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Synthesis and Insertion, both *in vivo* and *in vitro*, of Rat-Liver Cytochrome P-450 and Epoxide Hydratase into *Xenopus laevis* Membranes

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We described whole cell and cell-free systems capable of inserting into membranes cytochrome *P*-450 and epoxide hydratase made under the direction of rat liver RNA. The systems have been used to study the pathways followed by newly made secretory and integral membrane proteins. The cell-free system contains *Xenopus laevis* embryo membranes, and demonstrates competition for a common receptor between cytochrome *P*-450 and epoxide hydratase, and normal secretory proteins: evidence is provided for differential membrane receptor affinity. Thus, synthesis of secretory and membrane proteins appears to involve a common initial pathway. Microinjection of rat liver RNA into whole oocytes suggests that membrane insertion is neither cell type nor species specific, because functional rat liver enzymes are found inserted in the endoplasmic reticulum of the frog cell. Nonetheless, insertion is highly selective since albumin and several other proteins made under the direction of the injected liver RNA are sequestered within membrane vesicles and are then secreted by the oocyte, whilst epoxide hydratase and cytochrome *P*-450 are inserted into membranes but are not secreted.

The liver microsomal enzymes cytochrome *P*-450 and epoxide hydratase play a central role in the metabolism of both xenobiotics and endogenous compounds such as hormones [1–4]. These interesting enzymes also serve as model systems for the study of membrane synthesis and assembly [5, 6] and cytochrome *P*-450 is unusual in that it occurs on both sides of the endoplasmic reticulum [7]. It is clear that a substantial portion of at least some cytochrome *P*-450 molecules is exposed to the cytosol [8–11], but the precise orientation of the molecule within the phospholipid bilayer remains to be established. There are multiple forms of these two liver enzymes inducible by different agents [7, 12–15], but the symmetry of distribution of the different forms has not been studied, nor has their biosynthesis been examined in detail. There is evidence that some newly made cytochrome *P*-450 is found free in the lumen of the endoplasmic reticulum [16] although studies *in vitro* [6] suggest cotranslational insertion of the protein: nonetheless the existence of an hydrophobic N-terminal sequence in both primary [6] and inserted [17, 18] proteins suggests that the assembly within membranes of both epoxide hydratase and cytochrome *P*-450 involves at least the first stage [19, 20] of the secretory pathway [21].

In the present paper we describe whole cell and cell-free systems, both of which insert cytochrome *P*-450 and epoxide hydratase made under the direction of added messenger RNA: the systems have been used to study the initially confluent and subsequently divergent pathways followed by newly made secretory and integral membrane proteins.

EXPERIMENTAL PROCEDURE

RNA Extraction

RNA from rapidly sedimenting rat liver endoplasmic reticulum was prepared by phenol/chloroform extraction [22]. Chicken oviduct and guinea pig mammary gland poly(A)-rich RNAs were the kind gifts of Dr M. Doel and Dr R. Craig, respectively.

Oocyte Microinjection and Fractionation

30–40 *Xenopus laevis* oocytes were each injected with about 50 nl of rapidly sedimenting endoplasmic reticulum RNA (200 A_{260} units/ml), and were left in modified Barth X medium for about 16 h to permit messenger recruitment [23, 24] and removal of dying oocytes. The oocytes were then incubated at 19 °C for 20 h in medium containing 300 μCi [^{35}S]methionine (900–1100 Ci/mmol, Amersham, UK) at 2 μCi/μl. Uninjected control oocytes were treated similarly. The oocytes were then washed and homogenized in buffer T (20 mM Tris/acetate pH 7.6, 50 mM KCl, 10 mM magnesium acetate and 6 mM 2-mercaptoethanol) containing 0.3 M sucrose.

The yolk platelets and pigment were removed by layering the homogenate over a 400-μl cushion of 1.5 M sucrose made up in buffer T, and then centrifuging for 10 min at 12000 × *g*. The membranes were collected by centrifuging the combined interface and supernatant at 4 °C in a Beckman type-65 rotor at 35000 rev./min for 0.5 h; the remaining supernatant fraction contains the newly made cytosolic proteins of the oocyte. The oocyte membranes were finally washed with 0.5 M NaCl made up in homogenization medium, prior to immunoprecipitation.

Measurement of enzymes made under the direction of injected rat liver RNA required longer incubation periods (3 days at 19 °C) and larger batches (150 oocytes) of both injected and control cells. After removel of imperfect oocytes, membranes were prepared by the normal procedure.

Vesicles used for studies of membrane protein synthesis and insertion *in vitro* were prepared as above, but without the high salt wash, from dejellied (2% cysteine, pH 8.0) blastula stage embryos. The blastula vesicles were immediately frozen in

liquid nitrogen and stored at −70 °C. Membranes from whole oocytes or from translation systems *in vitro*, used for the preparation of integral membrane proteins, were washed once with 0.5 M NaCl, 0.05% sodium deoxycholate made up in buffer T.

