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Biogenesis of hepatocyte plasma-membrane domains

Incorporation of [^{3}H]fucose into plasma-membrane and Golgi-apparatus glycoproteins

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1. Rats were injected intracaudally with [^{3}H]fucose and its rate of incorporation into the fucoproteins of serum, Golgi and plasma-membrane subfractions was followed for up to 2h. 2. Incorporation into the Golgi dictyosome and secretory-vesicular fractions reached a maximum at 15min or less, but most of the radioactivity was associated with classes of secretory glycoproteins. Incorporation into sinusoidal plasma-membrane fractions reached a maximum at 30min, coinciding with the maximum release of fucoproteins into the serum. Contiguous and canalicular plasma-membrane fractions were labelled slightly later and at a lower rate and specific radioactivity. 3. Fluorography of fucoproteins separated by polyacrylamide-gel electrophoresis helped to distinguish between the major secretory and membrane-bound glycoproteins. The results show that a major biogenetic sequence is probably from Golgi dictyosomes to Golgi secretory elements to the sinusoidal plasma membrane. 4. The kinetics of incorporation make it unlikely that there is rapid and direct insertion of glycoproteins into the bile-canalicular plasma membrane. A route involving direct transfer of glycoproteins via a membrane-mediated intracellular path from the blood sinusoidal to the bile-canalicular plasma membranes is proposed.

One of the major problems in cell biology is to elucidate the mechanisms that direct newly synthesized soluble and membrane-bound proteins to various locations in the cell. In the context of the plasma membrane, the routes of transfer of proteins from their site of synthesis to the cell surface also require 'mapping' to the regional sites or domains where they carry out their functions. These functional site(s) need not necessarily coincide with the site of insertion into the plasma membrane. The correct routing of proteins and lipids to the cell surface is especially relevant in tissues and organs where the plasma membrane is differentiated into functional domains separated by intercellular junctions and interfacing at different external environments.

Three major surface regions are recognized anatomically and physiologically on the hepatocyte surface (Evans, 1980). The bile-canalicular membrane forms a small microvillar region that is segregated from the flat lateral region by tight junctions. The blood sinusoidal region extending from the lateral region is extensive and microvillar (Motta *et al.*, 1978). The present work takes advantage of the fact that subcellular-fractionation techniques are available that allow plasma-membrane subfractions to be prepared that derive predominantly from the three major functional domains (Wisher & Evans, 1975; Carey & Evans, 1977; Evans, 1980). The rates of incorporation of fucose into glycoproteins of the plasma-membrane fractions as well as Golgi-dictyosome and secretory-vacuole fractions were followed. The results show that the rates of incorporation of fucoproteins into the plasma-membrane regions are heterogeneous. It is also evident that there is a delayed incorporation of radioactivity into the bile-canalicular plasma-membrane region despite its close physical proximity to the Golgi apparatus.

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Experimental

Administration of radioactive isotopes

For each time point, two male Sprague–Dawley rats (100–120g) were used to prepare the fractions. Each rat was injected, via the tail vein with 400μl of a phosphate-buffered saline, pH7.4, containing 0.1μmol of L-[1-^{3}H]fucose (200μCi) (The Radiochemical Centre, Amersham, Bucks., U.K.). Rats

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were killed at the intervals specified (7–120 min), blood collected from the jugular vein and the livers were then homogenized.

