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I am submitting herewith a dissertation written by Theodore Arnold Gottlieb entitled "The Posttranslational Modification and Secretion of Vitellogenin in Xenopus laevis." I have examined the final copy of this dissertation for form and content and recommend that it be accepted in partial fulfillment of the requirements for the degree of Doctor of Philosophy, with a major in Biomedical Sciences.

Robin A. Wallace

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We have read this dissertation
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Accepted for the Council:

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Vice Chancellor
Graduate Studies and Research

THE POSTTRANSLATIONAL MODIFICATION AND SECRETION OF VITELLOGENIN IN *XENOPUS LAEVIS*

A Dissertation

Presented for the

Doctor of Philosophy

Degree

The University of Tennessee, Knoxville

Theodore Arnold Gottlieb

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DEDICATION

This dissertation is dedicated to my family, my hometown, and Stanley, each having contributed profound influences resulting in a complex melange of inspirations, and a not so delicate balance between courage and cowardice.

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Many people were directly responsible for both the intellectual and spiritual growth that I experienced during my stay in Oak Ridge, as well as the Biology Division. My thesis committee members, Drs. John Cook, M. L. Pan, Frank Gaertner, and Fred Snyder were endless sources of encouragement and other miscellaneous forms of first aid. Special gratitude is extended to Dr. Jim Dumont for allowing me carte blanche access to his electron microscope facility, and to Carole Sue Richter for teaching me all that I know of this useful art.

The friendship and assorted affections of Harry Ratrie III, Laurie Phyllis Pearlman, Marcy Ellen Wax, and Jane Elizabeth Mendel, have made life in this often dessicated and expressionless community, an undeniably satisfying and even beautiful experience. I could not have made it without them.

Of course the gratitude and warmth I feel for my rather elusive advisor cannot go unmentioned. I've not met many competent investigators who also fall into the category of "gentleman and scholar," therefore I express heartfelt appreciation and respect for Robin Wallace, a truly endangered species.

ABSTRACT

The thesis presented represents an attempt to elucidate the intracellular pathway linking the synthesis and secretion of the yolk precursor protein vitellogenin. More specifically, how the hepatocyte attaches both phosphate and carbohydrate to the peptide backbone prior to secretion is considered. A model which summarizes the accumulated data depicting various aspects of these processes is also presented.

A procedure was developed for the preparation of rough and smooth microsomes from small quantities of liver obtained from estrogen-stimulated Xenopus laevis females. The separation involves the centrifugation of a postmitochondrial supernatant over a CsCl-containing, discontinuous sucrose gradient. Morphological, biochemical, and enzymatic characterization of these fractions indicates that excellent separation of rough microsomes from smooth microsomes is achieved. In addition, pulse-chase experiments in which (5 min) pulses with [^{3}H]leucine are used to demonstrate that rough and smooth microsomes each exhibit the predictable patterns of incorporation characteristic of secretory protein synthesis and intracellular translocation.

This procedure was combined with suitable incubations for pulse-chase experiments which demonstrate the subcellular sites of vitellogenin phosphorylation. The results of these experiments indicate that approximately 70% of the phosphate residues are covalently attached to vitellogenin during its intracellular translocation through the smooth microsomes, while the rough microsomes can account for the remainder of

the total incorporated phosphate. This is further supported by the analysis of newly synthesized and assembled [^{3}H,^{32}P]vitellogenin on sodium dodecylsulfate-polyacrylamide gels and measurements of protein kinase activity in microsomal subfractions.

The presence of a divalent-cation requiring protein kinase (E.C. 2.7.1,37, ATP:protein phosphotransferase) is demonstrated in the microsomes derived from the liver of estrogenized female Xenopus laevis. Using Xenopus phosvitin, a proteolytic derivative of vitellogenin, as substrate, we have characterized various requirements and kinetic properties of this enzyme activity. In addition we present data showing that this protein kinase is responsible for phosphorylating hepatic precursors to serum vitellogenin in vivo.

Pulse-chase experiments measuring the rates of incorporation of radiolabeled glucosamine and galactose into intracellular vitellogenin show that glycosylation of this multicomponent protein occurs in a Golgi-enriched fraction isolated from homogenized liver slices. No apparent role for the rough endoplasmic reticulum was demonstrable. Kinestics of the intracellular translocation of glycosylated intermediates of vitellogenin indicate that the galactosylated form is secreted more rapidly than the glucosamine-labeled precursor. This was corroborated by measuring the rates of accumulation of various pulse-labeled forms of vitellogenin in the chase medium. In addition, a negligible amount of mannose was incorporated into intracellular or secreted vitellogenin.

The antibiotic tunicamycin was shown to inhibit [^{3}H]glucosamine incorporation into microsomal vitellogenin by 70%, without any

significant effects on the synthesis of the protein backbone. In addition, nonglycosylated vitellogenin showed normal secretion kinetics. After suitable pretreatment with the antibiotic followed by a labeling period in tunicamycin-free medium, mannose was still not incorporated into vitellogenin whereas glucosamine behaved in a typical manner. In contrast to this finding, gas-liquid chromatography of the alditol acetate derivatives of the neutral hexoses of vitellogenin showed that mannose was indeed a major component of the vitellogenin oligosaccharide side chain.

These results indicate that the oligosaccharide component of vitellogenin in Xenopus is a "complex" type of carbohydrate unit which is linked via an N-glycosidic bond between an asparagine residue and N-acetylglucosamine. With respect to subcellular localization of glycoprotein assembly in Xenopus liver, there is a significant departure from currently accepted models of glycoprotein synthesis.

PREFACE

This dissertation has been assembled into a format which reflects the earlier use of Chapter II-IV as individual manuscripts which were submitted for publication. Therefore, each of these chapters possesses both a general introduction and a section describing the experimental methods used within that chapter. I call this to the attention of the reader since he or she might not be acquainted with this format of organizing dissertation research.

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CHAPTER I

INTRODUCTION

A universal feature of oocyte growth in nonmammalian vertebrates is the accumulation within the cytoplasm, of nutritive substances collectively referred to as "yolk." It has been convenient in general to refer to the two major components of yolk as "protein yolk" and "fatty yolk," the former being rich in phosphoprotein while the latter is composed of varying amounts of neutral lipid and phospholipid. The relative quantities of each of these yolk components show considerable species variation. The sum of the processes by which yolk proteins are made and deposited within the oocyte is referred to as "vitellogenesis." In most cases, as will be documented below, the yolk protein precursors are derived from an anatomical site distinct from the oocyte (1, 2). It is from here that the yolk precursor protein is carried to the growing oocyte by the maternal circulation. For the sake of unifying the terminology of this field, the suggestion by Pan et al., that the term "vitellogenin" be used to identify the serum precursors to yolk proteins (3) has gained wide acceptance.

In vertebrates the site of synthesis and secretion of vitellogenin is the liver (4, 5). Serum vitellogenin is carried to growing oocytes through the bloodstream where it diffuses from follicular capillaries to the oocyte surface (6). It is then specifically sequestered by receptor-mediated micropinocytosis (7) which is followed by the conversion of the endocytosed vitellogenin to the yolk proteins lipovitellin and

phosvitin (7). These are then stored in specialized organelles known as yolk platelets, until their utilization during embryogenesis.

It was the work of Urist and Schjeide (8) that first demonstrated that estrogen injection into Rana catesbiana could induce serum components which were biochemically and biophysically similar to the yolk proteins. Several other laboratories then proceeded to purify vitellogenin from Xenopus laevis serum and to elucidate the hormonal control of its physiology (9-11). In brief, both estrogen and gonadotrophins can be shown to induce high serum levels of vitellogenin (12). In addition, human chorionic gonadotrophin stimulated both the uptake of vitellogenin by oocytes (12), and the production of estrogen by ovarian tissues (13). It appears therefore that vitellogenesis is regulated by the hypothalamo-hypophysial axis, whereby estrogen behaves as an intermediate under the strict control of gonadotrophin levels (1).

Wallace and co-workers showed that it was both the livers of gonadotrophin- or estrogen-stimulated females, or estrogen-treated males which was the site of vitellogenin synthesis (5). It was not until much later however, that other investigators proved that the primary target of estrogen action was in fact the hepatocyte (14, 15). Since that time, numerous laboratories have taken advantage of the hepatic induction of vitellogenin synthesis by estrogens in order to probe other processes normally occurring in most cells.

One major focus of attention is the regulation and structure of the vitellogenin gene (16). Since no vitellogenin mRNA can be detected in the livers of normal male Xenopus, the activation of this previously

inactive gene to an extent whereby its protein product represents 60-70% of the total proteins being synthesized makes this system an outstanding model for gene regulation.

An unresolved question recently posed by biochemists and cell biologists stems from the molecular structure of vitellogenin (9-11). The intact molecule has a molecular weight of about 450,000 daltons. Structurally, vitellogenin is composed of two peptide subunits each of approximately 200,000 daltons. The remainder of the mass is comprised of lipid (12%), phosphorous (1.5%) and carbohydrate (1%). How these posttranslational modifications come about is not known, though several unsubstantiated models have appeared (17, 18). In addition, recent work has demonstrated three electrophoretically distinct forms of vitellogenin peptide subunits derived from Xenopus serum (19). How this heterogeneity arises is also not known; therefore, their respective relationships within the intact dimer remains an unsettled question.

It should be pointed out that the domestic fowl, Gallus domesticus, is also an excellent and often exploited system for studying vitellogenesis. Many aspects of this process, such as hormonal control, the presence of multiple serum forms of vitellogenin and their composite prosthetic groups, and regulation at the gene level show overall similarities to these phenomena in Xenopus (20, 21). A recent review encompassing the data obtained on this and other less well understood vertebrates has recently appeared (2).

In mammals, vitellogenesis has been evolutionarily replaced by lactation, a process whereby milk proteins are directly secreted to

nourish the young, as opposed to the long-term storage and utilization of yolk materials. The endocrinology of lactation is strikingly complex. Development of the mammary gland requires both adrenal and ovarian steroids in addition to the protein hormones prolactin and growth hormone (22). The induction of milk protein synthesis and secretion seems to be under the control of the ovarian steroids estrogen and progesterone, as well as the protein prolactin (22). Some interesting comparisons can be made between the caseins on one hand, and Xenopus and hen phosvitin on the other (1, 23, 24). Both are groups of low molecular weight phosphoproteins (28,000-34,000 daltons) which possess a heavily glycosylated component. With respect to amino acid composition, both of these proteins possess little or no methionine and cysteine whereas they are both abundant in proline and glutamic acid. Perhaps further analysis of the genes in question could help draw conclusions on the evolutionary mechanisms leading away from vitellogenesis and toward lactation.

Since the early work of Palade and co-workers (25) it has become dogma that secretory proteins in animal cells are synthesized on membrane-bound polysomes (i.e., rough endoplasmic reticulum). During and after synthesis of the peptide, the protein remains sequestered within the lumen of the rough endoplasmic reticulum until transferred via smooth-surfaced vesicles (i.e., transition elements) to the Golgi apparatus. Here, the final processing and packaging of the secretory product occurs prior to its exocytosis at the cell surface.

Several systems have been used to probe these processes, each one having a different advantage. The first was the guinea pig pancreas (25).

Palade and his co-workers took advantage of the distinctive ultrastructure of the acinar cells of the exocrine pancreas to follow by electron microscope autoradiography the intracellular pathway involved in zymogen granule formation. These early studies were eventually followed by experiments which coupled pulse-chase incubations with subcellular fractionation (26). In this way, they were able to further demonstrate the roles of these various cell fractions, and to allow biochemical analyses of these structures and their contents. One potential source of ambiguity with the pancreas is that the content of the zymogen granules is a heterogeneous mixture of proteins. Therefore tissues which allowed investigators to focus on a single protein were sought.

The system most often chosen is the hepatic synthesis and secretion of serum albumin by the rat liver. Here, the intracellular pathway of secretory proteins was, for the most part, confirmed (27). A serious limitation is that ironically, serum albumin is perhaps the only major serum protein which is not either a lipoprotein or glycoprotein. Therefore, the role of the internal cytomembrane system in the post-translational modification of a single peptide species could not be elucidated by these investigations.

A major advance in the study of such phenomena was the introduction of immunoglobulin secreting cells as model systems (28). Specifically, the covalent attachment of carbohydrate to the various forms of immunoglobulins has been analyzed. This process, referred to as glycosylation, can start in the rough endoplasmic reticulum, and continues until a time just prior to secretion (29). The final stages of processing take place in the Golgi apparatus.

The phosphorylation of specific proteins (i.e., caseins) has not been explored by the pulse-chase type of experiment as mentioned above. Conclusions here have been based solely on inference from the subcellular distribution of casein kinase activity in the lactating mammary gland (30). Though in this case the conclusions are probably correct, a more direct approach is, in general, more favorable. It is my belief that the liver of estrogen-stimulated Xenopus laevis can encompass the features of all these above-mentioned systems.

The extensive chemical modifications of Xenopus vitellogenin mentioned earlier suggests that the hepatocyte chemically modifies the peptide backbone prior to secretion of the final assembled serum protein. The thesis presented here is aimed at elucidating some of the molecular mechanisms involved in the posttranslational modifications of this complex protein. That estradiol-17β can induce vitellogenin synthesis in a tissue which is readily available in gram quantities makes the use of an in vitro liver slice system ideal for our purpose. In addition, vitellogenin is by far the predominantly synthesized hepatic protein, representing 60-70% of the total, thereby making its detection relatively unambiguous. These facts together point to vitellogenin as an excellent probe to explore the functional inter-relationships of the component parts of the internal cytomembrane network of Xenopus liver.

In order to trace these events within Xenopus liver we have separated the main membrane components of this internal network. With the aid of suitable culture conditions we intend here to demonstrate

which molecular modifications of vitellogenin occur with respect to subcellular localization and their temporal sequence. In addition, some data on the actual molecular mechanisms involved are presented.

CHAPTER II

INTRACELLULAR PHOSPHORYLATION OF VITELLOGENIN IN THE LIVER OF ESTROGEN-STIMULATED *XENOPUS LAEVIS*

Introduction

After estrogen is administered to either sex of *Xenopus laevis*, liver parenchymal cells undergo profound cytological changes involving loss of glycogen and extensive elaboration of rough endoplasmic reticulum and Golgi elements (1-4). Concomitant with these morphological changes, the yolk precursor protein, vitellogenin, becomes the major protein synthesized and secreted into the bloodstream (4-7). Chemical analyses have shown that vitellogenin is a highly modified protein with phosphate and carbohydrate moieties covalently attached to, and lipid noncovalently associated with, the peptide backbone (8-10).

In attempting to describe the mechanism of vitellogenin phosphorylation in the amphibian and avian liver, the most commonly employed approach has been to perform kinetic studies of the incorporation rates of [^{3}H]leucine and [^{32}P]orthophosphate into phosphoprotein secreted by liver slices *in vitro* (11, 12). Though valuable information may be obtained in this manner, one can only speculate from kinetic data as to the nature of the various cellular protein processing compartments since no intracellular intermediates have been demonstrated or localized. Furthermore, these types of experiment have resulted in conflicting conclusions in the literature concerning the subcellular localization

of vitellogenin phosphorylation. Schirm et al. (11) conclude that in the estrogen-stimulated rooster, vitellogenin is phosphorylated after the peptide is released from the polysomes and during its transport to the bloodstream. Using the same approach in Xenopus, Merry et al. (12) indicated that phosphorylation of the newly synthesized peptide probably occurs in the rough endoplasmic reticulum. An analysis of intracellular events is needed to resolve this apparent conflict.

Any approach aimed at elucidating the synthesis and posttranslational modifications of secretory proteins such as vitellogenin must include a procedure for subfractionating microsomes into functionally distinct entities. We have found that existing procedures developed for rat liver (13) yield poorly separated subfractions when applied to estrogen-treated Xenopus liver. Accordingly, we have developed a procedure for microsomal subfractionation which allows for the temporal analysis of the posttranslational modification and intracellular distribution of vitellogenin intermediates within small amounts (approximately 1.5 g wet weight) of cultured Xenopus liver cubes. This procedure, in conjunction with suitable pulse-chase incubation conditions, has allowed us to perform experiments which directly elucidate a posttranslational process (i.e., phosphorylation) involving this complex steroid-induced multicomponent protein.

Materials and Methods

Animals and Isotopes. Xenopus females were obtained from the South African Snake Farm (Fish Hoek, Cape Province, South Africa). They were

maintained and fed as previously described (14), injected on two alternate days with 2.0 mg estradiol-17β, and used 10 to 21 days after the first injection. The serum obtained from such animals is referred to as vitellogenic serum. L-[4,5-^{3}H]leucine (62 to 65 Ci/mmol) and [5-^{3}H]orotic acid (19 to 20 Ci/mmol) were obtained from Schwarz/Mann, and [U-^{14}C]UDP-galactose (200 mCi/mmol) was purchased from New England Nuclear. [^{32}P]orthophosphate and [γ-^{32}P]ATP (>2000 Ci/mmol) were from the Radiochemical Centre of Amersham/Searle.

Culture of Liver Explants. Animals were bled from the heart and the livers were removed to divalent cation-free amphibian saline, O-R2 (15). Pieces were weighed out so that approximately 1.5 g were used for each culture. The livers were then placed on a dry glass plate and sliced with scalpel blades until cubes of approximately 2 mm per side were obtained. The cubes were transferred to a 50-ml conical tube and exhaustively washed with divalent cation-free O-R2 until the supernatant remained perfectly clear. When experiments which measured the incorporation of [^{32}P]orthophosphate were performed, the liver cubes were washed three more times with phosphate-free O-R2 which contained normal levels of divalent cations.

Conditions were established for obtaining high levels of isotope incorporation within a short period of time after initiation of the pulse and were based on the procedures of Jamieson and Palade (16). Liver cultures were cooled on ice in small Erlenmeyer flasks, and subsequently 10 ml of ice-cold pulse medium containing the appropriate isotopic precursors were added. The liver cubes were then left to

equilibrate in the cold for periods of time specified in the figure legends. The pulse medium was comprised of O-R2 supplemented with 30 mM 4-(2-hydroxyethyl)-1-piperazineethanesulfonic acid (Hepes), 2 mM glutamine, and all amino acids (except leucine) at concentrations identical to that of 50% Leibovitz L-15 medium (17, 18). For double-label experiments measuring both [^{32}P]orthophosphate and [^{3}H]leucine incorporation, orthophosphate was also omitted from the pulse medium.

We initiated the pulse by swirling the stoppered flask under warm tap water for about 1 min and then at room temperature (21°C) for a total of 15 min unless otherwise indicated. We terminated the pulse by washing the liver cubes three times in solution O-R2 supplemented with leucine at a concentration equal to 50% L-15 medium; then 10 ml of 50% L-15 supplemented with 30 mM Hepes and 2 mM glutamine (pH 7.8) was added as the chase medium.

Fractionation of Liver Homogenates. To liver cubes derived from 1.5 g starting material, 2.5 ml 0.4 M sucrose (brought to pH 7.4 with NaOH) were added, and the mixture [approximately 25 to 30% (w/v)] was homogenized with five gentle strokes of a motor-driven Teflon pestle rotating at 650 rpm. The homogenate was spun at 10,000 × g for 20 min, giving a pellet and a postmitochondrial supernatant.

Sucrose solutions were adjusted to refractive indices of 1.408 (1.54 M) and 1.394 (1.16 M) and made 15 mM with CsCl. As will be indicated below, omitting the CsCl from the latter solution gave superior results. A discontinuous gradient was made with 2.0 and 1.0 ml of the respective sucrose solutions, and this was overlain with 2.0 ml

of the postmitochondrial supernatant. The gradient was then spun in a Beckman type SW65 rotor at 47,000 rpm for 12 h. The pelleted rough microsomes and the banded smooth microsomes were removed and diluted with 0.3 M KCl (pH 8.0), homogenized lightly with a loose Dounce pestle, and pelleted in a Beckman type 40 rotor spun at 40,000 rpm for 60 min. Pellets were resuspended in 0.4 M sucrose.

Chemical and Enzymatic Methods. Protein was measured by the filter assay of Bramhall et al. (19) with bovine serum albumin as a standard. Cytochome c oxidase was measured as described by Beaufay et al. (20), at pH 6.8 and 21°C. The method for assaying NADPH-cytochome c reductase was also as described by Beaufay et al. (20), but at pH 7.6 and 21°C. UDP-galactose:N-acetylglucosamine galactosyltransferase was measured by the column procedure of Fleischer (21) at pH 7.0. To terminate the reaction at 35°C we plunged the tubes into ice and immediately pipetted the reaction mixture onto a 0.5-ml column of AG 2X-8 resin, 200 to 400 mesh (BioRad), packed into a 3.0-ml disposable syringe fitted with a short 25-gauge needle. A 1.0-ml distilled water wash of the reaction tube was also layered on the column, which was then slowly eluted with pressure from the plunger of the syringe. Everything except free galactose and newly synthesized lactosamine eluted from the column. Aliquots (0.4 ml) of each eluate were counted in 15 ml ACS (Amersham/Searle). Blanks were run without N-acetylglucosamine and consistently showed negligible hydrolysis of [^{14}C]UDP-galactose. All reactions were linear with protein concentration under the conditions specified.

Assay of Protein Kinase Activity. Membrane fractions treated with the detergent Nonidet P-40 at a final concentration of 1% were added to the following assay mixture: 50 mM 2[N-morpholino]ethane sulfonic acid (pH 7.0), 3 mM $MgCl_2$, 1 mM [^{32}P]ATP, 0.6 mg phosvitin, and enzyme in a final volume of 0.1 ml. At the end of 20 min at room temperature, 20-μl aliquots were removed and spotted on Whatman 3MM filter discs. The dried discs were washed overnight in 10% trichloroacetic acid made 10 mM in sodium pyrophosphate. After two short rinses in the same solution, the discs were washed in ethanol, ethanol:ether (1:1), and ether and then air dried. The discs were placed in scintillation vials with 10 ml ACS and counted.