Cell-Free Translation

Nuclease-treated reticulocyte lysates, programmed with 0.7 A_{260} unit of rat liver RNA (corresponding to 0.45 µg of polyadenylated RNA) per 100 µl of cell-free system [22], were incubated for 1.5 h. Insertion of membrane proteins *in vitro* was carried out by adding blastula membranes (100 µg protein/100-µl incubation volume) at the onset of translation, followed 2 min later by synchronization with pactamycin (1 µM). After the incubation, the membranes were recovered by centrifugation at 4 °C for 20 min at 12000 × *g* through 600 µl of 0.3 M sucrose in buffer T, and were then washed once in buffer T, containing 0.5 M KCl and 0.3 M sucrose. Sequestered products were defined by their resistance to proteolytic digestion [25].

Immunoprecipitation

Immunoprecipitations were performed in 400 µl of 20 mM Tris/HCl pH 8.3, 300 mM NaCl, 1% Nonidet NP40 containing 100 µg/ml phenylmethylsulfonyl fluoride. Albumin synthesized *in vitro* was immunoprecipitated as described previously [22], while cytochrome *P*-450 and epoxide hydratase were precipitated with antibodies raised against antigens from phenobarbital-treated rats [13, 15, 26]. Immunoprecipitates formed for 20 h at 4 °C were collected and washed five times in the above medium. Radioactivity in proteins purified by immunoprecipitation was measured by computer-integrated densitometry of gel autoradiograms.

Gel Electrophoresis

Dodecylsulphate polyacrylamide gel electrophoresis staining and destaining were performed according to Laemmli [27], while the procedure of Cleveland et al. [28] was used for proteolytic digestion of bands excised from dried gels. Digestion was carried out over a 60-min period in the spacer gel, using 0.1 µg/slot of *S. aureus* protease V8 (Miles Laboratories, Ltd, UK). Fluorography was performed by the method of Bonner and Laskey [29].

Enzyme Assays

Epoxide hydratase activity was measured using [7-^{3}H]styrene oxide as substrate [30]. 7-Ethoxycuomarin *O*-deethylase activity was determined as described elsewhere [13].

RESULTS

Synthesis of Cytochrome P-*450 and Epoxide Hydratase* in vivo *and* in vitro

The specificity of insertion into membranes and of post-translational modification of rat cytochrome *P*-450 and epoxide hydratase made under the direction of liver rapidly sedimentating endoplasmic reticulum RNA can be investigated using the *Xenopus* oocyte microinjection system: moreover, translation in the reticulocyte lysate, in the absence of added membranes, permits comparisons between free and

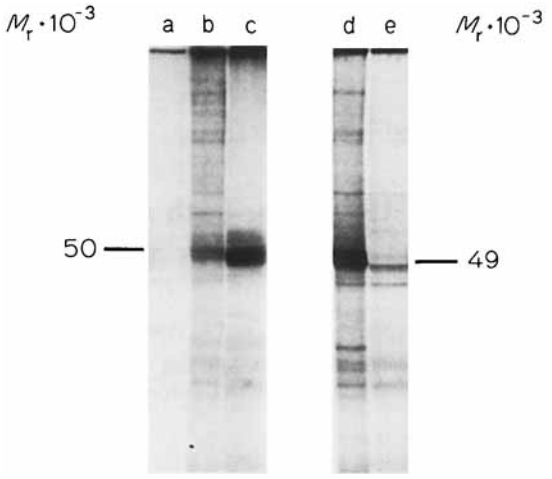

Fig. 1. *The association of newly synthesized rat liver cytochrome* P-*450 and epoxide hydratase with the* Xenopus *oocyte membrane fraction.* Batches of oocytes were injected with rat liver rapidly sedimenting endoplasmic reticulum RNA, labelled with [^{35}S]methionine and membrane vesicles were prepared by subcellular fractionation. (a, b) Amounts of immunoprecipitable cytochrome *P*-450 present in membrane fractions from control (track a) and RNA-injected (track b) oocytes. (c) Immunoprecipitable cytochrome *P*-450 made in the reticulocyte lysate under the direction of added liver rapidly sedimenting endoplasmic reticulum RNA. (d, e) Amounts of immunoprecipitable epoxide hydratase present in membrane fractions from control (track e) and RNA-injected (track d) oocytes. In (a) is shown the mobility of a marker of M_r 50000. Rat liver cytochrome *P*-450 is found in our gel system within the M_r range 48000 – 53000. In (e) is shown the apparent molecular weight of epoxide hydratase (49000) purified from the livers of phenobarbital-treated rats