Preparation of subcellular fractions

Plasma-membrane subfractions were prepared from the 'low-speed' pellets and microsomal pellets of rat liver homogenates essentially as described previously (Wisher & Evans, 1975; Poupon & Evans, 1979). Owing to the reduced amounts of liver homogenates used, the 'low-speed' pellet was prepared as follows. The livers (7–8 g total weight) were homogenized in 1 mM-$NaHCO_3$/0.5 mM-$CaCl_2$ in a loose-fitting Dounce homogenizer, and the filtered homogenate was centrifuged at $3000\,g_{av.}$ for 15 min. The loose pellet was washed by centrifugation at $3000\,g_{av.}$ for 15 min a further two times. The washed pellet was resuspended in 72% (w/v) sucrose to give a final concentration of 62% (w/v) sucrose, which was overlayered with 54, 48, 43 and 8% (w/v) sucrose layers. After centrifugation at $98\,000\,g_{av.}$ for 2 h in a Beckman SW27 rotor, plasma membranes were collected at the 8/43% and 43/48% interfaces (Neville, 1968). The plasma-membrane fraction resuspended in 8% sucrose was vigorously homogenized in a tight-fitting Dounce homogenizer and subfractionated in discontinuous sucrose gradients (Evans, 1970). The light subfraction, designated bile-canalicular plasma membranes, was collected at the 8/37% (w/v) sucrose interface and the heavy subfraction, designated contiguous, collected at the 37/49% sucrose interface. The first supernatant after collection of the initial 'low-speed' pellet was centrifuged at $105\,000\,g_{av.}$ for 1 h in a Beckman type 35 rotor and the pellet was resuspended in 8% sucrose. A plasma-membrane fraction was collected by flotation from the microsomal fraction as previously described (Wisher & Evans, 1975; Poupon & Evans, 1979). This was designated the blood sinusoidal plasma-membrane fraction.

Golgi light, intermediate and heavy fractions were prepared from rat liver homogenates as described by Bergeron (1979).

All fractions were washed by centrifugation after homogenization in a small tight-fitting Dounce homogenizer (Blaessig Glass Co., Rochester, NY, U.S.A.) in 8% sucrose, followed by 5 mM-Tris/HCl, pH 7.6.

Determination of radioactivity

Washed membranes were precipitated on to Whatman 3 mm filter papers by addition of trichloroacetic acid and then washed with ethanol/diethyl ether (1:1, v/v) followed by diethyl ether (Mans & Novelli, 1961). Radioactivity on the filter paper discs was measured in a toluene-based scintillant with an Intertechnique scintillation counter. The efficiency with this system was 6%.

Polyacrylamide-gel electrophoresis

Membranes were dissolved by boiling in sodium dodecyl sulphate/2-mercaptoethanol and polypeptides were separated as previously described (Wisher & Evans, 1975). Fluorography of dried gels was carried out as described by Bonner & Laskey (1974). The fluorographs were traced by using a Joyce–Loebl densitometer. To ensure that identical amounts of protein were loaded on to gels, membrane fractions solubilized in sodium dodecyl sulphate were centrifuged at $100\,000\,g_{av.}$ for 1 h and the protein content of the applied sample was redetermined.

Enzymic and protein estimations

5'-Nucleotidase and alkaline phosphodiesterase activities were estimated as previously described (Poupon & Evans, 1979). Galactosyltransferase activity was measured by the method of Vischer & Reutter (1978). Protein content of membrane samples was measured as described by Lowry *et al.* (1951) and serum proteins by the biuret method after washing in dioxan for 30 min (Beisenherz *et al.*, 1953).

Results

The rates of incorporation of [^{3}H]fucose into fucoproteins of plasma-membrane subfractions derived from each of the three major functional domains were followed over 120 min. Fucose was chosen as it is considered an excellent precursor of secretory and membrane glycoproteins and, to a minor extent, glycolipids (Vischer & Reutter, 1980). To follow the routes of glycoproteins to the plasma membrane, incorporation into Golgi fractions was followed over a shorter period. Analyses by polyacrylamide-gel electrophoresis of the fucoproteins present in Golgi fractions, in conjunction with those of serum, helped to distinguish between the membrane-bound and the occluded or loosely associated secretory fucoproteins, since it is well established that these are difficult to remove from subcellular fractions (Bergeron *et al.*, 1973; Castle *et al.*, 1975).

