Fatty Liver and Kidney Syndrome in Chicks
II.* Biochemical Role of Biotin

R. L. Hood, A. R. Johnson, A. C. Fogerty and Judith A. Pearson

Division of Food Research, CSIRO, P.O. Box 52, North Ryde, N.S.W. 2113.

Abstract

The role of biotin-dependent enzymes in the fatty liver and kidney syndrome of young chicks was studied. Under conditions of a marginal deficiency of dietary biotin, the level of biotin in the liver has differing effects on the activities of two biotin-dependent enzymes, pyruvate carboxylase and acetyl-CoA carboxylase. The activity of acetyl-CoA carboxylase is increased, but when the dietary deficiency of biotin produces biotin levels which are below $0 \cdot 8 \, \mu g/g$ of liver, the activity of pyruvate carboxylase may be insufficient to completely metabolize pyruvate via gluconeogenesis. There is an increase in liver size and in the activities of enzymes involved in alternate pathways for the removal of pyruvate. Blood lactate accumulates and there is increased synthesis of fatty acids, and an accumulation of palmitoleic acid; these steps are accomplished by increased activities of at least the following enzymes: acetyl-CoA carboxylase, malate dehydrogenase (decarboxylating) ($NADP^+$) and the desaturase enzyme. When the biotin level is below $0 \cdot 35 \, \mu g/g$ of liver and the chick is subjected to a stress, physiological defence mechanisms of the chick may be inadequate to maintain homeostasis and they finally collapse, resulting in accumulation of triacylglycerol in the liver and blood; the chick is unable to maintain blood glucose levels and death occurs, often only a few hours after the imposition of the stress.

Introduction

The role of dietary biotin in the fatty liver and kidney syndrome (FLKS) of young chicks was reported in the preceding paper (Pearson *et al.* 1976). The combination of a diet low in biotin (Blair and Whitehead 1974; Payne *et al.* 1974; Whitehead *et al.* 1974; Johnson *et al.* 1975; Pearson *et al.* 1976), of chicks from a young parent flock (Hemsley and Marshall 1973; Payne *et al.* 1974) and of stress (Johnson *et al.* 1972) will precipitate FLKS in commercial broiler flocks or in chickens under laboratory conditions. Birds severely affected with FLKS have lowered blood glucose and increased lipid deposition in the liver (Evans and Bannister 1974; Johnson *et al.* 1975; Pearson *et al.* 1976) suggesting malfunctions in carbohydrate and lipid metabolism respectively.

Pyruvate carboxylase, a biotin-dependent enzyme (Scrutton and Utter 1965), is an important link in the supply of four-carbon units for gluconeogenesis and in the replenishment of intermediates in the tricarboxylic acid cycle. Atwal *et al.* (1971, 1972) reported that increased dietary biotin resulted in higher pyruvate carboxylase activity in livers of 3-week-old chicks. In birds, as is the case in mammals, hepatic lipogenesis and the activity of associated enzymes are related to the composition of the diet (Balnave and Pearce 1969; Yeh and Leveille 1969; Yeh *et al.* 1970).

However, demonstration of the response of acetyl-CoA carboxylase, the first enzyme identified as biotin dependent (Wakil *et al.* 1958), to conditions of biotin deficiency has not been entirely successful (Donaldson 1964; Balnave and Brown 1967; Puddu *et al.* 1967; Dakshinamurti and Desjardins 1968), although most studies have shown a small decline in acetyl-CoA carboxylase activity.

This study was undertaken to investigate the biochemical role of dietary biotin in FLKS of young chicks and to examine the findings in relation to the observed abnormalities of carbohydrate and lipid metabolism in this disorder.

Materials and Methods

The husbandry procedures and several of the biochemical determinations used in the two experiments reported in this paper have been described in Part I (Pearson *et al.* 1976). In the present study, 1-day-old female chicks from young parent flocks had been maintained on the various diets for 4 weeks when studies on lipogenesis were undertaken. The low-biotin diets were prepared as previously described (Pearson *et al.* 1976). In the first experiment the control diet was identical to the low-biotin diet except that supplemental biotin had been added. In the second experiment four diets were prepared: a basal low-biotin diet and three other diets identical with the basal diet but with increasing levels of added biotin; the biotin levels (μg/kg feed) in these four diets were 76 for diet 1, 93 for diet 2, 108 for diet 3 and 257 for diet 4. In the first experiment some of the birds were fasted for 18 h before being killed, whereas in the second experiment some were fasted for 6, 12 or 18 h.

