein.

It was pointed out in the Results section that while the overall pattern of incorporation of precursors into ovomucoid was similar in several experiments, the absolute values varied from oviduct to oviduct. This may have reflected the stage of the egg laying cycle of the hen (Wyburn et al, 1970) and its hormone balance. The effects of estrogen and progesterone on oviduct are well documented. Ovalbumin synthesis may be induced by estrogen and avidin synthesis by progesterone (O'Malley, McGuire & Korenman, 1967; Kohler, Grimley & O'Malley, 1968; Korenman & O'Malley, 1968; Muller, Cox & Carey, 1970; Oka & Schimke, 1969). However Palmiter et al (1971) were unable to induce ovalbumin synthesis in explants of oviduct magnum in vitro. In one experiment using oviduct portions in vitro Mandeles and Ducay (1962) found that the addition of estrogen, progesterone and the hens own anterior hypophysis to the incubation medium resulted in ovalbumin and ovotransferrin with increased specific radioactivities. The incorporation of glycine into flavoprotein was completely abolished while the hormones had virtually no effect on ovomucoid synthesis. In the studies described here (Table 20) it was also found that no combination of progesterone and estrogen tested could stimulate ovomucoid synthesis or its secretion.

Hen oviduct has been shown to have the capacity to synthesise ovalbumin in vitro (Anfinsen & Steinberg, 1951; Hendler, 1956; Carey, 1966) and lysozyme (Canfield & Anfinsen, 1963). The synthesis in vitro of ovalbumin was confirmed by Mandeles & Ducay (1962) and the synthesis of lysozyme, ovotransferrin, flavoprotein and ovomucoid demonstrated. In the studies presented here it has been confirmed that hen oviduct

is the site of formation of ovomucoid. This would make the possibility that egg white proteins are synthesised elsewhere and concentrated in the oviduct (see Romanoff & Romanoff, 1949) unlikely.

It was found that lysine was incorporated linearly over a 5 hour period into the total trichloroacetic acid precipitable protein, while there was a lag of about an hour before lysine labelled acid precipitable protein appeared extracellularly (Figs. 42 and 44). This meant that the curves for the incorporation of lysine into total protein were similar to the curves for the incorporation of lysine into ovomucoid. There was a lag of about an hour before glucosamine was incorporated into intracellular total acid precipitable protein (Figs. 43 and 45) which was similar to the lag found for ovomucoid. However the total incorporation of glucosamine into acid precipitable protein extracellularly was not as much below its incorporation into intracellular protein as in the case of ovomucoid. As the incorporation of lysine into extracellular total protein was much lower than its incorporation into intracellular total protein, this may have reflected the large amount of glycosylated proteins in oviduct secretions. Hendler (1956) using radioactive CO_2 as precursor in tissue incubation studies also found that there was a lag in incorporation of radioactivity into extracellular total protein. The incorporation of radioactivity into the proteins from oviduct homogenate supernatant which were precipitable with 40% (w/v) ammonium sulphate showed only a very slight lag (Hendler, 1956).

With $[^{14}C]$ glucosamine as precursor the specific radioactivity of intracellular ovomucoid was always higher than the specific radioactivity of the intracellular acid precipitable protein fraction (Figs. 46 and 47).

Once again this may have reflected the extensive carbohydrate moiety of ovomucoid (Montgomery & Wu, 1963) and the possibility that there were oviduct proteins synthesised which had no or less carbohydrate attached. The specific radioactivities of extracellular ovomucoid and total protein in terms of glucosamine labelling were closer together than in the case for the intracellular proteins. Although ovomucoid is about 11% of the total egg white protein it has a much more extensive carbohydrate moiety (20 - 25%) than ovalbumin (4%) which forms about 54% of egg white protein (Table 1).

