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Biotin Deficiency and the Development of the Fatty Liver and Kidney Syndrome in Chickens: An Ultrastructural Study

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Abstract

The structure of the tissue from the liver, kidneys, pancreas and adrenal glands of 4-week-old chickens showing symptoms of the fatty liver and kidney syndrome (FLKS) was compared with that of normal tissue and related to the amount of biotin present in the liver tissue. These birds were reared with different levels of dietary biotin and were stressed by removal of food before being killed. Birds with less than 1·5 μg biotin/g liver were considered to be deficient in biotin. In the stressed birds the severity of FLKS increased with decreasing levels of biotin.

No lesions were found in the liver and kidney tissue of the birds with severe FLKS. Large quantities of fat were accumulated in the hepatocytes and intercellular spaces of the liver tissue, but the cell contents were not disorganized. In the kidney, conspicuous fat accumulation occurred in the proximal convoluted tubule cells and some ultrastructural disorganization of the cell contents was evident. No structural changes were found in the tissue of the pancreas or the adrenal glands of chickens suffering from severe FLKS.

Introduction

The fatty liver and kidney syndrome (FLKS) of 2- to 6-week-old chickens, which has caused considerable loss to the broiler industry in various countries, has been related to dietary causes and to stress, apparently healthy birds dying within a few hours of stress being applied. Affected chickens are characterized by pink carcass fat, fluid around the heart in severe cases, low blood glucose levels (hypoglycemia), enlarged fatty livers [a ratio of (liver weight / body weight) × 100 of approximately 5·0 instead of 2·0], enlarged fatty and pale kidneys, and a high level of palmitoleic acid in the livers (Pearson *et al.* 1976). There is no visible damage to the tissue in these birds.

It is now evident that FLKS can be controlled experimentally by increasing the level of dietary biotin (Whitehead *et al.* 1973; Payne *et al.* 1973, 1974; Pearson and Hemsley 1976; Pearson *et al.* 1976) and that the biotin content of the liver, which reflects the level of dietary biotin available, is related to the development and severity of FLKS during the imposition of stress (Hood *et al.* 1976). Hood *et al.* (1976) showed that chickens had no symptoms of FLKS and were unaffected by stress (removal of food) if the biotin level was above 1·5 μg/g liver; that stressed chickens had enlarged livers (hepatomegaly), but appeared healthy, if the biotin content of their livers was 0·5–1·5 μg/g; and that at lower levels of biotin, the stressed chickens died since homeostasis could be maintained no longer, the fat content of the enlarged livers increasing from 4–5% up to 12% on a fresh weight basis.

The purpose of this investigation was to use light and electron microscopy to examine the structure of liver, kidney, pancreas and adrenal gland tissue from 4-week-

old chickens which were fed diets containing various levels of biotin and stressed by the removal of food before killing.

Background information on the structure of the chicken liver was taken from Hickey and Elias (1954), Purton (1969*a*, 1969*b*) and Hodges (1972, 1974); on the chicken kidney from Siller (1971) and Hodges (1974); on the chicken pancreas from Langslow and Hales (1971) and Hodges (1974); and on the chicken adrenal gland from Fujita (1961), Sivaram (1965), Kondics and Kjaerheim (1966), Kjaerheim (1968), Wells and Wight (1971) and Unsicker (1973*a*, 1973*b*).

Table 1. Data available for the chickens used in the present investigation

Bird No.	Weight of bird (g)	Weight of liver (g)	$\frac{\text{Liver wt}}{\text{Body wt}} \times 100$	Fat content in liver (% fresh weight)	Biotin in feed (μg/kg)	Biotin in liver (μg/g)	Duration of fasting before killing (h)	Condition of bird at time of killing
				(*a*) *Normal birds*				
1	656	13·2	2·01	5·0	257	3·21	12	Healthy
2	666	12·3	2·10	4·3	257	2·71	12	Healthy
3	590	11·9	2·01	5·0	108	1·58	12	Healthy
4	695	14·4	2·08	4·0	108	2·72	12	Healthy
				(*b*) *Biotin deficient birds*				
5	495	17·7	3·58	5·5	93	0·44	12	Apparently healthy, but showing liver enlargement
6	493	22·6	4·58	9·8	93	0·38	12	Dying
7	451	19·5	4·32	6·1	93	0·43	18	Dying
8	451	22·7	5·03	11·1	76	0·12	12	Dying
9	355	16·5	4·65	12·3	76	0·12	12	Dying
10	489	23·8	4·87	9·2	76	—[A]	6	Dying

[A] No data available.

Materials and Methods

Source of Material

Chickens used in the investigation were 4 weeks old and were taken from the experiments of Hood *et al.* (1976). Ten chickens, which had been given a diet containing either 257, 108, 93 or 76 μg of biotin per kg of feed, were stressed by fasting for 6–18 h before being killed. The data provided by Hood *et al.* are set out in Table 1. These birds were divided into two classes according to the biotin content of their livers—those with less than 1·5 μg biotin/g liver were considered to be biotin deficient. Nos 1–4 remained healthy. Nos 6–10 had severe symptoms of FLKS and were dying at the time of killing. No. 5 had a slightly enlarged, but non-fatty liver and although considered to be biotin deficient it appeared healthy. It was not known whether this bird was suffering from mild FLKS or whether it had recovered partially from severe biotin deficiency symptoms.