inserted forms of these two liver enzymes. Thus, RNA-injected and control oocytes were labelled with [^{35}S]methionine, membrane and cytosol (supernatant) fractions were prepared and immunoprecipitated using specific antisera raised against the rat liver enzymes. Immunoprecipitable cytochrome *P*-450 and epoxide hydratase were only detectable in oocytes injected with liver RNA and, as shown in Fig. 1, there is an association between the newly made enzymes and the oocyte membrane fraction: neither enzyme is detectable in the cytosol fraction (data not shown) nor can they be seen in the membrane fraction from control oocytes. The cytochrome *P*-450 found in association with the oocyte membrane migrates with both immunoprecipitated cytochrome *P*-450 made in the rabbit reticulocyte lysate (track c), and with the enzyme purified from the livers of phenobarbital-treated rats. Thus there appears to be no precursor to either native or induced [31] cytochrome *P*-450. There is also no detectable difference in gel mobility between epoxide hydratase made in oocytes and that made in lysates, even if the cell-free system lacks added *Xenopus* membranes (see Fig. 2). The immunoprecipitable species of M_r 49000 made in translation systems both *in vivo* and *in vitro* migrates with epoxide hydratase purified from livers of phenobarbital-treated rats.

Insertion of rat liver cytochrome *P*-450 and epoxide hydratase *in vivo* into *Xenopus* membranes was correct as judged by two criteria. Firstly, as shown in Fig. 2, epoxide hydratase is assembled in the frog oocyte membrane as an integral protein. Tracks (a) and (b) show that some of the epoxide hydratase associated *in vivo* is resistant to extraction with buffer T containing 0.5 M NaCl and 0.05% sodium deoxycholate. Tracks (c) and (d) reveal the resistance to extraction of a portion of the enzyme inserted *in vitro* which was

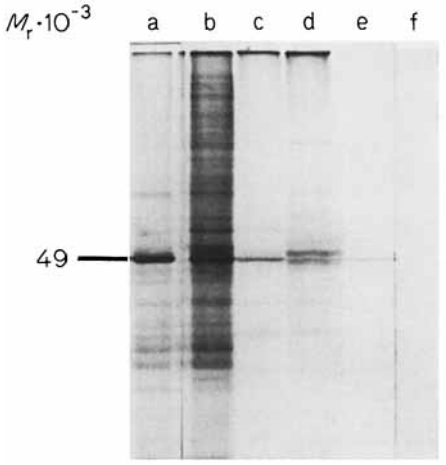

Fig. 2. *Insertion* in vitro *and* in vivo *of newly made rat liver epoxide hydratase into* Xenopus *membranes. Xenopus* oocyte membranes were prepared from [^{35}S]methionine-labelled oocytes injected with liver rapidly sedimenting endoplasmic reticulum RNA. Epoxide hydratase immunoprecipitates of whole membranes (track a) or those washed with buffer T containing 0.5 M NaCl and 0.05 % sodium deoxycholate (track b) were analyzed on gels. *Xenopus* embryo membranes were added to a reticulocyte lysate containing liver rapidly sedimenting endoplasmic reticulum RNA and were recovered by centrifugation. Epoxide hydratase content was estimated by immunoprecipitation both before (track c) and after (track d) washing with deoxycholate. Track (e) shows epoxide hydratase immunoprecipitated from a lysate containing liver RNA but no *Xenopus* membranes. The mobility of the 49000-M_r epoxide hydratase species found in the livers of phenobarbital-treated rats is denoted by a bar. Track (f) shows immunoprecipitates from a lysate containing no added RNA

Table 1. *Enzyme activity of membrane-associated cytochrome* P-*450 and epoxide hydratase made in oocytes under the direction of rat liver rapidly sedimenting endoplasmic reticulum RNA*
Xenopus laevis oocyte membranes from injected and uninjected oocytes were prepared and assayed for cytochrome *P*-450 and epoxide hydratase enzyme activities as described in the text. Results are expressed as the mean deviation of triplicate determinations

Source of *Xenopus* membranes	Activity of	
	7-ethoxy-coumarin *O*-deethylase	styrene oxide hydratase
	pmol min^{-1} mg protein^{-1}	nmol min^{-1} mg protein^{-1}
A RNA-injected oocytes	9.8 ± 1.1	0.65 ± 0.075
B Uninjected control oocytes	0	0.32 ± 0.075

obtained by translating rat liver RNA in a reticulocyte lysate supplemented with blastula membranes. Similar results have been obtained with cytochrome *P*-450, showing that a portion of forms inserted both *in vivo* and *in vitro* behave as integral proteins. More direct evidence of correct orientation requires demonstration of biological activity. Table 1 shows that a large and statistically significant increase in 7-ethoxycoumarin *O*-deethylase activity, an activity specific to cytochrome *P*-450, was evident in membranes from oocytes injected with liver RNA. The epoxide hydratase assay which was based on styrene oxide hydratase activity, was less clear out, for endogenous levels were high: nonetheless there was a twofold increase over the levels of uninjected control oocytes.