Labeling of Polysomal RNA. Animals received two injections of 0.5 mCi [^{3}H]orotic acid, 12 to 15 h apart. The animals were killed 20 h after the last injection, and rough and smooth microsomes were isolated. Aliquots of the microsomal subfractions were precipitated and washed two to three times with 10% trichloroacetic acid containing 5 mM uridine. The precipitates were then washed in ethanol, ethanol: ether (1:1), and ether. The dried precipitates were solubilized at 100°C in 0.5 ml H_2O_2 and counted after the addition of 10 ml ACS.

Electron Microscopy. Smooth and rough microsomes were fixed in suspension for 2 h in 3% glutaraldehyde in 0.1 M cacodylate buffer (pH 7.4). After being pelleted at 10,000 × g for 15 min, they were rinsed in buffer overnight and treated with 1% OsO_4 for 2 h, dehydrated in an alcohol series, and embedded in Epon. Thin sections were cut on

a PorterBlum MT-2 ultramicrotome, stained in uranyl acetate and lead citrate, and viewed on a Hitachi HU-11E electron microscope operating at 75 kV. The procedure for the rough microsomal preparation included a few drops of 2 M sucrose added to the bottom of the gradient as a cushion to facilitate the removal of glycogen granules which obscure visualization of ribosomes.

Incorporation Studies. The incorporation of radioactive precursors was measured by precipitation of the sample with 10% trichloroacetic acid made 10 mM in cold precursor followed by two to three washes with this solution. Precipitates were then washed with ethanol, ethanol: ether (1:1), and ether. The dried residues were solubilized by being boiled in 30% H_2O_2. Aliquots were placed into scintillation vials with 10 ml ACS and the radioactivity was measured.

SDS-Polyacrylamide Gel Electrophoresis. The gel and buffer systems used were those of Laemmli (22). All samples to be electrophoresed were precipitated with 10% trichloroacetic, washed with ethanol, with ethanol:ether (1:1), and with ether three times. The residue, usually still a bit moist from the last ether wash, was solubilized by addition of 0.2 ml of 10% SDS, two drops of β-mercaptoethanol, and 10 µl of 58% NH_4OH, then boiled for several minutes. Laemmli sample buffer was added to a final sample volume of about 1.0 ml with continued boiling. The sample was spun in an International centrifuge at 850 × g for 10 min, and an aliquot of the supernatant was loaded onto a 5% polyacrylamide gel. After electrophoresis, gels were frozen in Dry Ice, and 1-mm

slices were cut and dissolved in 0.3 ml H_2O_2. Radioactivity was measured after 10 ml ACS was added.

Results

Isolation and Characterization of Rough and Smooth Microsomes. The postmitochondrial supernatant prepared as described had a protein content of 7 to 8 mg/ml, which is high enough to prevent particle aggregation (13) but not so high as to overload the discontinuous sucrose gradient. Rough and smooth microsomes were isolated from the same pooled postmitochondrial supernatant on gradients either with or without 15 mM CsCl in the upper sucrose layer. In both cases, 15 mM CsCl was included in the lower sucrose layer since its omission from the denser sucrose solution resulted in a very small rough microsomal pellet. Enzymatic data obtained from two representative fractionations are presented in Table I.

The microsomal subfractions were, as expected, relatively free of the mitochondrial marker enzyme cytochrome c oxidase but enriched in NADPH-requiring cytochrome c reductase. Measurements made on whole microsomal pellets obtained from identically prepared postmitochondrial supernatants indicate a 5 to 6-fold enrichment of the NADPH-requiring cytochrome c reductase over the homogenate, with negligible levels of mitochondrial contamination (data not shown). This is supported by the fact that the pellet obtained from centrifugation of the homogenate for 20 min at 10,000 × g accounted for 120% of the total cytochrome c oxidase activity based on the total homogenate activity. We conclude, therefore, that our homogenization and centrifugation procedures yield

TABLE I

DISTRIBUTION OF ENZYME ACTIVITIES BETWEEN MICROSOMAL SUBFRACTIONS

Experiment	Microsomal Subfraction	Cytochrome c oxidase[a]	NADPH-Cytochrome c reductase[b]	Galactosyl-transferase[c]
1	Original homogenate	0.063	0.010	4.3
	Rough microsomes (+)[d]	0.016	0.016	6.1
	Smooth microsomes (+)	0.012	0.019	105.0
	Rough microsomes (-)	0.018	0.053	ND[e]
	Smooth microsomes (-)	0.018	0.025	90.0
2	Original homogenate	0.063	0.009	13.3
	Rough microsomes (+)	0.036	0.028	21.9
	Smooth microsomes (+)	0.012	0.026	123.0
	Rough microsomes (-)	0.045	0.043	ND
	Smooth microsomes (-)	0.048	0.027	181.0

[a] μMoles cytochrome c oxidized/min/mg protein.

[b] μMoles cytochrome c reduced/min/mg protein.

[c] nMoles [^{14}C]UDP-galactose transferred/30 min/mg protein.

[d] The presence (+) or absence (-) of 15 mM CsCl in the upper sucrose solution is indicated parenthetically.

[e] Not detectable.

a suitable starting material (postmitochondrial supernatant) from which microsomal subfractions can be isolated.

Galactosyltransferase, an enzyme shown to be localized in the Golgi apparatus (21), was also measured as a marker for smooth microsomes (Table I). As expected, the smooth microsome preparation was much more enriched with Golgi membranes than either the original homogenate or the rough microsomes. Also, omission of the CsCl from the upper sucrose layer diminished further the amount of galactosyltransferase activity cosedimenting with the rough microsomes. In the experiments shown here, the percentage of smooth microsomal protein contaminating the rough microsomal fraction went from 6 to 18% with CsCl in the upper layer to undetectable levels when CsCl was omitted. In general practice, we have found that smooth microsomal contamination of rough microsomal pellets varies from 0 to about 7% in the absence of CsCl in the upper layer (data not shown).

As a second indication of the extent to which the rough and smooth microsomes were separated from one another, the relative amount of labeled RNA was determined in the two microsomal subfractions after injection of animals with [^{3}H]orotic acid as described under "Materials and Methods" (Table II). In this case, the relative amount of labeled RNA in the rough as compared with the smooth microsomes, already high when CsCl was present in the upper sucrose layer, was about three times greater still when the CsCl was omitted. Hence, we concluded that reproducibly better separation of rough and smooth microsomes is achieved when the upper sucrose layer is free of CsCl; subsequent preparations incorporate this feature.

TABLE II

INCORPORATION IN VIVO OF [^{3}H]OROTIC ACID INTO MICROSOMAL SUBFRACTIONS

Experiment	Microsomal subfraction	Incorporation[a]	Rough/smooth ratio
1	Original homogenate	8.0	
	Rough microsomes (+)[b]	35.6	15.4
	Smooth microsomes (+)	2.3	
	Rough microsomes (-)	60.9	40.6
	Smooth microsomes (-)	1.5	
2	Original homogenate	4.1	
	Rough microsomes (+)	14.9	9.7
	Smooth microsomes (+)	1.5	
	Rough microsomes (-)	19.3	33.0
	Smooth microsomes (-)	0.6	

[a] Cpmx 10^{-3}/mg protein.

[b] The presence (+) or absence (-) of 15 mM CsCl in the upper sucrose solution is indicated parenthetically.

Isolated rough and smooth microsomes were prepared for transmission electron microscopy, and the micrographs obtained are shown in Fig. 1. The rough microsomal fraction (Fig. 1A) was highly enriched for ribosome-attached membranes, with no detectable smooth vesicles present. The smooth microsomal fraction (Fig. 1B), on the other hand, contained a heterogeneous population of smooth-surfaced vesicles, but no ribosome-studded vesicles.

Incorporation of [^{3}H]Leucine into Microsomal Subfractions. Since at least 60 to 70% of the total protein synthesized by estrogen-stimulated Xenopus liver is vitellogenin (4, 12, 23, 24), one should be able to observe, in a general way, the relative contribution of the rough and smooth microsomal subfractions to the synthesis and intracellular translocation of vitellogenin in a pulse-chase experiment with [^{3}H]leucine. Such an experiment, in which the specific activity of microsomal subfractions was measured as a function of time, is illustrated in Fig. 2. After a 5-min pulse with [^{3}H]leucine, the rough microsomes were highly labeled while the smooth microsomes were labeled to a negligible extent, thus reflecting the location of newly synthesized vitellogenin. With subsequent time in unlabeled medium, however, the specific activity of the smooth microsomes rose over that of the rough microsomes, reaching a maximum value at about 3 h. By this time most of the pulse-labeled vitellogenin should be in the smooth microsomes since a lag of 1.5 to 2 h occurs before labeled vitellogenin begins to appear in the medium (12). The large decrease in the labeling of the smooth microsomes by 4 h can be correlated with observations that secretion of pulse-labeled

FIG. 1. Electron micrographs of isolated rough and smooth microsomal fractions. (A) Rough microsomal pellet × 29,500; (B) Smooth microsomal pellet × 29,500. Both micrographs show that essentially no visible cross-contamination exists.

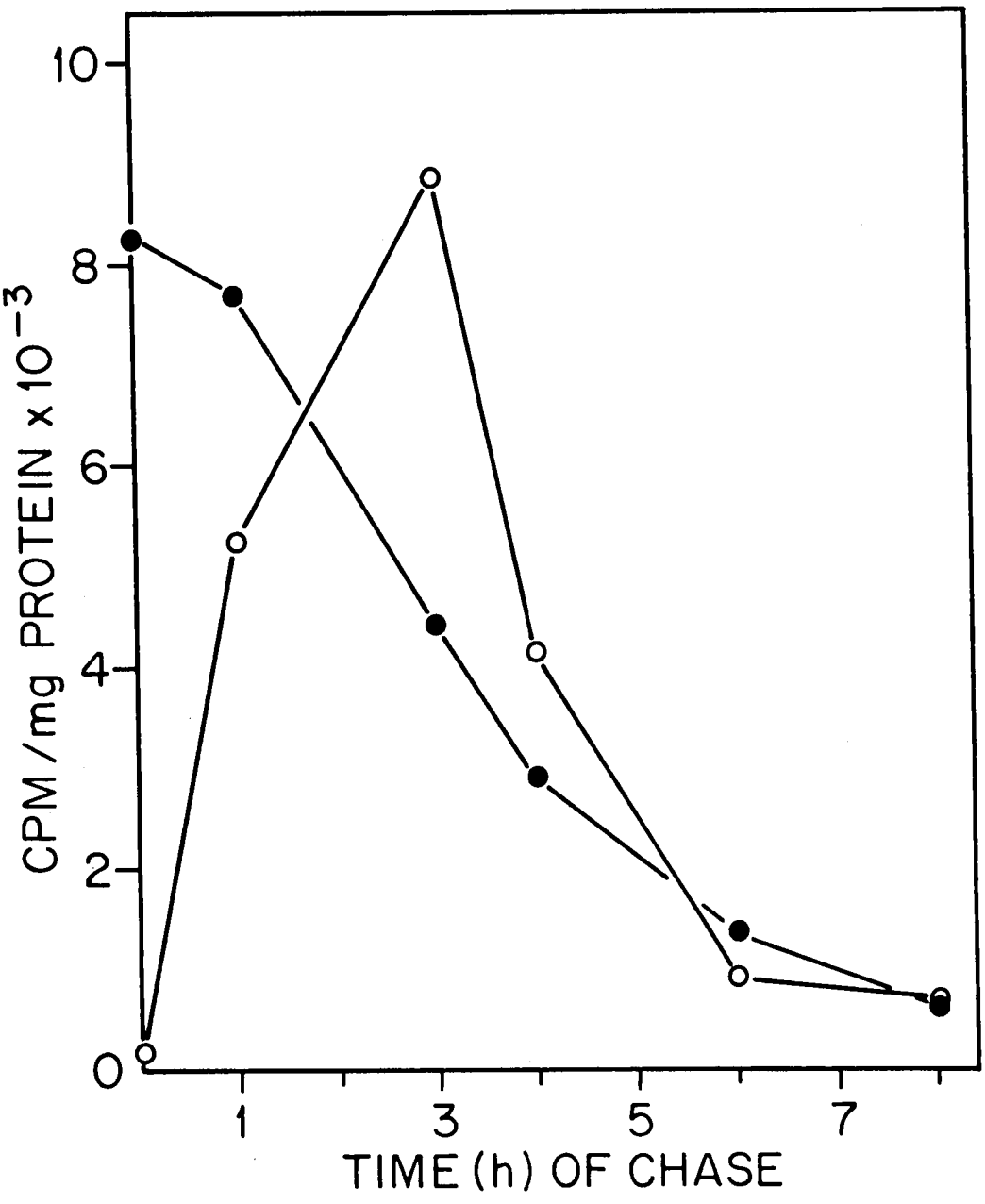

FIG. 2. Incorporation of [^{3}H]leucine into (●) rough and (o) smooth microsomes during brief pulse-chase periods of varying lengths. Liver cubes were equilibrated in the cold for 15 min with pulse medium containing [^{3}H]leucine (10 μCi/ml). The cultures were then incubated for 5 min at room temperature, washed, and incubated in chase medium for 1, 3, 4, 6, or 8 h. At each time point, liver cubes were collected and washed, and microsomal subfractions were isolated as described.

vitellogenin is complete by 4 to 5 h. By 6 h and persisting until 8 h, a low specific activity was observed in both microsomal subfractions, reflecting the labeling of endogenous microsomal components.

The experiment illustrated in Fig. 2 demonstrates that, after a 5-min pulse-labeling of secretory proteins, the intracellular translocation of newly synthesized products can be followed until a time when secretion is complete and only endogenous microsomal components are labeled. This indicates that the microsomal subfractions we have isolated are well suited for study of the posttranslational processing of secretory protein, which in estrogen-stimulated Xenopus liver is predominantly vitellogenin.

Characterization of Liver Culture Conditions. The conditions for pulse-labeling of liver cultures were assessed by measurement of the incorporation of [^{32}P]orthophosphate and [^{3}H]leucine into trichloroacetic acid-insoluble radioactivity during a continuous incubation for up to 3 h in pulse medium (which lacks both nonradioactive leucine and orthophosphate), and by analysis of whole liver phosphoproteins on SDS-polyacrylamide gels. In this way the ability of the liver cubes to sustain biosynthetic activity while employing only endogenous orthophosphate pools was tested.

After a cold equilibration period of 15 min, liver cultures were brought to room temperature and incubated for up to 3 h in pulse medium containing [^{3}]leucine and [^{32}P2orthophosphate. Fig. 3 illustrates the kinetics obtained. Both [^{3}H]leucine and [^{32}P]orthophosphate are incorporated into trichloroacetic acid-precipitable material with

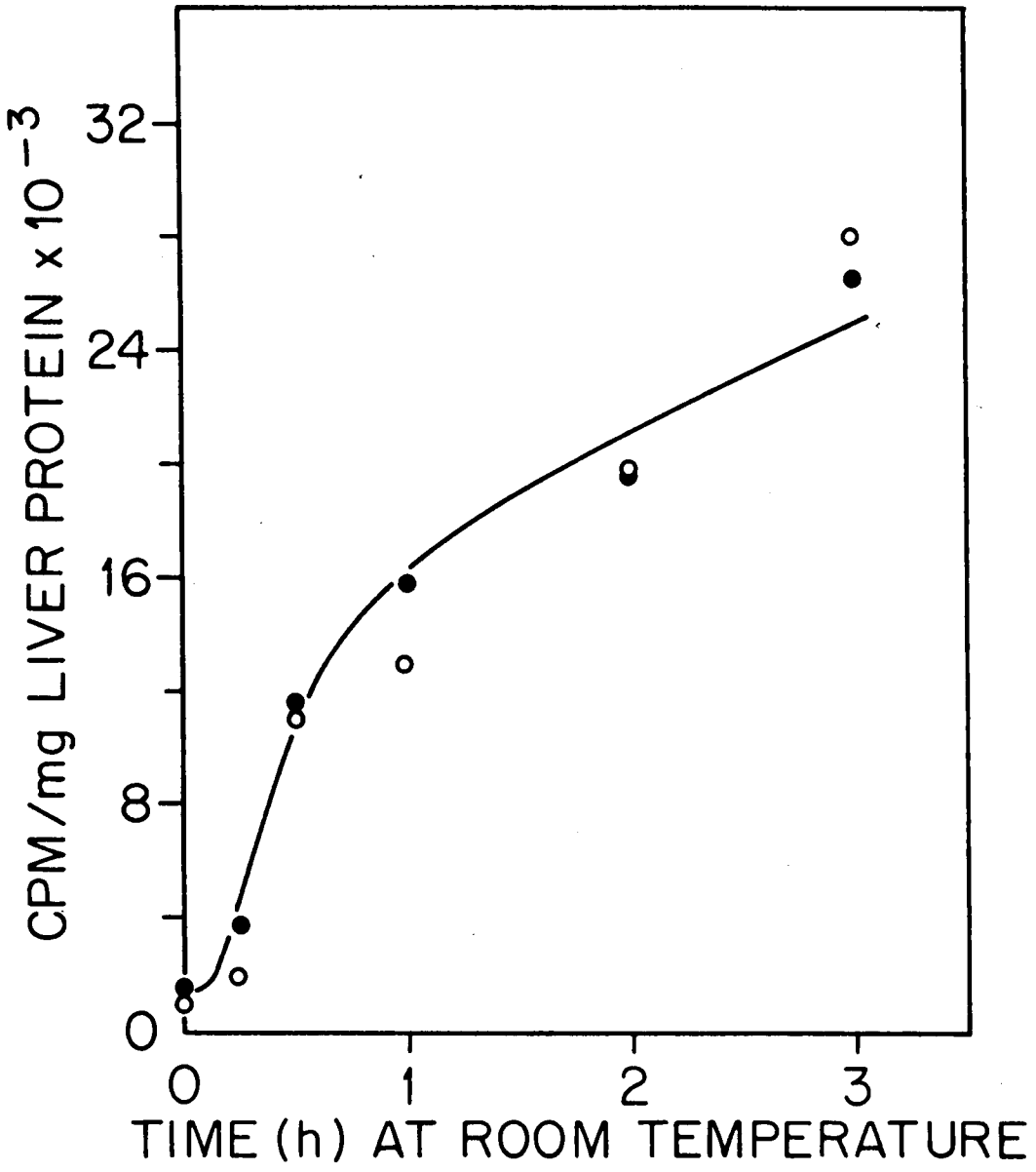

FIG. 3. Time course of incorporation of [^{3}H]leucine (5 µCi/ml) (•), and [^{32}P]orthophosphate (25 µCi/ml) (o), into total phosphoprotein of liver cubes from an estrogen-treated female. Liver cubes were cold equilibrated for 15 min as described, then continuously incubated at room temperature. At each time point, approximately three to four pieces of liver were homogenized in O-R2; then aliquots were used for protein determination and spotting on Whatman 3MM filter discs for radioactivity measurements.

similar kinetics during the entire incubation. The apparent tapering off of the rate of isotope incorporation which begins between 1 and 2 h is due to the fact that vitellogenin, the major phosphoprotein synthesized by the estrogen-stimulated liver (Fig. 4) has begun to be secreted (Figs. 5 and 6). Therefore the approach to a steady state between the incorporation of [^{3}H]leucine and [^{32}P]orthophosphate into acid insoluble material and the secretion of vitellogenin into the medium will be reflected in the labeling kinetics of liver phosphoprotein. We conclude that this medium is well suited for at least the brief pulse period as shown below.

Figure 5 illustrates the incorporation kinetics of [^{3}H]leucine into liver protein and secreted material by liver cubes under the pulse-chase conditions used throughout most of this work. The data for [^{32}P]orthophosphate incorporation which was identical are not presented here. After a 15-min cold equilibration and a 15-min pulse, incorporation into liver proteins continues for up to 45 min. A decline in the specific activity of labeled liver protein coincides with the progressive increase of radioactive secreted product in the medium. Both of these reciprocal processes level off at similar times during the chase, demonstrating that these pulse-chase conditions are well suited for study of vitellogenin processing. The inset of Fig. 5 shows the kinetics which were obtained in whole microsomes. The kinetics of whole microsomes and of whole liver slices are essentially identical, allowing us to assume that we can follow the complete progression of modifications through which vitellogenin is finally assembled and secreted by analyzing microsomal subfractions.