With [^{3}H] lysine as precursor in <u>in vitro</u> studies with oviduct tissue it was found that the specific radioactivity of ovomucoid was in one case (Fig. 48) about the same as that of total proteins over a 6 hour incubation period. This was true both intracellularly and extracellularly. In an experiment with a different oviduct it was found that the specific radioactivity of ovomucoid (intracellular or extracellular) was about half that of total protein over the same time course (Fig. 49) in terms of lysine labelling. The oviducts in these experiments were excised at different times post-oviposition and there may be a variation in the rate of formation of egg white proteins at different times in the egg laying cycle (Mandeles & Ducay, 1962; Wyburn <u>et al</u>, 1970). Mandeles and Ducay (1962) found in a 3 hour <u>in vitro</u> experiment that the specific radioactivities of ovalbumin and ovotransferrin were similar while the specific radioactivities of ovomucoid, lysozyme and flavoprotein differed from this value and from each other in terms of glycine labelling. When the oviduct was excised at different times

post-oviposition the specific radioactivities of all the proteins varied. However while the specific radioactivities of ovalbumin and ovotransferrin paralleled each other, the specific radioactivities of the other proteins bore no relationship to this. The results presented in this work seem to be similar to the findings of Mandeles and Ducay (1962). It would appear that at different times in the egg laying cycle the synthesis of ovomucoid may vary when compared to ovalbumin or total protein synthesis. Neither the results of the present work nor those of Mandeles and Ducay (1962) suggested that ovomucoid synthesis was stimulated by the addition of hormones to the incubation medium. However egg white proteins may be synthesised and secreted by different and specific cells (O'Malley et al, 1967; Kohler et al, 1968; Korenman & O'Malley, 1968; Wyburn et al, 1970).

The above results have shown that lysine was incorporated linearly into intracellular ovomucoid and that a small amount of newly synthesised material appeared in the medium after a lag of about an hour. There was a lag of about an hour before the linear incorporation of glucosamine into intracellular ovomucoid, and the incorporation of glucosamine into extracellular ovomucoid was lower over an 8 hour incubation period than the incorporation into intracellular ovomucoid. These results are similar to the results of other studies on glycoprotein biosynthesis.

In general it has been found that intracellular proteins are synthesised on free ribosomes while extracellular proteins are synthesised on membrane bound ribosomes (Redman, Siekevitz & Palade, 1966; Redman, 1969). The overall pattern of glycoprotein synthesis which has emerged

is that a growing glycoprotein moves from the rough membranes of the endoplasmic reticulum through the smooth membranes of the endoplasmic reticulum to the Golgi apparatus (Rambourg et al, 1969; Choi et al, 1971; Melchers, 1971; Zagury et al, 1970; Schenkein & Uhr, 1970). During this transport through the cisternae of the endoplasmic reticulum system carbohydrate is added to the glycoprotein by specific membrane bound sugar transferases (Ginsburg & Neufeld, 1969).

Liver particulate fractions were considered to contain precursors of plasma glycoproteins (Sarcione et al, 1964; Li et al, 1968). A particle bound precursor of thyroglobulin was found in particulate fractions of thyroid (Spiro & Spiro, 1966). It was found in these present studies that ovomucoid which was released from the 105,000g pellet of oviduct homogenate by deoxycholate had a higher specific radioactivity (d.p.m./mg of ovomucoid) than intracellular ovomucoid over a 5 hour incubation period (Figs. 50, 51, 53 and 54) both in terms of lysine labelling and glucosamine labelling. The specific radioactivities of the ovomucoids from both fractions were however fairly similar over the time course. Hendler (1956) also showed that cell debris proteins had a slightly higher specific radioactivity than the proteins from the supernatant which were precipitable with 40% (w/v) ammonium sulphate in one experiment and that the specific radioactivities were very similar in another experiment. There was no lag in the incorporation of isotope into cell debris proteins (Hendler, 1956) and similar observations were noted here for ovomucoid (Figs. 52 and 55). The ovomucoid from the deoxycholate extracted fraction seemed to have

different solubility properties from soluble intracellular ovomucoid (Table 15). It may have been that deoxycholate extracted ovomucoid did represent a precursor to intracellular ovomucoid. However the similarity of specific radioactivities of ovomucoids from both fractions may have meant that any precursor of ovomucoid was "contaminated" with ovomucoid of a lower specific radioactivity. It is possible that the 105,000g pellet contained secretory granules, but the low yield of ovomucoid from this fraction (Figs. 35 and 38) meant that it was only a small proportion of the total ovomucoid from the tissue (approximately 7%).

