lymph will therefore contain a mixture of dietary and endogenous sterols together with reduced or oxidized derivatives formed by the action of reductases (or cholesterol dehydrogenases) within the mucosa.

Recently Swell, Trout, Hopper, Field & Treadwell (1958) have also observed that dietary [$4\text{-}^{14}\text{C}$]cholesterol mixed with the endogenous sterols of the intestine of the rat during absorption. It should be noted, however, that this exchange and mixing process involves only the endogenous sterols of the mucosa and connective-tissue layers, and not those of the muscle layers, with which the dietary sterols scarcely come into contact. It was shown previously that the sterol compositions of these two neighbouring types of tissue are quite different and therefore do not take part in the above rapid-exchange system (Glover *et al.* 1952; Glover & Green, 1957).

SUMMARY

1. The distribution of total sterols, 7-dehydrocholesterol and lathosterol between the various cell fractions of intestinal mucosa of fasting rats and animals dosed with 7-dehydrocholesterol has been determined.

2. In both groups of animals, about 50 % of the total sterols in the tissues was associated with the microsomes and mitochondria, 30 % with the supernatant or groundplasm and 10 % with the 'cell debris and nuclei' fraction. The mitochondrial fraction of the dosed rats contained more of the total sterol (15 %) than the corresponding fraction from fasting animals (10 %).

3. In agreement with previous work on the guinea pig, the various sterols were uniformly

distributed among the components of the mucosal cells of both fasting and dosed groups, implying that the accumulated dietary sterol can exchange or mix with the endogenous sterols of the cell components.

4. It is also postulated that in the rat the uptake and exchange of sterols between the mucosal cell lipoprotein plays an important role in the absorption of sterols. The sterols in excess of the fasting level which are finally transferred to the lymph will, however, contain a mixture of the dietary and endogenous types displaced by the exchange process.

5. A large part of the 7-dehydrocholesterol absorbed from the intestine is also reduced within the mucosa to lathosterol.

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Sterol Metabolism

5. THE UPTAKE OF STEROLS BY ORGANELLES OF INTESTINAL MUCOSA AND THE SITE OF THEIR ESTERIFICATION DURING ABSORPTION

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The total sterols in the intestinal mucosa of the fasting guinea pig, previously maintained on a cholesterol-free diet, are invariably unesterified in the free alcohol form (Glover, Glover & Morton, 1952). On the other hand, it has been widely established since the work of Mueller (1916) that absorbed cholesterol appears in the lymph mainly as the ester, irrespective of the form in which it is

administered. Cholesterol esters are hydrolysed mainly in the lumen of the intestine before absorption, but the position in the gut wall where resynthesis of esters commences has not yet been satisfactorily settled. Favarger & Metzger (1952) administered single doses of deuterated cholesterol oleate to rats, and found that the proportion of the labelled sterol in the free form was greater in the

mucosa than in the lumen of the intestine, implying that the action of the esterase is mainly hydrolytic in the mucosa. They then suggested that re-esterification of the free sterol probably occurred in the lymph.

It is necessary to establish, however, if esterification can occur to any appreciable extent within the mucosa during the absorption of cholesterol so that the role of cholesterol esterase in the latter process may be more clearly defined. The presence of cholesterol esterase within the mucosa means only that cholesterol esters might be formed from absorbed cholesterol if other conditions were satisfied. Such conditions could be an adequate supply of fatty acids or that the cholesterol should not be bound as a lipoprotein complex. Recent work on the absorption of cholesterol and 7-dehydrocholesterol in the guinea pig (Glover & Green, 1954, 1957) and the rat (Glover & Stainer, 1956, 1959) suggests that absorption of sterols is mediated via an exchange and transfer process between the lipoproteins of the mucosa. Under the conditions of these experiments, where absorption is occurring only over a limited period, the sterol may not then be esterified within the mucosa. The present work was planned to examine this point.

At the same time the measurement of sterol/nitrogen ratios for various mucosal cell fractions of fasting and dosed animals was undertaken to determine the extent of the uptake of absorbed sterols by each fraction during absorption.

