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GOLGI FRACTIONS FROM LIVERS OF CONTROL AND ETHANOL-INTOXICATED RATS

ENZYMIC AND MORPHOLOGIC PROPERTIES FOLLOWING RAPID ISOLATION

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Summary

Following rapid isolation, it has been found that Golgi apparatus from ethanol-intoxicated animals contain high levels of galactosyltransferase but also detectable glucose-6-phosphatase and microsomal esterase, as well as 5′-nucleotidase activity. In experiments carried out in parallel on littermate animals but without intoxication, similar recoveries and specific activities of the four enzymes were observed. Morphologic analysis of Golgi fractions isolated from control animals demonstrated no striking morphologic difference to those from the ethanol-intoxicated animals. Indeed, using galloyl glucose-lead staining techniques to mark the lipoprotein particles in situ, it was found that all Golgi apparatus of hepatocytes from control animals were marked by very low density lipoprotein particles. It is therefore concluded that within the limits of the present analyses, Golgi fractions isolated from control animals are as valid as those isolated from ethanol-intoxicated rats.

Introduction

Isolation procedures for Golgi apparatus have involved reasonably lengthy manipulations, e.g. [1--3]. The recent availability of a straightforward enzymic assay for galactosyltransferase [4] and work clarifying (a) the lability of this enzymic activity [3] and (b) the complications arising from potent hydrolysis of the substrate [5] especially by the starting homogenate and microsomes, prompted the development of a more rapid modification of the Golgi isolation procedure from ethanol-intoxicated rats.

In addition, the finding [6] that ethanol intoxication had no effect on secretion of serum proteins prompted a reexamination of Golgi apparatus (in situ as well as after fractionation) from non-ethanol intoxicated (i.e. control) animals.

Thus, following rapid isolation, a reexamination of marker enzymes for Golgi (galactosyltransferase), endoplasmic reticulum (glucose-6-phosphatase, and microsomal esterase) and plasmalemma (5′-nucleotidase) was carried out. Further, the effect of ethanol on the distribution of the same enzymic activities was assessed by designing the procedure to accommodate parallel fractionation and enzymic assays on control and ethanol-intoxicated animals. Finally, morphologic analysis of fractions and livers from control and ethanol-intoxicated animals were carried out in parallel.

Methods

Isolation of Golgi fractions. Male Sherman rats from the McGill Department of Anatomy colony (similar but less extensive studies were carried out with Sprague-Dawley rats obtained from Canadian Breeding Farms and Laboratories Ltd., St. Constant, Quebec, and used immediately after purchase) were fasted overnight. Littermates (100—200 g body weight) were either ethanol intoxicated [1] or not (controls). Both sets of animals were killed simultaneously and 20% (w/v) liver homogenates (Potter-Elvejhem, six strokes) in 0.25 M sucrose were fractionated into a combined nuclear/mitochondrial pellet (8700 $\times g_{av}$ for 10 min, Sorvall SS-34 rotor). This pellet was washed and the combined supernatants sedimented (200 000 $\times g_{max}$ for 30 min; A321 rotor of IEC B 60) to obtain a microsomal pellet. Immediately the pellet was gently resuspended in 0.25 M sucrose with a loose-pestled Dounce (pestle 'A') and 2.0 M sucrose added to give a final concentration of 1.15 M sucrose (1.5 ml/g liver starting weight). The suspension (0.8 ml) was underlayed beneath a discontinuous gradient made up of 0.8 ml each of 0.86, 0.6 and 0.25 M sucrose. Following centrifugation (300 000 $\times g_{av}$ for 60 min) in the SB 405 rotor, bands appeared at each interface. The entire portion of each fraction was collected, diluted to 0.25 M sucrose and pelleted (200 000 $\times g_{max}$ for 30 min) with the A 321 rotor. Immediately the pellets were resuspended in 0.25 M sucrose and assayed for enzymic activities and protein determination (Biorad protein assay kit. Biorad Laboratories, Mississauga, Ontario). In separate experiments, small aliquots of each fraction were removed from the final gradient and processed for electron microscopy (vide infra).

Enzymic assays. Galactosyltransferase was assayed as described by Bretz and Staubli [4] with ovomucoid as receptor. The assay mixture contained 2 mM ATP to counteract hydrolysis of the [^{3}H]UDPgalactose substrate [5]. Glucose-6-phosphatase and 5′-nucleotidase were assayed as described previously [7] and microsomal esterase as described by Beaufay et al. [5].