Preparation for Electron Microscopy

Tissue was taken, as soon as possible after killing, from the liver, kidneys, pancreas and adrenal glands from each of the birds in Table 1 and prepared by a schedule based on that of Hayat and Giaquinta (1970) and Bain and Gove (1971). Pieces were cut approximately 1 mm^3; immersed in 3% glutaraldehyde in 0·1 M phosphate buffer (pH 7·3) for 30 min; washed in phosphate buffer; post-fixed in 1% OsO_4 in phosphate buffer for 30 min; washed, stained with 1% aqueous uranyl

acetate for 5 min; dehydrated rapidly in an alcohol series; placed into epoxy propane and then into a mixture of epoxy propane and Epon 812 (50 : 50) before being embedded in Epon 812 which was polymerized at 90°C overnight. Sections were stained on the grid with uranyl acetate and lead citrate (Reynolds 1963). Uranyl acetate was omitted from the liver tissue in some cases because of its known adverse effect on the staining of glycogen (see Bhatnagar and Leeson 1975), but as glycogen was reduced by fasting this omission was found unnecessary.

Preparation for Light Microscopy

Material, embedded as above, was sectioned at approximately 2 μm and stained with either toluidine blue (Mercer and Birbeck 1972) or with polychrome stain (Sato and Shamoto 1973).

Results

The severity of the disorder, as shown by the extent of liver enlargement and increase in fat content and the health of the bird following removal of food, was associated with a decreasing level of biotin in the liver (Table 1). Examination of the tissue from Nos 5–10 showed structural changes in the liver and kidneys when compared with those of Nos 1–4, but no changes were observed in the pancreas and adrenal glands of these birds. The ultrastructure of the liver and kidney tissue in the experimentally produced FLKS was similar to that examined in this laboratory in preliminary studies on chickens affected with FLKS in commercial flocks. Light micrographs (Figs 1–4) show the increased fat content in the livers and kidneys of the birds with FLKS.