EXPERIMENTAL

Materials

Recrystallized cholesterol (British Drug Houses Ltd., London) was purified further by forming the dibromide derivative and regenerating the free sterol as described by Schwenk & Werthessen (1952). Crystalline 7-dehydrocholesterol (obtained from Pebo Ltd., Liverpool) was freshly recrystallized from methanol as required.

Animals

Female guinea pigs (250–550 g.), divided into six pairs having approximately the same average weight (350 g.), were placed on a lipid-free diet (Glover *et al.* 1952) for 3 days before selection for experiment and then fasted for 24 hr. The control animals were examined at this stage. The test animals were given a dose of 300 mg. of cholesterol each in two stages: first, 150 mg. dispersed in 1 ml. of arachis oil was given mixed in approx 5 g. of lipid-free diet. After 24 hr. the remaining 150 mg. was given in the same way. The animals were killed 5½ hr. later. It was hoped that at this time the animals would be absorbing cholesterol along the greater part of the intestine.

Twelve groups of rats (140–265 g.) were prepared similarly. These animals were dosed with 7-dehydrocholesterol in two stages but each 150 mg. was mixed with 10 instead of 5 g. of lipid-free diet as used in guinea-pig experiments.

Methods

Cell-fractionation. The intestinal mucosal layer was scraped off the freshly removed intestine, placed in 25–30 ml. of 0.25 M-sucrose solution and comminuted for 90 sec. at 0° in a Potter-Elvehjem homogenizer fitted with a Teflon pestle. The cell fractions ['cell debris and nuclei', mitochondria, microsomes, supernatant groundplasm and F layer (fatty particles)] were prepared by high-speed centrifuging as previously described (Glover & Green, 1957).

A slight modification was introduced for the groups of rats used for determination of sterol/nitrogen ratios. The volume of sucrose added to the mucosa was only 10–15 ml. and the homogenate was centrifuged immediately at 20 000 *g* under a layer of light petroleum (b.p. 40–60°)–ether (50:50, v/v) at 5° for 90 min. Thus the F layer and supernatant, or groundplasm, fractions were obtained first, and the latter with a minimum amount of sucrose contaminant for the nitrogen assay. The pellet from the first high-speed centrifuging, containing all the particulate fractions, was then resuspended in 25–30 ml. of sucrose solution and fractionated by differential centrifuging as before. The supernatant from this fractionation labelled groundplasm (ii) was not assayed for nitrogen. Each fraction was divided into two equal parts, one of which was used for sterol assay and the other for nitrogen determination.

Determination of total, free and esterified sterols. The lipids were extracted from the various fractions as previously described (Glover & Green, 1957) and the total sterol content was determined on a suitable portion by the procedure described by Kenny (1952). It was considered better to look for small amounts of ester by a paper-chromatographic method than to estimate its presence in the filtrate from the precipitation of free sterol as the digitonide. A modification of the reversed-phase technique developed by Kodicek & Ashby (1954) worked well. The procedure was checked by carrying out some analyses on lipids from blood samples previously analysed by standard methods: the free cholesterol as digitonide (Sobel & Mayer, 1945) and total cholesterol by the methods of Abell, Levy, Brodie & Kendall (1952) and of Kenny (1952).

Paper-chromatographic procedure. Strips of Whatman no. 3 MM paper (1.5 in. × 10 in.) are immersed in a solution of liquid paraffin in light petroleum (5%, v/v), drained and dried at 60° for 10 min. The mobile phase is ethanol–water (95:5) mixture. The mixed lipid containing 1 mg. of total sterol is applied in chloroform solution from a micropipette (0.2 ml.) along a thin zone (0.5 cm. wide) about 4 cm. from one end of the strip. The paper is then mounted for ascending chromatography with this end just dipping in the mobile phase. The esterified cholesterol remains at the origin and the free material moves with R_f 0.8. The separation therefore takes place quickly and the paper may be examined about 1–2 hr. after commencing the chromatography, when the solvent front has moved 10–15 cm. An additional sample is usually run as a qualitative standard to check the position of the sterol zones, which may be located by spraying the dried paper with antimony trichloride reagent, and heating, as described by Kodicek & Ashby (1954). An alternative procedure is to cut the chromatogram into sections 2 cm. wide, which are folded and extracted with 3 ml. of chloroform in a test tube. The

paper is then lifted out and the Liebermann-Burchard test performed.