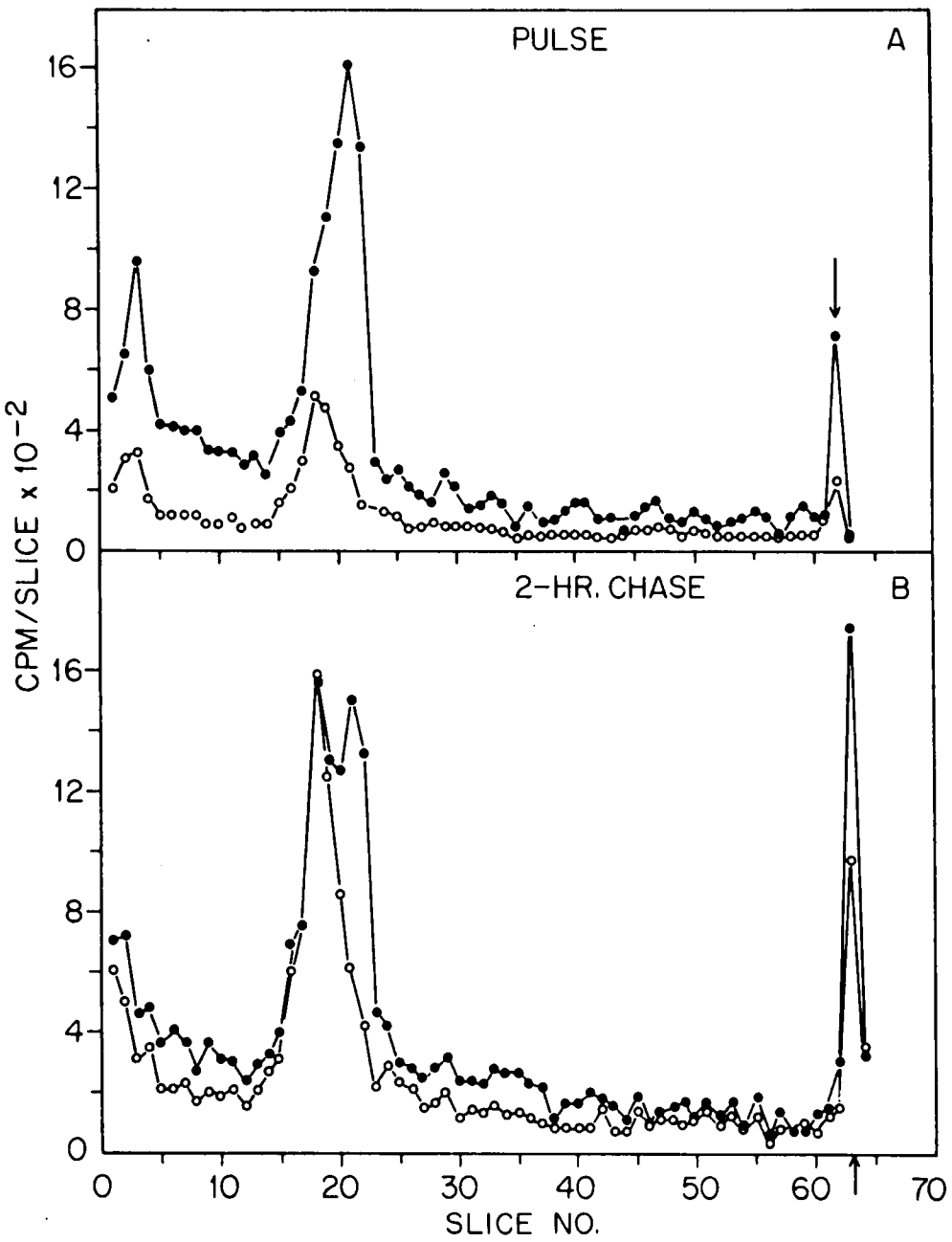

FIG. 4. SDS-polyacrylamide gel electrophoresis of whole liver proteins. Liver cubes from an estrogen-treated female were incubated for 1 h in pulse medium containing [^{3}H]leucine (10 μCi/ml) and [^{32}P]orthophosphate (25 μCi/ml). Some slices were removed at the end of the 1-h pulse and homogenized in O-R2, then precipitated with trichloroacetic acid. Others were incubated in chase medium for another 2 h, then treated as above. All trichloroacetic acid-precipitated samples were then processed for SDS-polyacrylamide gel electrophoresis. (A) Radioactivity profile of liver cubes pulsed for 1 h; (B) Profile of cultures subsequently incubated for 2 h in chase medium. (●) [^{3}H]leucine; (o) [^{32}P]orthophosphate.

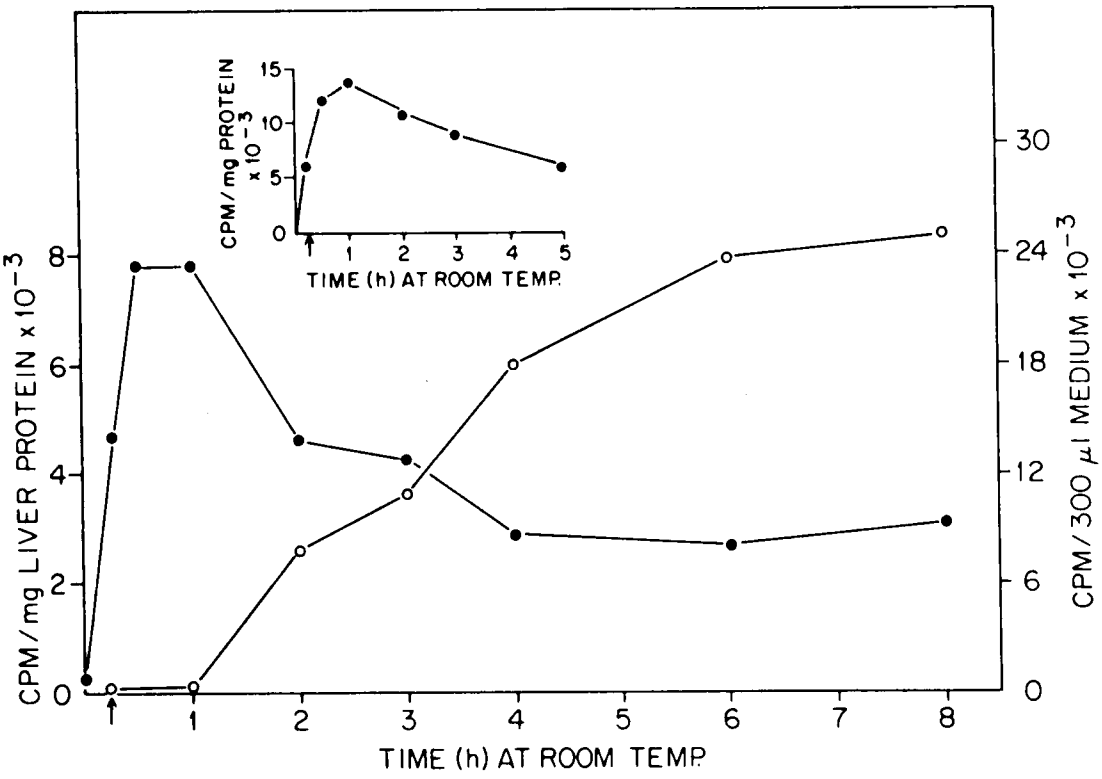

FIG. 5. Demonstration of the pulse-chase kinetics of liver cubes obtained from estrogen-treated females. Tissue was cold equilibrated for 15 min in pulse medium containing [^{3}H]leucine (5 µCi/ml) and then brought to room temperature for a 15 min pulse. At the end of this period (arrow), tissue slices were washed and the chase period was begun as described in "Materials and Methods." Tissue samples (●) were processed as described in Fig. 3. At various times, 300 µl of chase medium (o) were taken and mixed with 10 µl of carrier vitellogenic serum, precipitated with trichloroacetic acid, and processed for liquid scintillation counting.

Inset: Same experiment showing the pulse-chase kinetics of microsome labeling. Liver slices were homogenized in 0.3 M sucrose and spun at 6000 × g for 25 min. The resultant supernatant was centrifuged at 110,000 × g for 1.5 h to yield the microsomal pellet, which was washed and resuspended as described.

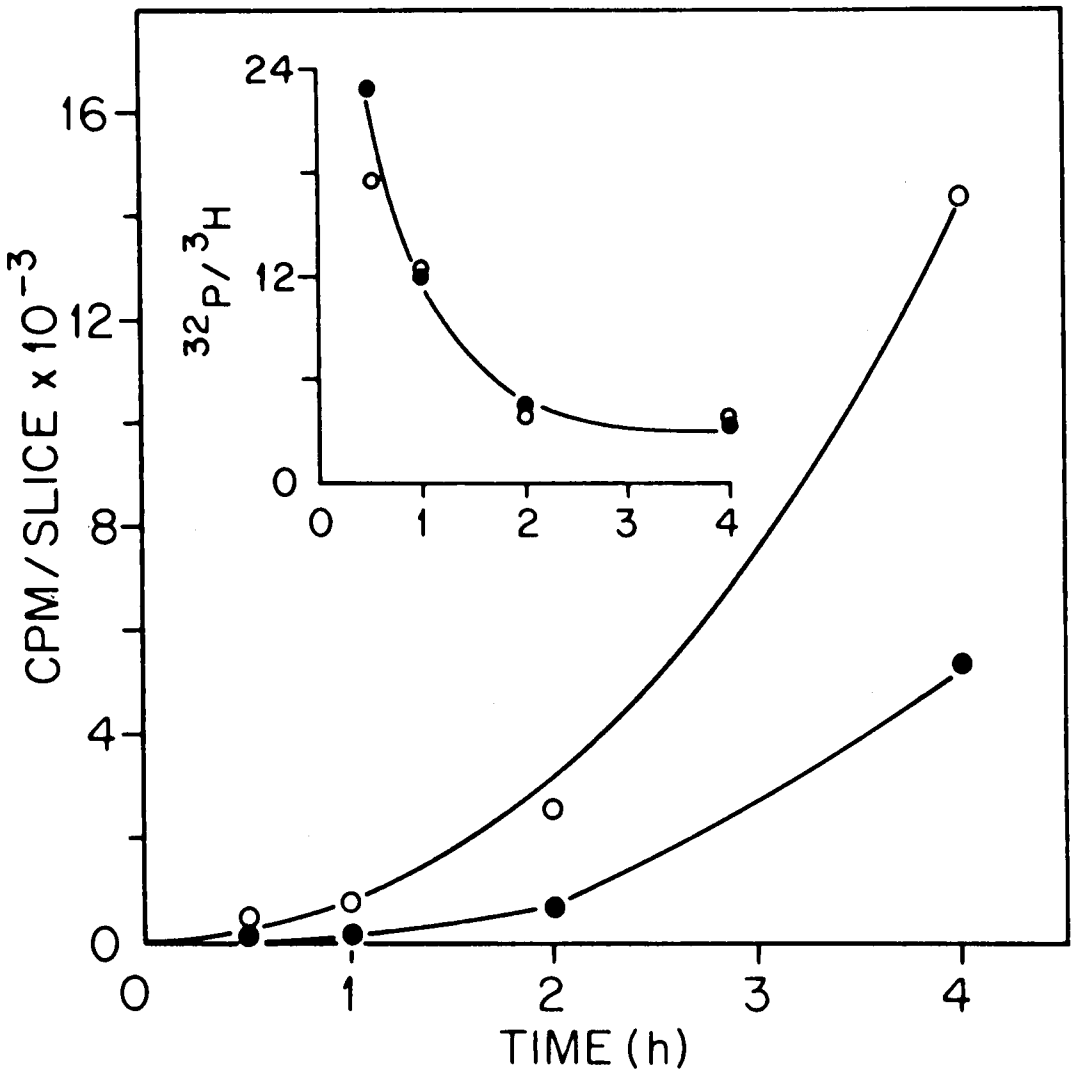

FIG. 6. Time course of secretion of (•) [^{3}H]leucine- and (o) [^{32}P]orthophosphate-labeled vitellogenin. Liver slices were incubated in pulse medium containing [^{3}H]leucine (5 μCi/ml) and [^{32}P]orthophosphate (10 μCi/ml). At various times 0.3 ml of medium were mixed with 10 μl of carrier vitellogenic serum, precipitated with trichloroacetic acid, and processed for SDS-gel electrophoresis on 5% polyacrylamide gels. The vitellogenin region of the gel was located, cut out, and dissolved in 30% H_2O_2; radioactivity was measured after the addition of 10 ml of ACS.

Inset: $^{32}P/^{3}H$ ratio in the gel slices (•) or material directly precipitated from the medium with trichloroacetic acid (o) at each time point.

If one compares the specific activity of [^{3}H]leucine-labeled liver protein at the time of peak incorporation (about 1 h) with the residual specific activity after the liver has completed secreting the newly made protein, one can calculate that at least 60% of the radioactivity was present in secreted product, almost all of which is vitellogenin (7). This is in good agreement with published data from other labs (12, 23, 24).

For qualitative analysis of the species of phosphoproteins synthesized by liver cubes, the following experiment was performed. Cultures were incubated for 1 h in pulse medium containing [^{3}H]leucine and [^{32}P]orthophosphate followed by an additional 2 h in 50% L-15. Three cubes were removed at the end of each incubation period, homogenized in divalent cation-free O-R2, and precipitated with cold trichloroacetic acid. Aliquots of the solubilized samples were analyzed on SDS-polyacrylamide gels (22) as described above. Fig. 4 shows the pattern of phosphoproteins synthesized by the estrogen-stimulated liver of Xenopus.

Clearly, the major peak of [^{3}H]leucine radioactivity, and the only peak of [^{32}P]orthophosphate incorporation, reside in the vitellogenin region of the gel. Closer examination of Fig. 4A shows that the [^{3}H]leucine peak is slightly asymmetric, with a slower moving shoulder. Interestingly, however, after the 1-h incubation the [^{32}P]orthophosphate-labelled vitellogenin is situated under this shoulder, not under the main peak of the newly synthesized peptide. More information can be obtained by observation of the pattern of incorporation after the liver slices

are incubated in chase medium for 2 h (Fig. 4B). It is evident that what was initially only a [^{3}H]leucine-labeled shoulder which represented a more highly phosphorylated form of vitellogenin has now increased in size relative to the faster migrating, less highly phosphorylated form of vitellogenin. That we can distinguish the intracellular intermediates of vitellogenin processing by electrophoretic methods, and can also control their relative amounts by manipulating the culture conditions of a tissue devoted almost exclusively to vitellogenin synthesis, demonstrates valuable advantages of this system which can be exploited to probe the assembly of this multicomponent protein.

Secretion Kinetics of [^{3}H]Leucine and [^{32}P]Orthophosphate-Labeled Vitellogenin. Our first approach was to examine the relative rates at which [^{3}H]leucine- and [^{32}P]orthophosphate-labeled vitellogenin is secreted during incubation in pulse medium lacking both nonradioactive leucine and orthophosphate. Earlier work indicated that no lag period existed between the appearance of [^{3}H]leucine- and [^{32}P]orthophosphate-labeled vitellogenin in the medium (12). These authors concluded that vitellogenin was phosphorylated at a time immediately after its synthesis. Since pool sizes and their rates of equilibration were not considered, we felt our system might yield additional information, since no nonradioactive precursors are present in the medium. Liver cubes were incubated under standard conditions, and at the indicated times 300 µl of medium were mixed with 10 µl of vitellogenic serum and precipitated with trichloroacetic acid. After the precipitates were washed as described, they were resuspended and boiled in SDS-gel sample

buffer and electrophoresed along with a vitellogenin standard. The appropriate regions of the gels were cut out, dissolved, and counted. Fig. 6 shows the result of this experiment.

[^{32}P]orthophosphate-labeled vitellogenin can be detected in the medium 30 min after the incubation is begun and 90 min prior to the appearance of newly synthesized [^{3}H]leucine- and [^{32}P]orthophosphate-labeled material. The resultant decrease in the $^{32}P/^{3}H$ ratio with time is a good indication of the rate of dilution of the rapidly secreted [^{32}P]orthophosphate-labeled vitellogenin by newly synthesized [^{3}H,^{32}P]vitellogenin. The inset shows that the $^{32}P/^{3}H$ ratios in both the medium and the vitellogenin bands of the gels are almost identical and decrease at the same rate. The time of approach to a steady-state $^{32}P/^{3}H$ ratio coincides with the [^{3}H]leucine-labeled material being secreted into the medium, which requires a lag time of 90 to 120 min (Figs. 5 and 6). Though this experiment is not in itself definitive evidence, a reasonable scenario may be advanced. Specifically, that at the time of initiation of the liver cultures there exists a small amount of synthesized but unphosphorylated vitellogenin which is labeled with [^{32}P]orthophosphate and secreted prior to the newly formed, double-labeled vitellogenin. The ability to detect this lag period probably means that this unphosphorylated form of vitellogenin exists in a membrane compartment which is both spatially and temporally closer to the final secretory step. This result differs from the previously cited work (12) on Xenopus in that no such lag period was detected by these authors. More direct evidence in support of this view of

vitellogenin processing comes from the subcellular fractionation studies described below.

Subcellular Fractionation. To ascertain whether our conclusions based on the secretion kinetics described above were correct, we fractionated Xenopus liver microsomes into rough and smooth components and directly analyzed the nature of the vitellogenin intermediates in each. The experimental approach consisted in incubating liver cubes in pulse medium containing [^{3}H]leucine and [^{32}P]orthophosphate for 15 min on ice and then for 15 min at room temperature. Subsequently, liver cultures were washed three times as described and placed in 50% L-15 medium for a chase period of either 1 or 3 h. Liver cubes were then collected and processed for subcellular fractionation according to the scheme documented above. These specific time points were chosen based on the experiments shown in Fig. 5, which indicated that at 1 h incorporation is maximal, but no secretion of label has occurred. At 3 h, however, secretion of label has started, indicating that the intracellular translocation of labeled vitellogenin must be well under way.

A typical result is illustrated in Fig. 7. Whether microsomal subfractions were isolated on the discontinuous gradient possessing 15 mM CsCl in the lighter sucrose layer or from gradients where it was omitted, essentially identical results are obtained. Experiments reported here, however, were performed on the improved gradient (Tables I and II, pps. 16 and 18), which has 15 mM CsCl present in the more dense sucrose layer only.

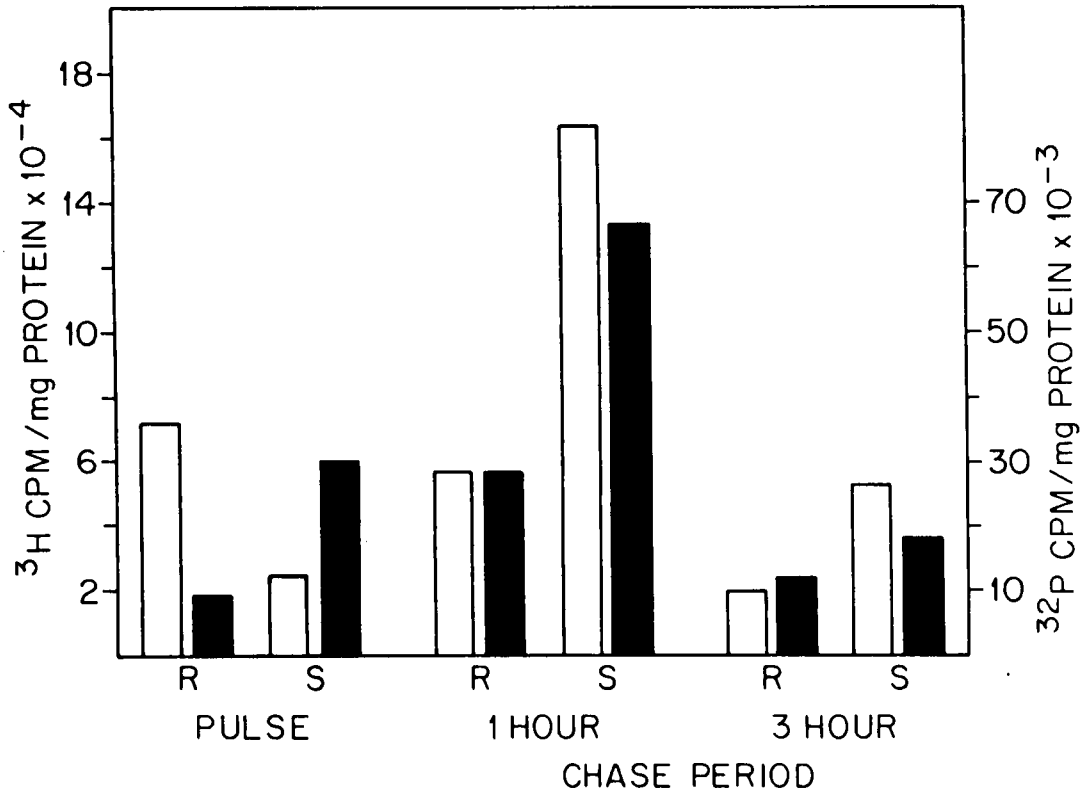

FIG. 7. Incorporation of [^{3}H]leucine (25 μCi/ml) and [^{32}P]orthophosphate (100 μCi/ml), during a 15 min pulse and 1- or 3-h chase, into the microsomal subfractions of cultured liver slices. For each time point, 1.5 g of liver were used. R = rough microsomes; S = smooth microsomes. Open bars = [^{3}H]leucine; solid bars = [^{32}P]orthophosphate.

After a 15-min pulse period the differential compartmentalization of newly synthesized and partially phosphorylated labeled vitellogenin within the rough microsomes as compared with the much more highly phosphorylated intermediate in the smooth microsomes is clearly demonstrated. We interpret this to mean that [^{32}P]orthophosphate is incorporated into newly synthesized vitellogenin first in the rough microsomes and then, to a greater extent, again in the smooth microsomal fraction. This experiment shows that the rough microsomes would account for about 30% of the [^{32}P]orthophosphate incorporated.

The data for the 1- and 3-h chase periods show that, as expected, incorporation of both isotopes continued after the 15-min pulse and that most of the newly formed [^{3}H,^{32}P]vitellogenin has been transferred to the smooth microsomes. By 3 h the microsomal subfractions are labeled slightly less than at the end of the 15-min pulse period and much less than the subfractions isolated from tissue incubated 1 h in chase medium. This indicates that the synthesis, phosphorylation, intracellular translocation, and secretion of vitellogenin may be analyzed at the subcellular level by our procedures.