Hendler (1956; 1957) demonstrated that microsome-like material sedimented at centrifugal fields of 600g in 10 minutes and Carey (1966) showed that RNA rich particulate fractions could be washed out of the 600g pellet to sediment at 10,000g in 10 minutes in centrifugation studies of oviduct homogenates. On differential centrifugation studies in this present work (Fig. 58, Table 22) it was found that there was no clear separation of cell fractions of oviduct homogenate. Fractionation of oviduct homogenates by stepwise or linear sucrose density gradients did not improve the separation of recognisable cell fractions (Figs. 59-64). It is not clear why RNA enriched fractions of oviduct sediment at low centrifugal fields, but this may be a reflection of a highly developed endoplasmic reticulum system (Hendler, 1956).

The temporal relationship of the synthesis of the peptide and carbohydrate portions of ovomucoid was investigated by carrying out tissue incubation studies in the presence of inhibitors of protein synthesis. Preliminary attempts at blocking the synthesis of the carbohydrate portion

of ovomucoid with 2-deoxyglucose (Tables 24 and 25) were not successful. However it was shown (Table 23) that cycloheximide virtually abolished the incorporation of lysine into intracellular ovomucoid after a 2 hour and a 7 hour incubation period while the incorporation of glucosamine was essentially unaltered. Similar results were found in ovomucoid from the extracellular and deoxycholate extracted fractions. Tissue incubations in the presence of puromycin also gave similar results. These results suggested that oviduct contained a pool of ovomucoid precursors with completed peptide chains but incompleted carbohydrate moieties to which carbohydrate could be added. It is also implied that carbohydrate addition followed protein synthesis and could go on for several hours in the absence of protein synthesis. Similar observations have been made in other tissues (Spiro & Spiro, 1966; Molnar & Sy, 1967; Cook et al, 1965). The inhibition of carbohydrate synthesis follows the inhibition of peptide synthesis more rapidly in some tissues than in others, presumably reflecting different pool sizes of precursor material to which carbohydrate can be added or a different rate of utilisation of these precursors.

It has been shown that newly synthesised ovomucoid can appear in the incubation medium after approximately 1 hour while it has been demonstrated that carbohydrate addition can go on for several hours in the absence of protein synthesis. It is perhaps possible that not all the ovomucoid has to go through the complete carbohydrate synthesising pathway before secretion. There is possibly a slow build up of the carbohydrate moiety and some elaboration of the carbohydrate part which

is not necessary for secretion. As glucosamine may be metabolised to sialic acid, part of the incorporation of radioactivity from glucosamine may be as sialic acid (Table 19).

Many studies have demonstrated the microheterogeneity of ovomucoid (Bier et al, 1953; Rhodes et al, 1960; Chatterjee & Montgomery, 1962; Melamed, 1967; Beeley, 1971a, b). Ovomucoid could be fractionated by isoelectric focusing (Figs. 3, 30, 31, 68 and 69) or by sulphoethyl (SE)-Sephadex C-50 chromatography (Figs. 10 - 15). The ovomucoid species varied in sialic acid content (Fig. 15) and in galactose content (Beeley, 1971a). However the trypsin inhibiting activities of the ovomucoid variants were similar (Table 7) and they had similar amino acid contents and immunochemical properties (Beeley, 1971a). Most other workers have demonstrated heterogeneity of egg white ovomucoid but the present studies have shown that intracellular ovomucoid from oviduct had similar charge heterogeneity.