For the quantitative assay the two zones containing the different forms are cut out (up to 3 cm. wide) together with a blank zone taken above the solvent front. These pieces are folded and placed in a test tube (0.5 in. diam.) and covered with 3 ml. of chloroform. The tubes are shaken a few times at room temperature and the extract is transferred to a 10 ml. graduated tube fitted with a glass stopper. The papers are then washed twice with 3 ml. of ether and the bulked ether extracts carefully evaporated to dryness in test tubes (1 in. diam.). The respective residues are then transferred to the main extracts, not more than 2 ml. of chloroform being used for each. The final volume of each extract in the graduated tubes is made up to 5 ml., and the sterol content of the solution estimated spectrophotometrically at 410 $m\mu$ (Green, Lowe & Morton, 1955). The blank reading is deducted from the test and the amount of free or esterified sterol estimated from standard curves for the free and ester forms respectively.

The various results on blood-lipid samples are set out for comparison in Table 1. In a few cases, additional cholesterol or cholesterol ester was added to the blood lipids and this material was recovered quantitatively in the correct fraction.

Nitrogen determination. The various cell fractions used for nitrogen assay were made up to known volume with water and an appropriate portion was taken for analysis by the standard micro-Kjeldahl procedure with the Markham (1942) distillation apparatus. Each fraction was assayed in duplicate, and with each set of determinations duplicate ammonium sulphate standards and triplicate blanks were run.

Vitamin A absorption. One experiment with two guinea pigs was performed in the same manner with vitamin A alcohol in order to compare its absorption with that of the sterols. Each animal was given 22 mg. of vitamin A alcohol in 0.5 ml. of arachis oil. The free and combined vitamin A were separated from the extracted lipid of the intestine by column chromatography on alumina (Peter Spence and Sons Ltd., Widnes, Lancs.; grade 0) weakened by the addition of 5% of water (v/w) (Macqueen, 1948).

RESULTS

Occurrence of sterol esters in intestinal mucosal cells

When the mucosal cell fractions of four groups of fasting guinea pigs were examined for esterified sterols none could be detected. In the same number of experiments with animals dosed with cholesterol, sterol ester was found in the groundplasm fraction of only one group.

In rat intestinal mucosa, on the other hand, sterol esters were encountered more often but sporadically. In only two out of five groups of fasting rats examined were sterol esters detected. In both instances, esters were found in the groundplasm and F-layer fractions and in one group also in the microsome fraction. Similarly, esterified sterols were found in only three out of five groups of rats dosed with 7-dehydrocholesterol. Again, it was detected in the groundplasm (one group) and

Table 1. *Comparison of methods of estimating free and esterified cholesterol*

Results for free and esterified cholesterol separated by reversed-phase paper chromatography are compared with those obtained from the measurement of total cholesterol and the free alcohol as digitonide (Sobel & Mayer, 1945).

Blood-lipid sample no.	Free sterol		Ester		Total*	
	Digitonide (mg.)	Chromatography (mg.)	By difference (mg.)	Chromatography (mg.)	Direct method (mg.)	Chromatography (mg.)
1	0.160	0.160	0.390	0.390	0.550	0.550
2	—	0.175	—	0.375	—	0.550
2	0.155	0.140	0.395	0.400	0.550	0.540
3	0.275	0.280	0.635	0.605	0.910	0.885
4	0.145	0.135	0.255	0.290	0.400	0.425
	—	0.155	—	0.265	—	0.420
5	0.220	0.200	0.460	0.450	0.680	0.650
	—	0.205	—	0.428	—	0.633
6	0.268	0.258	0.250	0.247	0.518	0.505
7	0.425	0.409	0.483	0.476	0.908	0.885
	—	0.415	—	0.470	—	0.885
8	0.311	0.296	0.127	0.130	0.438	0.426
	—	0.315	—	0.135	—	0.450
9	0.285	0.300	0.850	0.830	1.135	1.130
	—	0.295	—	0.827	—	1.122
10	0.765	0.760	0.480	0.476	1.245	1.236
	—	0.778	—	0.463	—	1.241

* Estimated in samples 1-5 by the method of Abell, Levy, Brodie & Kendall (1952) and in samples 6-10 by the Kenny (1952) procedure.