Electron microscopy. Aliquots of fractions from the final gradient were mixed with glutaraldehyde (1—2.5% in 0.05—0.1 M Sörensen's buffer, pH 7.4, or 0.05—0.1 M cacodylate buffer, pH 7.4) for 10 min. The suspension was pelleted (35 000 $\times g_{max}$ for 20 min) and the pellet postfixed in 1% OsO_4 in 0.05 M cacodylate buffer (pH 7.4) followed by galloyl glucose [8]. Following dehydration and embedding in Epon, thin sections of oriented pellets [1] were

cut, stained with lead citrate [8] and observed in the Philips 400 electron microscope.

Liver tissue from control and ethanol-intoxicated rats was morphologically analyzed following intracardiac whole-body perfusion with lactated Ringer's followed by glutaraldehyde as described previously [9]. Post fixation with OsO_4 and mordanting with galloyl glucose was also carried out as previously described [9].

Results

The distribution of galactosyltransferase, glucose-6-phosphatase, microsomal esterase, and 5'-nucleotidase was first determined on the simplified fractionation of the homogenate (Fig. 1) from control and ethanol-intoxicated animals.

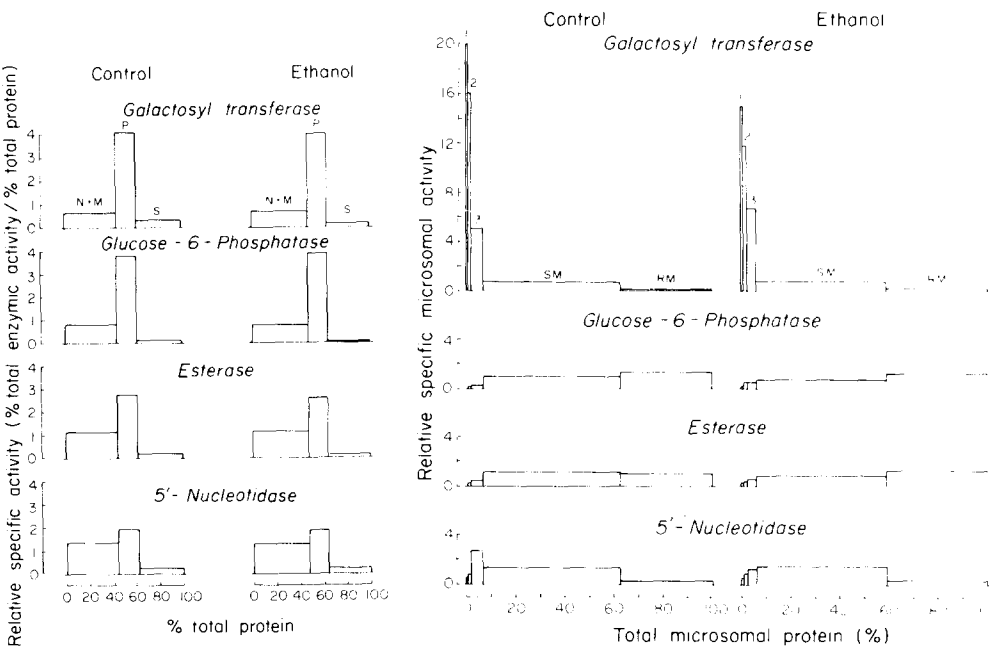


Fig. 1. De Duve plot representing the distributions of enzymic activities in fractions derived from starting homogenates of control or ethanol-intoxicated rats. The designation (N + M) (nuclear plus mitochondrial) is given to the 8500 × *g* pellet, P to the microsomal pellet and S to the final supernate. The results are the average of three fractionations carried out in parallel from livers of control and ethanol-intoxicated animals. The specific enzymic activities of microsomes (from control animals) were as follows: galactosyltransferase, $1.43 \cdot 10^6$ dpm [^{3}H]galactose (1.41 nmol) transferred $\cdot$ min^{-1} $\cdot$ mg^{-1} protein; glucose-6-phosphatase 0.48 μmol P_i liberated $\cdot$ min^{-1} $\cdot$ mg^{-1} protein; microsomal esterase, 20.8 ΔA (6.78 μmol *O*-nitrophenol produced $\cdot$ min^{-1} $\cdot$ mg^{-1} protein; 5'-nucleotidase, 0.09 μmol P_i liberated $\cdot$ min^{-1} $\cdot$ mg^{-1} protein.