Peptide Analysis

Peptide mapping confirmed the similarity to the two liver enzymes of proteins (made in *Xenopus laevis* oocytes and reticulocyte lysates under the direction of rat liver RNA) which react with specific cytochrome *P*-450 and epoxide hydratase antibodies. One-dimensional maps of the quality of those in Fig. 3 provide evidence of the identity of the proteins that react with the antibodies: this is important given the high background seen in some experiments (Fig. 1, track b) involving immunoprecipitation of small amounts of radioactive cytochrome *P*-450 formed in oocytes. However, maps of higher quality are required to demonstrate the presence of absence of specific peptides, such as putative signal sequences. Mapping studies are occasionally complicated by the resolution of multiple immunoprecipitable species (*in vivo* and *in vitro*) migrating in the regions of cytochrome *P*-450 and epoxide hydratase, all made under the direction of liver RNA. Fig. 3 shows dodecylsulphate gel autoradiograph tracks of protease-digested cytochrome *P*-450 (A) and epoxide hydratase (B). Fig. 3A shows peptide products from cytochrome *P*-450 synthesized *in vitro* (horizontal autoradiogram and dashed line) and the enzyme synthesized by oocytes *in vivo* (solid line). The mobilities of products from rat liver cytochrome *P*-450 (phenobarbital-induced form) are denoted by arrows. There is a broad similarity between peptides from cytochrome *P*-450 of liver, whole oocytes and reticulocyte lysate. Fig. 3B shows products from epoxide hydratase synthesized *in vitro* (dashed line) and the enzyme synthesized by oocytes *in vivo* (solid line). The mobilities of products from rat liver epoxide hydratase (phenobarbital-induced form) are denoted by arrows. Again there is broad similarity between peptides from enzyme made in the reticulocyte lysate, the whole oocyte or the rat liver. Epoxide hydratase made *in vivo* in both systems has an additional major peptide, perhaps as a result of additional secondary modification occurring in whole cells.

Aspects of the Mechanism of Insertion in vitro *into Membranes of Newly Made Cytochrome* P-*450 and Epoxide Hydratase*

The vectorial transfer of secretory proteins across the endoplasmic reticulum probably requires interaction between the N terminus of the nascent chain and a (putative) membrane receptor [19, 20, 25, 32]. It seemed possible that insertion of normal cellular integral membrane proteins involved the same initial pathway, and that competition experiments could provide evidence for a common membrane receptor, whilst kinetic studies might reveal any co-translational requirements of the insertion process.

We have therefore examined the ability of messengers coding for secretory polypeptides to reduce the association *in vitro* between *Xenopus* embryo membranes and newly made cytochrome *P*-450 and epoxide hydratase. The translation products of mammary and oviduct poly(A)-rich RNA were chosen as competitors and these mRNAs were used in amounts that caused little reduction (< 20 %) in the translation of liver RNA, except at the highest concentration used (< 40 %). The effects of such reductions were corrected by normalizing to constant rat albumin synthesis. Since the degree of association to the *Xenopus* membranes is 28 % and 46 % respectively of the total synthesized cytochrome *P*-450 and epoxide hydratase