F-layer (two groups) fractions. When sterol esters were detected, the amounts present were invariably quite small, less than 50 μ g.

The greater tendency for esterified cholesterol to appear in the F layer and groundplasm of the rat is possibly due to the fact that the rat absorbs fat a little better than the guinea pig (Cook & Thomson, 1951), and hence the immediate concentration of fatty acids within the mucosa may be higher during the absorption of the cholesterol.

In the comparative experiment in which vitamin A alcohol was examined the absorption pattern is different. In sharp contrast with the sterols this experiment shows the ester form present in every cell fraction of the guinea pig (Table 2). Approximately 0.5% of the original dose was in the mucosa at the time of examination. This is consistent with the observations of Glover, Goodwin & Morton (1947), who could not detect vitamin A in the mucosa of the fasting rat, and of Gray & Cawley (1942), who showed that it became esterified on absorption. It appears that the mucosal cell fractions have the capacity to take up lipids in addition to those present in the fasting

state just as vitamin A ester is taken up by liver-cell fractions (Schotz, Rice & Alfin-Slater, 1953).

Sterol/nitrogen ratios in mucosal cell fractions

Results for the total sterol/nitrogen ratios of the various mucosal cell fractions from fasting and dosed guinea pigs and rats are given in Table 3. The values for the corresponding fractions from the fasting animals of the two species are very similar. They are approximately double, however, the values for rat-liver fractions as calculated from the results of Spiro & McKibbin (1956). The generally higher ratios for the dosed animals indicate that the absorbed sterol is taken up by the lipoproteins of the organelles as well as simply exchanging with endogenous material (Glover & Green, 1957).

DISCUSSION

The present results show that cholesterol or 7-dehydrocholesterol esters are not formed to an appreciable extent in the mucosa of animals receiving a single dose. The fact that esters were detected only in trace amounts and sporadically implies that the esterification of the sterol is not essential for its passage across the outer-cell membrane or 'brush border'. Furthermore, the higher sterol/nitrogen ratios for the dosed animals compared with the controls indicate that the absorbed sterols have been taken up by the organelles. The implication is that although a readily available supply of fatty acids or neutral fat (from arachis oil in the dose) is also present in the cell, sterol absorbed in small amounts must be associated with protein or lipoprotein in a form which does not easily become esterified. In this connexion, Favarger & Metzger (1952) showed that a mucosal preparation in the presence of oleic acid readily esterified free cholesterol added in aqueous

Table 2. *Distribution of absorbed vitamin A in the cell fractions of the total intestinal mucosa from two guinea pigs examined 5.5 hr. after dosing*

Each animal was given 22 mg. of vitamin A alcohol in 0.5 ml. of arachis oil.

	Vitamin A		
	Total (μ g.)	Ester (μ g.)	Percentage as ester
Debris and nuclei	44	35	80
Mitochondria	46	22	48
Microsomes	45	35	78
Groundplasm	45	27	60
F layer	65	65	100
Whole mucosa (calc.)	245	184	75

Table 3. *Uptake of sterols by the various cell fractions of intestinal mucosa of the guinea pig and rat during the absorption of cholesterol and 7-dehydrocholesterol*

Two animals were used in each group and each was dosed with two 150 mg. portions of the sterol in 0.5 ml. of arachis oil, 24 hr. and 5½ hr. before examination.