Fig. 2. Modified De Duve histogram indicating the distribution of enzymic activities in subfractions derived from parent microsomes of livers from control or ethanol-intoxicated rats. The Golgi light, intermediate and heavy fractions are, respectively, indicated by 1, 2, 3. The residual load zone (smooth microsomes) and residual pellet (rough microsomes) are designated SM, RM, respectively. The data are averaged from five fractionations carried out in parallel for control and ethanol-intoxicated rats for all enzymic activities except that described for 5'-nucleotidase. For this latter enzymic activity the data are based on the average of two fractionations. The ordinate represents the ratio of the percent microsomal total enzymic activity found in the fraction divided by percent microsomal protein found in that fraction. The abscissa indicates the proportion of microsomal protein found in each fraction.

TABLE I

SPECIFIC ACTIVITY RELATIVE TO MICROSOMES OF ENZYMIC ACTIVITIES AND PROTEIN DISTRIBUTION IN MICROSOMAL SUBFRACTIONS

The specific enzymic activities of each fraction was compared to that of total recovered microsomes (set at 1.00) as described in the legend of Fig. 2. C, E, abbreviations referring to control and ethanol-intoxicated rats, respectively.

Fraction	Galactosyl transferase		Glucose-6-phosphatase		Esterase		5′-Nucleotidase		Protein (%)	
	C	E	C	E	C	E	C	E	C	E
Golgi light	20.2	15.1	0.07	0.2	0.20	0.34	0.52	0.57	0.4	0.8
Golgi intermediate	16.2	11.8	0.23	0.47	0.25	0.41	0.65	0.86	1.5	1.7
Golgi heavy	5.6	6.7	0.23	0.45	0.41	0.46	2.67	1.33	4.8	3.7
Smooth microsomes	0.7	0.8	0.89	0.94	1.03	0.91	1.32	1.49	56.1	52.7
Rough microsomes	0.07	0.07	1.30	1.17	1.07	1.22	0.33	0.35	37.1	41.1

In separate experiments with microsomes as the starting material, the distribution of the same enzymic activities was analyzed in the final gradient for purification of Golgi subfractions (Fig. 2, Table I).

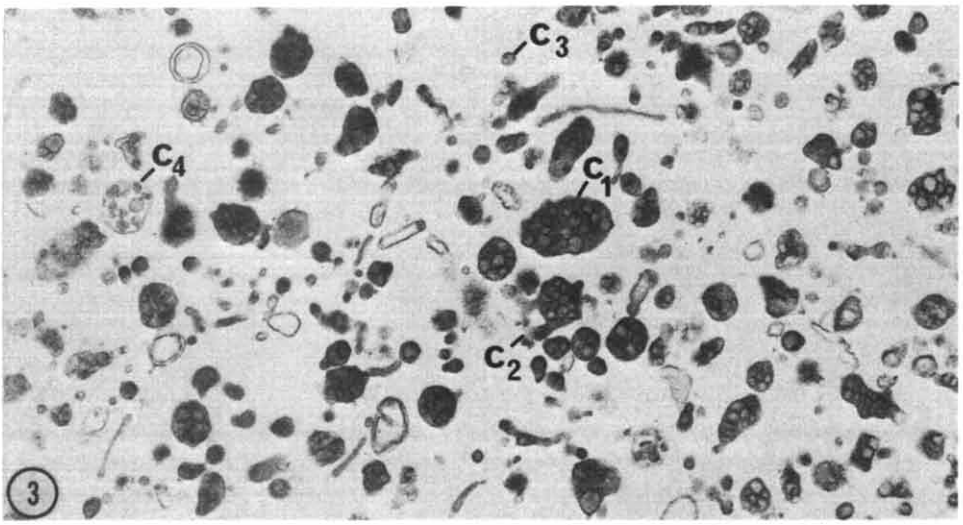
The minor differences between the fractionation from control and ethanol-intoxicated animals prompted an analysis of the morphology of Golgi fractions isolated from the control animals. Through the depth pellet analyses of each of the fractions showed (Figs. 3—5) similar morphology to that previously described for the fractions isolated from ethanol-intoxicated animals. These findings prompted the reexamination of the control liver in situ with use of techniques to render the very low density lipoprotein particles more visible *. In control fasted animals, all hepatocytes showed visible accumulations of very low density lipoprotein particles concentrated in Golgi regions (Fig. 6) which

Fig. 3. Golgi light fraction isolated from control, i.e. non-ethanol-intoxicated rats. The fraction viewed in the middle of the pellet showing very low density lipoprotein particle filled profiles. Most are large, round (c_1) and occasionally show portions continuous with tubular elements of the Golgi apparatus (c_2). Very small vesicles or tubules marked by a single-lipoprotein particle are also found (c_3). In addition some partially filled vacuoles are encountered (c_4). ×20 000.