Studies of lipogenesis with [1-^{14}C]acetate and [U-^{14}C]glucose (Radiochemical Centre, Amersham, England) were carried out using *in vitro* liver-slice techniques (Hood *et al.* 1972). Pyruvate carboxylase (EC 6.4.1.1) was assayed in a liver homogenate (pH 7·4) prepared in 0·25 M sucrose (Ballard *et al.* 1970). Acetyl-CoA carboxylase (EC 6.4.1.2), isocitrate dehydrogenase (NADP$^+$) (EC 1.1.1.42), malate dehydrogenase (decarboxylating) (NADP$^+$) (EC 1.1.1.40), glucose-6-phosphate dehydrogenase (EC 1.1.1.49) and phosphogluconate dehydrogenase (decarboxylating) (EC 1.1.1.44) were assayed as previously described (Hood and Allen 1973*a*, 1973*b*). Blood serum samples were analysed for glucose and triacylglycerol using standard clinical techniques (single channel Auto Analyzer I, Technicon Methods N-9P and N-78 respectively). Lactate in the blood serum was assayed using a standard test kit (Boehringer Mannheim, Germany). Biotin in the diets and livers was determined using a radiochemical assay (Hood 1975).

In the tables data are expressed as means ± standard error of the means.

Results and Discussion

Since hepatomegaly was a consistent symptom in chicks with FLKS, a liver weight to body weight ratio higher than the mean ratio for control chicks by at least two standard deviations was used as an index of FLKS (Pearson *et al.* 1976) in the first experiment. However, during the design of the second experiment it became apparent that the biotin status of the chick was the key factor in precipitating FLKS; therefore, the biotin level of the liver, the organ most affected in this disorder, was selected as the index of FLKS. When FLKS was present, the severity of the disorder was indicated by the level of blood glucose, since hypoglycemia is a likely factor contributing to mortality in biotin-deficient chicks (see Table 1 for limits of liver biotin and blood glucose).

Hyperfunctional Hepatomegaly

Smyth *et al.* (1952) have established that a comparison of the ratio of liver weight to body weight is a sensitive index of nutritional stress, which results in hypertrophy of the liver due to the metabolism and removal of the material under study. For

example, Brown *et al.* (1959) showed that hepatomegaly resulted from the feeding of antioxidant butylated hydroxytoluene (BHT) to rats. Gaunt *et al.* (1965) confirmed these observations and showed that in addition the liver enzymes responsible for the detoxification of BHT and other drugs were hyperfunctional. This increased activity results from an increased availability of substrates and may reflect an increased rate of synthesis of the enzymes or a decreased rate of their degradation (Schimke 1969). Most examples of increased activity of liver enzymes as a control mechanism to maintain homeostasis are produced by compounds normally foreign to the body.

In birds suffering from symptoms of FLKS, hyperfunctional hepatomegaly, which developed in the absence of histopathological changes (J. Bain, personal communication), was invariably present and was probably a physiological response aimed at removing the excess glucose metabolites that accumulate due to impaired gluconeogenesis.
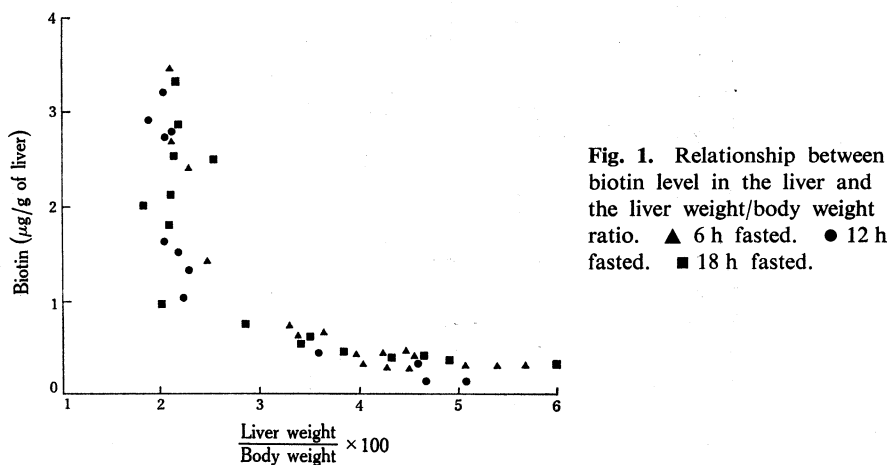