Cell fraction		Total sterol (mg.)/nitrogen (mg.)			
		Guinea pig		Rat	
		Controls (3 expts.)	Dosed (3 expts.)	Controls (4 expts.)	Dosed (8 expts.)
Cell membranes and nuclei	Range	0.11–0.15	0.30; 0.39	0.19–0.23	0.26–0.94
	Mean ($\pm$ s.d.)	0.13 ($\pm$ 0.02)	0.35	0.21 ($\pm$ 0.02)	0.52 ($\pm$ 0.25)
Mitochondria	Range	0.18–0.21	0.21–0.27	0.18–0.28	0.31–0.67
	Mean ($\pm$ s.d.)	0.19 ($\pm$ 0.02)	0.23 ($\pm$ 0.03)	0.23 ($\pm$ 0.05)	0.45 ($\pm$ 0.14)
Microsomes	Range	0.28–0.33	0.34–0.45	0.34–0.40	0.42–0.62
	Mean ($\pm$ s.d.)	0.31 ($\pm$ 0.03)	0.41 ($\pm$ 0.06)	0.35 ($\pm$ 0.03)	0.52 ($\pm$ 0.07)
Groundplasm	Range	0.02–0.03	0.11; 0.07	0.03–0.05	0.06–0.15
	Mean ($\pm$ s.d.)	0.03 ($\pm$ 0.005)	0.09	0.05 ($\pm$ 0.02)	0.08 ($\pm$ 0.03)

dispersion but not free cholesterol added in the form of the lipoprotein complex existing in plasma. It is also interesting to contrast the behaviour of vitamin A with that of the two sterols. The greater proportion (75 %) of the former is readily esterified within the mucosal cell, although here a different enzyme may be involved. The experiment with vitamin A, however, suggests either that a much greater pool of fatty acids must be required to bring about the esterification of intestinal sterols or that the nature of the binding of the two substances to the cell proteins is quite different.

Cholesterol, like vitamin A, eventually appears in the chyle and in the chylomicra mainly in the esterified form (Daskalakis & Chaikoff, 1955; Page, Lewis & Plahl, 1953), but when labelled cholesterol was administered to rats radioactivity was first detected in the free fraction of lymph (Hellman, Rosenfeld, Gallagher, Adlersberg & Wang, 1953). Thus it appears that some cholesterol at least may enter the chyle in the free form. This is consistent with the work of Hernandez, Chaikoff, Dauben & Abraham (1954), who found that although *epi*-(3 α -OH)cholesterol is not esterified by cholesterol esterase, it is readily absorbed into the lymph from the intestine although at only one-half of the rate characteristic of cholesterol. So, esterification of 3 β -OH sterols probably takes place as they leave the mucosal cells and enter the connective tissue or in the lymph. As the proportion of ester in the quite rapidly flowing lymph is fairly constant, around 70 %, the former site is favoured. It is here that the incoming sterol will first tend to accumulate, and the excess, over and above that which can be held in a complex with protein, will then be exposed to the action of esterase. Esterification could then be regarded as ancillary to the absorption process in that it can accelerate its rate but is not apparently obligatory. The occasional occurrence of cholesterol esters in the F layer and microsome fraction of both species might be explained by assuming that some of the mucosal cells in the immediate region of optimum absorption had accumulated more cholesterol than could be accommodated as a lipoprotein complex involving the free hydroxyl group; the excess would then become esterified. An additional factor is the concentration of fatty acids within the mucosa, which, as mentioned previously, is likely to be greater in the rat. Cholesterol esters accumulate in the mucosal cells of the guinea pig when administration of the sterol is continued over a number of days (Green, 1955). There is also little doubt that the lymph channels beneath the mucosal layer will also contain sterol esters, but in general single doses of sterol do not yield any appreciable ester in the mucosa.

The manner by which the additional free or

esterified sterols are held by the various organelles of the mucosa is not yet clear. From the sterol/nitrogen ratios it would appear that their lipoproteins can carry a variable quantity of sterols. Furthermore, it is possible to change the type of sterol associated with the organelles through exchange during prolonged feeding with a particular sterol (Glover & Green, 1956). However, it still remains to be determined if any marked change in the morphology or function of the mitochondria (or microsomes) is brought about by these qualitative and quantitative changes in sterol content.