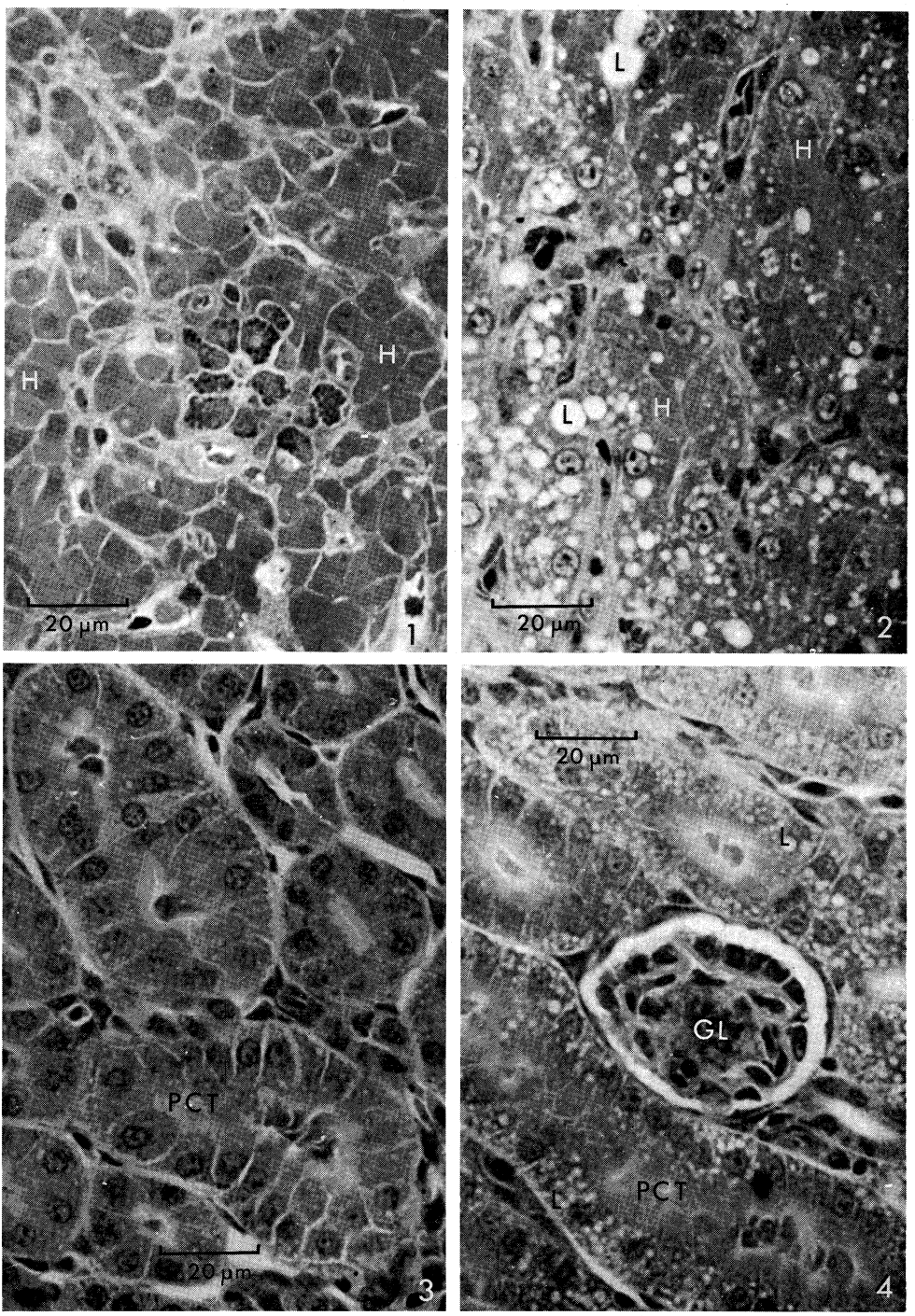
Structure of the Liver

(i) *Normal birds (Nos 1–4)*

The ultrastructure of the hepatocytes of these birds was similar to that described by Hodges (1972) for the immature fowl (Figs 5 and 6). The hepatocytes were commonly polygonal with a vascular and bilary pole, the plasmalemma contacting the bile canaliculus, the space of Dissé associated with the sinusoids, and the intercellular space. Two types of hepatocytes, 'light' and 'dark' cells according to the density of the cytoplasm, were noticeable throughout the tissue (Fig. 5). The light cells were fewer and were scattered singly or in groups within the section. The large nucleus was found usually at the vascular pole of the cell. Mitochondria were numerous in the cytoplasm. Rough endoplasmic reticulum was abundant, but composed of one or two short cisternae closely associated with the mitochondria; ribosomes were plentiful on the endoplasmic reticulum and in the cytoplasm; and smooth endoplasmic reticulum was found in areas of the cytoplasm amongst the depleted glycogen reserves. Golgi regions were seen infrequently in this material. When found, they were usually at the apical end of the cell and consisted of more than one Golgi complex. Each complex had only a few cisternae and two types of granules (amorphous granules within the cisternae and denser granules outside the cisternae) were associated with them (Fig. 6). Deposits of fat were not a feature of these cells, an occasional globule being seen in the cytoplasm. Large fat globules were prominent in the Kupffer cells found in the sinusoids.

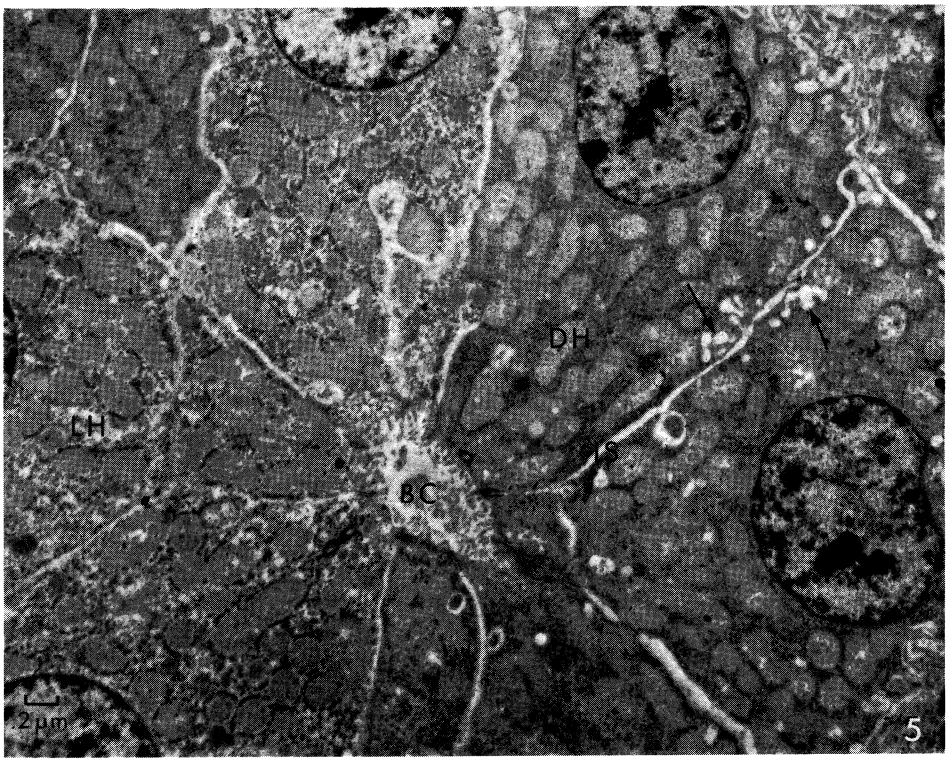
(ii) *Biotin deficient birds (Nos 5–10)*

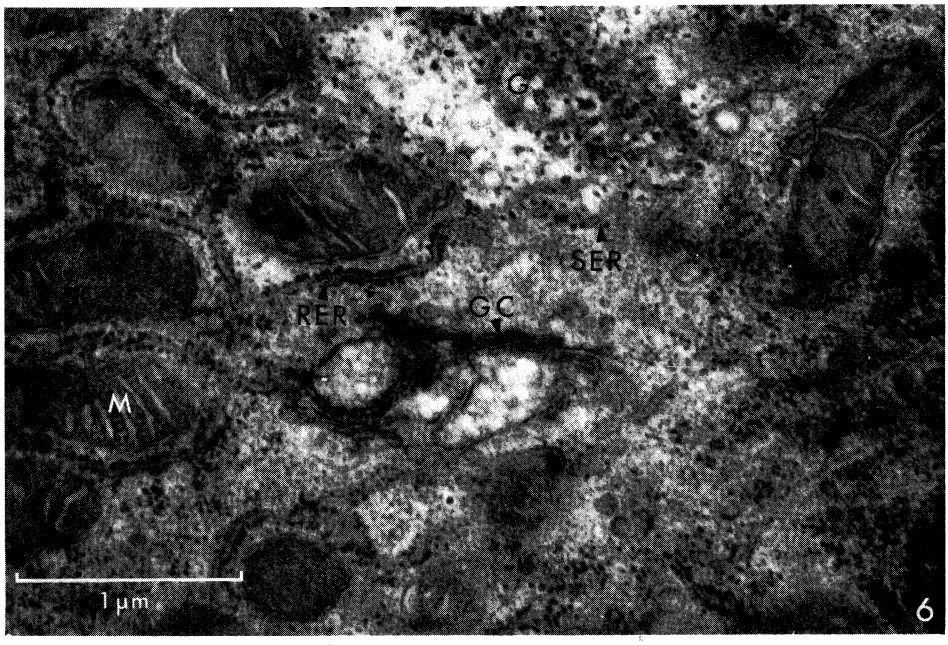
Figs 7, 8 and 9 show the details of the structure of the hepatocytes in bird No. 8, which was suffering from severe symptoms of FLKS at the time of killing. Large and small fat globules were prominent throughout the cytoplasm of both light and dark