In an experiment aimed at directly analyzing the differentially compartmentalized [^{3}H,^{32}P]vitellogenin intermediates, rough microsomes and smooth microsomes were isolated from appropriately labeled liver cubes and examined by SDS-polyacrylamide gel electrophoresis. The profile of radioactivity obtained for each subfraction is shown in Fig. 8. One important observation is that neither the rough microsomes (Fig. 8A) nor the smooth microsomes (Fig. 8B) possess the multiple

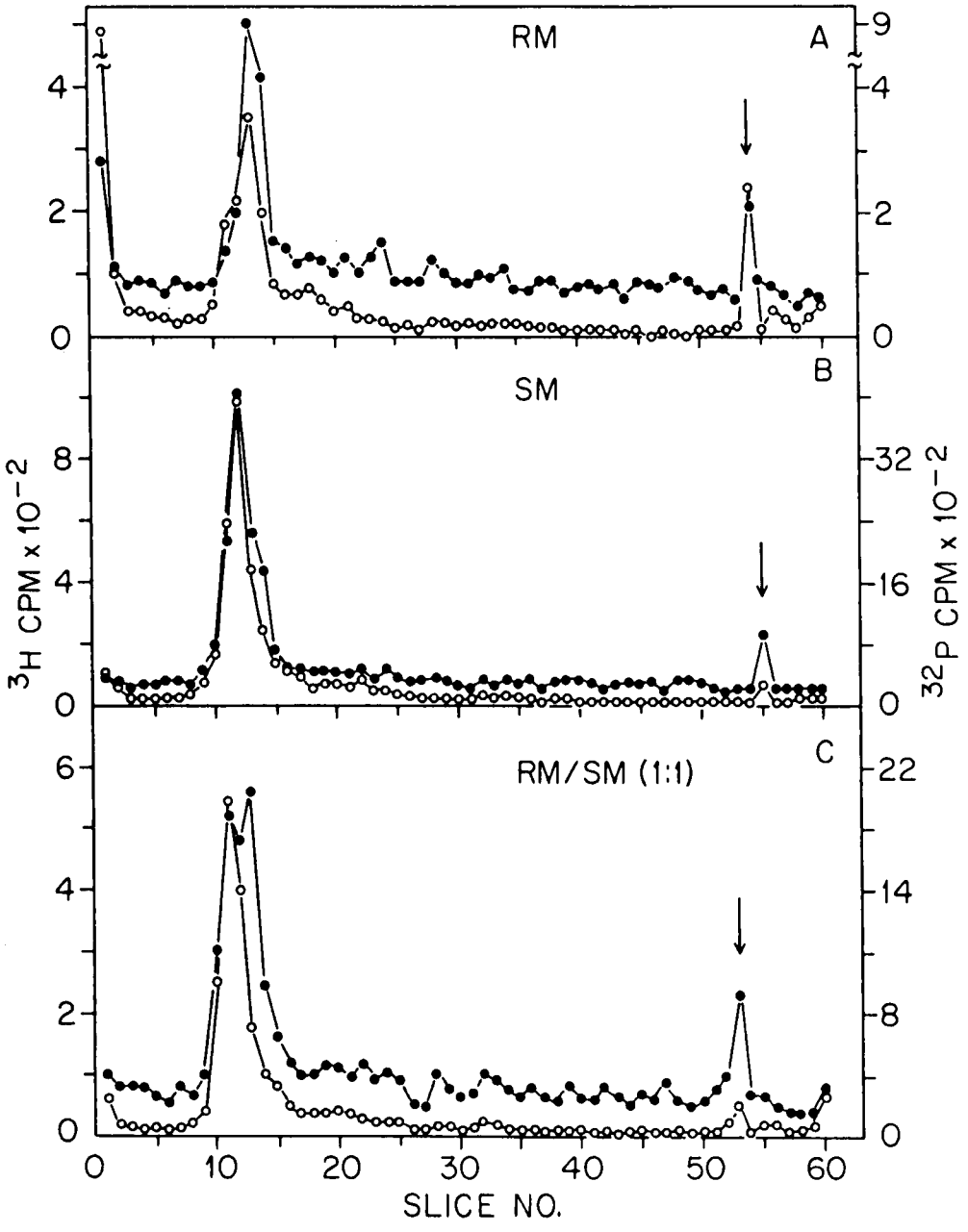

FIG. 8. SDS-polyacrylamide gel electrophoresis of radioactive phosphoprotein contained within the rough and smooth microsomes obtained from the liver of an estrogen-stimulated female. Liver cubes were cold equilibrated in pulse medium containing [^{32}P]orthophosphate (120 µCi/ml) and [^{3}H]leucine (10 µCi/ml) for 0.5 h. Slices were brought to room temperature for a 0.5 h pulse period, washed and chased for 1.0 h. The tissue was then collected, washed with divalent cation-free O-R2, and fractionated as described. (A) and (B) Radioactivity profiles of rough and smooth microsomes, respectively; (C) Obtained from co-electrophoresis of a sample composed of equal parts of the rough and smooth microsome samples used in (A) and (B). (●) = [^{3}H]leucine; (o) = [^{32}P]orthophosphate.

vitellogenin peaks shown in whole liver (Fig. 4B, p. 25) and whole microsomal fractions (not shown). Furthermore, the greater incorporation of [^{32}P]orthophosphate relative to [^{3}H]leucine into the vitellogenin peak of the smooth fraction (Fig. 8B) when compared with that of the rough microsomal precursor (Fig. 8A) substantiates our conclusion based on the subcellular fractionation experiments reported above (Fig. 7) that a major portion of the [^{32}P]orthophosphate is incorporated into the smooth microsomal intermediate.

Closer inspection of these gel profiles reveals that the rough microsomal intermediate possesses a higher electrophoretic mobility than the smooth microsomal intermediate (Fig. 8A and B). The greater mobility of the [^{32}P]orthophosphate-labeled peak of the rough microsomal fraction compared with that of the smooth rules out any possibility that we are measuring smooth microsomal contamination in the rough microsomes. If this contamination were significant the rough microsomes would possess the more highly labeled, and therefore more easily detected, [^{32}P]orthophosphate-labeled smooth microsomal intermediate, which in turn, would possess a slower mobility than the rough microsomal [^{3}H]leucine-labeled peak.

If a sample composed of solubilized rough microsomes is mixed with similarly treated smooth microsomes and coelectrophoresed, the pattern of Fig. 3C is obtained. A double peak of [^{3}H]leucine-labeled material with incorporated [^{32}P]orthophosphate comigrating under the slower peak is observed, as we have previously demonstrated in whole liver (Fig. 4B) and whole microsomes (not shown). We can say, therefore, that the

heterogeneity of intracellular vitellogenin precursors in estrogen-stimulated Xenopus liver is due to the differential compartmentalization of these intermediates within the hepatocyte.

Protein Kinase Activity in Isolated Microsomal Subfractions. Since the covalent attachment of phosphate to proteins occurs enzymatically by the activity of a class of enzymes commonly referred to as protein kinases, we measured the ability of rough and smooth microsomes to transfer [^{32}P] orthophosphate from [γ-^{32}P]ATP to endogenous substrates and exogenous phosvitin, a proteolytic derivative of vitellogenin (25). The results are shown in Table III.

Whether endogenous substrates alone are assayed or after phosvitin is added, the smooth microsomes possess a higher protein kinase specific activity than the rough: the latter possess 25 and 18% of the activity of the smooth microsomes when endogenous substrates or phosvitin are measured, respectively. This quantitatively supports the notion that though most vitellogenin phosphorous is added in the smooth microsomes, approximately 20 to 30% is attached by the rough.

Discussion

The results presented here document for the first time a subcellular fractionation scheme which yields rough and smooth microsomal subfractions from the liver of estrogen-stimulated Xenopus females in good yield and excellent purity. We have used this procedure coupled with pulse-chase incubations to follow the synthesis, assembly, and intracellular translocation of [^{3}H]leucine and [^{32}P]orthophosphate-labeled vitellogenin.

TABLE III

PROTEIN KINASE ACTIVITY IN ROUGH AND SMOOTH MICROSOMAL FRACTIONS

Fraction	Substrate	Specific activity[a]
Rough	Endogenous	122.5
	Phosvitin	248.3
Smooth	Endogenous	486.7
	Phosvitin	1395.6

[a] pMoles [^{32}P]orthophosphate transferred per mg protein during a 20 min assay period.

Starting material for the procedure is approximately 1.5 g tissue, which is a convenient amount for pulse-chase experiments designed to follow the posttranslational modifications of vitellogenin within liver cubes obtained from one or two females (liver wet weights are approximately 5 g each). Our concern was to obtain fractions of high purity and not necessarily high yield. Nevertheless, our recovery of microsomal marker enzymes is about 10%, which is close to what has been reported in many other laboratories (26).

Galactosyltransferase activity and [^{3}H]orotic acid incorporation into RNA provide two sensitive and specific assays for measuring the presence, and hence the cross-contamination, of smooth and rough microsomes, respectively. The distribution of galactosyltransferase activity in our final preparations indicates that smooth microsome contamination of rough microsomes ranges from 0 to 7%, while [^{3}H]orotic acid incorporation demonstrates that rough microsome contamination of smooth microsomes is less than 3%. These data, taken together with the electron micrographs shown in Fig. 1 (p. 20), indicate that the microsomal subfractions isolated are of high purity with respect to one another.

When CsCl is included in the upper sucrose solution, the separation of microsomal subfractions is at least as good as that achieved in other mammalian systems (26-28) and considerably better than previously reported for Xenopus liver (3). The separation is further improved by omission of CsCl from the top layer of the gradient (Tables I and II, pps. 16 and 18). The precise effect of CsCl is uncertain, but Dallner (13) has suggested that it causes an aggregation of rat liver rough

microsomal vesicles to yield faster sedimenting particles. This seems to hold true for Xenopus microsomes as well, since total omission of CsCl produces a very small rough microsomal pellet.

As has been shown to be the case for other secretory tissues (16, 27), our rough and smooth microsomes behave in a predictable manner. The experiment illustrated in Fig. 2 (p. 20) demonstrates that, after a 5-min pulse-labeling of secretory proteins, the intracellular translocation of newly synthesized products can be followed until a time when secretion is complete and only endogenous microsomal components are labeled. This indicates that the microsomal subfractions we have isolated are well suited for the study of the posttranslational processing of secretory protein, which in estrogen-stimulated Xenopus liver is predominantly vitellogenin.

The experiment in Fig. 6 (p. 27), which shows that [^{32}P]vitellogenin is secreted prior to [^{3}H]leucine-labeled material, is our first indication that at least part of the phosphorylation process occurs after synthesis of the completed vitellogenin peptide. This was corroborated in a more direct manner by the incorporation of [^{32}P]orthophosphate and [^{3}H]leucine into newly synthesized and assembled vitellogenin within microsomal subfractions. By the end of the 15 min pulse period one could calculate that approximately 31% of the incorporated [^{32}P]orthophosphate was contributed by a rough microsomal process, the remainder of the [^{32}P]orthophosphate being attached in the smooth microsomes.

Analysis of the [^{3}H,^{32}P]vitellogenin intermediates on SDS-polyacrylamide gels shows that both coincident peaks of radioactivity

within the rough microsomes have faster electrophoretic mobilities relative to their smooth microsomal analogues. We take this as good evidence for a lack of significant smooth microsomal contamination in the rough microsomal pellet. Further, the fact that only one peak of ^{32}P-labeled material is evident in the rough microsomal fraction indicates that it is the intact vitellogenin peptide which is the substrate recognized by the appropriate protein kinase. This is in agreement with experiments indicating that the phosphorylated regions of vitellogenin are located near its carboxy terminus (29, 30), which is the final segment of vitellogenin to be released into the lumen of the endoplasmic reticulum.

If one considers the related $^{32}P/^{3}H$ ratios of vitellogenin in the rough and smooth microsomes (Fig. 8A and B, p. 34) one can calculate that between 18 and 32% of the [^{32}P]orthophosphate is incorporated into the rough microsomal intermediate. The latter figure takes into account the presence in the smooth microsomes of [^{32}P]vitellogenin which originated in rough microsomes. This is in good agreement with the data in Fig. 7 (p. 32) which indicated a contribution of about 31%.

A microsomal protein kinase activity was partially characterized (data not shown) and was subsequently assayed under optimized conditions. We measured the transfer of [^{32}P]orthophosphate from [$\gamma^{32}P$]ATP into endogenous substrates or exogenous phosvitin by isolated microsomal subfractions. Rough microsomal phosphorylation of endogenous acceptors or phosvitin was 25 or 18%, respectively, of the activity found in the smooth microsomes, indicating that one can localize the responsible

enzymatic activity at concentrations quantitatively harmonious with the conclusions based on Figs. 7 and 8 (pps. 32 and 34). We feel, therefore, that a multisite phosphorylation of vitellogenin within the hepatocyte is the basis for the mechanism of this phosphorylation process.

The most difficult task of this scenario is to explain the actual cellular organization of these membrane-delineated functions. More specifically, is the protein kinase responsible for vitellogenin phosphorylation present in all rough endoplasmic reticulum membranes at levels approximately 20 to 30% of that present in smooth microsomes, or is it only a small fraction of the total rough endoplasmic reticulum engaged in "transition element" formation (31) but which still cosediments with "typical" rough microsomes, thus imparting the measured [^{32}P]orthophosphate incorporation? Finally, the heterogeneity of the smooth microsomes has not been analyzed. Though the presence of galactosyltransferase activity in this fraction implies that these membranes have a Golgi-like character, the presence of other types of membranes involved in vitellogenin processing cannot be ruled out. Further subfractionation of the smooth microsomes should clarify this issue.

CHAPTER III

MICROSOMAL PROTEIN KINASE ACTIVITY IN THE LIVER OF ESTROGEN-STIMULATED *XENOPUS LAEVIS*

Introduction

Under the influence of estrogen, the livers of both male and female *Xenopus laevis* are stimulated to synthesize and secrete the yolk precursor protein vitellogenin (9, 14, 15, 34, 35). Chemical analyses of purified vitellogenin have shown that covalently bound carbohydrate and phosphate, as well as noncovalently associated lipid, are attached to the peptide backbone (9-11). These data indicate that vitellogenin is chemically modified prior to its secretion from the hepatocyte into the bloodstream.

We have recently begun experiments aimed at determining both the subcellular localization and molecular mechanisms involved in the posttranslational modifications of this highly complex multicomponent protein. One approach is to characterize the enzyme activities associated with the specific chemical modification in question. To this end, we report here on a protein kinase activity (E.C. 2.7.1.37, ATP: protein phosphotransferase) localized within the microsomes obtained from estrogen-stimulated *X. laevis*.

Materials and Methods

General. The maintenance (40) and injection of animals (56) have

been described. Our procedure for the fractionation of livers from these animals has also been described in detail (56).

Protein Kinase Assay. Protein kinase activity was assayed at room temperature for 20 min in the following mixture: 50 mM 2(N-morpholino)-ethane sulfonic acid, pH 7.0, 3 mM $MgCl_2$, 1 mM (γ-^{32}P)ATP, 10 mM NaF, and 0.6 mg phosvitin in a final volume of 0.1 ml. The reaction was started with the addition of microsomal membranes solubilized in 1% Nonidet-P40. At the end of 20 min, 20-µl aliquots were spotted on Whatman 3MM filter discs. The discs were processed for liquid scintillation counting as previously described (56). The reaction was linear with up to 100 µg of protein for 45 min.

Immunoprecipitation. When immunoprecipitation of [^{32}]vitellogenin from the assay mixture was performed, phosvitin and nonradioactive ATP were omitted from the reaction. To 35 µg of solubilized microsomal protein was added the protease inhibitor Trasylol (Mobay Chemical Co.) to a final concentration of 2%. The kinase reaction was initiated by adding this preparation to the reaction mixture described above. The reaction was terminated by the addition of 0.3 ml of phosphate-buffered saline containing both 1 mM disodium EDTA and 1 mM ATP. An aliquot (p.1 ml) of monospecific antivitellogenin antiserum (brought to 2% Trasylol) was added, and the immunoprecipitation reaction left overnight. No carrier vitellogenin was added. The resulting immuno-precipitate was collected by centrifuging at 850 × g for 30 min and the pellet washed 3 times in the phosphate buffered saline previously

described. The immunoprecipitate was then solubilized in sample buffer and electrophoresed on SDS polyacrylamide gels according to Laemmli (47). After electrophoresis, the gel was frozen in dry ice, sliced into segments 1 mm thick, and dissolved at 50°C by placing them into scintillation vials with 0.3 ml of 30% H_2O_2. After the addition of 10 ml of aqueous counting scintillant (ACS, Amersham/Searle), the gel slices were analyzed by liquid scintillation counting.

Results

In the presence of the nonionic detergent Nonidet-P40, the microsomal protein kinase showed an approximately 7-fold increase in specific activity over intact membranes (data not shown). We characterized the enzyme activity by omitting or adding various compounds to the reaction mixture (Table IV). Most notable was the dependence of the enzyme activity on the presence of divalent cations. The omission of $MgCl_2$ from the reaction allowed only 12% of the activity demonstrated by the complete assay. This requirement for divalent cations was almost totally restored by the addition of $MnCl_2$, whereas $CaCl_2$ had no effect.

Of special interest is the last entry of Table IV that indicates that the enzyme is specific for ATP as its phosphate donor. Substitution of ATP with 1 mM (γ-^{32}P)GTP resulted in a transfer of phosphate to phosvitin of only 15% of that obtained with (γ-^{32}P)ATP as phosphate donor. In addition, the data presented in Table V suggest that this protein kinase activity is not regulated by cyclic nucleotides.

Under our assay conditions we have been able to characterize the protein kinase activity with respect to both substrates, ATP (Fig. 9)

TABLE IV

EFFECT OF VARIOUS AGENTS ON MICROSOMAL PROTEIN KINASE ACTIVITY

Condition of assay	Activity (% of complete assay mixture)
Complete	100
- Phosvitin	63
- $MgCl_2$	12
- $MgCl_2$ + 5 m$\underline{\underline{M}}$ $CaCl_2$	12
- $MgCl_2$ + 5 m$\underline{\underline{M}}$ $MnCl_2$	84
- NaF	106
+ Dithiothreitol (1 m$\underline{\underline{M}}$)	99
- ATP + 1 m$\underline{\underline{M}}$ [γ-^{32}P]GTP	15

TABLE V

EFFECT OF CYCLIC NUCLEOTIDES ON PROTEIN KINASE ACTIVITY

Cyclic nucleotide	Activity (% of control)
None	100
Cyclic AMP (10^{-6} M)	82
Cyclic AMP (10^{-5} M)	93
Cyclic AMP (10^{-4} M)	105
Cyclic GMP (10^{-6} M)	93
Cyclic GMP (10^{-5} M)	101
Cyclic GMP (10^{-4} M)	88

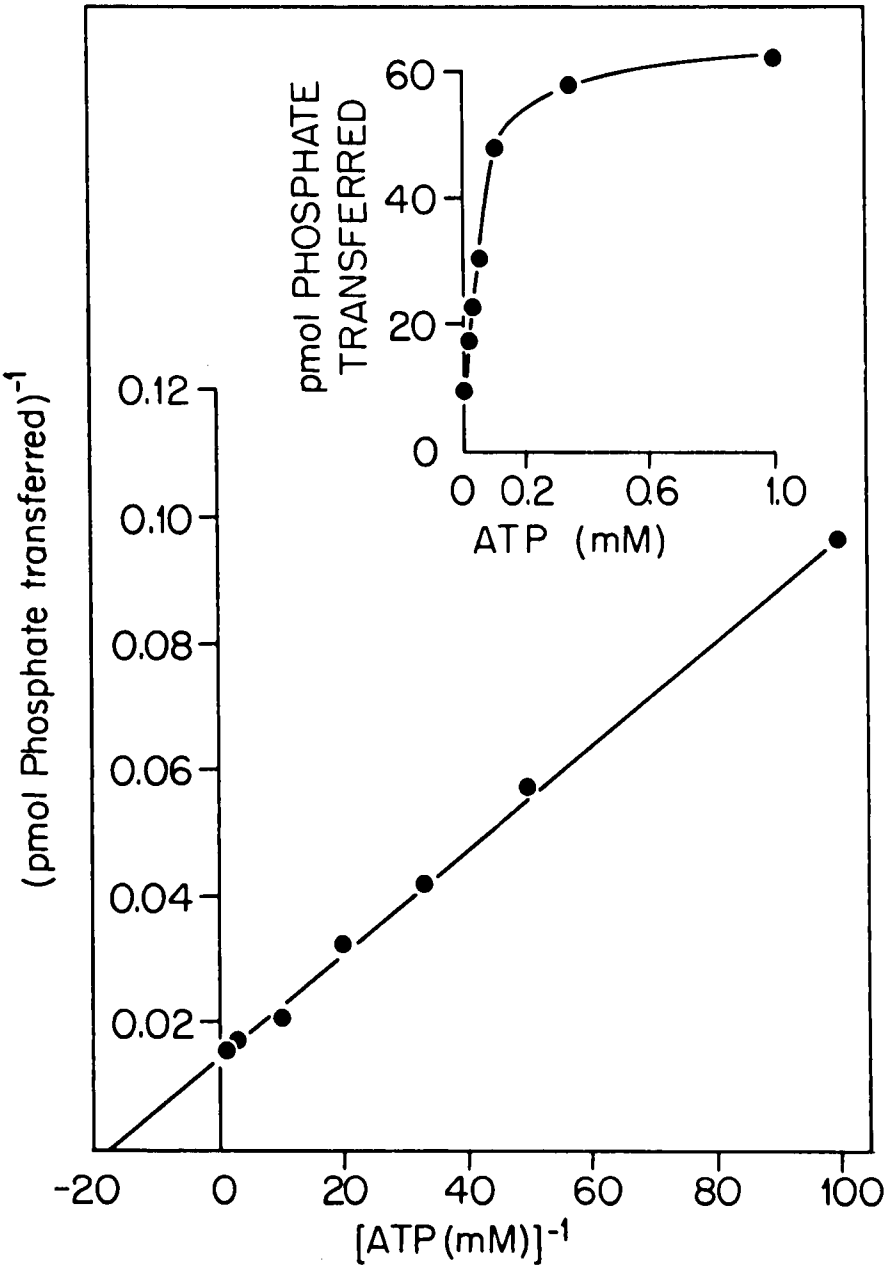

FIG. 9. Lineweaver-Burk analysis of the protein kinase activity with varying concentrations of ATP. The line represents least-squares fit of the points derived from the Michaelis-Menten plot (inset).

and Xenopus phosvitin (Fig. 10). Lineweaver-Burk analysis of the data shows that the K_m for ATP is 54.9 μM (Fig. 9) and that for phosvitin (MW 34,000) is 13.8 μM (Fig. 10). These K_m values are very similar to that of the casein kinase activity derived from the lactating mammary gland of rats (57), a tissue that also actively secretes phosphoproteins.

Next, we determined that this protein kinase is in fact the one responsible for phosphorylating vitellogenin in vivo. Our approach was to incubate a small amount (35 μg) of solubilized extract with 10 μCi (γ-^{32}P)ATP. The second entry of Table IV indicates that such incorporation into endogenous acceptors can be substantial. We then specifically immunoprecipitated labeled microsomal vitellogenin precursors from the mixture and analyzed the immunoprecipitable radioactivity after electrophoresis on SDS-gels. The profile of radioactivity is shown in Fig. 11.