Fig. 1. Relationship between biotin level in the liver and the liver weight/body weight ratio. ▲ 6 h fasted. ● 12 h fasted. ■ 18 h fasted.

Liver Biotin

The relationship between liver biotin and the liver weight/body weight ratio is shown in Fig. 1. Biotin levels in the liver decrease in response to decreased dietary intakes but liver enlargement occurs only when liver biotin approaches a critical level of approximately $1 \cdot 5$ μg/g of liver. At levels of biotin between $1 \cdot 5$ and $0 \cdot 5$ μg/g liver small increases in liver weight become apparent. However, at very low biotin levels in the liver the weight of this organ increases dramatically.

In Table 1, chicks are grouped at each fasting period solely on the basis of the presence or absence of FLKS, i.e. chicks within these classifications may come from any of the four dietary treatments. Chicks classified as affected by FLKS on the basis of liver biotin were found in three of the four dietary treatments. Diet 4 (257 μg biotin/kg feed) was the only treatment which did not produce instances of FLKS. The variation in the severity of FLKS within chicks on the same dietary intake of biotin is presumably related to the biotin status of the chick at hatching, which is presumably related to the biotin status of the parent flock (Hemsley and Marshall 1973; Payne *et al.* 1974). The chicks with low initial biotin levels could be expected to be more susceptible to FLKS when given diets containing low levels of

Table 1. Effect of fasting and FLKS on various biochemical parameters in the liver and blood of 4-week-old chicks

PC, Pyruvate carboxylase; ACC, acetyl-CoA carboxylase; MDH, malate dehydrogenase (decarboxylating) (NADP$^+$)

Fasting period (h)	FLKS[A]	No. of chicks	Liver biotin (µg/g)	Blood parameters (mg/100 ml)			$\frac{\text{Liver wt}}{\text{Body wt}} \times 100$	% Liver lipid	% 16:1 in fatty acids	Substrate converted [nmol min^{-1} (g liver)$^{-1}$]			Substrate converted to total lipid [nmol h^{-1} (g liver)$^{-1}$]	
				Glucose	Triacyl-glycerols	Lactate				PC	ACC	MDH	Acetate	Glucose
0	Absent	5	2·11±0·12	256±8	90±10	49±6	2·93±0·14	5·4±0·6	6·0±0·6	6226±749	89±17	3448±193	84±11	29±2
	Mild	7	0·38±0·05	278±17	149±39	72±3	4·80±0·23	4·5±0·2	10·0±1·3	1005±262	115±16	4776±758	117±12	29±5
6	Absent	4	2·49±0·43	235±12	65±7	45±4	2·23±0·09	4·8±0·2	2·9±0·2	6585±1306	59±21	3707±197	63±19	39±7
	Mild	4	0·45±0·09	240±30	83±20	93±26	3·94±0·23	4·2±0·2	6·6±1·2	1804±1075	72±8	3990±526	65±3	28±5
	Severe	4	0·46±0·13	66±18	355±141	107±9	4·17±0·39	7·5±0·8	14·5±3·3	827±162	33±17	5005±228	60±10	34±7
12	Absent	8	2·13±0·30	226±3	53±4	41±5	2·08±0·05	4·6±0·1	2·4±0·2	8077±823	18±4	3693±142	32±3	42±3
	Severe	4	0·26±0·08	85±28	300±70	111±16	4·46±0·31	9·7±1·5	16·4±3·6	1091±316	42±9	4665±391	27±2	51±3
18	Absent	8	2·24±0·25	211±3	52±3	42±5	2·13±0·07	4·8±0·2	2·1±0·1	7698±524	9±5	3692±484	11±2	22±5
	Mild	4	0·59±0·06	212±4	53±3	77±9	3·40±0·21	5·8±1·6	5·9±2·0	1683±445	15±1	3945±603	12±6	25±3
	Severe	4	0·32±0·06	134±19	206±99	64±13	4·96±0·36	12·6±2·5	15·8±2·0	771±27	19±8	4087±320	34±12	40±7