Fig. 4. Golgi intermediate fraction also from control rat liver and viewed at the middle of the pellet. Generally smaller profiles to those encountered in the Golgi light fraction are found. Both circular profiles (c_1) but also dumbbell-shaped elements (c_2) are encountered as well as small vesicles marked by single-lipoprotein particles (c_3). Membraneous profiles of unknown origin are found as indicated by an arrow. ×20 000.

Fig. 5. Golgi heavy fraction from control rat liver viewed at the middle of the pellet. Small vesicles marked by single lipoprotein particles are prominent (v) as are the less frequent circular profiles with several lipoproteins in their content (c). Tubular profiles (t) and empty collapsed cisternae (f) are frequently found. Occasional lysosome contaminants are labeled (ly) and empty profiles of uncertain origin are marked by arrows. ×20 000.

* As first described in the paper identifying the mordanting properties of galloyl glucose [8], the very low density lipoprotein particles (at least in the small intestinal absorptive cells) are readily distinguished even at relatively low magnifications.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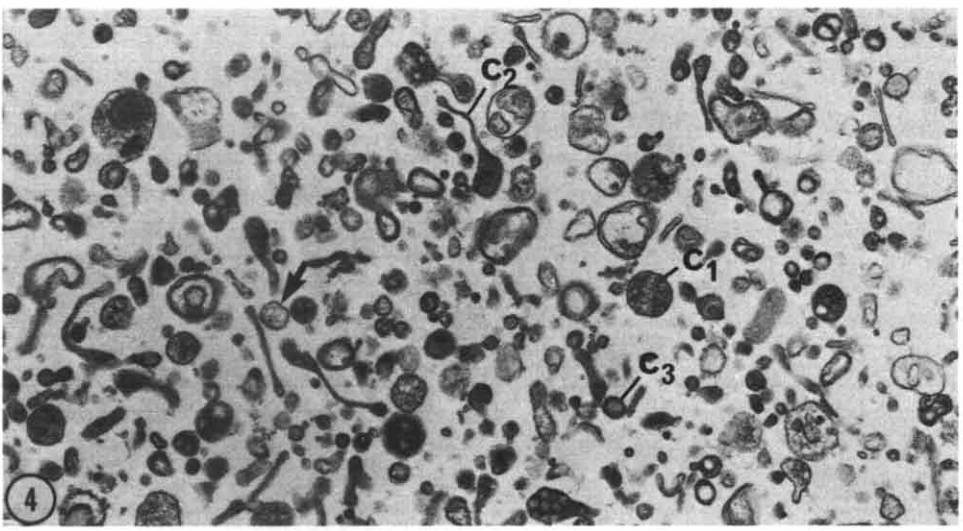
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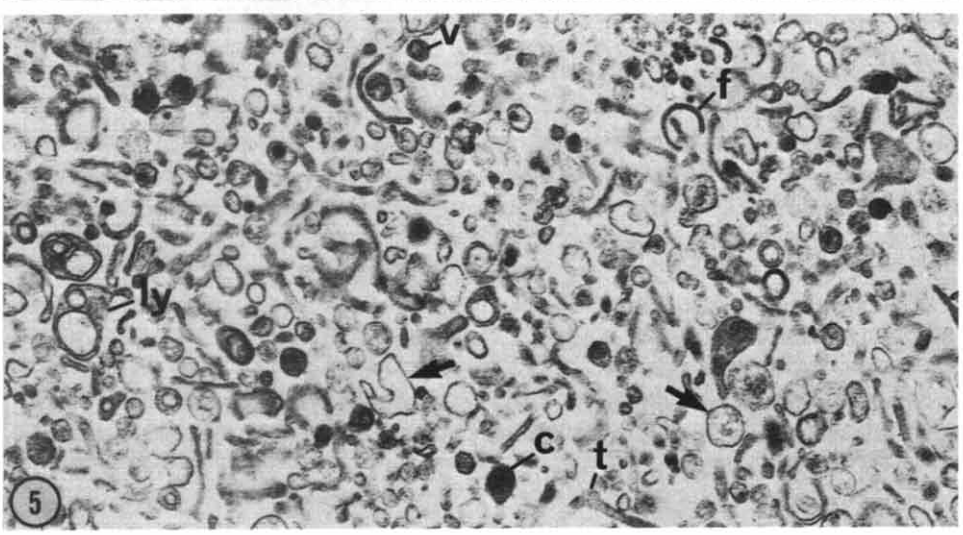
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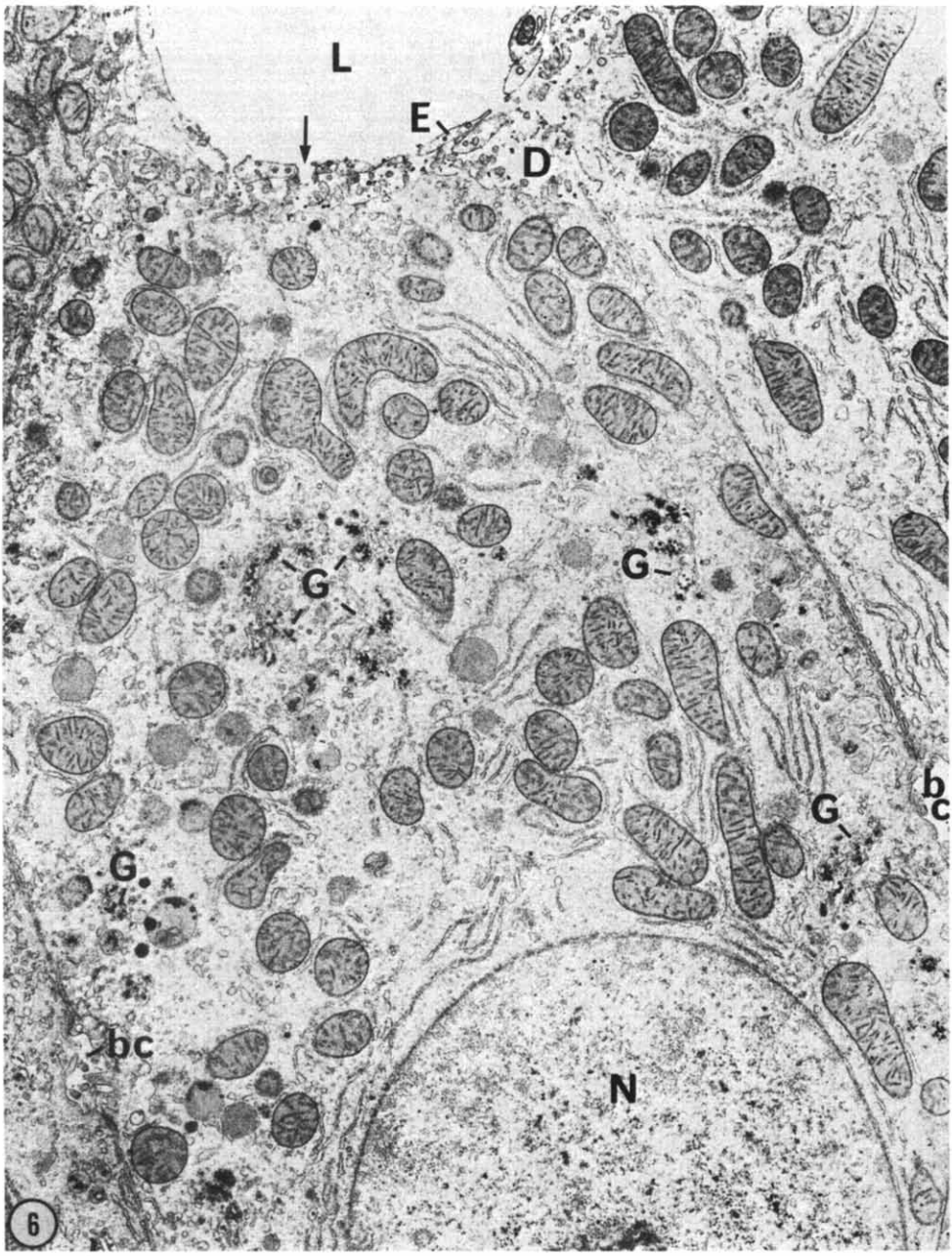