Properties of the plasma-membrane subfractions

The light and heavy plasma-membrane subfractions prepared from the 'low-speed' pellet originate mainly from the bile-canalicular and contiguous regions respectively, whereas there is extensive evidence that the microsomally derived fraction originates from the sinusoidal face of the hepatocyte (Wisher & Evans, 1975; Carey & Evans, 1977; Doyle *et al.*, 1979). In the present experiments, the plasma membranes were isolated on a reduced scale from a washed 'low-speed' pellet of the homogenate by flotation (Neville, 1968) instead of rate-zonal centrifugation. Table 1 shows that the enzymic

Table 1. *Recovery of protein and radioactivity, and distribution of enzymic markers in the plasma-membrane subfractions*

	Enzyme activity (μmol of substrate hydrolysed/mg of protein per h)			
	Homogenate	Canalicular fraction	Contiguous fraction	Sinusoidal fraction
Protein (% recovery) ...	—	0.08	0.43	0.53
Radioactivity (% recovery)* ...	—	0.16–0.30	0.88–1.75	2.85–3.74
5′-Nucleotidase	1.7	214	56	38
Alkaline phosphodiesterase	1.9	251	34	41
Galactosyltransferase†	1	0.75	0.22	40

* Range of fucoproteins recovered 30–120 min after injection of isotopes.
† Values are activities relative to that in the homogenate.

differences between the light and heavy subfractions were similar to those previously described (Wisher & Evans, 1975; Poupon & Evans, 1979). Furthermore, electron microscopy (result not shown) confirmed that the light subfraction was predominantly vesicular, whereas the heavy subfraction contained vesicles and membrane strips punctuated by intercellular junctions, as shown for the zonal-rotor-derived subfractions (Poupon & Evans, 1979). The presence of galactosyltransferase activity in the sinusoidal plasma-membrane fraction is due in part to Golgi contamination (Wisher & Evans, 1975), although it has been argued that the enzyme is also present at the sinusoidal region, reflecting constant exocytosis involving Golgi-apparatus components interacting with the plasma membrane (Merrit *et al.*, 1977).

Incorporation of fucose into serum, Golgi and plasma-membrane-fraction fucoproteins

The time course of incorporation of fucose into the glycoproteins of serum and Golgi subfractions was investigated first. The Golgi heavy and light subfractions are derived mainly from the Golgi dictyosomes and secretory vacuoles respectively; the Golgi intermediate fraction is considered as a mixture of both (Ehrenreich *et al.*, 1973; Bergeron *et al.*, 1973). The secretory vesicles integrate into the sinusoidal plasma membrane concurrently liberating the secretory products into the space of Disse (Fig. 7) (Meldolesi *et al.*, 1978).

Release of fucoproteins into serum reached a maximum 30 min after injection of isotopes; maximum incorporation of fucose in the Golgi light subfraction occurred at 15 min, whereas maximum incorporation had already occurred in the Golgi heavy and intermediate fractions at 7 min (Fig. 1). Incorporation into liver plasma-membrane fucoproteins reached a maximum at 30 min or later, thus extending the biogenetic sequence (Fig. 2). A clear peak of incorporation into the sinusoidal plasma

