Structural Lipoproteins of the Mucosal Cell

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All subcellular components from guinea-pig intestinal mucosa contain the same mixture of sterols, suggesting that an equilibrium is maintained throughout the cell. During the absorption of sterols, the absorbed material mixes with the endogenous sterols in all the cell fractions (Glover & Green, 1957), most of it appearing in the mitochondria and microsomes (Green, 1963). To obtain information on the way in which the different endogenous and exogenous sterols are bound in the structural lipoproteins, mitochondria and microsomes from starved and dosed guinea pigs have been disrupted in various ways. A preliminary account of this work has been published (Green, 1962).

EXPERIMENTAL

Materials

7-Dehydrocholesterol was obtained from Peboc Ltd., Liverpool, and recrystallized from methanol. Sodium deoxycholate was from Hopkin and Williams Ltd., digitonin from L. Light and Co. and Carbowax 20M from G. T. Gurr Ltd.

Methods

Guinea pigs of mixed breed weighing 500–800 g. were used. Control animals were starved for 24 hr. before use; dosed animals were starved for 24 hr., then given 150 mg. of 7-dehydrocholesterol in 1 ml. of arachis oil and killed 4–5 hr. later. The intestinal mucosa was obtained and fractionated as described by Glover & Green (1957), except that the microsome fraction was obtained by centrifugation for 60 min. in a Spinco model L ultracentrifuge (rotor 40) at 105 000g.

Lipid was extracted from packed pellets by the method of Folch, Lees & Sloane-Stanley (1957) and from aqueous suspensions by that of Glover & Green (1957). Total sterol, 7-dehydrocholesterol, total nitrogen and phospholipid were estimated as previously described (Glover & Green, 1957; Glover, Green & Stainer, 1959; Ashworth & Green, 1963).

Treatment of mitochondrial and microsomal fractions. All operations were carried out at 4°. Each fraction was extracted as follows (Dallam, 1958) and not with 0.14M-NaCl alone as previously stated (Green, 1962). The mitochondrial or microsomal pellet was dispersed in a Potter-Elvehjem homogenizer in 0.14M-NaCl (13 ml.) and, after 10 min., centrifuged at 105 000g for 20 min. The precipitate was extracted again in the same way. A third extraction with 13 ml. of M-NaCl was carried out, the three extracts being combined for analysis. The residue was either suspended in 0.14M-NaCl (13 ml.) and treated for 20 min. with ultra-

sonic vibrations (Measuring and Scientific Equipment Ltd. disintegrator; 18–20 kcyc./sec.) or homogenized with one of the following solutions (13 ml. per pellet): (1) 0.5% sodium deoxycholate, pH 7.5–8.0; (2) 1% digitonin; (3) mixed detergents (Green, Tisdale, Criddle, Chen & Bock, 1961). After centrifugation for 20 min. at 105 000g, the total nitrogen, total sterol, phospholipid and 7-dehydrocholesterol contents of the supernatant and sediment were determined. The material present in the supernatant after such treatment was considered to be solubilized.

Paper electrophoresis. Solutions were concentrated eight- to ten-fold by dialysis against polyethylene glycol (Carbowax 20M) by the method of Kohn (1959). The buffer used was borate, pH 9.2 (6.2 g. of boric acid and 50 ml. of N-NaOH, diluted to 1 l.). A portion (0.5–1.0 ml.) of the concentrate was applied to large sheets of Whatman no. 3 filter paper and electrophoresis continued for 16 hr. at 150 v (5 mA). The sheets were dried in a stream of air, and narrow strips were cut out and stained with Naphthalene Black 12B to locate the protein zones. The rest of the sheet was then cut up into suitable pieces and each piece extracted separately with boiling chloroform-methanol (2:1, v/v). The mixture was filtered, the solvent was evaporated off in a stream of nitrogen and the lipid dissolved in 3 ml. of ethanol for the determination of 7-dehydrocholesterol, followed by determinations of total sterol or phospholipid or both.