Fig. 6. Low-power micrograph of an hepatocyte from control liver following whole-body perfusion fixation. Golgi apparatus (G) are all clearly marked by the content of dark very low density lipoprotein particles. Even at this low magnification however, single-lipoprotein particles are evident in the space of Disse (D) intermingled with the sinusoidal microvilli of the hepatocyte. The sinusoidal lumen (L) has been washed of all content. Openings (indicated by arrow) in the endothelium (E) are clearly of sufficient dimension and should have enabled easy passage of lipoprotein particles into the sinusoidal lumen. The bile canaliculi are noted (bc) as is the nucleus (N). ×9500.

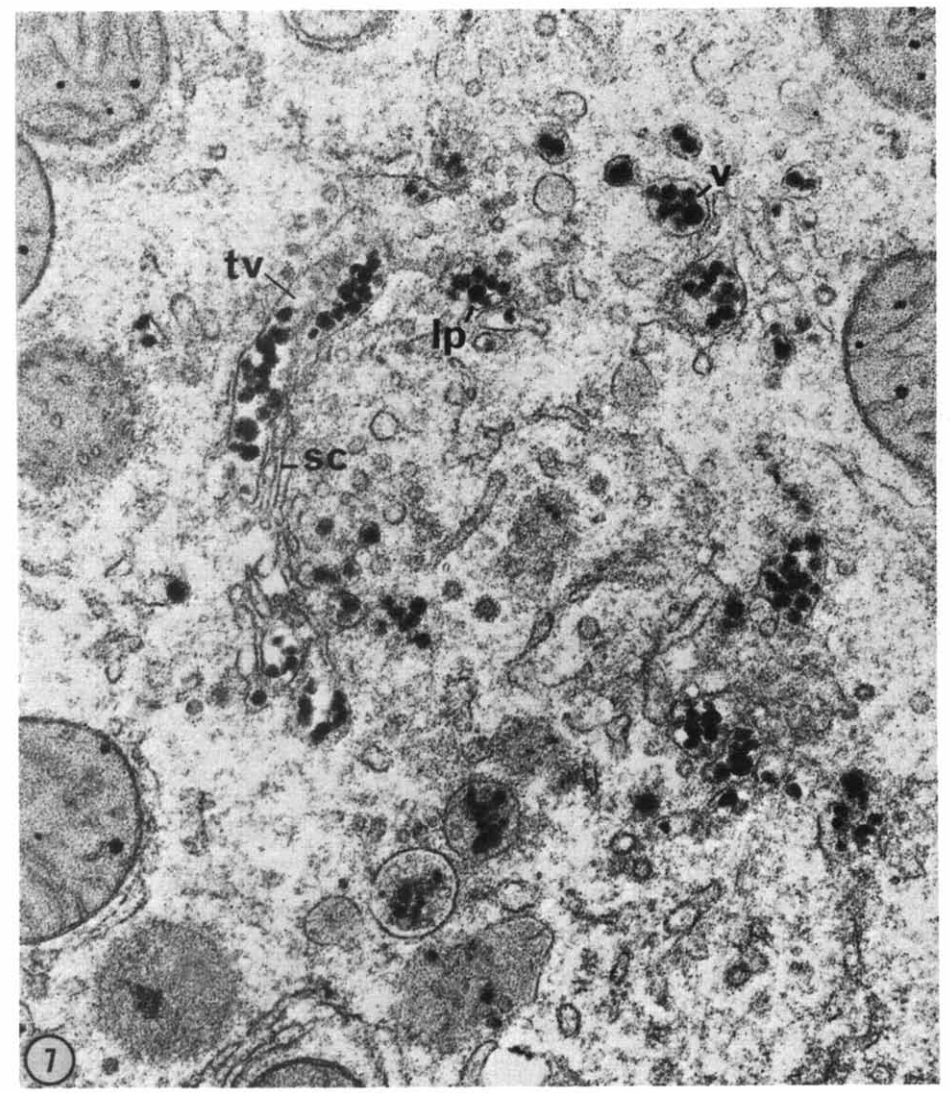

Fig. 7. Higher power view of the Golgi apparatus centrally located in Fig. 6. Only the most *cis* elements of the stacked cisternae (sc) are unmarked by lipoprotein particles (lp). Vesicles (v) and *trans* Golgi cisternae (tr) are heavily laden with the lipoprotein particles. ×39 000.

under higher power were in all (*cis* and *trans*) components of the Golgi apparatus (Fig. 7) as well as Golgi secretory droplets (Fig. 8).

A noteworthy feature of both control and ethanol-intoxicated animals was the accumulation of the lipoprotein particles in the space of Disse (Fig. 8) in close association with the sinusoidal surface of the plasmalemma.