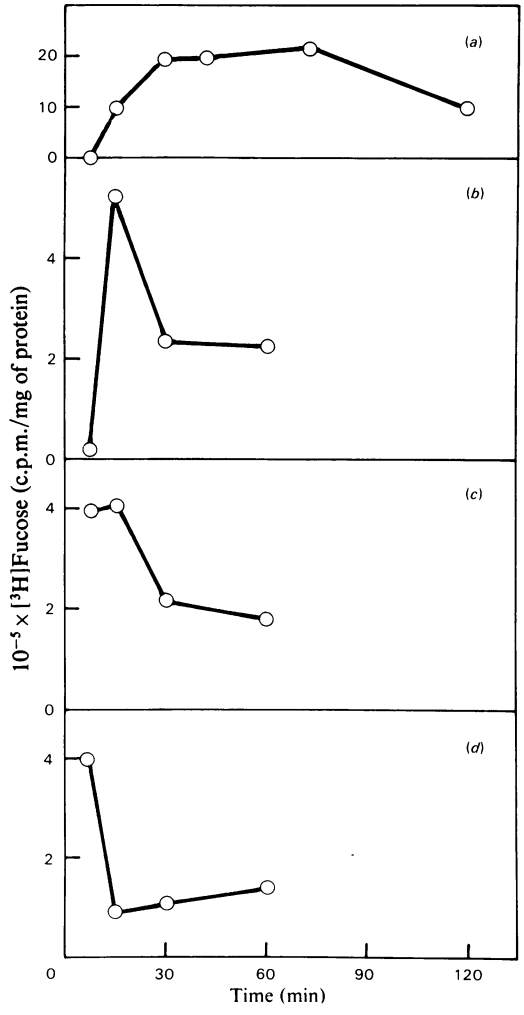


Fig. 1. *Rate of incorporation of [^{3}H]fucose into fucoproteins of (a) serum, (b) Golgi light subfraction, (c) Golgi intermediate subfraction and (d) Golgi heavy subfraction*

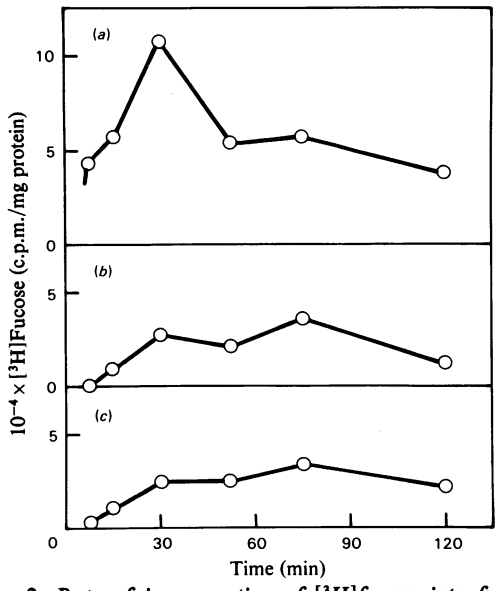


Fig. 2. *Rate of incorporation of* [^{3}H]*fucose into fucoproteins of* (*a*) *sinusoidal plasma-membrane subfraction,* (*b*) *contiguous plasma-membrane subfraction and* (*c*) *canalicular plasma-membrane subfraction*

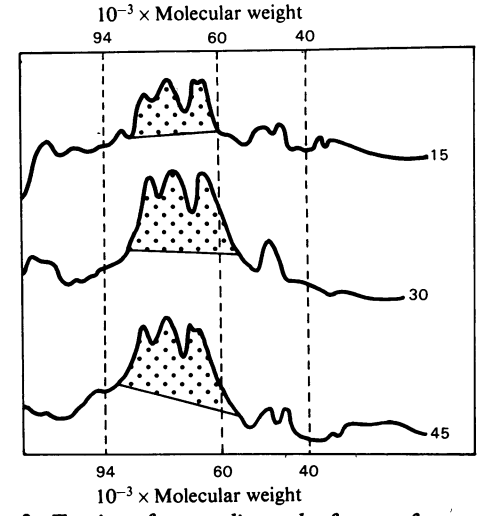


Fig. 3. *Tracing of autoradiograph of serum fucoproteins separated by polyacrylamide-gel electrophoresis at various time intervals*

Stippled peaks are for comparison and identification with probable secretory fucoproteins in Figs. 4 and 5. Time intervals are indicated (in minutes) by each curve.