RESULTS

The compositions of mitochondria and microsomes from the intestinal mucosa of starved guinea pigs are given in Table 1. For comparison, the results of one analysis of guinea-pig liver are included. The total sterol:total nitrogen and total sterol:7-dehydrocholesterol ratios are similar to those previously reported (Glover & Green, 1957; Glover *et al.* 1959). The total sterol includes cholesterol, dihydrocholesterol, 7-dehydrocholesterol and Δ^7 -cholesterol, the latter two making up 20% of the total. As these two sterols give a more intense Liebermann-Burchard colour than cholesterol, the estimated value for total sterol may be too high by 20%. No correction has been applied for this as the amount of dihydrocholesterol (which gives no colour with the Liebermann-Burchard reagents) is not known. In other tissues of the guinea pig it represents 2–16% of the total sterol (Werbin, Chaikoff & Imada, 1962).

The results show that the two subcellular fractions are very similar. The microsomes contain a higher percentage of lipid than the mitochondria

but its composition remains the same. The intestinal organelles are distinctive in possessing a relatively low concentration of phospholipid and a high concentration of sterol. The phospholipid:nitrogen ratios are only about half that of guinea-pig-liver microsomes and are much lower than the ratios (2:1–4:1) that can be calculated from published data on mitochondria and microsomes from a wide range of tissues and species (Dallam, 1955; Toschi, 1959; Ernster, Siekevitz & Palade, 1962; Dickman, Madison & Holtzer, 1962; Wherret & McIlwain, 1962; Roodyn, 1962). On the other hand, sterol makes up a much smaller proportion of the lipid in other tissues (Dallam, 1955; Spiro & McKibbin, 1956; Clement, Clement & Le Breton, 1956).

Extraction of mitochondria and microsomes. The combined saline extracts contained 32.5% of the total nitrogen of the mitochondria and 50.1% of that of the microsomes. These values are lower than those found by Dallam (1955, 1958) and Bartley, Getz, Notton & Renshaw (1962) for rat-liver fractions. Only very small amounts of lipid appeared in these extracts. From mitochondria less than 5% of sterol and phospholipid, and from microsomes less than 8% of sterol and phospholipid, were removed. The results indicate that the time of centrifugation chosen is sufficient to sediment intact organelles and hence that the removal of nitrogen represents a true extraction.

Many different means (mechanical, enzymic and chemical) have been used to disrupt subcellular particles. In the present studies some of the more common ones were employed. Their effects on the composition of the mitochondrial and microsomal residues are shown in Table 2. The different methods reveal no significant differences between the two fractions. Ultrasonic vibrations proved to be least efficient in solubilizing all constituents. If the time of exposure to the vibrations had been lengthened, the efficiency would probably have increased. However, 7-dehydrocholesterol was rapidly destroyed during this treatment, and the time chosen (20 min.) was a compromise between increasing destruction of this sterol and increasing solubilization of the other constituents. All detergents solubilized most of the nitrogen but they differed in their effects on the lipids. Sodium deoxycholate removed the highest proportion of all components. The very low yield of sterol in the supernatant after treatment with digitonin was expected since digitonin combines specifically with 3 β -hydroxy sterols. The mixture of detergents used by Green *et al.* (1961) in extracting the structural protein of mitochondria solubilized most of the nitrogen but only about half of the lipids.

The proportions of the various constituents of deoxycholate extracts are given in Table 3. The total sterol:total nitrogen ratios of both mitochondrial and microsomal extracts are those that

Table 1. *Composition of mitochondria and microsomes of guinea-pig liver and intestinal mucosa*

Experimental details are given in the text. The ratios (by wt.) are expressed as means $\pm$ S.E.M., with the numbers of experiments in parentheses.