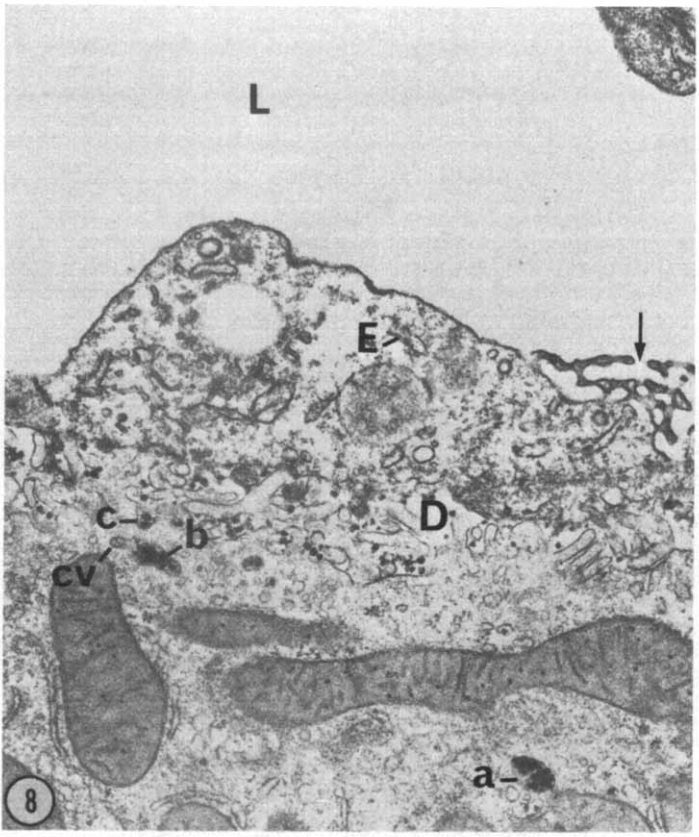

Fig. 8. View of the sinusoidal pole of an hepatocyte showing secretion droplets (a and b) probably migrating to the plasmalemma for exocytosis (c); the vesicle (b) is in close proximity to coated vesicles (cv) at either end. The space of Disse (D) is full of lipoprotein particles closely associated with the hepatocyte plasmalemma. A particularly favorable olbique view indicates the large open 'fenestrae' (indicated by an arrow) of the endothelium (E). Such openings should have allowed for free flow of the lipoprotein particles into the lumen of the sinusoid (L) which has been clearly washed free of content. X17 500.

Discussion

The rapid isolation of the fractions enabled enzymic assays to be conveniently carried out on the same day, i.e. within 3 h of killing the animals. In general, the high concentration of galactosyltransferase activity and low concentration of glucose-6-phosphatase activity in the Golgi light and intermediate fractions confirms the original study [7]. However, there are several details at variance with the original report.

Thus with respect to the fractions isolated from alcohol-intoxicated animals, it was found that 63% of the homogenate galactosyltransferase activity was recovered in microsomes. Of this, an average of only 56% of the microsomal enzymic activity (but only 6% of microsomal protein) was recovered in the three Golgi fractions (Figs. 1 and 2). In addition, the enzymic activity was on average of 64-, 47- and 27-fold enriched over the homogenate activity for the

Golgi light, intermediate and heavy fractions, respectively. This relationship is in the opposite trend to that originally described [7] *.

By far the most surprising aspect of the present study, however, concerned the fractions isolated from control animals. Thus a near identical proportion (65%) of galactosyltransferase activity was recovered in microsomes as was the case for ethanol-intoxicated animals. In addition, 59% of the microsomal galactosyltransferase activity (but only 6.7% of the microsomal protein) was recovered in the three Golgi fractions. The same trend observed in the fractions isolated from ethanol-intoxicated animals was found for those from controls with an 83-, 66- and 23-fold enrichment of galactosyltransferase over homogenate for the Golgi light, intermediate and heavy fractions, respectively. Electron microscope analysis of the Golgi fractions isolated from control animals showed few noteworthy differences to those from ethanol-intoxicated animals. Therefore, it was considered of importance to return to the in situ liver and utilize new staining techniques [8] which render lipoprotein particles more distinct. With galloyl glucose mordanting and lead citrate staining, the near exclusive location of putative very low density lipoprotein particles [1,10,11] within all elements of the Golgi apparatus as well as Golgi secretory droplets was confirmed in control as well as ethanol-intoxicated animals **.

One noteworthy finding was the demonstration of lipoprotein particles bound to interstices of the sinusoidal microvilli. The possibility that these represent lipoprotein particles bound to the hepatocyte very low density lipoprotein receptor [12] is therefore suggested. That the lipoprotein particles were merely trapped within the complicated geometry of the microvilli is unlikely. The lumina of all sinusoids were devoid of lipoprotein particles and erythrocytes thereby indicating an efficient perfusion with the Ringer's buffer (prefixation). The dimensions of the discontinuities or openings (or 'fenestrae') in the endothelium are large enough (see Fig. 8 and Ref. 13) to enable the easy passage of lipoprotein from the space of Disse to the sinusoidal lumina. The consequence of this finding is relevant to the present study. The finding indicates that even the lipoprotein morphologic marker is not inviolate. Thus, during homogenization, the sinusoidal plasmalemma could vesiculate, thereby encompassing single lipoproteins. Such small single-lipoprotein-containing vesicles are in fact present in Golgi fractions and especially in the Golgi heavy fraction, although they are not a major component of the fractions. Thus both morphology of the fractions and liver in situ and biochemistry of the Golgi fractions confirm that the fractions isolated from control animals are at least

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* Part of the difficulty in the previous study [7] concerned the inactivation of galactosyltransferase in the lighter Golgi fractions, problems with differential hydrolysis of the substrate (UDPgalactose) for galactosyltransferase, and finally a misconception that the residual microsomal pellet (virtually devoid of galactosyltransferase activity) was representative of total residual microsomes. As only realized later, the residual microsomes were segregated into a smooth fraction (residual load zone) and [6] a rough ER fraction (the residual pellet).