SUMMARY

1. Sterol esters are absent from the intestinal mucosal cells of fasting guinea pigs and occur only occasionally in the groundplasm fraction of cells from animals dosed with cholesterol. In the rat, sterol esters were found sporadically in the mucosa of both fasting animals and animals dosed with 7-dehydrocholesterol, mainly in the groundplasm and F-layer fractions. The results imply that esterification is not directly involved in the absorption of sterols across the outer-cell membrane.

2. By contrast, a single dose of vitamin A is mainly (approximately 75 %) esterified during absorption across the intestinal mucosa of the guinea pig. The vitamin becomes associated with each cell fraction.

3. A comparison of the sterol/nitrogen ratios for the cell fractions of fasting animals with those of sterol-dosed animals indicates that additional sterol can be taken up by the organelles. The latter therefore appear to play an important part in sterol absorption.

4. It is postulated that in these two species esterification of single doses of cholesterol occurs mainly in the connective tissue before its entry into the lymph vessels, and that the action of esterase, although not obligatory for absorption, greatly accelerates the process.

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Biochemical Aspects of Bovine Ketosis

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Although studies on ketosis arising from diabetes, starvation and other causes have occupied a host of workers in the past, comparatively little is known of the biochemical aspects of bovine ketosis. Apart from the increased occurrence of ketone bodies in the blood and urine, bovine ketosis is characterized by abnormally low concentrations of blood glucose and liver glycogen (Shaw, 1955). It was decided to analyse the blood of diseased animals for the presence of abnormal quantities of metabolites, such as amino nitrogen, urea, ammonia and (in particular) members of the citric acid cycle. Of the 12 constituents estimated, which included cholesterol, in the serum of approximately seventy normal and diseased animals, the investigation of the levels of pyruvate, citrate, α -oxoglutarate and succinate proved the most fruitful. The highly significant abnormalities in the levels of these four components in the serum of ketosed animals represent the main result of this work.

Only one team of workers was found to have reported studies on the levels of pyruvate and α -oxoglutarate in the literature. In these Carlström, Myrbäck, Holmin & Larsson (1939) suggested that in bovine ketosis the blood levels of the two keto acids were above normal, though the opposite view was taken with regard to pyruvate in later work (Carlström, 1944, 1950).

One of the tasks undertaken was the elaboration of specific methods for the estimation of quantities of serum constituents as low as 1–2 μ g. Another point considered was the decomposition of the blood components during the transport and clotting period in which they were exposed to ambient temperature. This problem has been studied extensively in preceding work (Bach & Hibbitt,

1958), when storage conditions were found which would ensure the stability of the serum constituents until their estimation was possible.

From the results of the present work it was concluded that in bovine ketosis an interference may take place with certain reactions of the Krebs cycle, and further that these disturbances may assist in the explanation of the other biochemical symptoms of the disease. Though no significant differences were found between the serum levels of the nitrogenous components and of cholesterol in normal and ketosed animals, it is thought that the record of results for these constituents in normal and abnormal serum may fill a gap in the literature.

METHODS

Animals. All the blood samples from normal and ketosed animals were obtained from cows of the Jersey breed. Some of the normal samples were collected from the Veterinary Field Station of the University of Bristol and the remainder of the normal samples as well as all samples from animals suffering from ketosis were collected from Jersey Island.

Collection of samples and analytical methods. The procedure for the collection of blood samples was identical with the one described in previous work (Bach & Hibbitt, 1958), as were the methods used for the estimation of amino nitrogen, urea, ammonia, ketone bodies, cholesterol and glucose. In view of the findings in the above-mentioned work about the lability of the various serum components, care was taken that these were estimated within that period of time after collection during which the components had previously been found to be stable at ambient temperature (Bach & Hibbitt, 1958).

Estimation of pyruvate and α -oxoglutarate. Hydrazones of these compounds were prepared according to standard methods, the modification described in previous work