[A] FLKS symptoms

FLKS symptoms	Liver biotin (µg/g)	Blood glucose (mg/100 ml)
Absent	> 0·8	> 180
Mild	< 0·8	> 180
Severe	< 0·8	< 180

biotin. Biotin levels varying from 2·3 to 3·8 μg/g of liver were found in 1-day-old chicks used in this experiment.

After 2 weeks on the diets containing varying biotin levels, four chicks, randomly selected from each group, were killed, and the levels of biotin in the liver were determined. The average liver biotin levels for diets 1, 2, 3 and 4 were 0·26, 0·38, 1·02 and 2·77 μg biotin/g of liver respectively; the biotin levels in the liver were quite variable within a treatment, particularly for diets 2 and 3 which contained intermediate biotin levels. This variability was also reflected in the severity of the FLKS symptoms (e.g. liver weight, percentage liver lipid) and justified the classi-fication of the results into groups based on biotin level in the liver rather than dietary biotin. All the chicks which died of FLKS during the experiment had their livers analysed for biotin and these were found to contain less than 0·35 μg/g of liver. In chicks clinically affected with FLKS, Pearson and Hemsley (1976) have also reported low levels of biotin in the liver.

Biotin-dependent Enzymes

The activities of pyruvate carboxylase and acetyl-CoA carboxylase, biotin-dependent enzymes involved in gluconeogenesis and lipogenesis respectively, were investigated. Inhibition of pyruvate carboxylase presumably decreases the conversion of pyruvate to oxaloacetate, which is an intermediary step in the synthesis of glucose from pyruvate and lactate, whereas inhibition of acetyl-CoA carboxylase should decrease the synthesis of fatty acids from acetyl-CoA.

The activity of pyruvate carboxylase decreased with decreasing biotin levels in the liver, whereas no decline in acetyl-CoA carboxylase activity was observed when liver biotin levels were low (Table 1). After 4 weeks on the low-biotin diet, six chicks were given the diet which was highest in biotin. After 4 days on this diet, pyruvate carboxylase activity had increased approximately fourfold in response to the increased intake of dietary biotin, whilst the activity of acetyl-CoA carboxylase was not altered. Correlation of liver biotin level with pyruvate carboxylase activity resulted in a significant ($P < 0·01$) correlation coefficient of 0·89. In chicks not affected with FLKS, pyruvate carboxylase activity increased slightly with fasting, thus maintaining blood glucose levels. The livers of chicks are reported to have smaller glycogen reserves than those of guinea pigs and rats (Sarkar 1971) and, there-fore, may be more susceptible to impairment of gluconeogenesis. Acetyl-CoA carboxy-lase activity decreased in response to fasting but was generally higher in chicks which were affected with FLKS (Tables 1 and 2).

The effect of biotin deficiency on the activity of pyruvate carboxylase was quite different from its effect on acetyl-CoA carboxylase, although both enzymes are biotin dependent. It is apparent that various biotin-dependent enzymes require different amounts of biotin to be completely active and their activities are affected to varying degrees by a marginal deficiency of biotin in chicks. In biotin deficiency of rats, propionyl-CoA carboxylase activity was decreased to one-tenth of control levels, whereas acetyl-CoA carboxylase activity was reduced by one-half (Dakshina-murti and Desjardins 1968). In 3-week-old chicks which were fed a purified diet and were selected for biotin deficiency on the basis of obvious dermal lesions, Mason and Donaldson (1972) reported a reduction in the activity of acetyl-CoA

carboxylase.   The response of acetyl-CoA carboxylase to biotin deficiency is therefore dependent on the severity of the deficiency.