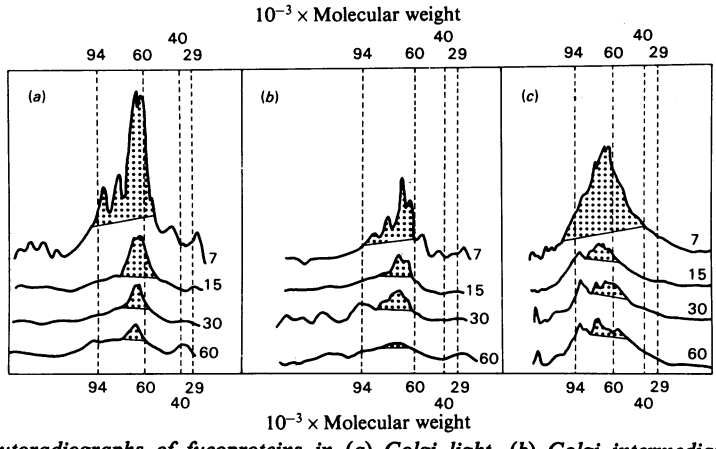
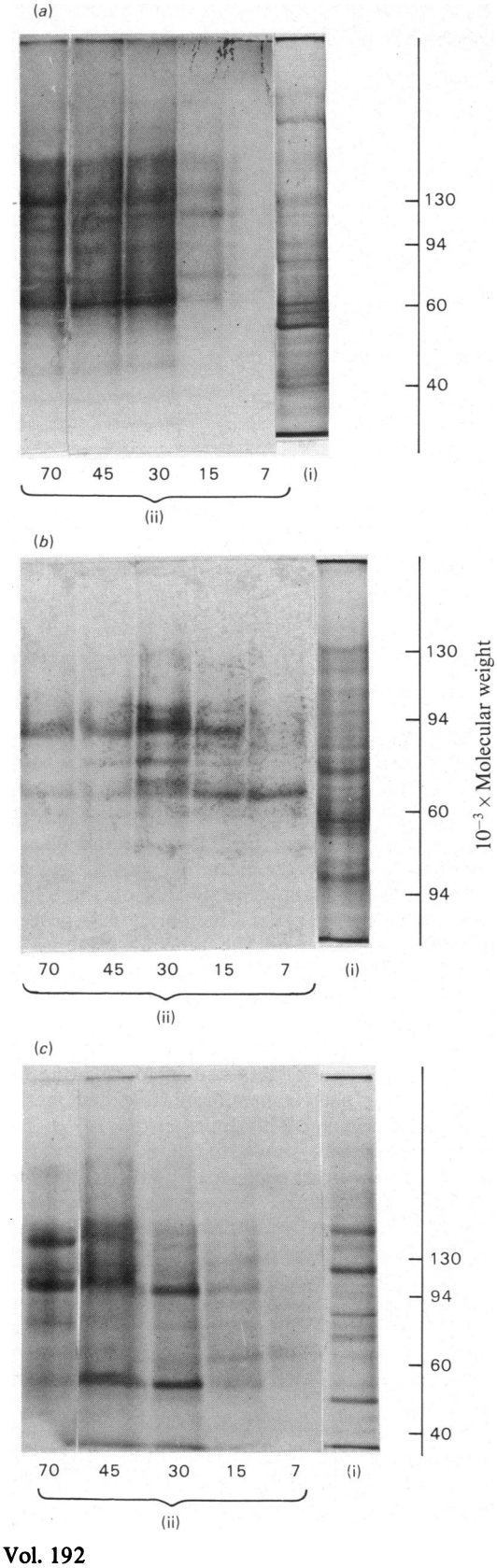


Fig. 4. *Tracing of autoradiographs of fucoproteins in* (*a*) *Golgi light,* (*b*) *Golgi intermediate and* (*c*) *Golgi heavy subfractions separated by polyacrylamide-gel electrophoresis*

Stippled peaks identify some major fucoproteins with similar electrophoretic mobility to those in serum, and designated as secretory fucoproteins. Time intervals are indicated (in minutes) by each curve.

membranes occurred at about 30 min, which coincided with the release of fucoproteins into serum, whereas incorporation into contiguous and canalicular plasma-membrane fractions was lower and appeared to reach a peak later (Fig. 2).

The incorporation results are in agreement with the accepted sequence of biogenetic events, showing that fucose is incorporated into glycoconjugates mainly in the Golgi apparatus and that these are then inserted primarily into the sinusoidal plasma membrane. Incorporation into the other plasma-membrane regions occurred later. However, conclusions based on isolated subcellular fractions, especially when prepared from secretory tissues, are unreliable owing to difficulties in distinguishing between the contribution made by membrane-bound (intrinsic) and secretory fucoproteins and this problem can be especially acute in Golgi fractions.