The major peaks of (γ-^{32}P)ATP derived radioactivity lies within the region of the gel where serum vitellogenin migrates (arrow). The presence of two peaks of [^{32}P]vitellogenin is consistent with the previous demonstration of two differentially phosphorylated forms of vitellogenin within liver microsomes (56). However, in this experiment a small degree of proteolysis of a single component, even in the presence of protease inhibitor, cannot be ruled out. No peaks were demonstrated when extracts labeled with (γ-^{32}P)GTP were examined, a finding consistent with the last entry in Table IV. We feel, therefore, that this is a direct indication of the involvement of this protein kinase activity in phosphorylating vitellogenin in vivo.

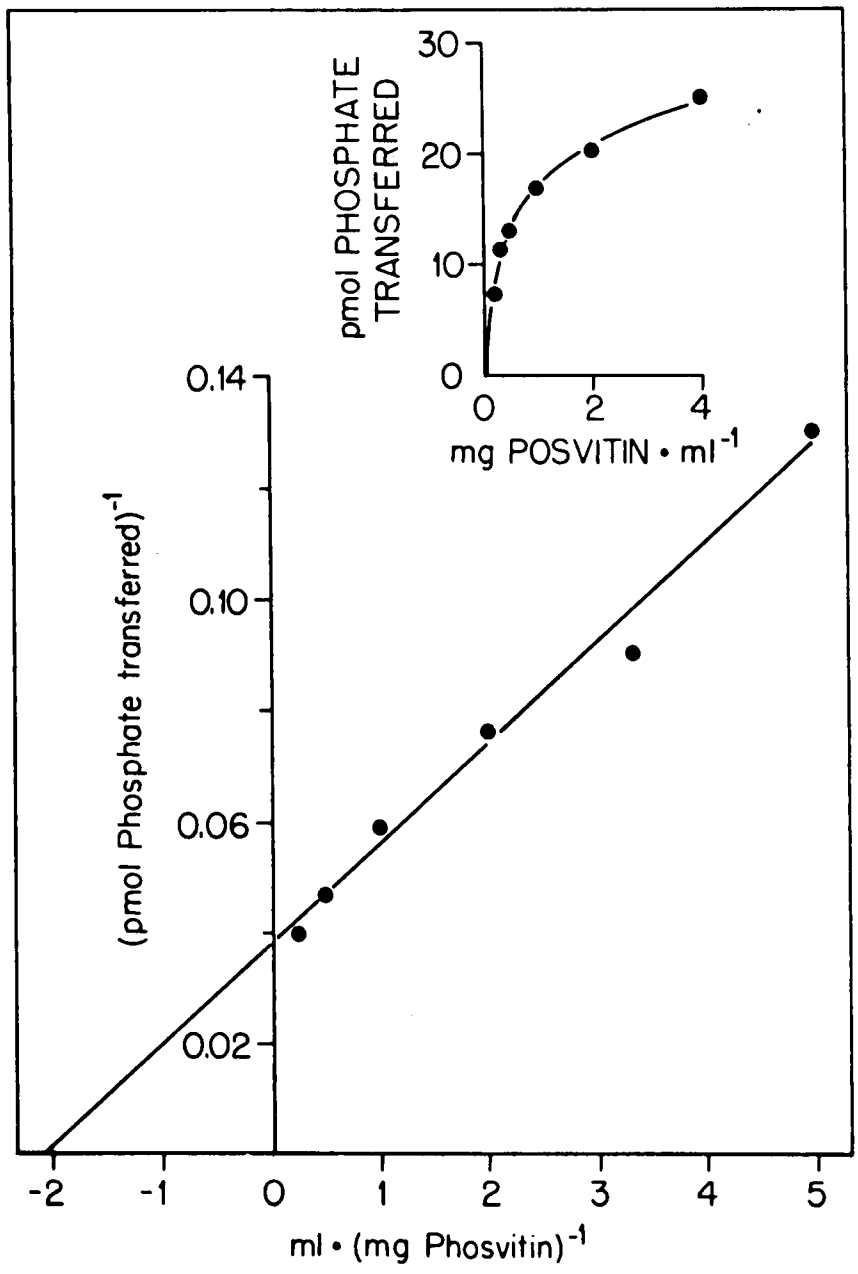

FIG. 10. Lineweaver-Burk analysis of the protein kinase activity with varying concentrations of Xenopus phosvitin. The line represents least-squares fit of the points derived from the Michaelis-Menten plot (inset). The activity demonstrated in the absence of exogenous phosvitin was subtracted from all other values.

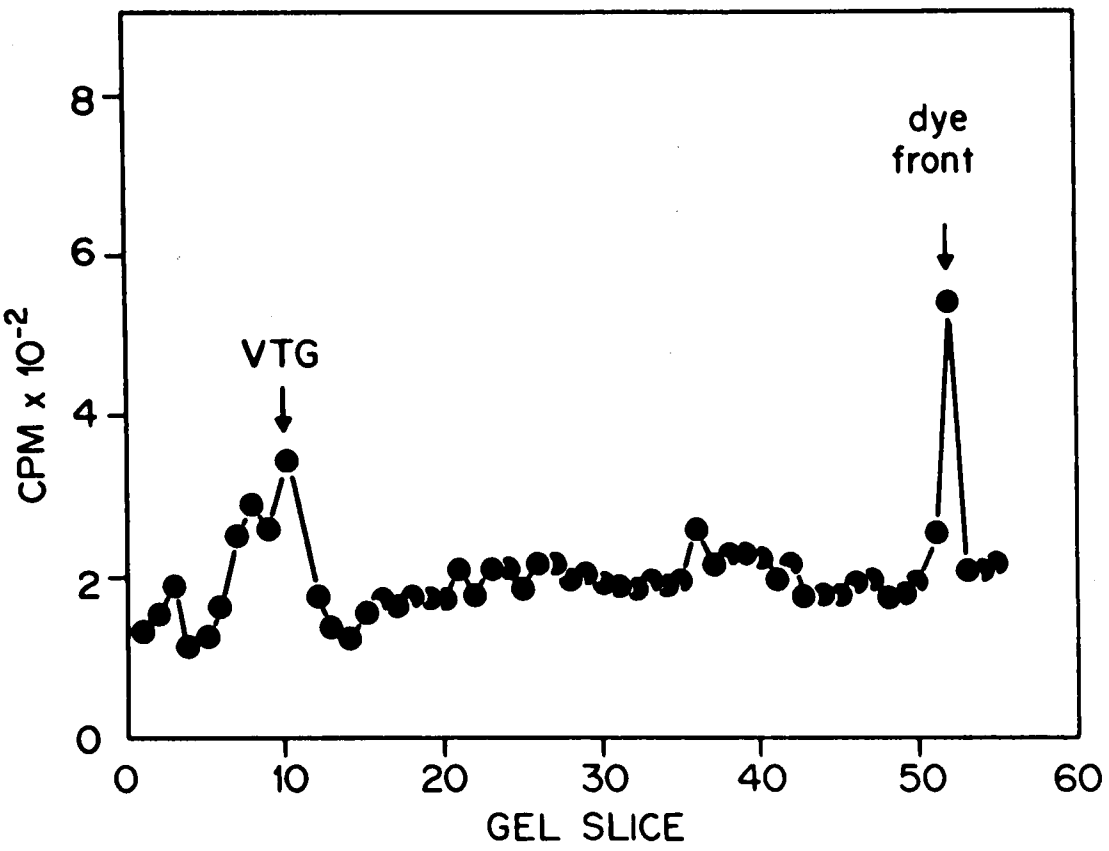

FIG. 11. SDS-polyacrylamide (7%) gel electrophoresis of [^{32}P]vitellogenin immunoprecipitated from the labeled endogenous acceptors of solubilized microsomes.

Discussion

The protein kinase activity we describe in this report is responsible for phosphorylating vitellogenin *in vivo*. A previous report from our laboratory indicated that the majority of this microsomal protein kinase lies within the smooth surfaced, Golgi-enriched subfraction of Xenopus liver microsomes (56). This was in good agreement with kinetic and electrophoretic data showing that the smooth microsomes accounted for approximately 70% of the total incorporated phosphate (56).

The lactating mammary gland secretes large quantities of casein, a group of phosphoproteins present in milk. Casein kinase activities have been characterized in Golgi membranes obtained from this tissue (57-59). The ability of this enzyme to phosphorylate the endogenous casein present in the luminal content of these Golgi vesicles has not been demonstrated. Therefore, one can only infer its probable role in the posttranslational modification of casein.

A phosphoprotein kinase has previously been purified from Xenopus liver (55). However, its localization within the soluble fraction of the hepatocyte, together with its ability to use GTP as a phosphate donor, indicates that this cannot be the same enzyme we present here. Another earlier report focused on the effect of estrogen on a phosvitin kinase of rooster liver (16). However, that enzyme was also derived from the soluble fraction of the tissue, and, therefore, could not be the one responsible for phosphorylating vitellogenin. Unpublished observations from our lab show that estradiol-17β increases total liver microsomal protein kinase activity 3- to 6-fold. This is due to an overall increase

in total microsomal protein per gram liver, not the preferential induction of synthesis of this one enzyme.

In summary, it appears that one step in the assembly of the final secreted vitellogenin molecule involves the transfer of phosphate from ATP via a divalent-cation requiring protein kinase to vitellogenin. This event does not seem to be regulated by cyclic nucleotides.

CHAPTER IV

INTRACELLULAR GLYCOSYLATION OF VITELLOGENIN IN THE LIVER OF ESTROGEN-STIMULATED *XENOPUS LAEVIS*

Introduction

When an adult *Xenopus laevis* of either sex is injected with estrogen, its liver is stimulated to synthesize and secrete the multicomponent yolk precursor protein vitellogenin (9, 35, 36). That the hormone acts directly on the hepatocytes has been demonstrated by achieving the primary induction of vitellogenin synthesis in liver cubes *in vitro* (14, 15). Chemical analysis of vitellogenin has shown that it is a highly modified protein (9-11). Specifically, phosphate and carbohydrate are covalently bound to the peptide backbone, whereas lipid is noncovalently associated with it. These data indicate that significant posttranslational modifications of vitellogenin occur prior to its secretion into the bloodstream.

Previous work in our lab and in others has shown that at least 60% of the total protein synthesized by livers of estrogen-treated animals is vitellogenin (34, 38, 48, 49, 56). We have taken advantage of this large induction of a single protein by studying vitellogenin synthesis and secretion using liver slices *in vitro*. This is an excellent model system for probing the assembly of highly modified proteins (56).

Our approach in examining the glycosylation of vitellogenin has been to use continuous and pulse-chase incubations with various

radiolabeled sugars in conjunction with subcellular fractionation. To this end we employ a fractionation scheme that we developed specifically for homogenates obtained from the small amount of tissue used for each incubation (56). The rough endoplasmic reticulum and smooth membrane fractions obtained allow both temporal and topological analysis of the intracellular processes leading to the secretion of the completely assembled vitellogenin molecule.

The preliminary experiments reported here are the first for an amphibian glycoprotein and suggest that Xenopus vitellogenin possesses an N-glycosidically linked carbohydrate prosthetic group which is structurally of the "complex" type (61). In many respects, therefore, this glycoprotein is probably assembled in a manner described by models based on avian and mammalian systems (61, 62). A significant difference, however, is that the core region of the oligosaccharide is attached to the peptide backbone of Xenopus vitellogenin within a smooth membrane compartment as opposed to the rough endoplasmic reticulum (61, 62).

Materials and Methods

Animals and Isotopes. Xenopus females were obtained from the South African Snake Farm (Fish Hoek, Cape Province, South Africa). They were maintained and fed as previously described (40), injected on two alternate days with 2.0 mg of estradiol-17β, and used 10 to 21 days after the first injection. L-[4,5-^{3}H]leucine (62 to 65 Ci/mmol) was obtained from Schwarz/Mann. D-[2-^{3}H]mannose (14.5 to 17.3 Ci/mmol), D-[1-^{3}H]galactose (9.3 to 14.2 Ci/mmol, and L-[5,6-^{3}H]fucose (60 ci/mmol)

were from New England Nuclear. [^{32}P]Orthophosphate and D-[6-^{3}H]-glucosamine hydrochloride (22.6 Ci/mmol) were from the Radiochemical Centre of Amersham/Searle.

Culture of Liver Explants. Animals were bled from the heart, and the livers were removed to divalent cation-free O-R2 (41). Pieces were weighed to yield the amount of tissue required as indicated in the figure legends. The pieces of liver were then sliced with scalpel blades and exhaustively washed as previously described in detail (56).

For pulse-chase experiments, liver cultures were cooled on ice in small Erlenmeyer flasks. Then 10 ml of ice-cold pulse medium containing [^{3}H]leucine or [^{32}P]orthophosphate was added. The procedure for pulse-labeling with radiolabeled sugars was identical except that 3 or 4 ml of ice-cold pulse medium was added. In all cases, cultures were left to equilibrate on ice for 30 min. The pulse medium was comprised of O-R2 supplemented with 30 mM 4-(2-hydroxyethyl)-1-piperazineethanesulfonic acid (Hepes) plus sodium pyruvate and all amino acids at concentrations identical to that of 50% Leibovitz L-15 medium (42, 43). No nonradioactive sugars were present during pulse periods. When pulse-labeling was performed with [^{3}H]leucine or [^{32}P]orthophosphate for 15 min, nonradioactive leucine or orthophosphate was also omitted from the medium (56).

We initiated the pulse by swirling the stoppered flask under warm tap water for about 1 min and then at room temperature (21°C) for the indicated times. We terminated the pulse by washing the cultures three times in O-R2 supplemented with the nonradioactive precursor at a concentration of 10 mM. Then 10 ml of 50% L-15 supplemented with 30 mM

Hepes and made 10 mM with nonradioactive precursor was added as the chase medium. All pulse and chase media had a final pH of 7.8.

Where indicated, tunicamycin (Calbiochem) in 25 mM NaOH was added to media at a final concentration of 1 or 3 μg/ml. Control media (0 μg/ml) received an equal amount of 25 mM NaOH, yielding a final NaOH concentration of 75 μM.

Fractionation of Liver Homogenates. Whole microsomes were isolated from cultures containing from 0.5 to 1.0 g of tissue. The tissue slices were washed in divalent cation-free O-R2 and then in 0.4 M sucrose (brought to pH 7.4 with NaOH). Disruption of the tissue was achieved with five strokes of a loose Dounce pestle. The postmitochondrial supernatant obtained after centrifugation for 10 min at 10,000 × g was spun in a Beckman type 40 rotor at 40,000 rpm for 90 min. The microsomal pellet was resuspended in 0.3 M KCl (pH 8.0), homogenized with a loose Dounce pestle, and pelleted as above.

Rough endoplasmic reticulum and smooth microsomes (enriched in Golgi membranes) were isolated and washed exactly as described previously (56).

Chemical Methods. Protein was measured by the filter assay of Bramhall et al. (44) with bovine serum albimun as a standard.

SDS-Polyacrylamide Gel Electrophoresis. Smooth microsomes were precipitated with ice-cold acetone. Precipitates were collected by centrifugation at 850 × g for 15 min. The pellets were solubilized by being boiled in sample buffer, and electrophoresed according to Laemmli

(47). Gels were frozen, sliced, and analyzed for radioactivity exactly as previously described (56).

Immunoprecipitation. Washed microsomes and microsomal subfractions were resuspended in phosphate-buffered saline made 1 mM in disodium ethylenediaminetetraacetate and 10 mM in the nonradioactive precursor whose radioactive counterpart was being used. To aliquots of this suspension were added the detergent Nonidet P-40 and the protease inhibitor Trasylol (Mobay Chemical Company), each to a final concentration of 1%. The mixture was centrifuged at 10,000 × g for 5 to 10 min. To a 0.5-ml aliquot of the solubilized extract was added 100 µl of rabbit antiserum known to be monospecific for vitellogenin. No carrier vitellogenin was added. The reaction continued for 1 h at room temperature, then overnight in the cold room (4°C). The immunoprecipitates were collected by centrifugation at 850 × g for 30 min. They were resuspended by addition of the phosphate-buffered saline solution described above with vortexing to disperse the immunoprecipitates. This was repeated for a total of four washes. The pelleted material from the last wash was solubilized in 0.5 ml of 10% SDS, and aliquots were counted after the addition of 10 ml of aqueous counting scintillant (Amersham/Searle). Blanks consisted of solubilized extract plus aliquots of preformed antigen-antibody complex added prior to the initial centrifugation.

Immunoprecipitation of labeled vitellogenin from chase media was achieved by addition of 800 µg of carrier vitellogenin to 1.0 ml of medium. To this was added 100 µl of sheep antiserum shown to be

monospecific for vitellogenin. This mixture was processed exactly as described above.

Neuraminidase Treatment of Solubilized Microsomes. Microsomes were solubilized in divalent cation-free O-R2 (pH 5.0), 1% Nonidet P-40. Aliquots received either no addition or neuraminidase (Sigma) or bovine serum album (Sigma), each at a final concentration of 5 mg/ml. After 1 h at 37°C, an equal volume of divalent cation-free O-R2 (pH 11.0) was added, followed by 100 µl of rabbit antiserum. This mixture was processed as described above.

Incorporation Studies. The incorporation of radioactive leucine and glucosamine into acid-insoluble components secreted into the culture media was measured as follows. A 0.5-ml aliquot of medium was mixed with 5 µl of serum obtained from an estrogen-treated animal. The serum served as carrier for the precipitation reaction with 10% trichloroacetic acid which was made 10 m$\underline{\underline{M}}$ in both leucine and glucosamine. The precipitate was collected by low-speed centrifugation, then resuspended and washed with this solution three more times. The precipitate was then washed with ethanol, ethanol:ether (1:1), and ether. The dried residue was dissolved by being boiled in 30% H_2O_2, and aliquots were removed for liquid scintillation counting.

Analysis of Sugar Fraction. Purified vitellogenin was hydrolyzed in 1 $\underline{\underline{N}}$ H_2SO_4 for 8 h at 80°C. The hydrolysate was neutralized with saturated barium hydroxide, and the insoluble material was removed by centrifugation. The clear supernatant was brought to pH 9.5 with NH_4OH

and passed over a column of AG1-X8 (Bio-Rad) resin in the acetate form. The column was then washed with distilled water, and the eluted material was tekan to dryness by lyophilization.

The alditol acetate derivatives of the sugar fraction were prepared according to the method of Lehnhardt and Winzler (63), except that the acetylation was performed for 1 h in a boiling-water bath. The mixture was dried under N_2 and redissolved in carbon disulfide. Aliquots were then injected into a column (6 ft × 1/8 in.) packed with 3% SP-2330 on 100/120 Supelcoport (Supelco Inc.) which was maintained at 225°C.

Results

Compartmentalization and Kinetics of Intracellular Translocation of Pulse-Labeled Vitellogenin. Our first experiments were aimed at obtaining an overall view of the intracellular translocation of vitellogenin after pulse-labeling of liver slices with either leucine, glucosamine, or galactose. These results are shown in Fig. 12. It is apparent that leucine- and glucosamine-labeled vitellogenin demonstrate a similar kind of kinetics, whereas the galactose-labeled material shows very different behavior. Specifically, both leucine- and glucosamine-labeled intermediates show peak accumulations of radioactivity within hepatocytes after a 1-h chase period; this is followed by a steady decline in specific activity which continues for at least 5 h after the chase period begins. In contrast, galactose-labeled vitellogenin within the hepatocyte shows a continuous decrease in specific activity after only 1 h in chase medium, and levels off after 3 h.

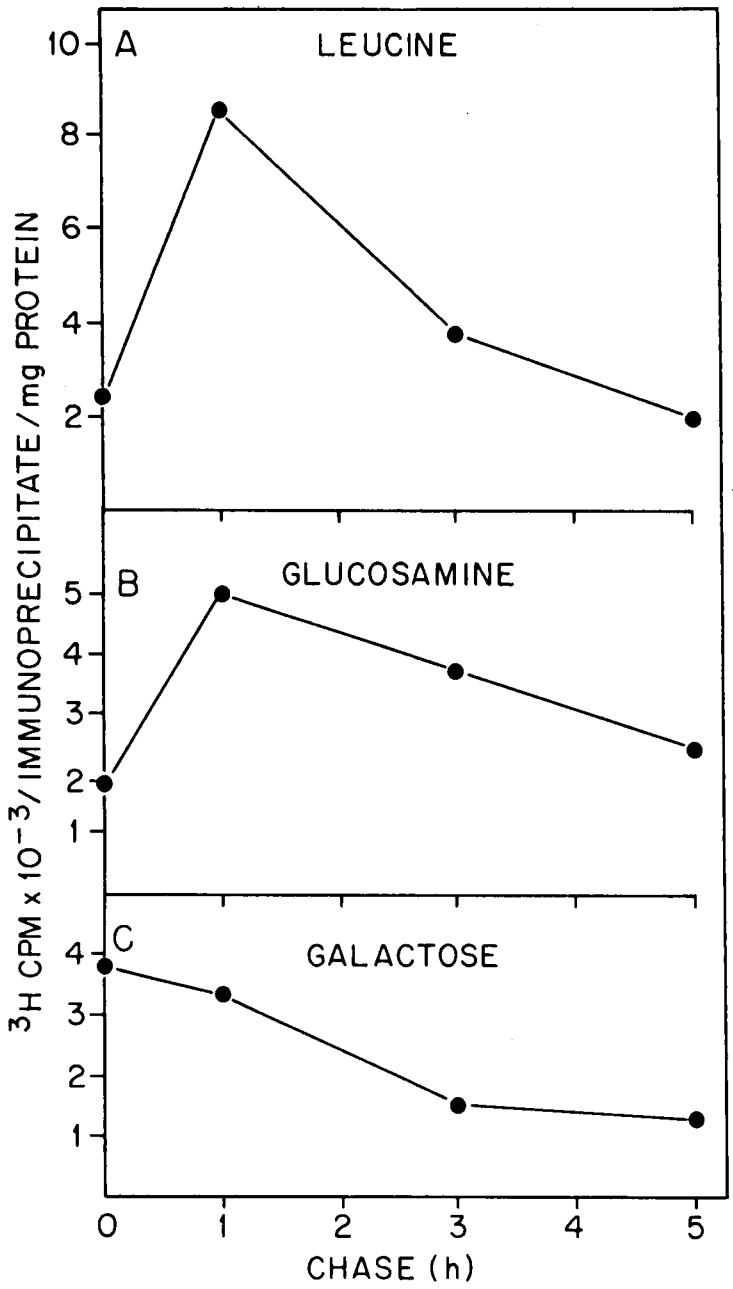

FIG. 12. Incorporation of (A) [^{3}H]leucine, (B) [^{3}H]glucosamine, and (C) [^{3}H]galactose into microsomal vitellogenin after a brief pulse, and chase periods of 1, 3, and 5 h. Liver slices (0.7 g) were pulse-labeled for 15 min with [^{3}H]leucine (10 µCi/ml), or 30 min with [^{3}H]glucosamine (30 µCi/ml) or [^{3}H]galactose (20 µCi/ml). At the end of the pulse period, cultures were washed, and the chase period was begun as described in "Materials and Methods." At each time point, whole microsomes were isolated and analyzed for immunoprecipitable radioactivity.