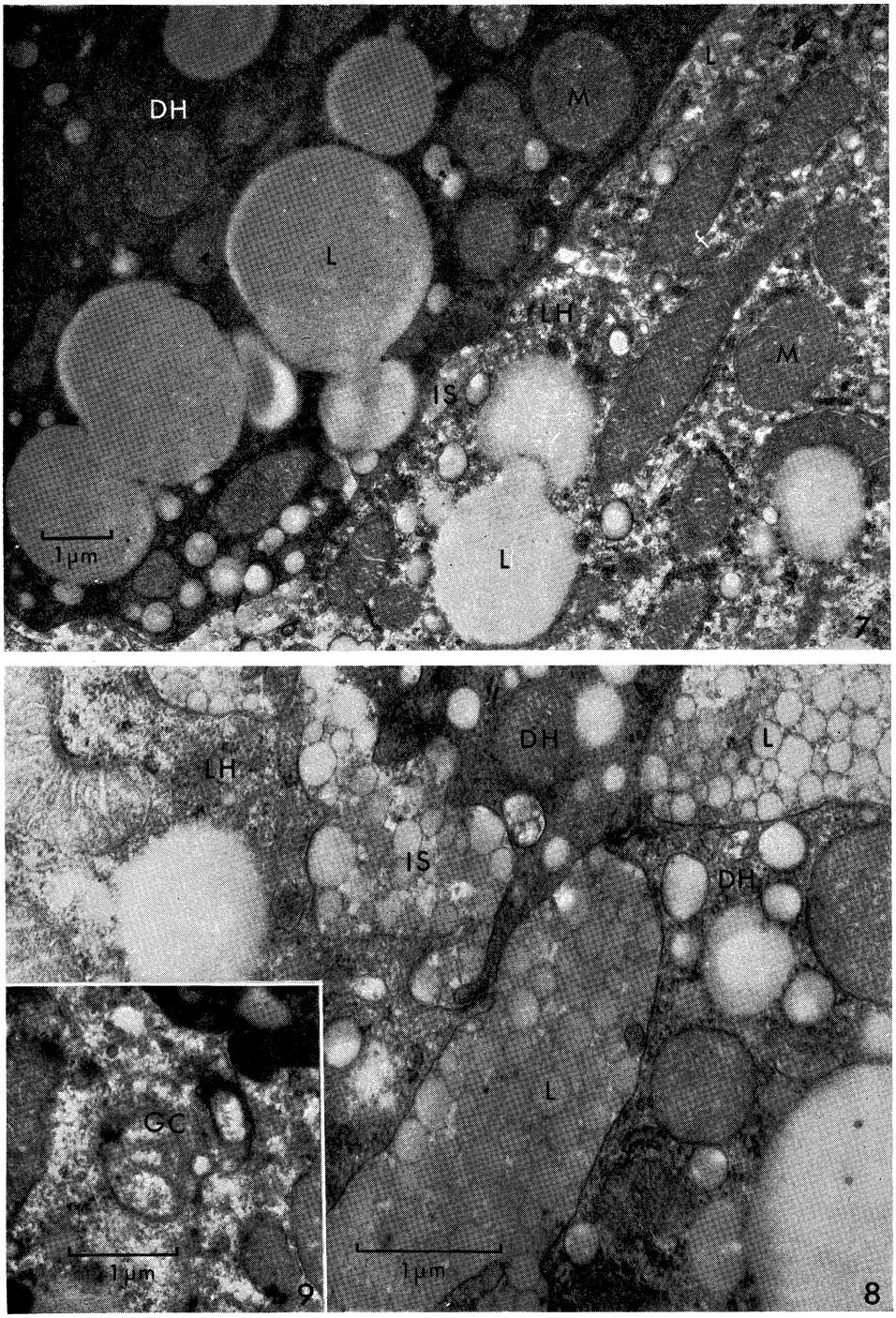
Figs 1 and 2. Light micrographs of chicken liver tissue showing increased fat content (*L*) in the hepatocytes (*H*) with the development of FLKS. Stained with toluidine blue. *1*, Normal tissue from bird No. 1. *2*, Tissue from the enlarged liver of bird No. 8.

Figs 3 and 4. Light micrographs of chicken kidney tissue showing increased fat content (*L*) in the cells of the proximal convoluted tubule (*PCT*) with the development of FLKS. A glomerulus is shown (*GL*). *3*, Normal tissue from bird No. 2. *4*, Tissue from a biotin deficient bird (No. 8).