	Phospholipid:total nitrogen ratio	Total sterol:total nitrogen ratio	Total sterol:7-dehydro- cholesterol ratio
Mucosal mitochondria	0.87 $\pm$ 0.26 (6)	0.21 $\pm$ 0.02 (6)	11.0 $\pm$ 1.3 (5)
Mucosal microsomes	1.08 $\pm$ 0.31 (5)	0.25 $\pm$ 0.02 (5)	11.7 $\pm$ 1.5 (5)
Liver microsomes	1.8 (1)	0.16 (1)	(1000)*

* From Glover, Leat & Morton (1957).

Table 2. *Action of detergents and ultrasonic vibrations on saline-extracted mitochondria and microsomes of guinea-pig intestinal mucosa*

Each mitochondrial or microsomal pellet was extracted twice with 0.14 M-NaCl and once with M-NaCl. The residue (R) was then treated with detergents or ultrasonic vibrations (as described in the text) and centrifuged at 105000g for 20 min. The percentage of a constituent solubilized is the proportion of the total in R present in the supernatant after centrifugation. Results are expressed as means, with the numbers of experiments in parentheses; where appropriate, the S.E.M. is given.

Treatment	Percentage solubilized					
	Mitochondria			Microsomes		
	Nitrogen	Total sterol	Phospholipid	Nitrogen	Total sterol	Phospholipid
Digitonin	69.5 (3)	11.2 (3)	55.8 (3)	78.3 (3)	12.7 (3)	65.8 (3)
Mixed detergents	71.1 (2)	42.8 (2)	49.7 (2)	79.6 (2)	50.3 (1)	62.5 (1)
Ultrasonics	33.5 (3)	26.5 (2)	37.0 (2)	53.2 (3)	42.4 (3)	20.3 (3)
Sodium deoxycholate	80.0 $\pm$ 2.5 (4)	78.7 $\pm$ 4.0 (5)	93.6 $\pm$ 1.8 (4)	83.2 $\pm$ 3.4 (4)	75.2 $\pm$ 4.5 (5)	83.9 $\pm$ 6.5 (4)

Table 3. Removal of lipids by sodium deoxycholate from saline-extracted mucosal mitochondria and microsomes prepared from starved guinea pigs and from animals absorbing 7-dehydrocholesterol

Experimental details are given in the text. The ratios (by wt.) are expressed as means $\pm$ S.E.M., with the numbers of observations in parentheses.

Starting preparation	Treatment of animal	Extract			Residue
		Phospho-lipid:total nitrogen ratio	Total sterol:total nitrogen ratio	Total sterol:7-dehydro-cholesterol ratio	Total sterol:7-dehydro-cholesterol ratio
Mitochondria	Starved	1.13 $\pm$ 0.29 (5)	0.30 $\pm$ 0.11 (8)	16.8 $\pm$ 1.5 (7)	11.0 $\pm$ 1.8 (5)
	Given 7-dehydrocholesterol	—	0.56 (2)	7.9 (2)	5.6 (2)
Microsomes	Starved	1.47 $\pm$ 0.27 (4)	0.47 $\pm$ 0.10 (8)	15.2 $\pm$ 1.5 (7)	12.8 $\pm$ 3.9 (4)
	Given 7-dehydrocholesterol	—	0.86 (2)	8.8 (2)	5.7 (2)

could be predicted from the saline-extraction values and the results given in Table 1. The phospholipid: nitrogen ratios of the two are lower than expected. It appears that 20–25% of the original phospholipid phosphorus does not appear in the lipid extracts. This may be because of difficulty in extraction in the presence of the detergents but is more probably due to breakdown of the phospholipids during the prolonged period required for the various manipulations. Bartley *et al.* (1962) found that hydrolysis of phospholipid occurred during treatment of rat-liver mitochondria with digitonin. Some destruction of 7-dehydrocholesterol also occurred, but, as the total sterol:7-dehydrocholesterol ratio in the residues obtained with deoxycholate is the same as that of the intact organelles, there is no discrimination between the two sterols by the detergent.