This was resolved by analysis by fluorography of the fucoproteins separated by polyacrylamide-gel electrophoresis.

Analysis of fucoprotein biosynthesis by fluorography

Polyacrylamide-gel electrophoretic analysis of the labelled serum fucoproteins present up to 45 min after [^{3}H]fucose injections showed that these were mainly of apparent mol.wt. 60000–90000 (Fig. 3). Analysis of the Golgi subfractions at early time points (Fig. 4) showed that fucoproteins of similar electrophoretic mobility were by far the major products observed by autoradiography and these corresponded to the stippled peaks shown in Fig. 3. When the plasma-membrane sinusoidal contiguous and canalicular fractions were analysed electrophoretically (Figs. 5 and 6), the prominent peaks that corresponded in mobility to secretory fucoproteins were present only in the sinusoidal plasma-membrane fraction, these again being most prominent at earlier time periods. Thus although the blood sinusoidal plasma-membrane fraction contained fucoprotein radioactivity peaks corresponding to those major ones in the serum and Golgi fractions, the amount was much decreased and other peaks corresponding to membrane fucoproteins in the other plasma-membrane fractions were identified (Fig. 5). To measure the rates of insertion of various fucoproteins into the three plasma membrane fractions, it is now possible to concentrate on certain classes of radiolabelled products.

A number of lines of evidence show that liver plasma-membrane glycoproteins are mainly in the mol.wt. range 80000–150000 (Vischer & Reutter, 1978; Evans, 1980) and these include, for example, 5′-nucleotidase (Evans & Gurd, 1973), alkaline phosphodiesterase (Evans *et al.*, 1973; Bischoff *et al.*, 1975), insulin receptors (Jacobs *et al.*, 1979; Wisher *et al.*, 1980) and a major glycoprotein (Kreisel *et al.*, 1980). Fig. 6 shows that the 'filled' peaks in the 80000–150000-mol.wt. range coinciding with the positions of membrane glycoproteins are labelled as early as 15 min in the sinusoidal plasma-membrane fraction, whereas these become labelled after about 30 min in the other two

Fig. 5. *Coomassie Blue staining (i) and autoradiographs (ii) of the proteins of (a) contiguous, (b) sinusoidal and (c) canalicular plasma-membrane subfraction*

Autoradiographs of the plasma membranes prepared 7–70 min after injection of [^{3}H]fucose were exposed for 3–12 weeks. For each track, identical amounts of protein were loaded on to the gels. For further details, see the Experimental section. Time intervals are indicated (in minutes) below the gels.

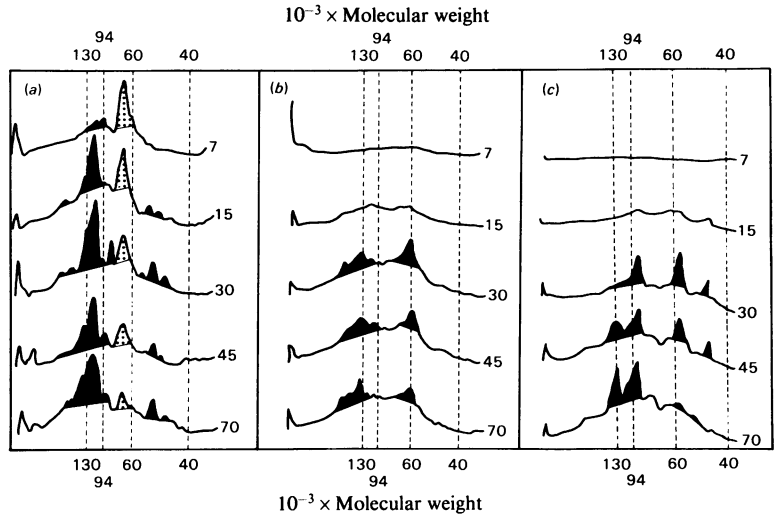


Fig. 6. *Tracings of autoradiographs of fucoproteins in (a) sinusoidal plasma-membrane fraction, (b) contiguous plasma-membrane fraction and (c) canalicular plasma-membrane fraction*