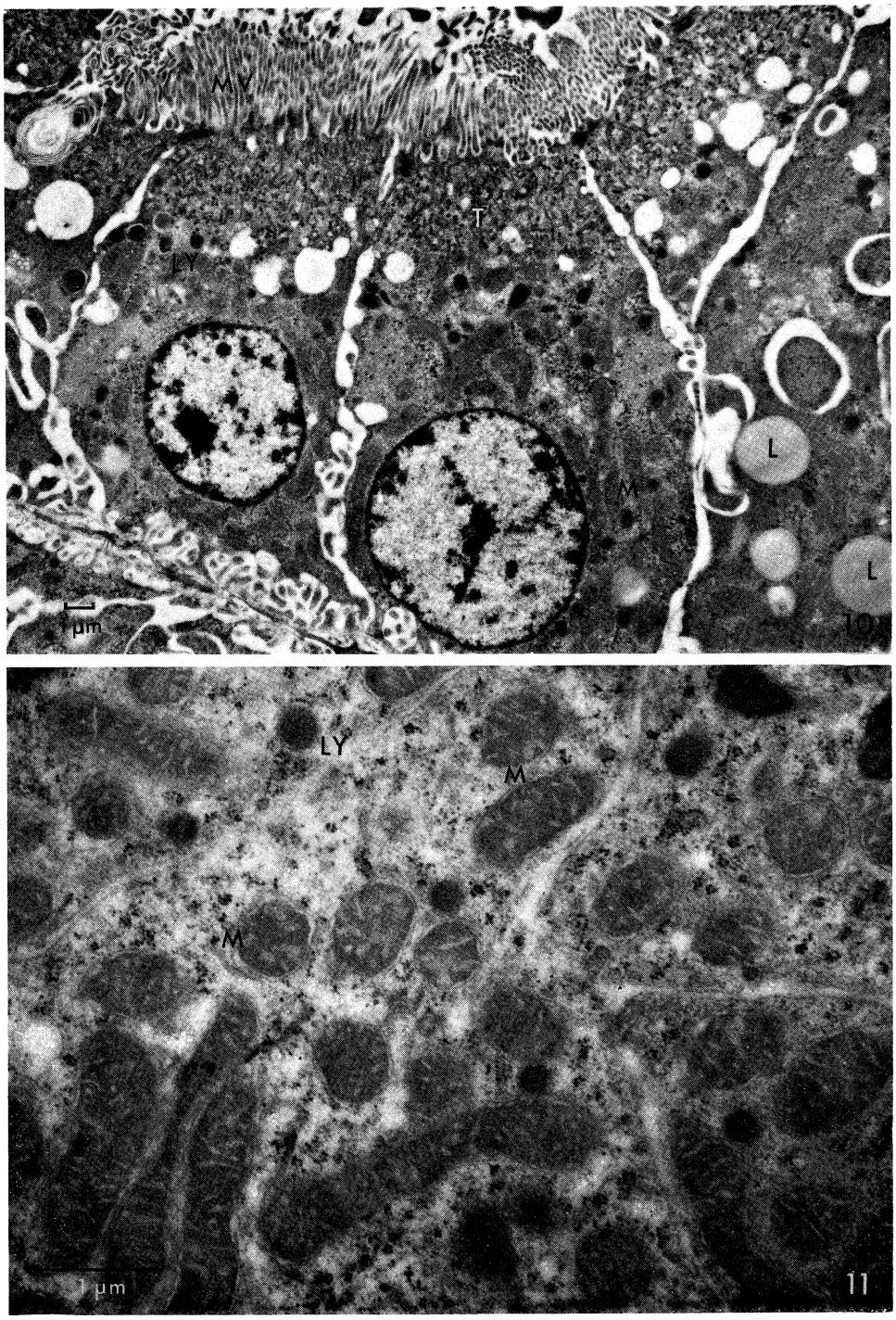




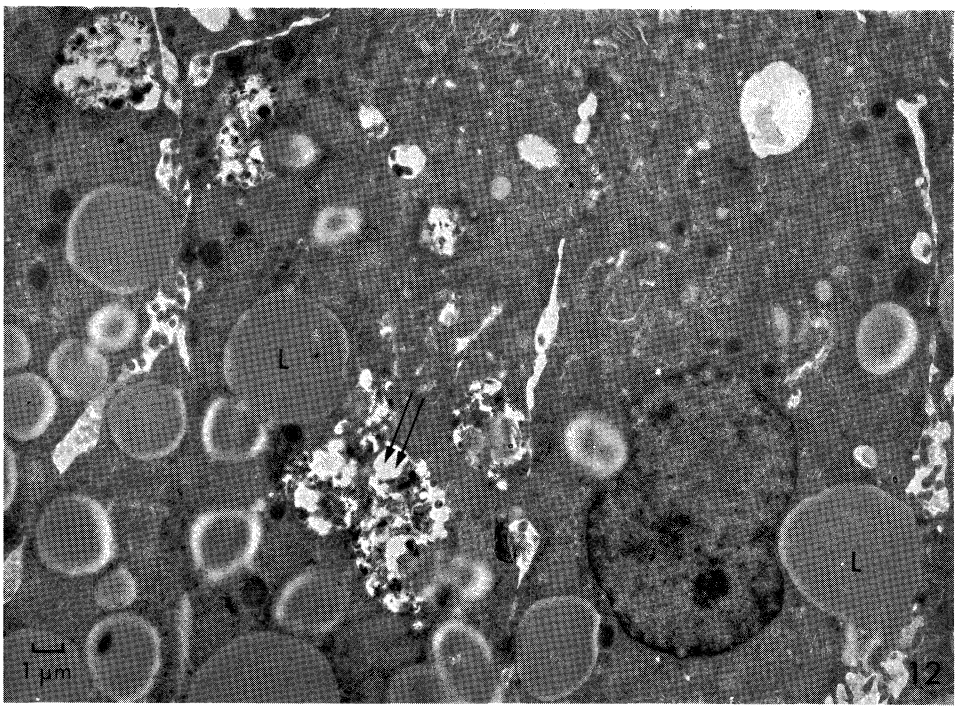
Figs 5 and 6. Normal chicken liver tissue. *5*, Bird No. 2. Light (*LH*) and dark (*DH*) hepatocytes are shown surrounding a bile canaliculus (*BC*). No large fat deposits are present. Small deposits of fat occur as light areas in the cell (→). These are close to the intercellular space (*IS*). *6*, Bird No. 3. Part of an hepatocyte showing rough endoplasmic reticulum (*RER*), mitochondria (*M*), smooth endoplasmic reticulum (*SER*) associated with some glycogen (*G*), and a functioning Golgi complex (*GC*).