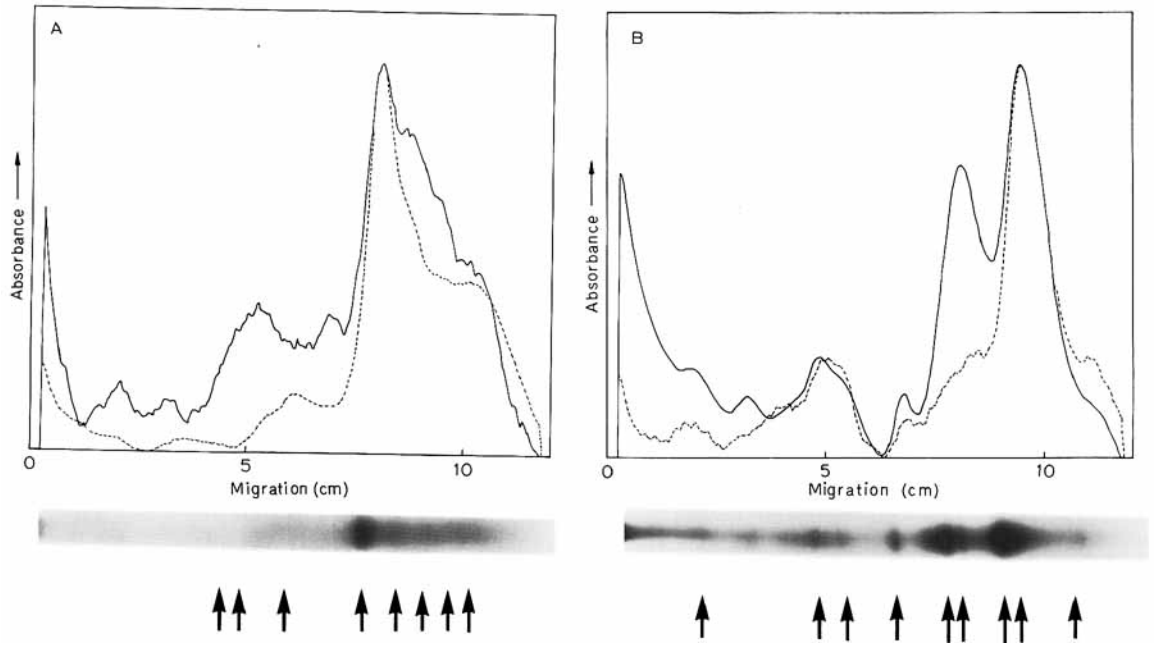

Fig. 3. *One-dimensional peptide maps of oocyte and liver cytochrome* P-*450 and epoxide hydratase synthesized* in vitro *and* in vivo. Immunoprecipitated proteins were separated on dodecylsulphate gels and bands were digested with *S. aureus* protease V8 [28]. The maps are shown as superimposed densitometer tracings of autoradiographs. (A) Digest of cytochrome *P*-450 (M_r 48000) synthesized *in vitro* (– – – and horizontal autoradiograph) and of cytochrome *P*-450 (M_r 50000) synthesized by oocytes *in vivo* (——). (B) Digest of epoxide hydratase (M_r 49000) synthesized *in vivo* (—— and horizontal autoradiograph) and epoxide hydratase (M_r 49000) synthesized *in vitro* (– – – –). The arrows denote the mobilities of proteolytic products from (A) phenobarbital-induced rat liver cytochrome *P*-450 (purified to homogeneity) and (B) rat liver epoxide hydratase obtained under identical digestion conditions to those used above. The scale denotes the distance migrated by a given peptide

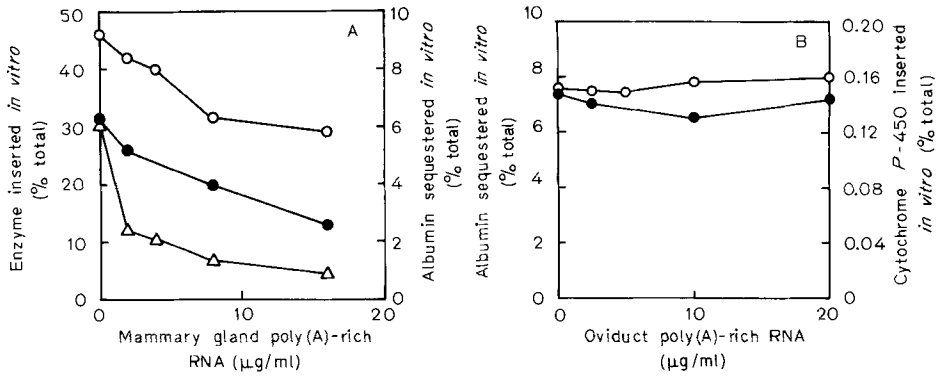

Fig. 4. *Competition* in vitro *between the sequestration of secretory proteins and the membrane insertion of both cytochrome* P-*450 and epoxide hydratase.* Rabbit reticulocyte lysates containing added *Xenopus* membranes were programmed with a constant amount of rat liver rapidly sedimenting endoplasmic reticulum RNA and a variable amount of either guinea pig mammary gland poly(A)-rich RNA (A) or chicken oviduct poly(A)-rich RNA (B). After the incubation, supernatant and membranes were separated by centrifugation and components present in the membrane fraction were defined as inserted. Newly synthesized albumin was measured directly by gel analysis followed by densitometry and served as the internal marker used for normalization of data; prior immunoprecipitation was required for measurement of epoxide hydratase and cytochrome *P*-450. (A) The amount of cytochrome *P*-450 (●) and epoxide hydratase (○) insertion into the membrane and albumin (△——△) sequestration as a percentage of the total amount of that protein synthesized in the lysate, plotted against the amount of mammary gland poly(A)-rich RNA added to the lysate. (B) The percentage insertion *in vitro* of cytochrome *P*-450 (●) and percentage sequestration *in vitro* of albumin (○) plotted against the amount of chicken oviduct poly(A)-rich RNA added to the lysate

(Fig. 4A), it is evident that 'receptor' numbers are limiting, a prerequisite for the competition study. As shown in Fig. 4A, with increasing amounts of poly(A)-rich RNA coding for milk proteins, there is a concomitant and distinct decrease both in albumin sequestration and cytochrome *P*-450 and epoxide hydratase membrane association. A corresponding increase in casein A sequestration is also obtained. Thus, we have indirect evidence that guinea pig casein A (the major mammary gland protein sequestered), rat albumin and the two liver enzymes, compete for the same site or receptor: given the low abundance of the mRNAs coding for the integral membrane proteins, the liver enzymes should have very high 'receptor' affinities.