As heterogeneity of glycoproteins could arise from the secretion of glycoprotein with incompleted carbohydrate moiety or from lack of complete specificity of glycosyltransferases or from the degradation of glycoprotein by glycosidases in the tissue (Gottschalk, 1969), an investigation was made into the specific radioactivities of the ovomucoid variants synthesised by oviduct in vitro. With lysine as precursor it was shown, after a 2 hour incubation period, that the specific radioactivity of the most basic ovomucoid (O_1) from the intracellular fraction was higher than the specific radioactivities of the sialic acid containing variants O_2 - O_5 (Figs. 65 and 66a). At times longer than 2 hours (Figs.66b,c and d)

the specific radioactivities of all the variants were similar. As the lysine contents of the species are virtually identical (Beeley, 1971a) this suggested that the sialic acid free variant O_1 was synthesised first. At all times the specific radioactivities of the intracellular species in terms of glucosamine labelling were similar to each other (Fig. 66). As carbohydrate is added to completed peptide chains this was probably a reflection of the late build up of the carbohydrate moiety of ovomucoid. At 2 hours (Fig. 66a) the ratio of $[^{14}C]$ glucosamine : $[^{3}H]$ lysine would be higher in the sialic acid containing variants than in the sialic acid free ovomucoid. This would be consistent with the addition of carbohydrate to completed peptide chains and that ovomucoid O_1 was the first to be labelled with lysine. The results suggested that O_1 could be the precursor to the other species intracellularly, but that the more acidic species were not the precursor to O_1. Essentially similar observations were found with extracellular ovomucoid (Fig. 67). This suggested that the most basic ovomucoid did not result from the removal of sialic acid from a more acidic species prior to secretion. It would also imply that the sialic acid free ovomucoid may be secreted without having had sialic acid added. As all the ovomucoid species were found in the tissue prior to secretion and there was differential labelling of the species with regards to lysine labelling, both intracellularly and extracellularly, it would appear that heterogeneity could partly be explained by secretion of ovomucoid with incompleted carbohydrate moiety. Other workers have proposed similar explanations for the observed microheterogeneity of glycoproteins (Gottschalk, 1969;

Dunn & Spiro, 1967; Kabasawa & Hirs, 1972).

Beeley (1971b) and Melamed (1967) found that there was a residual charge heterogeneity in egg white ovomucoid after neuraminidase treatment. The present results confirmed these observations and demonstrated that this residual heterogeneity remained in oviduct intracellular ovomucoid after neuraminidase treatment (Figs. 68 and 69). This residual heterogeneity may be due to a difference in amino acid content of the species (Melamed, 1967) or to carboxyl side chains with abnormal pK values (Melamed, 1967; Donovan, 1967). However the immunochemical properties and amino acid analyses of the ovomucoid variants are very similar (Beeley, 1971a). Fetuin also shows residual heterogeneity after neuraminidase treatment, but the microheterogeneity was considered to result primarily from the differences in the number and arrangement of the sialic acid residues (Oshiro & Eylar, 1968). Positional isomerism of sialic acid residues attached to galactose residues of α_1- acid glycoprotein (Jeanloz, 1966) have been proposed as an explanation of the observed polymorphism of this protein on starch gel electrophoresis (Schmid, 1968). It would seem likely that the charge heterogeneity of ovomucoid resides primarily in the sialic acid residues. If this is the case, then the observed micro-heterogeneity of ovomucoid can be partly explained from the results presented in this work by the secretion of ovomucoid with incompleted carbohydrate moiety.

SUMMARY

1. Ovomucoid was isolated from egg white or the homogenates of oviduct tissue by adsorbtion on and elution from an insolubilised trypsin derivative. Ovomucoid isolated this way had the same hexose, hexosamine and sialic acid content as ovomucoid prepared by the method of Fredericq and Deutsch (1949) and the same trypsin inhibiting activity. Immunochemical studies suggested that ovomucoid isolated by adsorbtion on and elution from G-200-trypsin was immunologically homogenous, while ovomucoid isolated by ethanol precipitation (Fredericq & Deutsch, 1949) contained more than one antigenic species.

2. Ovomucoid was fractionated into differently charged variants by sulphoethyl-Sephadex chromatography or isoelectric focusing. The 5 variants of egg white ovomucoid observed on isoelectric focusing were present in oviduct tissue before secretion, making it unlikely that the multiplicity of species arose from degradation following secretion. The isoelectric points of 4 of the variants were determined to be 4.45, 4.30, 4.15 and 4.0 and the isoelectric point of the most acidic species estimated to be less than 4. All had the same molecular weight, estimated by sodium dodecyl sulphate gel electrophoresis to be 30,000 $\pm$ 2,500. After neuraminidase treatment the number of variants was reduced from 5 to 3 suggesting that part of the charge heterogeneity resided in the sialic acid residues.