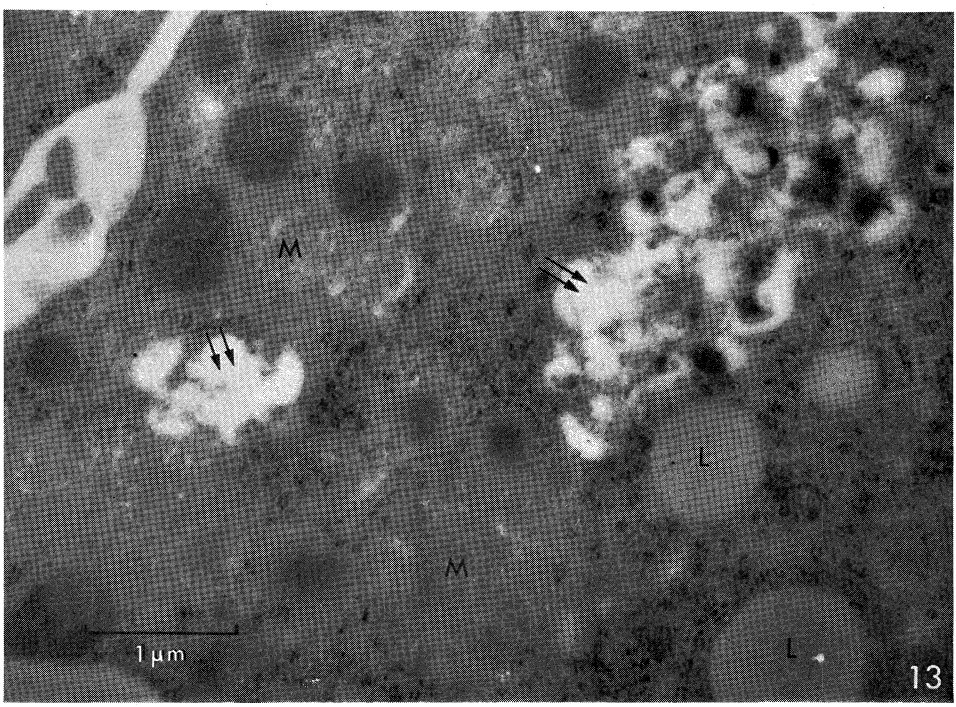


Figs 7, 8 and 9. Liver tissue of a biotin deficient bird with FLKS (bird No. 8). *7*, Fat (*L*) has accumulated in the cytoplasm of light (*LH*) and dark (*DH*) hepatocytes and in the intercellular space (*IS*). The cell contents are not disorganized. The mitochondria (*M*) have normal cristae structure. *8*, Detail of the small fat globules (*L*) which have accumulated in the much enlarged intercellular space (*IS*) between a light hepatocyte (*LH*) and two dark hepatocytes (*DH*). *9*, The Golgi complex (*GC*) is disorganized in this hepatocyte. A few small dense particles are present in the surrounding cytoplasm but no amorphous granules are associated with the cisternae. Compare with Fig. 6.



Figs 10 and 11. Normal kidney tissue (bird No. 2). *10*, The microvilli (*MV*) of the cells of the proximal convoluted tubule cells form a brush border to the tubule. Many small tubules (*T*) and lysosomes (*LY*) are found in the cell apex beneath the microvilli. Mitochondria (*M*) are numerous. *11*, Detail of the cytoplasmic structure of a cell of the proximal convoluted tubule. Numerous mitochondria (*M*) and lysosomes (*LY*) are shown.





Figs 12 and 13. Kidney tissue from a biotin deficient bird with FLKS (bird No. 8). *12*, Fat (*L*) has accumulated markedly in the cells of the proximal convoluted tubule. Compare with Fig. 10. Disorganized areas (double arrows) are evident in the cytoplasm. *13*, Detail of the cytoplasmic structure showing the disorganized appearance of the mitochondria (*M*), large accumulations of fat (*L*) and areas of breakdown (double arrows) in a proximal convoluted tubule cell.

cells (Figs 7 and 8). No membranes were associated with the fat globules, but ribosomes sometimes appeared very close to the surface of the small globules. In some instances the globules appeared very close to the mitochondria. There were instances when both large and small globules appeared to fuse with each other (Fig. 7). Small globules, which resembled those within the cells, were obvious in the intercellular space between the hepatocytes (Figs 7 and 8). The fat accumulations did not appear to cause internal disorganization of the hepatocytes (Fig 7). Fewer segments of rough endoplasmic reticulum were evident, but the internal structure of the mitochondria was unaltered. Lysosomes were numerous at the bilary pole of the cells. The Golgi complex was identified in a few instances, but it was disorganized with little evidence of a secretory function (Fig. 9).