A weak link in our argument is that we do not know the effect of the less abundant mRNA species present in the milk protein poly(A)-rich RNA. To circumvent this problem we have performed another competition experiment, this time using chicken oviduct poly(A)-rich RNA. The major product *in vitro* of this RNA sample is ovalbumin, and as expected [35]

Table 2. *The cotranslational requirements of association between rat liver proteins and* Xenopus *blastula membranes* in vitro
Lysates were programmed with rat liver RNA and blastula membranes were added either at the beginning (0 – 5 min) or after 90 min of translation. Lysates were incubated for a total of 100 min, as opposed to 90 min, when additions were made at the 90-min time point. Membranes were then spun out of the lysates, were washed and analysed on gels. Albumin, identified by its abundance and molecular weight, was measured by densitometery of gel autofluorograms (method A). In a separate series of experiments, membranes after isolation were solubilized and then immunoprecipitated with antibodies against either cytochrome *P*-450 or epoxide hydratase: the resulting immunoprecipitates were measured for their content of acid-insoluble radioactivity (method C) and then analysed on gels following which the appropriate bands were quantified by densitometery (method B). Unsynchronized lysates were used in the four experiments shown as pactamycin was unavailable. Protein synthesis was approximately linear for the first 45 – 60 min, but decreased progressively over the next 30 min. Less than 5% of total protein synthesis occurred between 90 and 100 min

Rat protein species associated with frog membranes	Measurement method	Amount of membrane-associated protein after membrane addition at:		Membrane-associated protein at 90 min cf. 0 – 5 min
		0 – 5 min	90 min	
				%
Albumin	A	44.9	3.2	7
Albumin[a]	A	37.5	1.8	5
Cytochrome *P*-450	B	12.2	1.5	12
Epoxide hydratase (expt. I)	B	> 60	18.2	< 30
		counts/min		
Epoxide hydratase (expt. I)	C	44700	13700	31
Epoxide hydratase (expt. II)	C	75000	15000	20

[a] After protease treatment of membrane vesicles to reduce nonspecific association

sequestration of albumin is not reduced appreciably by increasing amounts of oviduct RNA, whilst ovalbumin sequestration is low if liver RNA is present. Moreover, cytochrome *P*-450 membrane association is also unaffected by oviduct RNA (Fig. 4B); given that less abundant mRNA species are present in the oviduct RNA preparation, it seems likely that minor species (assumed to fulfil similar housekeeping functions in the two tissues) are not the cause of the competition seen with mammary gland RNA. The oviduct RNA competition experiment also serves to control against non-specific inhibitory effects of increasing the total amount of messenger RNA within the lysate.

The shared molecular features which lead to competition could be expressed at the level of either the nascent chain or the completed polypeptide, or at both levels. Kinetic studies can decide between some of these possibilities. Thus, unsynchronized lysates were programmed with liver RNA and at various times after the onset of translation *Xenopus* membranes were added: as shown in Table 2, without concomitant translation there is very little sequestration of albumin, and only limited (12%) association of cytochrome *P*-450 with membranes. Epoxide hydratase yielded variable and less clear-cut results, although in all experiments there was evidence for a co-translational requirement for at least two-thirds of membrane association. Fig. 5A and 5B show the kinetics of membrane interaction of rat liver RNA total translation products and of immunoprecipitated cytochrome *P*-450, the latter having been purified by immunoprecipitation and gel analysis following translation. Thus, for example, when mammary gland RNA is added it is likely that the competitors formed interact with nascent albumin chains and at least some nascent cytochrome *P*-450 chains.

Oocytes Secrete Albumin but not Epoxide Hydratase and Cytochrome P-450

The divergent fates of the different newly made liver proteins subsequent to their interaction with the common membrane receptor can be studied in the whole oocyte, simply by labelling with [^{35}S]methionine and analyzing the oocyte incubation medium. The mechanisms involved appear to be neither cell type nor species specific, but are nonetheless highly selective for, as shown in Fig. 6, albumin and at least 15 other rat liver proteins are exported by the oocyte (as are some oocyte or follicle cell proteins), whilst the two liver enzymes cannot be detected in the incubation medium. The exported albumin was identified tentatively by its abundance and molecular weight.