The most reasonable interpretation of these results is that when [^{3}H]galactose is incorporated into pulse-labeled vitellogenin it is at a point in the intracellular scheme of secretory protein processing that is much closer temporally, if not also spatially, to the actual exocytotic event. Several studies have indicated that the attachment of galactose to secretory proteins occurs during the terminal stages of intracellular processing of glycoproteins within Golgi-derived compartments (64-68).

Glucosamine, however, seems to follow a sequence of kinetic events similar to that of leucine (Fig. 12). Based on this observation and numerous reports in the literature (69-74), one might conclude that glucosamine is added to the oligosaccharide moiety of vitellogenin during or soon after synthesis of the polypeptide in the rough endoplasmic reticulum. The result which indicates that this is not the case is presented in Fig. 13. Vitellogenin labeled with [^{3}H]glucosamine after a 30-min pulse is localized exclusively in a smooth membrane compartment. The small amount of incorporation present in the rough endoplasmic reticulum is most probably due to contaminating smooth membranes (56). In addition, the kinetics obtained in glucosamine-labeled whole microsomes during pulse-chase experiments (Fig. 12) can be accounted for by the behavior of the isolated smooth microsomal subfraction (Fig. 13A, inset). SDS-polyacrylamide gel electrophoresis of the smooth microsome fraction (Fig. 13B) shows that essentially all of the incorporated [^{3}H]glucosamine radioactivity in this fraction migrates with an internal standard of [^{32}P]orthophosphate-labeled vitellogenin (derived from appropriately labeled smooth microsomes) whose position is indicated by the arrow.

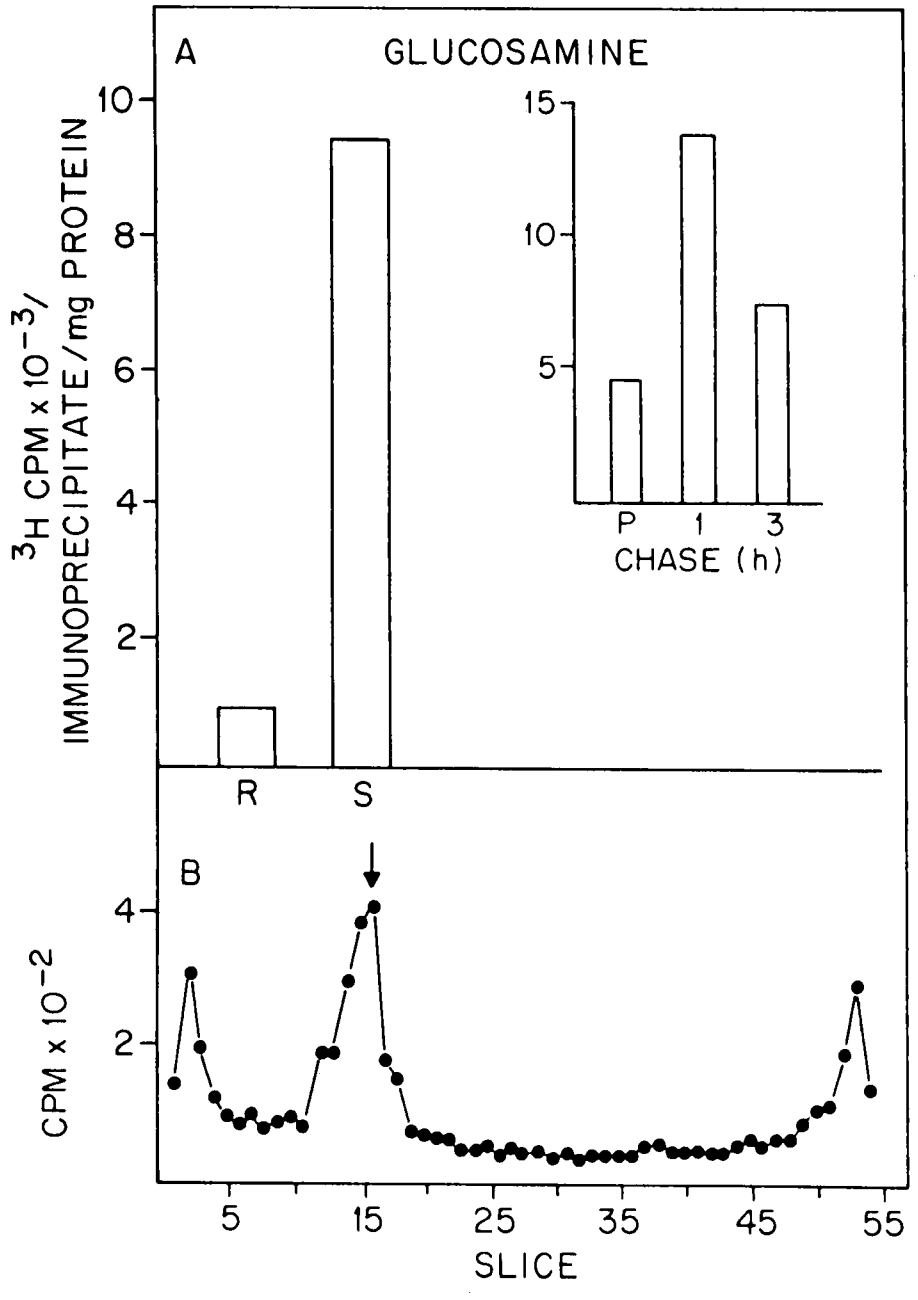

FIG. 13. Incorporation of [^{3}H]glucosamine (30 μCi/ml) into microsomal subfractions during a 30 min pulse and 1- or 3-h chase. Liver slices (1.5 g) were incubated as described in "Materials and Methods." At each time point, the tissue was homogenized and the rough endoplasmic reticulum and smooth membrane fractions were obtained and analyzed for immunoprecipitable radioactivity. (A) Distrition of radioactivity between rough and smooth membranes.

Inset: Labeling kinetics of smooth membranes during a pulse-chase incubation (R, rough endoplasmic reticulum; S, smooth microsomes). (B) Radioactivity profile of smooth microsomes labeled with [^{3}H]glucosamine analyzed by SDS-polyacrylamide gel electrophoresis. Arrow: Comigration of [^{32}P]vitellogenin as an internal standard.

The magnitude of the involvement of the smooth microsomes with respect to vitellogenin glycosylation and the omission of a role for the rough endoplasmic reticulum indicate that the attachment of glucosamine to vitellogenin occurs by a mechanism somewhat different than that operating in mammalian and avian systems (61, 62).

The identical experiment was performed with [^{3}H]galactose (Fig. 14). As with glucosamine, incorporation of galactose occurs within the smooth microsomal compartment (Fig. 14A). The inset of Fig. 14A shows that the overall kinetics of intracellular translocation of [^{3}H]galactose-labeled vitellogenin observed in whole microsomes (Fig. 12) can also be accounted for by the movement of labeled material through this smooth, Golgi-enriched compartment. The previous demonstration of Golgi elements within this isolated fraction (56) together with observed kinetics are consistent with the notion that the addition of galactose to vitellogenin in Xenopus liver is a terminal event, occurring very close to the final secretory step.

As a positive control for the functional integrity of our rough microsomal fraction, we pulse-labeled a culture of liver slices for 15 min with [^{3}H]leucine. At the end of this period the tissue was processed for subcellular fractionation (56). At each step in the procedure an aliquot of each of the indicated fractions was analyzed for acid-insoluble radioactivity as previously described (56). The results are shown in Fig. 15. As would be expected for a tissue almost exclusively devoted to the synthesis of secretory protein, the rough microsomes are clearly the most highly labeled fraction. The smooth microsomes are labeled to

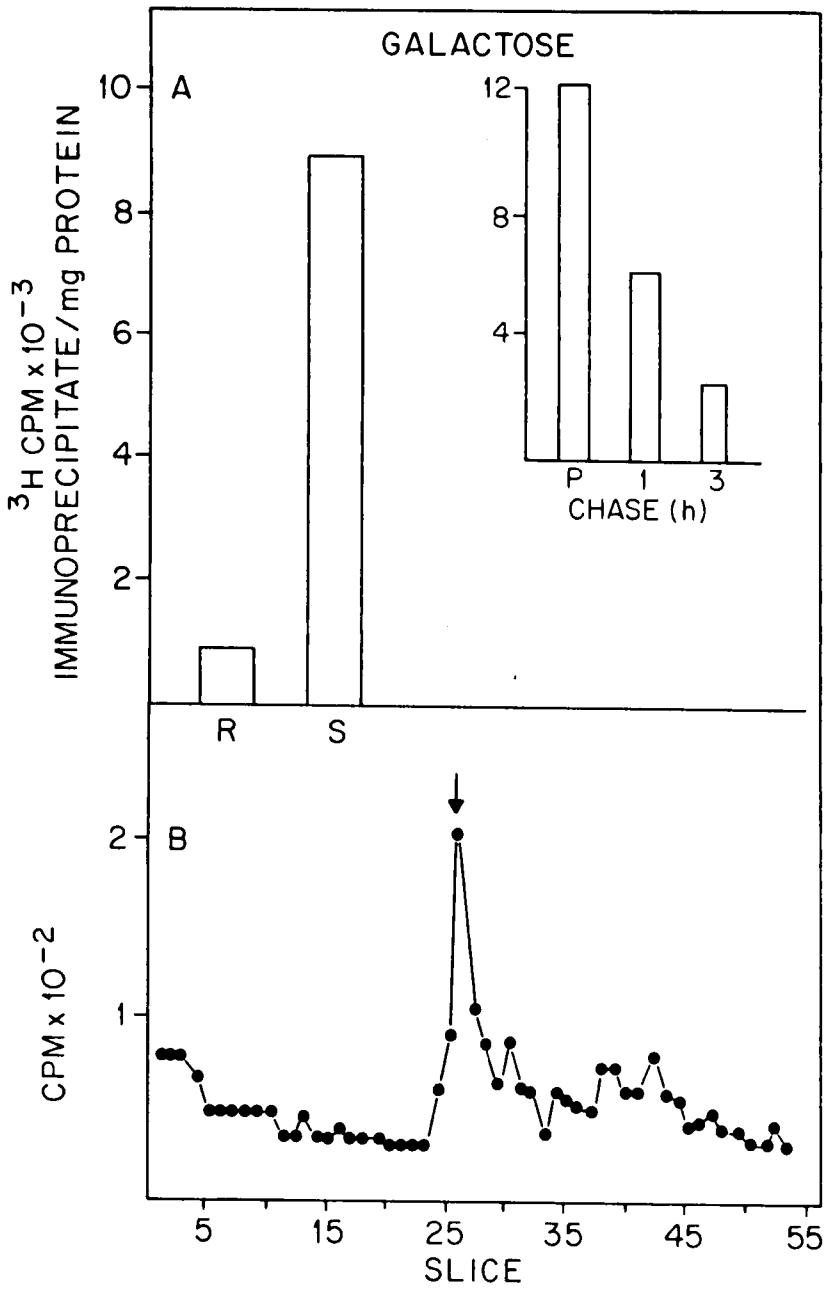

FIG. 14. Incorporation of [^{3}H]galactose (30 μCi/ml) into microsomal subfractions during a 30 min pulse and 1- or 3-h chase. The procedures and explanation of the symbols are as described in Fig. 13.

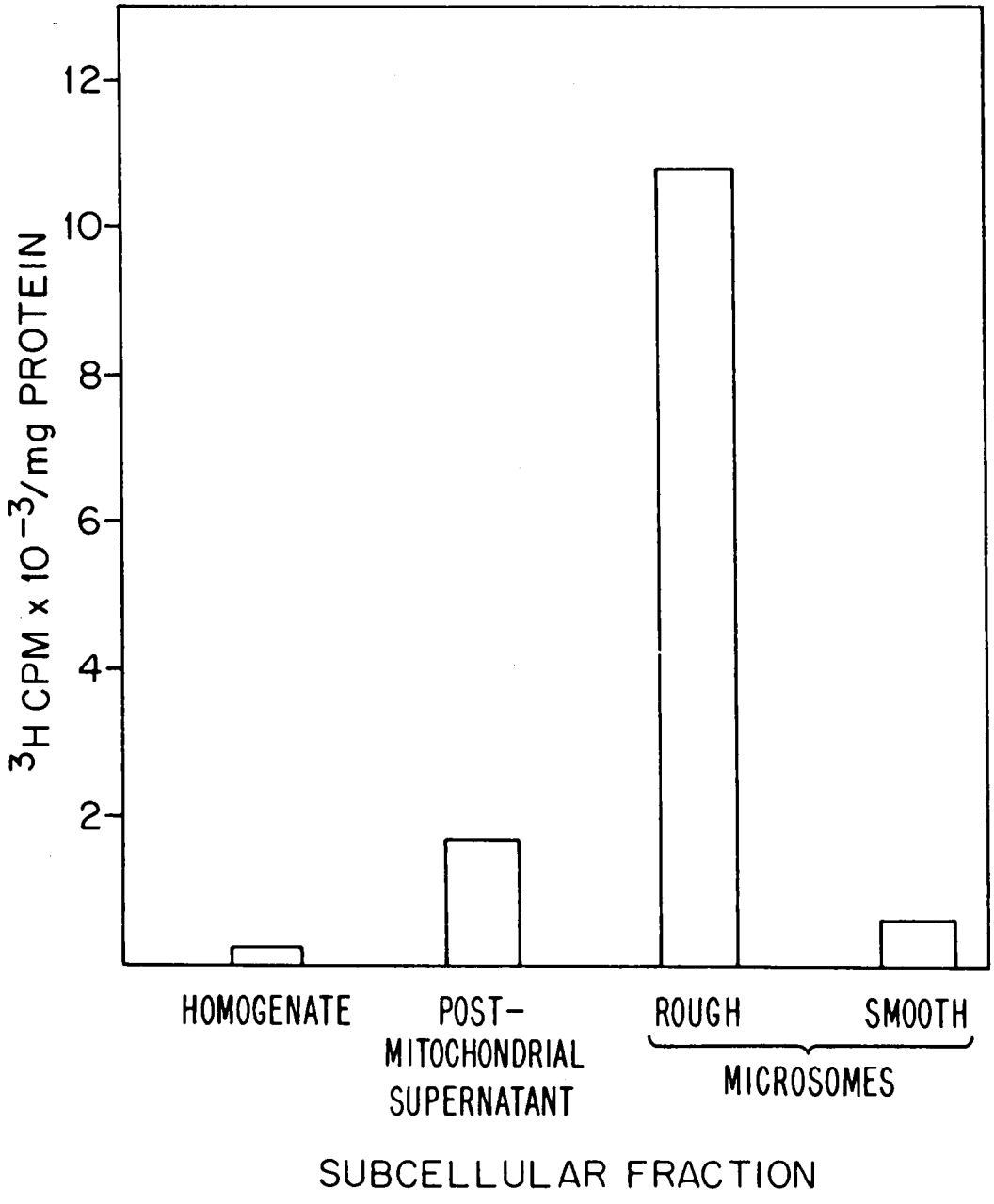

FIG. 15. Incorporation of [^{3}H]leucine (10 μCi/ml) into liver subcellular fractions after a 15 min pulse. Liver slices (2.0 g) were pulse labeled as described in "Materials and Methods." Subcellular fractions were obtained and analyzed for acid-insoluble radioactivity.

an extent of only 6% of that demonstrated by the rough fraction. Therefore, we conclude that our procedure for preparing microsomal subfractions maintains the functional characteristics of these membranes. More specifically, any attempt at explaining the results of Fig. 13 by suggesting that our smooth microsomes are contaminated by membranes of the rough endoplasmic reticulum which were artifactually stripped of their ribosomes is not valid.

One can infer from the different kinetics obtained for galactose and glucosamine incorporation that the addition of galactose is representative of a terminal glycosylation, whereas glucosamine incorporation results from a glycosylation step more proximal to the actual glycopeptide linkage. The results shown in Table VI indicate that [^{3}H]glucosamine-derived radioactivity is not decreased after neuraminidase treatment. Therefore, the potential conversion of glucosamine to sialic acid (69, 75), a terminal glycosyl unit, has not occurred, which supports our conclusion that, with respect to kinetics, glucosamine behaves more as a core glycosyl unit than as a terminally located one.

A question that may be raised at this point is whether the intracellular kinetic data can be explained by the differential efficacy of the wash and chase conditions favoring a premature termination of the galactose pulse, with respect to the length of the glucosamine pulse period. We approached this question by measuring the rates of accumulation of various pulse-labeled vitellogenins in the chase media. The rationale was that the lag period between incorporation of the

TABLE VI

NEURAMINIDASE DIGESTION OF [^{3}H]GLUCOSAMINE-LABELED MICROSOMAL EXTRACTS

Addition	^{3}H cpm/OD_{280}	Percent of control
None	2600	100
Neuraminidase	2480	95
BSA	2308	89

Microsomes from liver slices (2.0 g) labeled with [^{3}H]glucosamine (40 μCi/ml) were analyzed for immunoprecipitable radioactivity as described in "Materials and Methods." Aliquots of solubilized extract received either no addition, or neuraminidase (Sigma) or bovine serum albumin at a final concentration of 5 mg/ml. The latter addition showed that somewhat larger immunoprecipitates resulted from the increased protein concentration. The specific activity is therefore expressed per OD_{280} of the washed and solubilized immunoprecipitate. The digestion with neuriminidase was for 1 h at 37°C.

precursor and its appearance in the medium is a function only of the intracellular organization of these processes, not how rapidly the effective pulse is terminated. The results of these experiments are shown in Fig. 16.

Galactose-labeled vitellogenin appears in the chase medium by 30 min, whereas both glucosamine- and [^{32}P]orthophosphate-labeled vitellogenin appear within 2 h after the start of the chase. This corroborates our conclusion based on intracellular translocation studies that, though glucosamine is incorporated into a smooth membrane compartment, it is not behaving as a terminal glycosyl moiety. The observed rapid accumulation of galactose in chase medium indicates that it is, in fact, involved in one of the last posttranslational modification processes prior to secretion from the hepatocyte. The similarity in lag period between [^{32}P]vitellogenin and [^{3}H]glucosamine-labeled vitellogenin will be discussed below, based on our previous observations of the phosphorylation of vitellogenin (56).

An unexpected observation in Fig. 16 was that only a very little amount of [^{3}H]mannose was incorporated into secreted vitellogenin even after a 4-h chase period. This is further illustrated in Table VII, which surveys several tritiated precursors for their ability to be incorporated into microsomal vitellogenin. The major point to be made is that relative to galactose, [^{3}H]mannose is incorporated into vitellogenin at very low levels. That fucose also showed low incorporation is less surprising since it is present in other N-glycosidically linked oligosaccharides less frequently and, when present, it is quantitatively less significant (61, 76).