Paper electrophoresis. Concentrates of all extracts listed in Table 2 were subjected to paper electrophoresis but only the extracts obtained with deoxycholate gave discrete zones of protein and lipid. Several experiments were carried out with concentrates of extracts obtained with deoxycholate. The results were always similar for mitochondrial and microsomal preparations (Fig. 1). In each case, one migrating protein zone was seen, with some material remaining at the origin. Sterol and phospholipid were associated with both protein regions, the bulk being with the migrating material. 7-Dehydrocholesterol could not be detected after electrophoresis of mitochondrial extracts, and in only two out of seven experiments was it found in microsomal extracts. In both cases it was found at the origin. The amount present, however, was only a small fraction of that applied to the paper. Attempts to improve the recovery by altering the time, voltage and temperature of electrophoresis were unsuccessful.

Absorption of 7-dehydrocholesterol. The action of sodium chloride and deoxycholate on mitochondrial and microsomal fractions from the intestinal mucosa of guinea pigs absorbing 7-dehydrochole-

Fig. 1. Electrophoretograms of mitochondrial (a) and microsomal (b) extracts obtained with deoxycholate. The preparations were from starved guinea pigs. The horizontal inserts represent strips cut from the sheet and stained with Naphthalene Black 12B. The remainder of the sheet was divided into pieces 1 or 2 cm. wide by lines parallel to the origin. Each piece was cut out and eluted with chloroform-methanol and the amounts of the various lipids present were estimated. □, Total sterol; ▨, phospholipid; ▤, 7-dehydrocholesterol.

sterol was also investigated. The extracts obtained with saline contained only the same small amounts of sterol and phospholipid as those of preparations from starved animals. After treatment with deoxycholate, a creamy layer was recovered

from the top of the centrifuge tube. Although this contained a small amount of sterol, it was not studied in detail since experiments with [^{14}C]-cholesterol (Green, 1963) established that it was not of significance in absorption. Similar fatty layers have been recovered from organelles of other tissues (Ernster *et al.* 1962; Craston & Manery, 1963). They may contain the contents of fat droplets that can be seen to associate with the subcellular particles (Napolitano & Fawcett, 1958; Palade, 1959).

The total sterol:total nitrogen ratios given in Table 3 show that during the absorption of sterols both mitochondrial and microsomal structural lipoproteins double their sterol contents. Despite this large increase, treatment with deoxycholate does not discriminate between endogenous and absorbed sterol. Thus the proportion of sterol (and phospholipid) solubilized from saline-extracted mitochondria and microsomes was the same after giving 7-dehydrocholesterol as after starvation. Further, the total sterol:7-dehydrocholesterol ratios fell to the same extent in both solubilized and residual material.

DISCUSSION

The most striking fact to emerge from the present investigation is the similarity between the structural lipoproteins of mucosal mitochondria and microsomes. None of the treatments used brought out any difference in behaviour of any component of the two fractions. The microsomal fraction contains contributions from rough- and smooth-walled reticulum, the plasma membrane and probably from other sources. Although studies on other tissues indicate that mitochondrial preparations do consist mainly of mitochondria, they are never free from other particles. Thus the similarity may be more apparent than real. Carnie & Porteous (1962) found that particles from the brush border were present in mitochondrial and microsomal pellets obtained from rabbit mucosa by a technique similar to that employed in the present study. This gave rise to a distorted picture of intracellular enzyme distribution. However, this sort of consideration is of less importance when the materials being studied are major constituents of the cell. Previous work on sterol distribution strongly suggested that the sterols of all cellular lipoproteins (structural and soluble) were in equilibrium and that all behaved in the same way during absorption (Glover & Green, 1957; Glover *et al.* 1959; Glover & Stainer, 1959). The present investigation supports those conclusions. Studies on other tissues, both with the electron microscope and by chemical analysis, have also emphasized the great similarities between membrane structures of

cellular components (Dallam, 1955; Getz & Bartley, 1961; Levin & Thomas, 1961; Robertson, 1961; Ernster *et al.* 1962; Thomas & Levin, 1962).