Light micrographs from the liver tissue of bird No. 5 showed that most of the tissue was similar to that of the liver of the normal birds (Fig. 1), but fat accumulation had occurred in some hepatocytes. Electron micrographs showed that the increased fat content occurred as both large and small globules in the cytoplasm of these hepatocytes. Small fat globules were evident also in the widened intercellular spaces. Few Golgi complexes were seen and their activity appeared reduced when compared with their activity in the normal birds (Fig. 6), only a few cytoplasmic granules being associated with them.

Structure of the Kidney

(i) *Normal birds (Nos 1–4)*

The investigation was restricted to the proximal convoluted tubule (PCT) of the nephron. The PCT was characterized ultrastructurally as being made up of cells which had apical unbranched microvilli forming a brush border, small conspicuous tubules in the cytoplasm beneath the microvilli, a nucleus in the lower third of the cell, numerous microbodies and lysosomes, a rough endoplasmic reticulum less developed than in the hepatocytes, and numerous mitochondria with a well defined cristae structure (Figs 10 and 11). Golgi complexes were observed infrequently. They consisted of a few stacked cisternae and no secretory function was observed associated with them. A few large fat globules were evident in some of the PCT cells in both light micrographs (Fig. 3) and electron micrographs (Fig. 10).

(ii) *Biotin deficient birds (Nos 5–10)*

Fat globules were plentiful in the PCT cells in this tissue from birds showing severe symptoms of FLKS (Fig. 4). Electron micrographs showed that they occupied a large volume of the cell, with some large fat accumulations in contact with both the cytoplasm and the intercellular spaces (Fig. 12). It is possible that some small globules of fat-like material were present in the intercellular spaces of the PCT cells, but this quantity was small compared with the globules in the intercellular spaces of the liver tissue in the same birds. Areas of breakdown occurred in the cytoplasm (Fig. 12) and the structure of the cristae was indistinct in the rounded mitochondria (Fig. 13). A considerable increase in fat deposits was seen in the PCT cells of bird No. 5. Fat increase was more obvious in the kidney tissue than in the liver tissue of this bird, fat globules being scattered throughout the cytoplasm of the PCT cells. Their internal structure showed the same disorganization as described above.

Discussion

Fatty livers have been induced in a variety of animals by a variety of methods, e.g. the use of toxic fat (Allen and Carstens 1966), aflatoxin (Rao 1971), ethanol (Gordon and Lough 1972), manganese deficiency (Bell and Hurley 1973), DDT (David 1973), and clofibrate and orotic acid diets (Novikoff *et al.* 1974). These conditions caused an increase in the levels of triglycerides (triacylglycerols) in the liver and this was usually accompanied by the formation of lesions in the liver tissue and ultrastructural changes in the cell components. No macroscopic lesions were associated with FLKS in this investigation. This was in agreement with Wight and Siller (1975) who investigated the histopathology of FLKS in Scottish chickens and reported that there was no degenerative or inflammatory reaction associated with the syndrome. Lohr (1975), however, did describe pathological lesions in the hepatocytes when establishing that Q Disease in New Zealand chickens was similar to outbreaks of FLKS elsewhere.

Fixation of chicken liver tissue by immersion is reported to be inferior to that carried out by perfusion (Rothwell 1974), but experimental conditions necessitated its use in the present investigation. It could be expected that the preservation of the excessive amounts of fat material produced in the liver and kidneys of a chicken with severe symptoms of FLKS might present problems, but the quick processing methods used in the preparation of the present material for microscopical examination appeared to give adequate preservation of fat in the material examined. Fat accumulations were usually globular in section in this material, which was embedded in Epon 812, whereas Maxwell (1975) has shown irregularly shaped deposits of fat when the liver and kidney tissue of chickens with FLKS was embedded in Durcupan. The occurrence of light and dark hepatocytes, as found in all liver tissue examined here, is well documented (Allen and Carstens 1966; Ericsson and Biberfeld 1967; Fahimi 1967; Ganote and Moses 1968) and has been related to fixation by immersion (Ganote and Moses 1968). Drochmans *et al.* (1975), however, isolated hepatocytes from rat liver and obtained cells of different electron density (similar to light and dark cells in chicken liver) by isopycnic gradient ultracentrifugation. These differed in size, morphology and glycogen content.

Although the name of the syndrome indicates that the liver and kidneys are the main tissues affected in FLKS, other components show changes. Wight and Siller (1975) showed that the heart, pancreas, skeletal muscles, alimentary tract and central nervous system contained increased amounts of lipid and that the chromaffin cells of the adrenal glands showed decreased basophilia. In the present investigation no increase of fat was observed in the exocrine cells of the pancreas and glucagon granules and insulin crystals were present in the endocrine cells. It was expected that the liver would be the organ most affected in FLKS as liver enlargement is indicative of the early development of the disorder. The ultrastructure of the hepatocytes, however, did not show any obvious change, apart from increased fat accumulation, whereas that of the PCT cells in the kidney did show some change in the biotin deficient birds. Fat increase in the apparently healthy bird with 0·44 μg biotin/g liver (No. 5) appeared greater in the kidney tissue than in the liver tissue, indicating perhaps that the kidney could be more affected than the liver in FLKS.