3. The insolubilised trypsin isolation method was shown to be suitable for isolating labelled ovomucoid from tissue

incubation studies. Ovomucoid isolated from the tissue after a 7 hour incubation period *in vitro* was shown to be indistinguishable from ovomucoid from an unincubated piece of tissue or from egg white ovomucoid. Radiological purity was indicated by the lack of change in specific radioactivity (d.p.m./mg of ovomucoid) when additional purification steps, e.g. gel filtration or ethanol precipitation were included in the isolation.

4. [^{3}H] Lysine was incorporated linearly into intracellular ovomucoid over a 5 hour period *in vitro*. After about an hour a small amount of newly synthesised ovomucoid appeared in the medium. There was a lag of approximately an hour before the linear incorporation of [^{14}C] glucosamine into intracellular ovomucoid. The incorporation of [^{14}C] glucosamine into extracellular ovomucoid was lower over an 8 hour incubation period than its incorporation into intracellular ovomucoid. Although only a very small amount of newly synthesised material appeared in the medium, about 30% of the total ovomucoid isolated from the tissue was in the extracellular fraction after 8 hours.

5. There was no combination of progesterone and estrogen tested that could stimulate ovomucoid synthesis or secretion. However the rate of synthesis of ovomucoid varied when compared to the synthesis of the total trichloroacetic acid precipitable protein fraction when the oviduct was excised at different times post-oviposition.

6. Ovomucoid which was released from the 105,000g pellet of oviduct homogenates had a slightly higher, though fairly similar, specific radioactivity in terms of both lysine and glucosamine labelling to that of intracellular ovomucoid over a 5 hour incubation period. The deoxycholate extracted ovomucoid seemed to have different solubility properties from intracellular ovomucoid. Only a very small proportion of the total ovomucoid isolated from the tissue (approximately 7%) was released by deoxycholate. Fractionisation of oviduct homogenates by centrifugation suggested that there was no clear separation of subcellular components. Part of this difficulty was that RNA enriched material sedimented at lower centrifugal fields than that found for other tissues, liver for example.

7. Tissue incubations in the presence of puromycin or cycloheximide suggested that carbohydrate addition followed protein synthesis and could go on for several hours in the absence of protein synthesis. The tissue presumably contained a pool of ovomucoid precursors with completed peptide chains but incompleted carbohydrate moiety to which carbohydrate could be added.

8. By fractionisation of labelled intracellular ovomucoid it was indicated that the sialic acid free ovomucoid variant (O_1) was synthesised first. The specific radioactivity of lysine labelled extracellular O_1 was also higher than that of the sialic acid containing variants extracellularly in a 2 hour incubation study. This implied that O_1 did not result

from the removal of sialic acid from a more acidic species prior to secretion, and that O_1 may be secreted without having had sialic acid added. The results suggested that heterogeneity of ovomucoid could be partly explained by the secretion of ovomucoid with incompleted carbohydrate moiety.

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BIOSYNTHESIS OF OVOMUCOID

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Summary of thesis submitted to the

University of Glasgow, November, 1972.

Ovumocoid was isolated from egg white or the homogenates of oviduct tissue by adsorbtion on and elution from an insolubilised trypsin derivative (Sephadex G-200-trypsin). Ovomucoid bound to the insolubilised trypsin at pH 7, but could be eluted by washing the complex at pH 1.5. The method was shown to be selective for ovomucoid if a preliminary precipitation of other components with 5% (w/v) trichloroacetic acid, pH 3.5, was included in the isolation.

Ovomucoid isolated using insolubilised trypsin had the same trypsin inhibiting activity and the same hexosamine , hexose and sialic ac content as ovomucoid prepared by conventional methods, such as ethanol precipitation. Immunochemical studies suggested that ovomucoid isolated by adsorbtion on and elution from G-200-trypsin was immunologically homogenous, while ovomucoid prepared by ethanol precipitation, following a preliminary pH 3.5 trichloroacetic acid precipitation of other component contained more than one antigenic species. Ovomucoids from egg white or the homogenates of washed oviduct tissue were indistinguishable in the above studies.