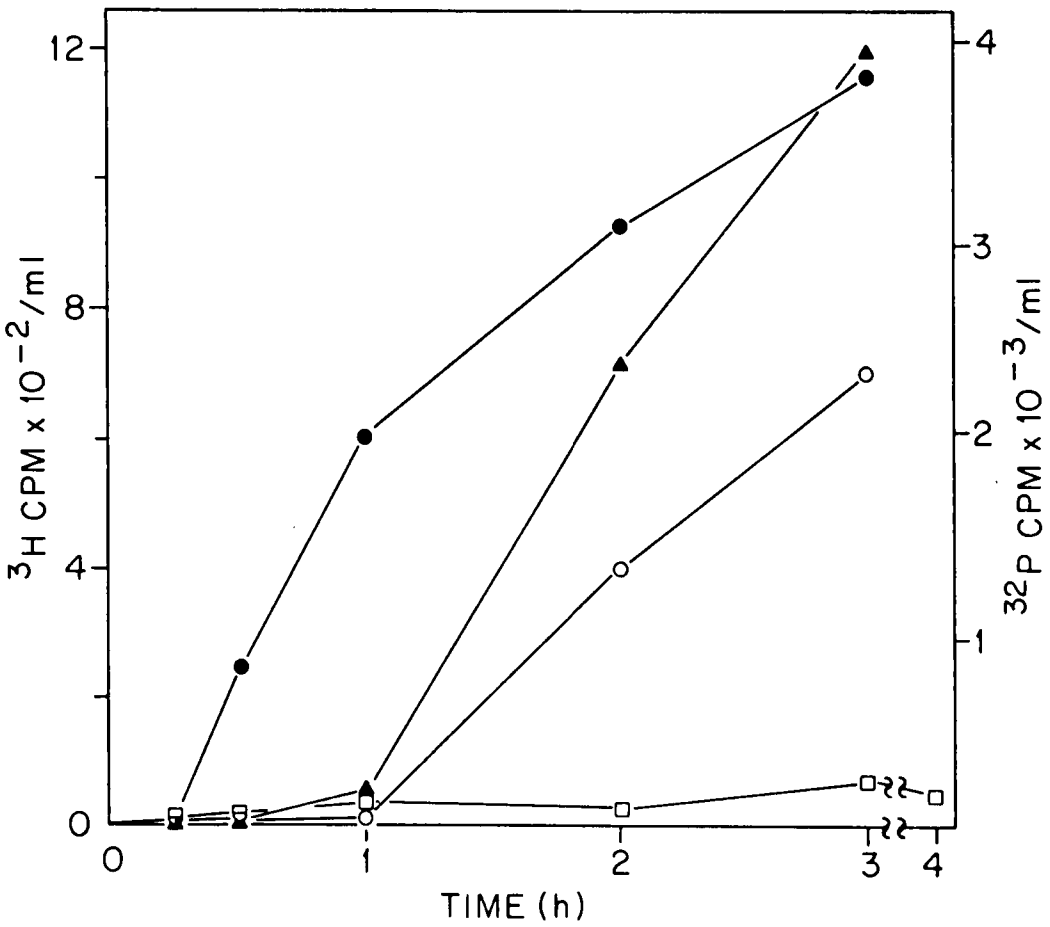

FIG. 16. Secretion kinetics of vitellogenin that was pulse-labeled with either [^{3}H]galactose (30 µCi/ml, ●), [^{3}H]glucosamine (30 µCi/ml, o), [^{3}H]mannose (50 µCi/ml, □), or [^{32}P]orthophosphate (50 µCi/ml, ▲). Either 1.0 g ([^{32}P]orthophosphate) or 1.5 g (radiolabeled sugars) of liver slices were pulse-labeled as described in "Materials and Methods," washed, and then incubated in chase medium. At the indicated times, 1.0 ml of chase medium from each culture was analyzed for immunoprecipitable vitellogenin as described.

TABLE VII

INCORPORATION OF VARIOUS TRITIATED PRECURSORS
INTO MICROSOMAL VITELLOGENIN

Tritiated precursor	cpm/immunoprecipitate
Leucine	89,510
Galactose	8,345
Fucose	945
Mannose	105

Liver slices (0.7 g) were incubated with each precursor at a concentration of 25 μCi/ml. Incubations were for 1 h at room temperature in a volume of 3.0 ml. At the end of this period, microsomes were isolated and analyzed for immunoprecipitable radioactivity as described in "Materials and Methods."

Effect of Tunicamycin on Vitellogenin Glycosylation. The antibiotic tunicamycin has been used in several laboratories as an inhibitor of the glycosylation of proteins which exhibit N-glycosidic linkages between asparagine and N-acetylglucosamine (77-80). Its mode of action seems to be very specific in that it prevents the transfer of the first N-acetylglucosamine to the lipid acceptor, dolicholphosphate (reviewed in ref. 62). This, in turn, prevents the formation of the oligosaccharide core which would normally be transferred en bloc to the appropriate asparagine residue of vitellogenin. If a pool of this lipid-oligosaccharide intermediate was both large and also slow in its turnover rate, then the impairment of mannose incorporation might be explained by these contributing factors. We have used tunicamycin to inhibit glycosylation and therefore deplete the pool size of the lipid-oligosaccharide intermediates involved in the glycosylation of vitellogenin.

Fig. 17 shows that liver slices incubated with tunicamycin (1 or 3 μg/ml) show essentially no inhibition of [^{3}H]leucine incorporation into acid-insoluble radioactivity secreted into the media (Fig. 17A). This secreted product has been shown to be almost totally vitellogenin (5). Under the same conditions, however, [^{3}H]glucosamine incorporation is decreased by about 60% after a total of 10 h in the presence of the antibiotic (Fig. 17B). When liver cultures were harvested after at least 7 h in tunicamycin and assayed for immunoprecipitable [^{3}H]leucine or [^{3}H]glucosamine in solubilized microsomes, the results in Fig. 18 were obtained. After incubation in tunicamycin (1 or 3 μg/ml) for the

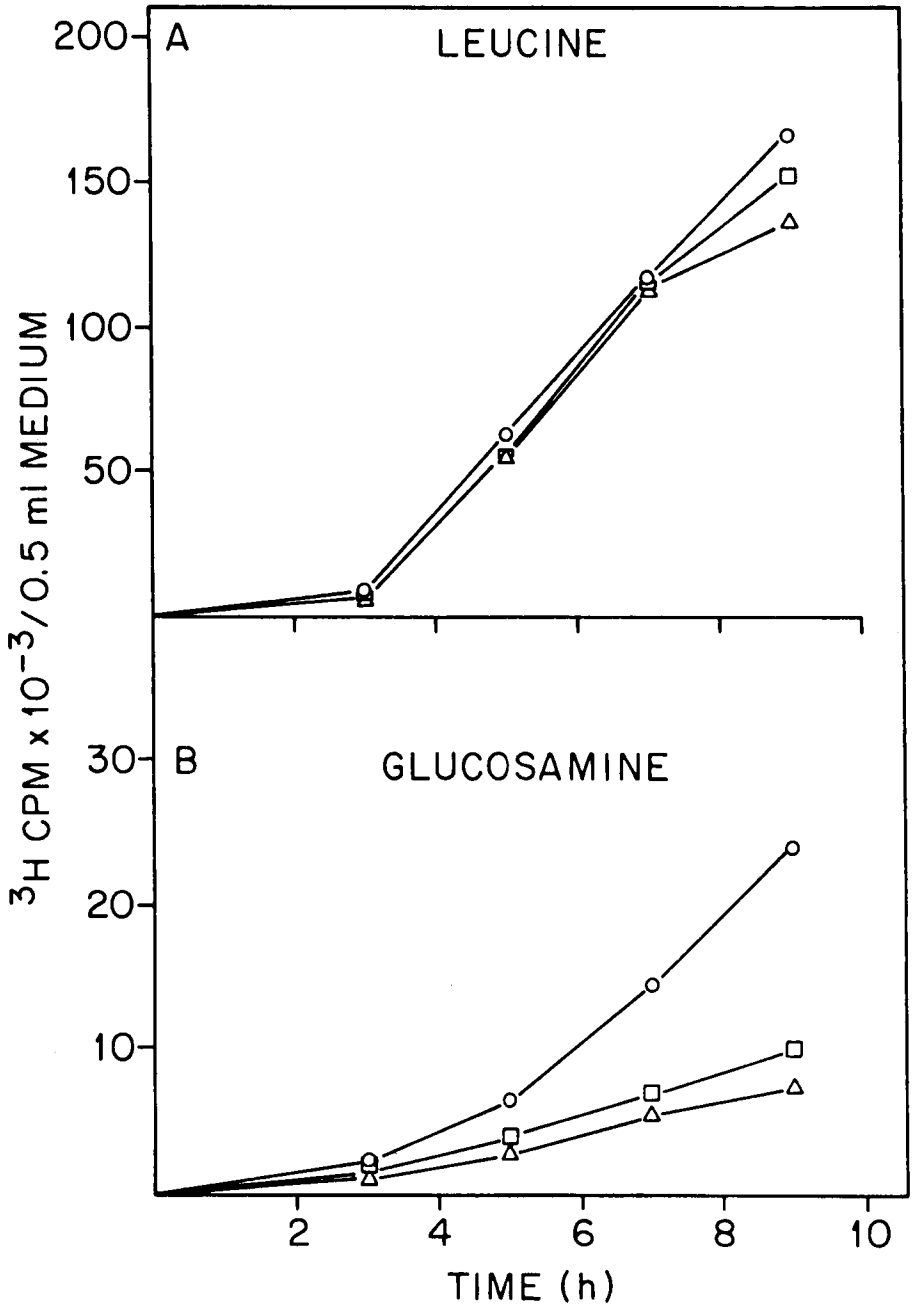

FIG. 17. Incorporation of (A) [^{3}H]leucine (5 μCi/ml) and (B) [^{3}H]glucosamine (5 μCi/ml) into acid-insoluble materials secreted into the medium. Liver slices (0.5) were preincubated for 1 h in nonradioactive medium containing 0, 1, or 3 μg/ml of tunicamycin. After 1 h the culture medium was replaced with the appropriate radioactive medium so that each culture possessed the same concentration of tunicamycin that was present during the 1 h preincubation. At the indicated times, 0.5 ml of the medium was analyzed for acid-insoluble radioactivity as described in "Materials and Methods." Tunicamycin concentrations were 0 (o), 1 (□), or 3 (Δ) μg/ml.

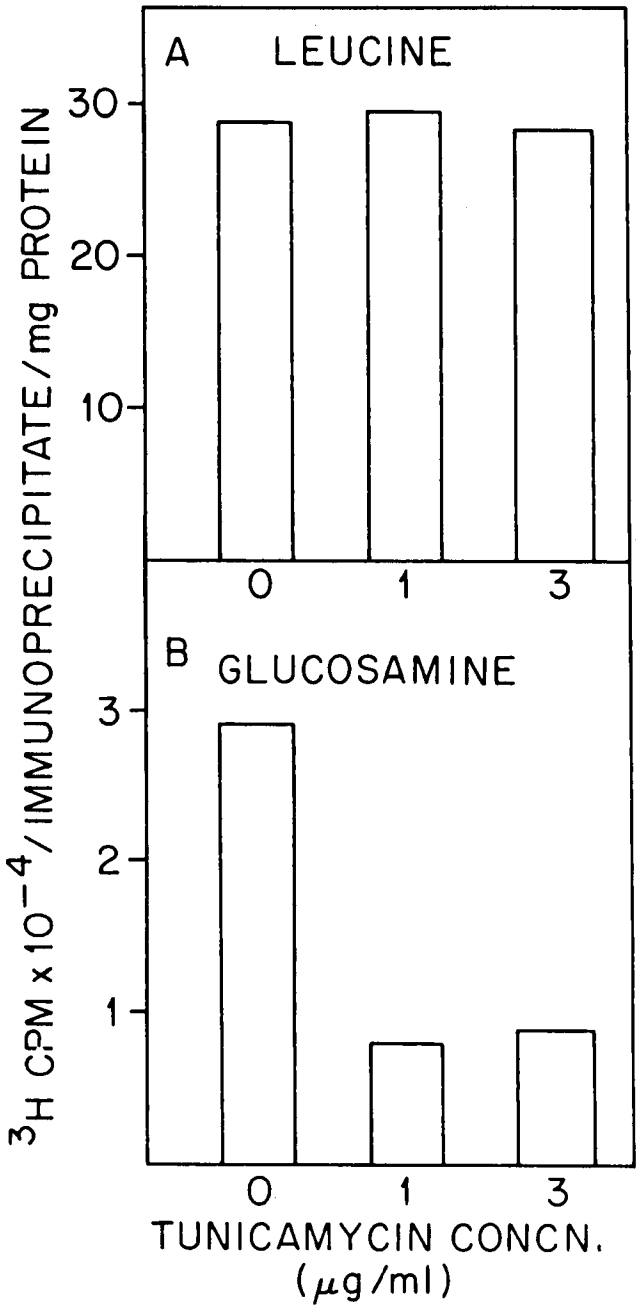

FIG. 18. Effect of tunicamycin on both (A) [^{3}H]leucine (10 μCi/ml) and (B) [^{3}H]glucosamine (10 μCi/ml) incorporation into microsomal vitellogenin. Liver explants (0.5 g) were preincubated in nonradioactive medium containing tunicamycin at concentrations of 0, 1, or 3 μg/ml. At the end of 1 h the explants were switched to medium containing [^{3}H]leucine or [^{3}H]glucosamine which included tunicamycin at the same concentration present during the 1-h preincubation. The cultures were labeled for 6 h. At the end of the labeling period, the slices were processed so that whole microsomes could be obtained and analyzed for immunoprecipitable radioactivity.

allotted time, the cultures exhibited essentially no effect on the synthesis of the vitellogenin peptide (Fig. 18A). The glycosylation of vitellogenin, however, was inhibited by 70% (Fig. 18B). We conclude, therefore, that treatment of liver slices for at least 7 h with tunicamycin under these conditions should significantly deplete the lipid-oligosaccharide pool, without affecting the rate at which vitellogenin is synthesized.

We proceeded to incubate four flasks of liver slices in tunicamycin (3 μg/ml) for 9 h. At the end of this period, we washed the tissue slices and placed them on ice with ice-cold medium containing [^{3}H]mannose or [^{3}H]glucosamine. The incorporation of each sugar was measured in the absence and presence of tunicamycin (3 μg/ml) after a 30-min equilibration at 0 to 2°C followed by a 2-h incubation at room temperature. At the end of this incubation we isolated microsomes and assayed for immunoprecipitable radioactivity. The results are shown in Table VIII. Under conditions whereby vigorous incorporation of [^{3}H]glucosamine was demonstrated with a 56% sensitivity to tunicamycin, we still observe negligible amounts of [^{3}H]mannose incorporated into vitellogenin. That [^{3}H]glucosamine was incorporated well after removal of the tunicamycin-containing medium indicates that the tissue slices recover well from the long pretreatment with the antibiotic. The inability to achieve significant [^{3}H]mannose incorporation even under conditions in which lipid-oligosaccharide pools have been depleted, led us to perform a structural analysis of the vitellogenin oligosaccharide.

Analysis of the neutral hexoses of vitellogenin by gas-liquid chromatography of the alditol acetate derivatives (Fig. 19) showed that

TABLE VIII

EFFECT OF TUNICAMYCIN PRETREATMENT ON [^{3}H]GLUCOSAMINE AND [^{3}H]MANNOSE INCORPORATION INTO MICROSOMAL VITELLOGENIN

Tritiated sugar	cpm/immunoprecipitate	
	Without tunicamycin	With tunicamycin
Glucosamine	6545	2895
Mannose	108	81

Liver slices (0.5 g) were pretreated with tunicamycin (3 µg/ml) at room temperature for 9 h. The cultures were then washed, and their ability to incorporate [^{3}H]glucosamine and [^{3}H]mannose, each at 20 µCi/ml, into microsomal vitellogenin in the absence and presence of tunicamycin (3 µg/ml) was tested. Microsomes were isolated and analyzed as described in "Materials and Methods."

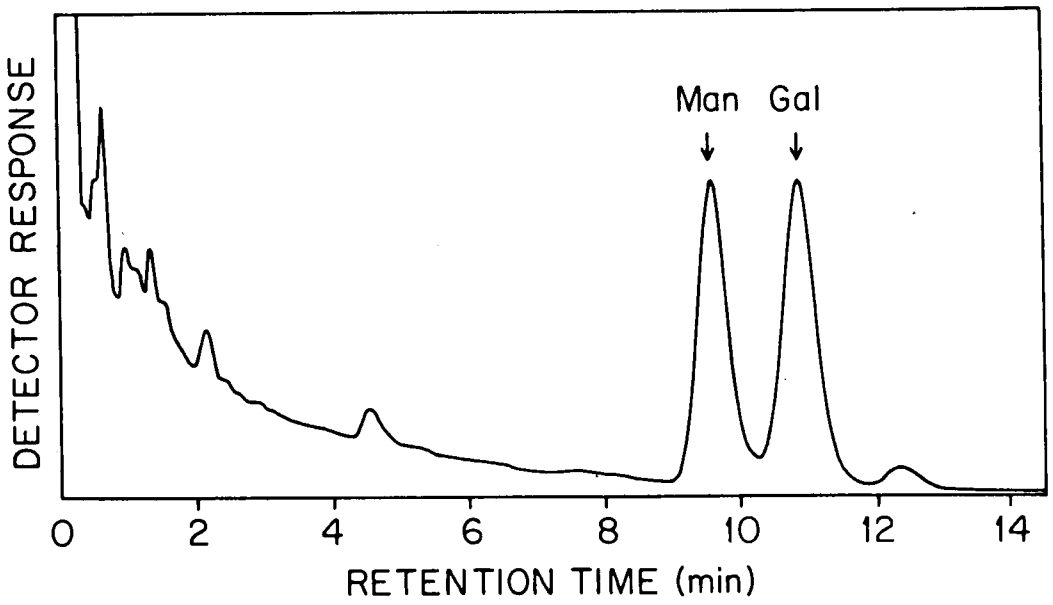

FIG. 19. Gas-liquid chromatography of the alditol acetate derivatives of the neutral hexoses derived from vitellogenin. The preparation and analysis of the derivatives are described in "Materials and Methods." Arrows indicate the retention times of the appropriate standards.

serum vitellogenin possesses approximately equimolar amounts of both mannose and galactose. As a control a commercial preparation of ovalbumin was treated identically to vitellogenin. As expected (76), this preparation yielded only one peak, which had a retention time identical to that of mannose (not shown). As will be discussed below, these data provide much insight into the probable class of oligosaccharide structure to which the glycosyl moiety of vitellogenin belongs. It appears then that the unexplainable inability of Xenopus liver to use [^{3}H]mannose as a precursor to the oligosaccharide chain of vitellogenin is an unfortunate idiosyncrasy of the system.

The experiments reported above shed no light upon the possibility that pretreatment with tunicamycin might deplete a pool of lipid-sugar intermediates localized within the rough endoplasmic reticulum. To this end, cultures of liver slices were incubated in the absence and presence of tunicamycin (3 μg/ml) for 12 h. The tissue slices were then washed and placed on ice. After a 30-min equilibration at 0 to 2°C in radioactive medium containing [^{3}H]glucosamine but no tunicamycin, the tissue was incubated for 1 h at room temperature. The incubation was ended by several washes in ice-cold, divalent cation-free O-R2 (41) followed by three washes in 0.4 M sucrose at pH 7.4. Rough endoplasmic reticulum and smooth microsomes were isolated as previously described (56) and analyzed for immunoprecipitable radioactivity. It is clear that the distribution of radioactivity between fractions is not altered by pretreatment of liver slices with tunicamycin (Fig. 20). In both cases, the rough endoplasmic reticulum shows negligible incorporation, as originally shown (Fig. 13A, p. 61).

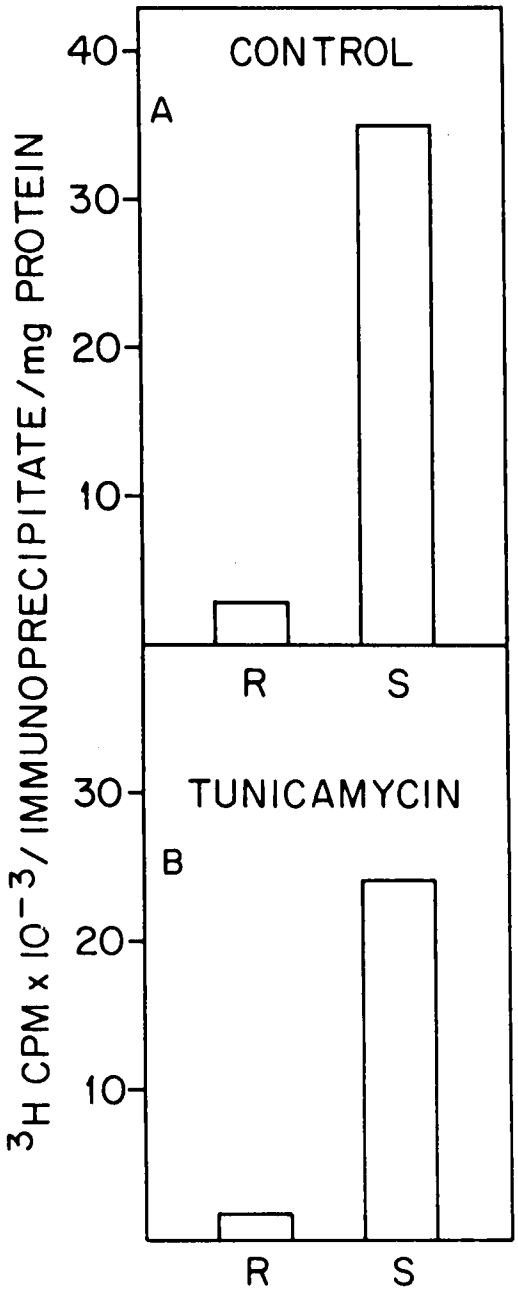

FIG. 20. The effect of tunicamycin (3 μg/ml) on the intracellular distribution of [^{3}H]glucosamine (30 μCi/ml) incorporated into vitellogenin. Liver slices (1.5 g) were incubated for 12 h in the (A) absence and (B) presence of tunicamycin (3 μg/ml). Both cultures were washed and equilibrated on ice for 30 min with 4.0 ml of radioactive medium lacing tunicamycin. After 30 min they were brought to room temperature and incubated for 1 h. At the end of this period the liver slices were processed for subcellular fractionation. The resultant rough endoplasmic reticulum and smooth microsomes were analyzed for immunoprecipitable radioactivity. R, rough endoplasmic reticulum; S, smooth microsomes.

A similar experiment was performed to show that the inhibition by tunicamycin of [^{3}H]glucosamine incorporation into vitellogenin could be localized within the smooth microsomes. Again cultures were incubated in the absence and presence of tunicamycin (3 μg/ml) for 12 h. At the end of this period, the slices were cold equilibrated as usual, then subjected to a brief 30-min pulse at room temperature in medium containing [^{3}H]glucosamine. The control culture (i.e., no tunicamycin present during the 12-h preincubation) was labeled in tunicamycin-free medium. Explants which were preincubated in tunicamycin for 12 h were pulse-labeled in medium which also possessed tunicamycin. At the end of 30 min, the explants were processed as in the experiment described in Fig. 20, and the microsomal subfractions were isolated and analyzed. Fig. 21 shows the results obtained. Tunicamycin inhibited [^{3}H]glucosamine incorporation into the smooth microsomes by 75%. The demonstration that tunicamycin sensitivity in Xenopus liver resides in a smooth microsomal compartment, in conjunction with the experiments presented in Figs. 13 and 14 (pps. 61 and 63) and Fig. 20, thus indicates that vitellogenin is glycosylated within the smooth membranes of the hepatocyte.