** On a qualitative basis, it has always been noticed that lipoprotein particles in livers of control animals appear less well-defined than those in ethanol-intoxicated animals (see also Ref. 1). This could be explained by ethanol acting as a substrate for lipid synthesis, thereby enabling a more lipidic and therefore larger and more osmiophilic very low density lipoprotein particles than controls. In addition this would help explain the 'fluffier' Golgi light fractions obtained from the ethanol-intoxicated animals compared to controls.

as valid as those from the ethanol-intoxicated rats. It is, therefore, worthwile to analyze the biochemical results in more depth.

In this laboratory, all assayed enzymic activities were detected in the Golgi fractions. whether from ethanol-intoxicated or control animals. Considerably different distributions were, however, noted for the few enzymes assayed. Thus, considering the final microsomal gradient, galactosyltransferase (a microsomal group a_3 marker [14]) was highly concentrated in the Golgi light fraction > Golgi intermediate fraction > Golgi heavy fraction. Glucose -6-phosphatase and microsomal esterase (group c marker [14]) indicated the opposite relationship. However, low but detectable activities were found in even the Golgi light fraction. In this fraction (the putatively purest fraction) a low of 7 to a high of 34% of the microsomal specific activity was detected. These new findings are due to the highly labile nature (especially of glucose-6-phosphatase [22,23]) in the Golgi light fraction (but not in the total microsomes). As has been repeatedly found [7,15—17] and confirmed in the present findings, high specific activities of 5'-nucleotidase (a group a_2 microsomal marker [14]) was detected in all three Golgi fractions (even the Golgi light fraction). This, however, was only a minor proportion of the homogenate activity (0.06%, 0.3% and 3.8%) for the light, intermediate and heavy Golgi fractions, respectively, of control animals and 0.13%, 0.43% and 1.4% of that for the respective fractions from ethanol-intoxicated animals, similar to that previously described [17].

Other investigators have reported endoplasmic reticulum type enzymes in Golgi fractions [18—22]. In contrast, previous data [7] on the Golgi fractions from ethanol-intoxicated animals concluded that at least one (glucose-6-phosphatase) was probably absent and at least one (cytochrome b_5) present. The present data now indicates that both microsomal esterase and glucose-6-phosphatase are also present in confirmation of the findings of Leelavathi et al. [18] and the more recent work of Hino et al. [19—21] as well as Howell et al. [22] and Ito and Palade [23]. That this represents endoplasmic reticulum is unlikely * (although not impossible). However, direct molecular and morphologic dissection of the Golgi fractions in the vein of the studies of Ito and Palade [23] is required to settle this point.

In conclusion, information has been gained from the rapid isolation technique. Golgi fractions contain not only very high levels of galactosyltransferase but significant activity characteristic of endoplasmic reticulum enzymes. In addition ethanol intoxication is not a requirement for obtaining the Golgi fractions. Near identical fractions can be obtained from control animals.

Acknowledgements

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* Chemical [7,17] and morphologic [6] determinations indicate that the proportion of non-membraneous content protein in the Golgi fractions is ten times or more greater than that of membrane protein. Thus if endoplasmic reticulum contamination were an explanation, the endoplasmic reticulum must be a very special subcategory of endoplasmic reticulum membranes possessing glucose-6-phosphatase and esterase enzymic activities in vastly higher (five times greater) concentrations than the remaining endoplasmic reticulum (as found in the smooth and rough microsomal fractions).

the galactosyltransferase assay as well as providing details of her studies prior to publication. Thanks are also extended to Dr. P. Lazarow (Rockefeller) for helpful details on the microsomal esterase assay and to Dr. R. Sikstrom (McGill) for much advice and encouragement. The assistance of Frances Power and Mme. Jeanne Fournel in biochemical assays, and Mr. R. Rachubinski, Mrs. Kathy Teng and Mrs. Ruth Partridge for assistance in morphological analyses is acknowledged. This research was supported by the M.R.C. of Canada and the U.S.P.H.S.

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