Ovomucoid was fractionated into differently charged variants by sulphoethyl-Sephadex chromatography or isoelectric focusing. The 5 variants of egg white ovomucoid observed on isoelectric focusing were present in oviduct tissue before secretion making it unlikely that the multiplicity of species arose from degradation following secreti The isoelectric points of 4 of the variants were determined to be 4.45, 4.30, 4.15 and 4.0 and the isoelectric point of the most acidic species estimated to be less than 4. All had the same molecular weight, estimated sodium dodecyl sulphate gel electrophoresis to be 30,000 ± 2,500. After neuraminidase treatment the number of variants was reduced from 5 to 3 suggesting that part of the charge heterogeneity resided in the sialic acid residues.

The insolubilised trypsin isolation method was shown to be suitable for isolating labelled ovomucoid from tissue incubation studies. Ovomucoid isolated from the tissue after a 7 hour incubation period *in vitro* was shown to be indistinguishable from ovomucoid from an unincubated piece of tissue or from egg white ovomucoid. Radiological purity was indicated by the lack of change in specific radioactivity (d.p.m./mg of ovomucoid) when additional purification steps, e.g. gel filtration or etha precipitation were included in the isolation.

$[^{3}H]$ Lysine was incorporated linearly into intracellular ovomucoid over a 5 hour period *in vitro*. After about an hour a small amount of newly synthesised ovomucoid appeared in the medium. There was a lag of approximately an hour before the linear incorporation of $[^{14}C]$ glucosamine into intracellular ovomucoid. The incorporation of

[^{14}C] glucosamine into extracellular ovomucoid was lower over an 8 hour incubation period than its incorporation into intracellular ovomucoid. Although only a very small amount of newly synthesised material appeared in the medium, about 30% of the total ovomucoid isolated from the tissue was in the extracellular fraction after 8 hours.

There was no combination of progesterone and estrogen tested that could stimulate ovomucoid synthesis or secretion in vitro. However the rate of synthesis of ovomucoid varied when compared to the synthesis of the total trichloroacetic acid precipitable protein fraction when the oviduct was excised at different times post-oviposition.

Ovomucoid which was released from the 105,000g pellet of oviduct homogenates by deoxycholate had a slightly higher, though fairly similar, specific radioactivity in terms of both lysine and glucosamine labelling to that of intracellular ovomucoid over a 5 hour incubation period. The deoxycholate extracted ovomucoid seemed to have different solubility properties from intracellular ovomucoid. Only a very small proportion of the total ovomucoid isolated from the tissue (approximately 7%) was released by deoxycholate. Fractionation of oviduct homogenates by centrifugation suggested that there was no clear separation of subcellular components. Part of this difficulty was that RNA rich material sedimented at lower centrifugal fields than that found for other tissues, liver for example.

Tissue incubations in the presence of puromycin or cycloheximide resulted in almost complete inhibition of the incorporation of lysine into ovomucoid in all tissue compartments after a 2 hour and a 7 hour

incubation period, while the incorporation of glucosamine was essentially unaltered. This suggested that carbohydrate addition followed protein synthesis and could go on for several hours in the absence of protein synthesis. The tissue presumably contained a pool of ovomucoid precursors with completed peptide chains but incompleted carbohydrate moiety to which carbohydrate could be added.

Fractionation of labelled intracellular ovomucoid indicated that the sialic acid free ovomucoid variant (O_1) was synthesised first as the specific radioactivity of lysine labelled O_1, was higher than that of the sialic acid containing variants after a 2 hour incubation period. The specific radioactivity of lysine labelled extracellular O_1 was also higher than that of the sialic acid containing variants extracellularly in a 2 hour incubation study. This implied that O_1 did not result from the removal of sialic acid from a more acidic species prior to secretion, and that O_1 may be secreted without having had sialic acid added. The results suggested that heterogeneity of ovomucoid could be partly explained by the secretion of ovomucoid with incompleted carbohydrate moiety.