DISCUSSION

We describe two new and complementary systems for investigating the insertion into membranes of newly made endoplasmic reticulum proteins. Microinjection into *Xenopus* oocytes or early embryos can be used to study the specificity and regulation of membrane assembly *in vivo*, whilst the precise mechanisms involved can be revealed using cell free systems: employing this approach, both mechanisms and control can be studied in the same cell type using the same RNA template. Thus in the present paper we have compared the pathways followed by newly made secretory and integral membrane proteins, examining their initial confluence and subsequent divergence.

Rat liver cytochrome *P*-450 and epoxide hydratase made under the direction of added messenger can be inserted *in vivo* or *in vitro* into *Xenopus* membranes. Moreover, the rat cytochrome embedded in the frog membrane appears biologically active. We have often observed, both in whole cell and in cell-free systems, three immunoprecipitable forms of cytochrome *P*-450: similarly, epoxide hydratase antibody often precipitates a minor band, of unknown identity, as well as the major form of M_r 49000. One-dimensional proteolytic maps of the latter, shown in Fig. 3, are very similar to the epoxide hydratase maps obtained by Gonzalez and Kasper [39]. Thus the identity of some of the immunoprecipitated forms obtained

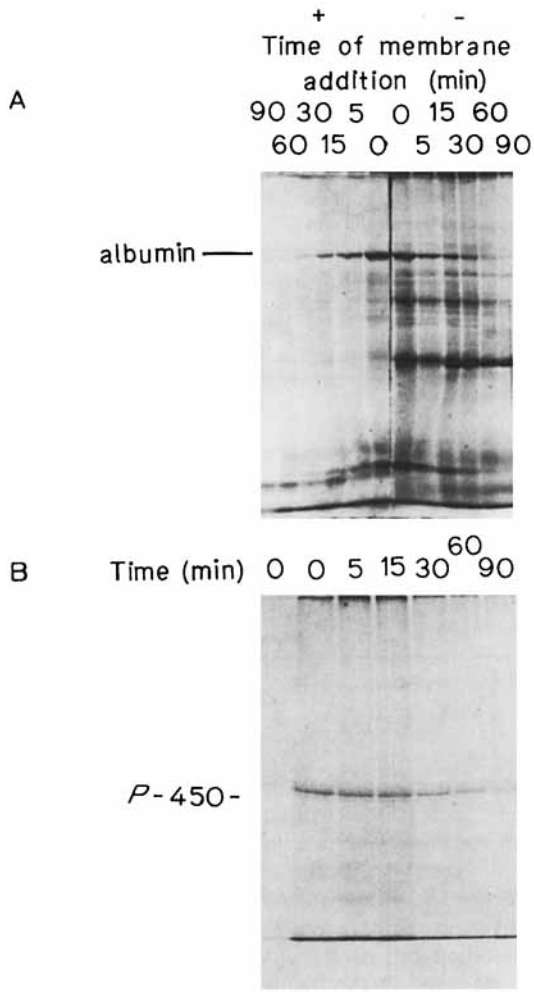

Fig. 5. *The temporal requirements for insertion of rat cytochrome* P-*450 and of sequestration of albumin* in vitro *within frog membranes.* Unsynchronised rabbit reticulocyte lysates were programmed with rat liver RNA: at various times after the beginning of the incubation *Xenopus* embryo membranes were added. The total time of reaction was held constant at 90 min, unless additions were made at the 90-min time point when the incubation was continued for a further 10 min. The membranes were spun out, treated with proteases where indicated (by the symbol +) and then solubilized. (A) The association of total translation products with membranes added at various times, associated products resistant to proteases are indicated (+). (B) Gel analysis of cytochrome *P*-450 immunoprecipitates obtained from membranes added to lysates at times after the onset of translation. The time of membrane addition is shown above each slot; the first slot shows lysate lacking added liver RNA. The calculated mobilities of cytochrome *P*-450 (M_r 50000) and rat albumin (M_r 66000) are shown