Acetyl-CoA carboxylase contains 1 mol of covalently bound biotin per active molecule (Moss and Lane 1971) whilst pyruvate carboxylase contains approximately four biotin prosthetic groups (Scrutton and Utter 1965), thus indicating that more biotin is required for the synthesis of the pyruvate carboxylase holoenzyme than the acetyl-CoA carboxylase holoenzyme.   Acetyl-CoA carboxylase is a cytoplasmic enzyme, whereas in chicken liver pyruvate carboxylase is predominantly a mitochondrial enzyme (Madappally and Mistry 1970).   Biotin may therefore be more accessible for the synthesis of the cytoplasmic acetyl-CoA carboxylase holoenzyme than for the formation of the pyruvate carboxylase holoenzyme.

Table 2.   Enzyme activities in liver from 4-week-old, non-fasted chicks

Biotin in diet[A] (μg/kg)	FLKS[B]	No. of chicks	$\dfrac{\text{Liver wt}}{\text{Body wt}} \times 100$	Substrate converted [nmol min^{-1} (g liver)$^{-1}$]		
				Pyruvate carboxylase	Acetyl-CoA carboxylase	Malate dehydrogenase (decarboxylating) (NADP$^+$)
341 ± 12	Absent	6	$2 \cdot 27 \pm 0 \cdot 11$	4742 ± 210	65 ± 4	2980 ± 151
68 ± 3	Absent	2	$2 \cdot 00 \pm 0 \cdot 14$	1820 ± 171	74 ± 4	3133 ± 391
	Present	4	$3 \cdot 98 \pm 0 \cdot 09$	162 ± 94	129 ± 12	3929 ± 122
				Isocitrate dehydrogenase (NADP$^+$)	Glucose-6-phosphate dehydrogenase	Phosphogluconate dehydrogenase (decarboxylating)
341 ± 12	Absent	6	$2 \cdot 27 \pm 0 \cdot 11$	4196 ± 313	28 ± 4	291 ± 33
68 ± 3	Absent	2	$2 \cdot 00 \pm 0 \cdot 14$	3893 ± 113	69 ± 13	213 ± 26
	Present	4	$3 \cdot 98 \pm 0 \cdot 09$	2879 ± 406	44 ± 7	297 ± 8

[A] Mean of triplicate determinations.
[B] FLKS was considered to be present when the liver weight/body weight ratio was greater than the mean value for the non-affected chicks by at least two standard deviations.
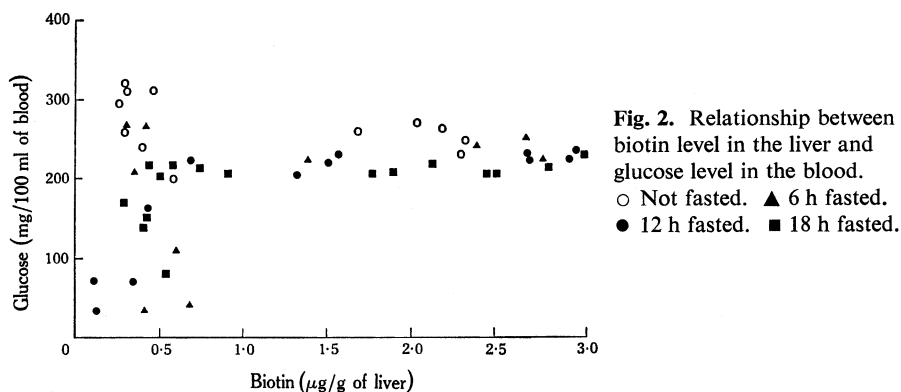
Stress and Fasting

During the fasting periods a number of chicks died with symptoms of FLKS. Food was taken away from the chicks at 0230 h and even before 0800 h several chicks had died.   Examination of these chicks showed that food was present in the crop and stomach, indicating that they were not in a state of starvation;   therefore the sequence of events leading to sudden death of the chick must have been initiated by stress imposed by nocturnal disturbance and the removal of food. Observations in field situations have confirmed that other forms of stress such as noise and changes in temperature can also induce sudden death through FLKS. In chicks which are partially deficient in biotin and have been fasted only for 6 or 12 h the overlapping effects of stress and fasting on intermediary metabolism do not allow a simple interpretation of the results.