Stippled peaks are those of similar electrophoretic mobility to the secretory glycoproteins in serum and Golgi subfractions. 'Filled' peaks identify membrane-bound fucoproteins.

plasma-membrane fractions. This result is in agreement with the overall incorporation kinetics shown in Fig. 2, and reinforces the conclusion that this early peak of incorporation in the sinusoidal plasma-membrane fraction is only partly contributed by secretory glycoproteins.

A further feature of the incorporation of glycoproteins into the three plasma-membrane fractions is that there are bands of similar and dissimilar electrophoretic mobility (Figs. 5 and 6). Thus, glycoproteins of similar electrophoretic mobility (mol.wt. 50000) are found in the sinusoidal and canalicular plasma-membrane fractions, and a group of glycoproteins of mol.wt. >60000 and <60000 is found in the contiguous and canalicular plasma membranes respectively. Therefore heterogeneous incorporation of fucoproteins is shown by the three major plasma-membrane fractions.

Discussion

The route of insertion of glycoproteins into the plasma membrane has been well established in many cells and tissues (Bennett *et al.*, 1974; Bennett & Leblond, 1977; Meldolesi *et al.*, 1978; Morré *et al.*, 1979). However, the mechanisms operative and routes adopted for positioning of glycoproteins at the plasma-membrane region where they become operational is less clear. The segregation in tissues by tight junctions of the cell surface into regions that interface at different environments emphasize the need for correct routing of plasma-membrane precursor molecules. It appears that many membrane proteins are synthesized mainly by membrane-bound ribosomes (Meldolesi *et al.*, 1978; Elder & Morré, 1976) and that they are glycosylated at the endoplasmic reticulum and the Golgi apparatus before insertion into the plasma membrane, probably concurrently with the release of secretory products. The fusion of the Golgi membranes with the plasma membrane is a process that allows each membrane type to preserve its molecular individuality. In hepatocytes, the Golgi apparatus is found in close proximity to the bile canaliculus (Motta *et al.*, 1978) and it is often argued that it takes part in the transport and processing of bile products and in formulating the bile-canalicular plasma membrane (e.g. see Jaeken & Thines-Sempoux, 1979; Jones *et al.*, 1979). The present experiments show that it is unlikely that there is a major direct transfer of newly synthesized membrane glycoproteins between the Golgi apparatus and the bile-canalicular plasma membrane (Fig. 7, route *b*). The major route of fucoproteins to the sinusoidal plasma membrane appears to be via the Golgi-apparatus components (Fig. 7, route *a*), although the high radioactivity of the secretory components made it difficult to identify radioactive bands common to Golgi membranes and sinusoidal plasma membranes.

The routes of insertion of glycoproteins into the contiguous and canalicular plasma membranes are not clarified in the present work, but some conclusions emerge (Fig. 7). Undoubtedly, some lateral

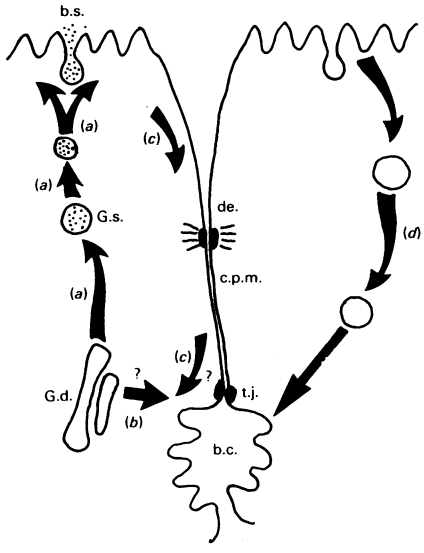


Fig. 7. *Routes of insertion of glycoproteins from the Golgi apparatus [Golgi dictyosomes (G.d) and Golgi-secretory elements (G.s.)] to the plasma membrane*