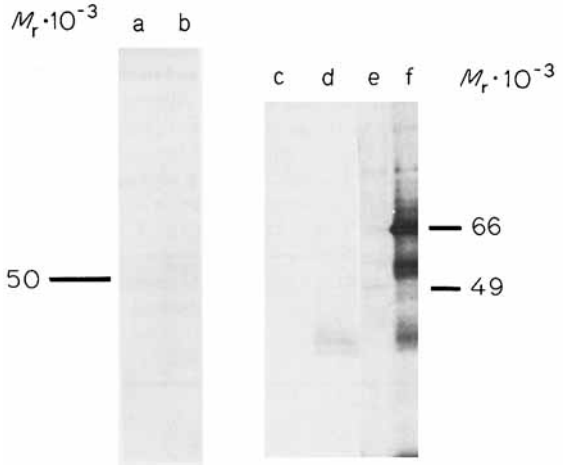

Fig. 6. *Export of proteins from* Xenopus laevis *oocytes injected with rat liver RNA.* Oocytes were injected and left overnight before labelling for 19 h with [^{35}]methionine. Gel analysis of both total medium (tracks e and f), and immunoprecipitates of cytochrome *P*-450 (tracks a and b) and epoxide hydratase (tracks c and d) is shown. Tracks a, c and e originate from control (uninjected oocytes) whilst tracks b, d and f originate from RNA-injected oocytes. Mock-injected control oocytes gave the same pattern as that shown in track e. Tracks a–d were exposed to film for five times longer than tracks e and f. The mobility of a marker of M_r 50000 is shown adjacent to track (a); tracks c–f, which come from a different gel, were run with markers of M_r 49000 (epoxide hydratase) and 66000 (rat albumin)

in vivo and *in vitro* has been confirmed by gel electrophoresis of peptides but more detailed analysis is required to prove conclusively the absence, as suggested by the mobilities of the primary products, of detachable signal sequences. Such an analysis is, in the case of the phenobarbital-induced form of cytochrome *P*-450, provided by sequencing studies [6] which show that the N terminii of the primary and native species are identical. Nonetheless the details of molecular structure do not affect the main conclusion of the present study, namely that heterologous proteins can be inserted both *in vivo* and *in vitro* into frog cell membranes.

It is easy to prepare active oocyte and embryo endoplasmic reticulum vesicles capable of sequestering a variety of proteins, such as chicken ovalbumin, frog and rat albumin, frog vitellogenin and honey bee promelittin, and able to carry out a variety of post-translational modifications such as glycosylation and signal sequence removal (Ohlsson and Lane, unpublished observations). Mixed messenger experiments performed in lysates containing blastula membranes are consistent with competition for a common receptor or site between secretory proteins and inserted liver enzymes: thus conclusions drawn from studies with viral proteins [33] can probably be extended to enzymes of the endoplasmic reticulum. Our results reveal that both cytochrome *P*-450 and epoxide hydratase have the functional equivalent of a domain possessed by milk protein secretory polypeptides. It remains to be proven both that this competitive domain is the signal sequence of the mammary protein and, consequently, that the liver enzymes have an equivalent sequence. However, if these enzymes do have signal peptides, one can deduce [6] that, as with ovalbumin [34, 35], the sequences are not detachable: nonetheless mature cytochrome *P*-450 [17, 36] and epoxide hydratase [18], unlike mature ovalbumin, have long hydrophobic N-terminal regions.

At what level *in vitro* does competition occur? Common molecular features could manifest their presence at either or both translational and post-translational levels. Albumin sequestration within blastula membranes clearly requires some co-translational event (Table 2 and Fig. 5), and it is reasonable [19, 20, 35] to assume this to be true of other secretory proteins. Cytochrome *P*-450 insertion is to a significant extent (88%, Table 2 and Fig. 5) dependent on concomitant translation. Thus at least some of the competition observed between mammary gland RNA products and cytochrome *P*-450 probably occurs at the nascent chain level, although direct proof is lacking. Moreover, there is no compelling evidence that the reduction of epoxide hydratase insertion is the result of competition at the nascent chain level for as much as 30% of membrane insertion can occur post-translationally: nor, however, does this uncertainty invalidate the concept of there

being shared molecular features between secretory and membrane proteins.

Epoxide hydratase and cytochrome *P*-450, although abundant compared to most other endoplasmic reticulum proteins, are rare species in terms of the total membrane-bound messenger population: in lysates programmed with rapidly sedimenting endoplasmic reticulum RNA these two enzymes account for 0.1–0.5% of total methionine incorporation, whilst rough microsomal RNA yields values twofold lower and free polysomal RNA gives values about 80-fold lower. Competition experiments show therefore that both enzymes have relatively high affinities for the receptor. We have only preliminary evidence that differential receptor affinity exists in whole oocytes, but nonetheless speculate that it may be very important in determining the relationship between the supply of messenger RNA and the fate of newly made proteins. Thus minor mRNA species coding for membrane-associated enzymes might be expected to have very high receptor affinities: in a competitive environment such preferred species could influence profoundly the fate of proteins coded for by abundant low-affinity species. The rapid destruction of primary translation products present in the wrong compartment has been revealed using the oocyte system [37].

For studies of regulatory mechanisms and of later events in membrane assembly, sequestration [24] or secretion [38] the whole oocyte is the system of choice. The insertion of rat epoxide hydratase and functional cytochrome *P*-450 into frog oocyte membranes appears to be neither species nor cell type specific; yet the association is highly selective since, unlike albumin and at least 15 other rat liver proteins, the cytochrome *P*-450 and epoxide hydratase are not secreted in detectable amounts, less than 5% of that inside the oocyte being found in the medium.

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