It is difficult to compare the experiments with detergents and ultrasonic vibrations with those of other workers since (1) the mucosal preparations were first extracted with sodium chloride solutions, (2) different workers use different conditions (concentration, temperature, time of treatment etc.), and (3) criteria of solubilization vary. In general, however, the effects of detergents and ultrasonic vibrations are the same on mucosal preparations as on those from other tissues. Many workers have found that deoxycholate and digitonin solubilize most of the protein and phospholipid of mitochondria and microsomes. Although Carruthers & Baumler (1962) found that sodium dodecyl sulphate solubilized more nitrogen from mouse-liver microsomes than did deoxycholate, the results of Roodyn (1962) indicate that the mixture of cholate, deoxycholate and dodecyl sulphate used in the present work was relatively inefficient in disrupting mitochondria. Wherret & McIlwain (1962) found more protein and phospholipid of guinea-pig-brain microsomes solubilized by detergents than by treatment with ultrasonic vibrations. Thus the mucosal fractions respond in the same way as other tissues despite their higher sterol and lower phospholipid contents.

The action of detergents on membranes is not fully understood and the state of the solubilized material is unknown. Toschi (1959), from studies with the ultracentrifuge on Lubrol W extracts of brain microsomes, concluded that they contained relatively small lipoprotein macromolecules, and the results of Jungblut (1963), who investigated the action of deoxycholate on liver microsomes, supported this view. On the other hand, Ernster *et al.* (1962) considered that extracts obtained with deoxycholate may contain any of a range of particles, including individual molecules, micelles and/or vesicles of various sizes. That the membrane is not necessarily solubilized intact is shown by the results given in Table 2. Phospholipid and protein are well solubilized by digitonin, although the sterol has been removed, and in other cases lipid and protein are solubilized in different proportions. In extracts obtained with deoxycholate, it appears that protein, phospholipid and sterol remain together on electrophoresis. The failure to find 7-dehydrocholesterol with the main bulk of protein and lipid may mean that there is some difference in the binding of cholesterol and 7-dehydrocholesterol in the membranes. Heterogeneity of microsomal extracts obtained with detergent has been demonstrated by several physical techniques (Toschi, 1959; Moulé, Bouvet & Chauveau, 1963; Jungblut, 1963). It seems more likely, in view of the great

difficulty in detecting the sterol after electrophoresis, that 7-dehydrocholesterol is better protected against oxidation in the material remaining at the origin. This most probably includes the larger membrane fragments or vesicles. Support for this view is given by the results given in Table 3 which show that 7-dehydrocholesterol is destroyed in the extracts obtained with deoxycholate but not in the residues from such extraction.

The experiments on animals absorbing 7-dehydrocholesterol show that the absorbed sterol in the mitochondrial and microsomal fractions is not present in soluble lipoproteins or in associated fat droplets. It is bound to the membrane structure and responds to deoxycholate in the same way as the endogenous sterol. The separation of endogenous and absorbed sterol by electrophoresis of extracts obtained with deoxycholate is discussed by Green (1963).

SUMMARY

1. Mitochondria and microsomes of guinea-pig intestinal mucosa were treated with sodium chloride and detergent solutions and with ultrasonic vibrations.

2. Sodium chloride solutions removed 33 % of the total N from mitochondria and 50 % from microsomes, but only very small amounts of lipids were removed.

3. Treatment with deoxycholate solubilized a greater proportion (75–94 %) of total N, total sterol and phospholipid from the saline-extracted particles than any other method.

4. Paper electrophoresis of the extracts obtained with deoxycholate revealed one protein band migrating toward the anode. Most of the sterol and phospholipid was associated with the migrating zone with some remaining at the origin. The recovery of 7-dehydrocholesterol was very poor and it could be detected only at the origin.

5. During the absorption of 7-dehydrocholesterol the absorbed sterol present in the particles was firmly bound to the lipoprotein membrane.

6. Mitochondrial and microsomal particles behaved similarly in all experiments.

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