Route (*a*) is to the blood sinusoidal plasma membrane (b.s.). Routes (*b*) and (*c*) are the direct pathways to the bile-canalicular region (b.c.) from the Golgi apparatus or the contiguous plasma membrane (c.p.m.) respectively. Route (*d*) is a route from the blood sinusoidal to the bile-canalicular plasma-membrane region, which traverses the hepatocyte interior. Other abbreviations used: t.j., tight junction; de., desmosene.

movement over the contiguous plasma-membrane regions is possible, but the presence of a radiolabelled band of apparent mol.wt. 60000 in the contiguous plasma membranes, which is absent in the sinusoidal fraction, suggests that there may exist other direct routes to the contiguous region bypassing the sinusoidal region.

Tight junctions, which form a belt surrounding hepatocytes, limit or prevent lateral diffusion of plasma-membrane components, and indeed are believed to contribute towards the maintenance of unequal activities of enzymes and concentrations of receptors on epithelial tissue cell surfaces (Pisam & Ripoche, 1976). The extent of lateral movement from the contiguous to the canalicular plasma-membrane regions in hepatocytes evident from autoradiographical studies of tissue sections (Bennett *et al.*, 1974; Bennett & Leblond, 1977) is unknown. The results also show that some (e.g. mol.wt. 90000–150000), but not all, fucoproteins are found in all the subfractions. Two reasons for this can be forwarded. The first is methodological, since some degree of cross-contamination of the plasma-membrane subfractions may occur. The second explanation gives regard to interdomain dynamics in a functional epithelium (Evans, 1980). In liver, the generation and maintenance of cell-surface polarity is probably a resultant of many forces. Among these is the constant transport of metabolites from blood to bile across the hepatocyte interior. For example, intravenously injected Thorotrast (a colloidal suspension of ThO_2) is rapidly extracted by liver into bile via a transcellular route, reaching a peak at 30 min (Hampton, 1958). Similarly, the transfer of immunoglobulin A from blood to bile takes place via an intracellular route, a peak of release into bile being reached at about 30 min (Orlans *et al.*, 1978). Furthermore, there is evidence that the transfer of polymeric immunoglobulin A from its binding site at the sinusoidal plasma membrane (possibly secretory component; Socken *et al.*, 1979) into the hepatocyte may occur inside vesicles that possess some enzymic properties in common with plasma membranes (Mullock *et al.*, 1979). Since this transepithelial transport process is likely to involve plasma-membrane-derived compartments, it also provides a pathway that can result in the transfer of specific plasma-membrane components, including the fucoproteins now investigated from the sinusoidal to the canalicular plasma-membrane regions, thereby by-passing the contiguous region and the tight-junctional boundary (Fig. 7, route *d*). This route connecting plasma-membrane domains, as previously suggested (Evans, 1980), may involve receptor mechanisms that prevent interiorized plasma-membrane fragments from intermixing with various intracellular membrane compartments during movement inside the hepatocyte and allow recognition of the inner surface of the bile-canalicular plasma-membrane region. Indeed, vesicles observed in the peri-bile-canalicular region and suggested to derive from the Golgi apparatus (Jones *et al.*, 1979) may well originate from the blood sinusoidal plasma-membrane region.

The present paper describes and discusses the routes of insertion of fucoproteins into the hepatocyte's plasma-membrane domains. Non-glycosylated plasma-membrane proteins may follow different routes (Evans, 1980), and there is evidence, for example, that cholesterol and lanosterol are rapidly transferred to the blood sinusoidal plasma membrane after synthesis (Pascaud *et al.*, 1980). Clearly, the routes and the mechanisms of plasma-membrane biogenesis operational in cells with polarized surfaces appear to be complex.

Note added in proof (Received 30 September 1980)

Similar kinetics of incorporation of fucose into liver plasma membranes was reported by Buchsel *et al.* (1980).

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