Hood *et al.* (1976) and Pearson *et al.* (1976) have related abnormalities in carbohydrate and lipid metabolism in the livers of chickens with symptoms of FLKS to

the functioning of the biotin-dependent enzymes, pyruvate carboxylase and acetyl CoA carboxylase. They concluded that the activity of pyruvate carboxylase is insufficient to completely metabolize pyruvate via gluconeogenesis if a bird is stressed and the biotin content falls below 0·8 μg/g liver, that the liver increases in size in an attempt to remove the pyruvate and to maintain homeostasis, and that the activities of enzymes in alternative pathways for the removal of pyruvate increase, that is increased synthesis of fatty acids and accumulation of blood lactate occur. They concluded also that if the bird is stressed when the biotin level is below 0·35 μg/g liver, the mechanisms involved in hyperfunctional hepatomegaly are inadequate to maintain homeostasis and that they collapse, triglycerides accumulating in the liver and blood and the bird dying because it can no longer maintain blood glucose levels. The present electron micrographs showed that the accumulation of large quantities of fat in the hepatocytes of birds 6–10 was not associated with their malfunction. Lesions were not found in the tissue and there were no structural changes in the cell organelles. The accumulation of fat in the liver appeared to be a feature of the death of the bird, but not the cause of it.

The presence of small globules of fat material in the intercellular spaces of the hepatocytes was unusual, especially as chylomicrons have not been detected in the blood of chickens, even after they were supplied with a diet relatively high in fat (Annison 1971). Fat synthesized in the liver is transported normally to other tissues of mammals and birds as very low density lipoproteins (VLDL) which are derived from the Golgi complex and moved within vesicles towards the space of Dissé, being emitted from the hepatocyte by emiocytosis (Jones *et al.* 1966, 1967; Hamilton *et al.* 1967; Constantinides 1974). Proteins and phospholipids, synthesized in the rough endoplasmic reticulum, are coupled with triglycerides in the rough endoplasmic reticulum before entering the Golgi complex to be pinched off as VLDL. No electron micrographs have shown how the lipid is transported into or out of the endoplasmic reticulum (Jones *et al.* 1967; Stein and Stein 1967; Novikoff *et al.* 1974). No lipid-like material has been observed entering the cell at the space of Dissé or been seen between the endoplasmic reticulum and the lipid globules in the cytoplasm. Bar-On *et al.* (1971) have shown biochemically that the triglyceride in lipid droplets must be hydrolysed and then be resynthesized before being released into circulation as VLDL. Lombardi and Oler (1967) and Novikoff *et al.* (1974) related the development of fatty livers in rats to impairment of the release of VLDL resulting in an increase in liver triglycerides.

Golgi complexes, which are small in the fowl when compared with those of other animals, were not easy to find in the liver tissue used in this study. Rouiller and Jézéquel (1963) have stated that the Golgi complex is rarely visible in the fasting animal. This fact could explain its scarcity in the material under examination. Chodnik (1948) stated that the Golgi network was disorganized in the starving bird and that considerable rearrangement occurred after feeding to return it to normal after 6 h. Although birds 1–4 had been starved for 12 h, some functioning Golgi systems were observed in their hepatocytes, amorphous granules and smaller denser particles being associated with the cisternae and possibly representing stages in the formation of VLDL (Fig. 6). When Golgi complexes were recognized in the hepatocytes of birds with severe FLKS (Nos 6–10) they appeared disorganized and there was little evidence of a secretory function (Fig. 9). If the formation of lipoprotein particles was affected in this way, this could account for some of the accumulation of

triglycerides in the hepatocytes of birds with FLKS. The Golgi complex in bird No. 5 (enlarged non-fatty liver) showed decreased activity when compared with that in Fig. 6 (normal) but less disorganization than the complexes from the tissue of birds with severe FLKS.

It is known that stress is an important factor in the development of FLKS (Johnson *et al.* 1975; Whitehead *et al.* 1975). Payne *et al.* (1974) suggested that symptoms of FLKS may have been caused by malfunction of the adrenal glands under conditions of stress. It was thought that FLKS in the present experimental birds could have been related to biotin deficiency in the adrenal glands, as normal concentration of biotin in these glands is high (Eisenstein 1967), but no ultrastructural changes were observed to relate their function under stress conditions to the level of dietary biotin—even though it is known that there is a decrease in adrenaline and noradrenaline in the adrenal glands of chickens subjected to experimental stress (Zachariasen and Newcomer 1974). Wight and Siller (1975) did not find increased lipid deposition in the adrenal glands of chickens with FLKS, but Wight (1975), on further investigation, found that 60% of the cases with FLKS had depleted amounts of catecholamines in the chromaffin cells. The effect of biotin levels on the functioning of the adrenal glands could provide a valuable contribution to the understanding of the causes of this syndrome, perhaps making the widely accepted nomenclature, fatty liver and kidney syndrome, even more of a misnomer than at present.

Note

Since this manuscript was prepared, a paper entitled 'An ultrastructural study of the liver, kidney and myocardium in the fatty liver and kidney syndrome in the fowl' has been published (Siller, W. G., and Wight, P. A. L., *Res. Vet. Sci.*, 1976, **21**, 79–89). That study showed similar intracellular and extracellular accumulation of lipid material in the liver and kidneys of birds with FLKS as was described in the present investigation.

Acknowledgments

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