Discussion

The data presented in this report allow us to make some conclusions about how vitellogenin, an estrogen-induced serum glycolipophosphoprotein, is glycosylated. We have examined the subcellular localization of these events (Figs. 13, 14, 20, 21) and have, in addition, been able to implicate a dolicholphosphate-type intermediate in the transfer of the

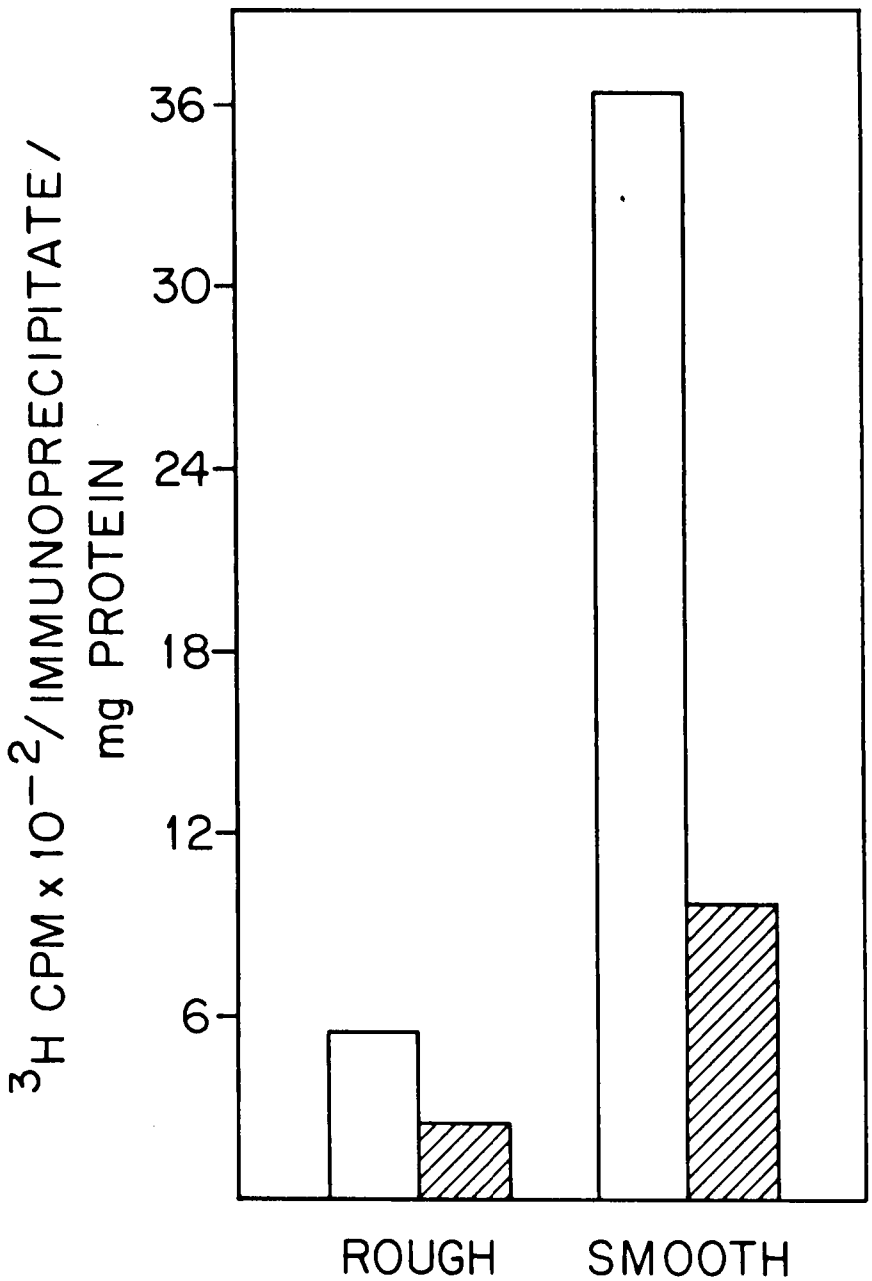

FIG. 21. Effect of tunicamycin (3 µg/ml) on [^{3}H]glucosamine (30 µCi/ml) incorporation into the smooth microsomes. Liver slices (1.5 g) were preincubated for 12 h in the absence and presence of tunicamycin (3 µg/ml). Both cultures were washed and equilibrated on ice for 30 min with 3.0 ml of radioactive medium containing the same concentration of tunicamycin present during the 12 h pretreatment. After 30 min on ice, the flasks were brought to room temperature for a brief 30 min pulse. At the end of this period, the liver slices were processed for subcellular fractionation. The resultant rough endoplasmic reticulum and smooth microsomes were analyzed for immunoprecipitable radioactivity. Open bars, radioactivity from control cultures; hatched bars, radioactivity from tunicamycin-treated cultures.

oligosaccharide unit(s) to the vitellogenin peptide (Figs. 17 and 18, pps. 71 and 72; Fig. 21).

Using [^{3}H]galactose as an indicator for terminal glycosylation, we could correlate the rapid clearance of [^{3}H]galactose pulse-labeled vitellogenin from hepatocytes (Figs. 12 and 14A, inset; pps. 59 and 63) with the rapid appearance of this material in the chase medium (Fig. 16, p. 68). Both of these observations would result if [^{3}H]galactose had been incorporated at a time when the secretory protein was near to being secreted from the cell (65, 68, 81). We have also pulse-labeled liver slices for 30 min with [^{3}H]galactose, processed thin sections for electron microscopic autoradiography, and noted that the silver grains were more highly concentrated over the Golgi-derived, electron-dense, secretory granules than the cisternae (data not shown). Some grains were also present in intercellular spaces and probably represent secreted material. These morphological observations strongly support our conclusion that [^{3}H]galactose conjugation to the vitellogenin peptide is a very late event in vitellogenin processing.

By contrasting the above results with the kinetics of intracellular translocation (Figs. 12 and 13A, inset; pps. 59 and 61) and secretion (Fig. 16) of [^{3}H]glucosamine-labeled vitellogenin, we can conclude that glucosamine is not a terminal monosaccharide, nor is it converted into one (Table VI, p. 66). This is to be expected since N-acetylglucosamine is generally located closer to the actual glycopeptide linkage of glycoproteins than is galactose (61, 76) and therefore must be attached prior to galactose.

One can only speculate as to why, after galactose-labeled vitellogenin begins to be secreted, there exists a lag of at least 1 h before the appearance of the glucosamine-labeled protein. One possibility is underlined by autoradiography at the ultrastructural level which shows that [^{3}H]galactose is incorporated into vitellogenin within secretory vesicles (data not shown). That this stage in vitellogenin packaging occurs 90 to 120 min after synthesis of the peptide backbone (38, 56) indicates that a large span of time during which various posttranslational modifications of the protein may occur does, in fact, exist. Another factor contributing to this lag is that a sequence of interdependent events might be required to transpire prior to the attachment of galactose. For example, in our Golgi-enriched fraction there exists an enzyme activity which transfers galactose from UDP-galactose to N-acetylglucosamine (56). If, in vivo, a minimum amount of N-acetyl-glucosamine transfer to vitellogenin is required to provide a suitable substrate for galactose transfer, the time required for the formation of this substrate might be a significant factor in the production of the lag period. Other processing steps undetected in our studies might also be involved.

We have also observed that approximately 86% of the incorporated glucosamine radioactivity in vitellogenin is not released by dilute alkali as acid-soluble material (data not shown). In addition, tunicamycin inhibits glucosamine incorporation by 70% (Figs. 17B and 18B, pps. 71 and 72; Fig. 21). By these criteria one can conclude that almost all of the vitellogenin carbohydrate is linked to the polypeptide

chain by an N-glycosidic bond between the amide nitrogen of asparagine and the core sugar N-acetylglucosamine (61, 76). The N-glycosidic linkage is not alkali-labile under mild conditions (76). Also, the formation of this bond can be specifically inhibited in vivo or in cell-free systems by the antibiotic tunicamycin (reviewed in ref. 61).

The above data, coupled with our radiolabeling and structural studies, show that Xenopus vitellogenin possesses the "complex" type oligosaccharide unit (60). This means that in addition to the core sugars N-acetylglucosamine and mannose, vitellogenin also possesses terminal monosaccharides. These were detected in cultures as both tunicamycin-resistant (i.e., noncore or terminal) [^{3}H]glucosamine (Fig. 18, p. 72 and Table VIII, p. 74) and [^{3}H]galactose incorporation (Figs. 12, 14, 16; pps. 59, 63, 68). An early report showed that sialic acid was also present (11). The presence of glucosamine and galactose was also confirmed by paper chromatography of the sugars derived from vitellogenin (not shown). This analysis incorrectly yielded a negative result for mannose, and led us to the more sensitive and quantitative method of gas-liquid chromatography (Fig. 19, p. 75). As can be seen, approximately equal amounts of mannose and galactose can be demonstrated. Taken together, the data indicate that the glycosyl moiety of Xenopus vitellogenin closely resembles other N-glycosidically linked "complex" units (60).

Much work has been aimed at understanding the cellular mechanisms involved in the assembly of this class of glycoprotein. Models which summarize data obtained in mammalian and avian systems have recently

appeared (60, 61). In brief, the initial step in protein glycosylation is the transfer of a high mannose-containing oligosaccharide from a preassembled oligosaccharide-dolicholpyrophosphate intermediate, thus forming an N-glycosidic bond between N-acetylglucosamine and an appropriately oriented asparagine residue. Both kinetic and subcellular fractionation data (65, 73, 74, 81-86) indicate that this step occurs in the rough endoplasmic reticulum. In some cases it has been shown that the incomplete nascent peptide is glycosylated before its release into the lumen of the rough endoplasmic reticulum (69-71). After transfer to the protein, the newly attached oligosaccharide core may remain relatively unaltered, or it can undergo various transformations, beginning with the removal of several mannose residues (73, 74, 82). This "trimming" of mannosyl groups may then be followed by the sequential addition of N-acetylglucosamine and the terminal glycosyl units galactose, sialic acid, and fucose. Most of these later stages occur in the smooth endoplasmic reticulum and Golgi apparatus. The results we present here suggest that this model can probably account for most of the events involved in the assembly of the glycosylated subunit of vitellogenin in Xenopus liver.

The major divergence from the proposed models lises in our inability to demonstrate glycosylation of vitellogenin in the rough endoplasmic reticulum (Figs. 13, 14, 20, pps. 61, 63, 77; Fig. 21). To explore this further, we took advantage of the fact that after pretreatment with tunicamycin, the liver slices recover and incorporate [^{3}H]glucosamine into both the tunicamycin-sensitive (i.e., core) and tunicamycin-resistant

(i.e., terminal) portions of the vitellogenin oligosaccharide (Table VIII, p. 74). The experiment presented in Fig. 20 (p. 77) showed that after pretreatment with the antibiotic, the liver slices incorporate [^{3}H]glucosamine into the smooth membrane fraction at almost control levels. The slightly diminished labeling is probably due to the inability to totally remove by washing the residual tunicamycin trapped within the liver slices. If after pretreatment with the antibiotic the cultures are pulse-labeled for only 30 min in the presence of tunicamycin, incorporation of [^{3}H]glucosamine into the smooth membrane fraction is inhibited by 75% (Fig. 21). Therefore we conclude that tunicamycin sensitivity and, more specifically, the attachment of the core region of the oligosaccharide to the peptide backbone of vitellogenin occur within the smooth membrane compartment of the hepatocyte. This is in direct contrast to studies in other systems which show labeling of glycoproteins with radiolabeled glucosamine and mannose within the rough endoplasmic reticulum both in vivo and in vitro (65, 69-71, 83, 85).

In hen oviduct, the enzyme activity responsible for transferring mannose from GDP-mannose to endogenous and exogenous acceptors was located primarily in the rough endoplasmic reticulum (87). In rat liver, however, comparable amounts of this and other enzyme activities involved in the same process have been demonstrated in smooth endoplasmic reticulum and Golgi fractions (83, 88). Determining the distribution of these enzymes within Xenopus liver is clearly the next step in elucidating the mechanisms governing vitellogenin glycosylation.

In a previous report we described the phosphorylation of vitellogenin (56). [^{32}P]Orthophosphate-labeled vitellogenin shows almost identical

kinetics of intracellular translocation and intracellular compartmentalization with respect to [^{3}H]glucosamine-labeled vitellogenin. The two precursors also share identical secretion kinetics (Fig. 16, p. 68). It is reasonable to conclude that both phosphorylation and glucosaminylation of vitellogenin are occurring simultaneously within a smooth membrane compartment but involving different regions of the vitellogenin molecule, i.e., glycosylation in the lipovitellin region (10) and phosphorylation in the phosvitin region (10, 89). The addition of galactose to vitellogenin occurs later, during packaging into secretory vesicles, and just prior to secretion.

The data presented indicate that the liver of estrogen-stimulated Xenopus represents an excellent model system for studying the assembly of multicomponent proteins. Further work will be required to elucidate in detail the steps involved in the posttranslational modifications of vitellogenin. For example, the use of detergent extracts of the various microsomal subfractions should provide insight into the nature and regulation of the enzymes involves in these processes. Experiments using this approach are now under way.

CHAPTER V

CONCLUSIONS

The events involved in the posttranslational modifications of vitellogenin can be summarized graphically by the model presented in Fig. 22. The intact newly synthesized peptide is partially phosphorylated after its release into the lumen of the rough endoplasmic reticulum. The subsequent segregation of vitellogenin within the budding smooth-surfaced regions of the rough endoplasmic (referred to as "transition elements") is inferred from the ultrastructure of these hepatocytes. Additional phosphorylation of vitellogenin occurs within its carboxy-terminal region during this vesicular transit to the Golgi apparatus. The kinetic evidence would indicate that by the time vitellogenin is being packaged into secretory vesicles, phosphorylation has ceased.

With respect to the protein kinase involved in this process, it appears to be an integral membrane protein whose catelytic site is accessible only to the luminal side of the microsomal vesicle. It requires either magnesium or manganese ions for activity, and is specific for ATP as phosphate donor. This phosphorylation process does not appear to be regulated by cyclic nucleotides.

Experiments have also focused on the glycosylation of vitellogenin. The presence of both core and terminal monosaccharides has led us to conclude that vitellogenin possesses an oligosaccharide which is linked by an N-glycosidic bond between asparagine and N-acetlyglucosamine. This was substantiated by the stability of this bond toward mild alkaline

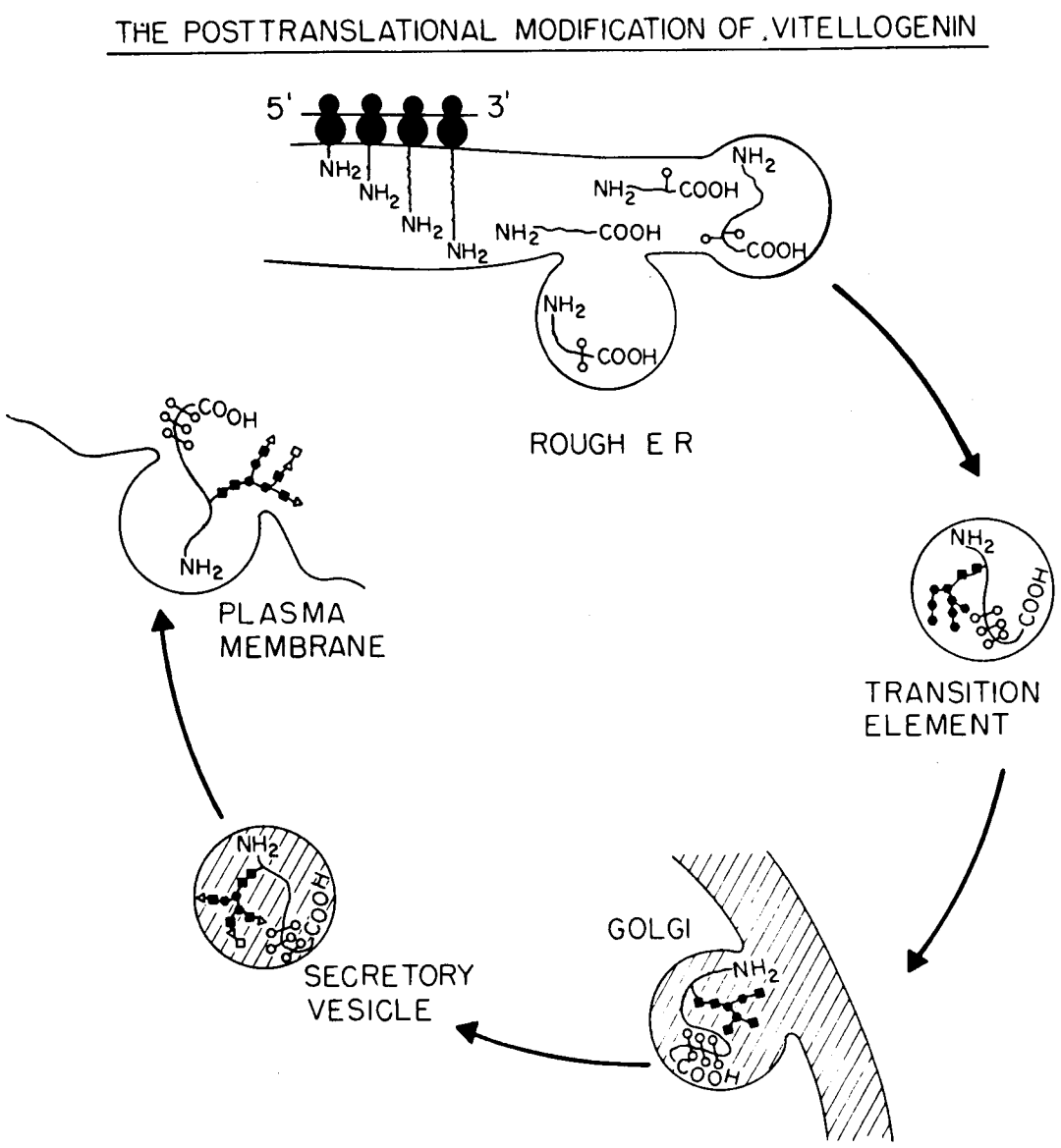

FIG. 22. Summary: The posttranslational modification and secretion of vitellogenin. (o) phosphate; (■) N-acetylglucosamine; (●) mannose; (Δ) galactose; (□) sialic acid.

hydrolysis. With what is known about glycoprotein assembly in other systems we can devise a scheme for the glycosylation of vitellogenin. The assembly of the core region occurs within the transition elements since [^{3}H]glucosamine incorporation is kinetically identical to [^{32}P]orthophosphate. The presence of mannose in vitellogenin would also indicate that the preassembled dolicholphosphate linked oligosaccharide possesses a high-mannose structure. After transfer to vitellogenin the oligosaccharide undergoes an enzymatic removal of several, but not all mannosyl groups. This trimming step is relatively slow, and is thought to occur within the Golgi. In my model the sequential addition of N-acetylglucosamine, galactose and sialic acid follows trimming. Kinetic evidence indicates that galactose (and probably sialic acid) is added just prior to secretion.

The lag in time between the secretion of [^{3}H]galactose- and [^{3}H]glucosamine-labeled vitellogenin indicates that some rate-limiting processes are occurring. One possibility is that correct assembly and processing of the core oligosaccharide might be a time consuming event. The sequential addition of monosaccharides to a growing side chain produces specific substrates acted upon by specific glycosyltransferases. Therefore in Xenopus the assembly of the proper substrate for the appropriate galactosyltransferase may occur as much as 60-75 min after the initial core glycosylation. That tunicamycin does not alter the secretion kinetics of vitellogenin shows that these processes are not necessary for secretion. Therefore other possibilities must be considered in order to explain the long lag period (90-120 min) between

vitellogenin synthesis and secretion. One such possibility might be the Golgi-dependent condensation of secretory products into the electron dense secretory granule. In any event, it appears that the rate-limiting steps in the hepatic processing of vitellogenin occur within the Golgi.

In most ways it seems that the vitellogenin peptide is processed very similarly to other proteins. The conspicuous lack of additional functions of the rough endoplasmic reticulum is however difficult to explain. This certainly is not the case with plasma cells, mammalian liver, or hen oviduct. In these cases the rough endoplasmic reticulum is capable of glycosylating newly made proteins, whereas Xenopus vitellogenin is glycosylated in a smooth membrane compartment. This might be behavior peculiar to Xenopus, amphibians in general, or even all vertebrates considered evolutionarily more primitive to the homeothermic species. Xenopus is in fact the first poikilotherm to be analyzed in such detail.

As a last note in this regard, I would like to bring attention to the fact that I have not dealt here with the lipidation of vitellogenin. Due to various technical problems, I would have had to rely heavily on data describing the subcellular distribution of the enzymes involved in lipid synthesis. My prediction is that these enzymes would in fact be present (though not necessarily exclusively) in the rough endoplasmic reticulum. Their absence would almost certainly make the task of coordinating the assembly of newly made membrane components into transferrable units very inefficient indeed.

The estrogen-stimulated liver of Xenopus would also be amenable to other kinds of experiments involving this induction of protein synthesis and secretion. The ability to purify vitellogenin in large amounts would make it relatively easy to prepare the actual glycopeptide from the intact protein. By sequential treatment of the glycosyl moiety with various glycosidases a series of substrates upon which the liver glycosyltransferases are tested can be prepared. This makes it possible to examine both the regulation of each of the glycosylation steps, and to determine which substrates (i.e., intracellular intermediates) are the important ones in vivo.

The role of the vitellogenin oligosaccharide can be analyzed in a very novel manner not easily available in other systems. Using liver slices, sufficient quantities of high specific activity vitellogenin (normal and nonglycosylayed) can be isolated in pure form. The role of the carbohydrate in the uptake and processing of vitellogenin by growing oocytes can then be analyzed using existing procedures.

It is apparent that many other problems in cell biology and biochemistry may be probed by analyzing various aspects of vitellogenesis. The dissertation presented here is a step in this direction.

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