Gluconeogenesis

The reduction in pyruvate carboxylase activity in the livers of those chicks severely affected with FLKS was accompanied by a decline in blood glucose (Table 1).   When

pyruvate carboxylase activities were above approximately 1600 units for fasted chicks, blood glucose levels were maintained by the chick (Table 1). When pyruvate carboxylase activity decreased below this critical level and the chick was stressed, hypoglycemia occurred and was a major factor contributing to death (Bannister *et al.* 1975). In chicks free of FLKS symptoms and fasted for 18 h, blood glucose levels declined by about 20%. This finding agrees with that of Sarkar (1971).

Fig. 2. Relationship between biotin level in the liver and glucose level in the blood. ○ Not fasted. ▲ 6 h fasted. ● 12 h fasted. ■ 18 h fasted.

The relationship between blood glucose and biotin levels in the liver is shown in Fig. 2. A similar relationship exists between liver biotin and pyruvate carboxylase activity. In Fig. 2 it is apparent that under stress of fasting, blood glucose can be maintained unless liver biotin is below $0 \cdot 8$ μg/g liver. Below this level, most chicks show symptoms of FLKS. However, in those chicks in which blood glucose falls below 180 mg/100 ml, the symptoms become so severe that death normally results. In non-fasted chicks, blood glucose was not influenced by dietary biotin or by the level of biotin in the liver (Fig. 2). Whitehead *et al.* (1974) observed a relationship between dietary biotin and plasma biotin levels, and also concluded that in non-fasted chicks plasma glucose was unaffected by the level of dietary biotin. Rats which are biotin deficient have also been reported to show no change in blood sugar levels (Wagle 1963). The activity of pyruvate carboxylase has been reported to drop to very low levels by 28 days in chicks fed from hatching on a purified diet which was deficient in biotin (Atwal *et al.* 1971). When liver and kidney slices from chicks affected with FLKS were incubated *in vitro*, their ability to form glucose from non-carbohydrate precursors was reported to be impaired (Bannister *et al.* 1975).

Impaired gluconeogenesis results in accumulation of substrate, such as pyruvate, which is metabolized further to lactate and acetyl-CoA. In birds affected with FLKS, blood lactate levels increased (Table 1).

Lipogenesis

In discussing lipogenesis from acetate and glucose it must be remembered that these two substrates are incorporated into fatty acids through separate pathways, the initial steps of which are quite different. Acetate is converted to acetyl-CoA by acetyl-CoA synthetase in the cytoplasm, the site of fatty acid synthesis. However, when glucose provides the two-carbon units for fatty acid synthesis, acetyl-CoA is generated in the mitochondria. Intramitochondrial acetyl-CoA condenses with

oxaloacetate to form citrate, which is free to diffuse into the cytoplasm. The citrate is cleaved by citrate cleavage enzyme, releasing oxaloacetate and acetyl-CoA. The latter is then available for fatty acid synthesis. The oxaloacetate formed in the cytoplasm is probably used to generate pyruvate and NADPH which can be utilized as a cofactor for lipogenesis. Therefore the citrate cleavage pathway requires the continual replenishment of oxaloacetate within the mitochondria. This is normally achieved by the carboxylation of pyruvate through pyruvate carboxylase.

The rates of *in vitro* lipogenesis using [U-^{14}C]glucose and [1-^{14}C]acetate as substrates are shown in Table 1. However, owing to the small number of chicks in each experimental group, statistically significant differences between means for affected and non-affected chicks were not always present, although a definite trend towards increased lipogenesis was apparent in chicks affected with FLKS at 0 and 18 h of fasting. Analysis of variance on the means from three experiments (results from one experiment included from the preceding paper; Pearson *et al*. 1976) showed that livers from FLKS chicks incorporated more labelled acetate into total lipid than did the livers of non-affected chicks (Table 3). This difference was significant when averaged over both 18-h fasted and non-fasted treatments, and for each of these treatments when analysed separately. Increased lipogenesis from glucose was observed only in FLKS chicks which had been fasted for 18 h (Table 3). These results are of greater importance when the total amount of lipid synthesized by the liver is considered, since the chicks with FLKS had enlarged livers.

Table 3. Summary of results from three FLKS experiments

Fasting period (h)	FLKS[A]	No. of chicks	$\dfrac{\text{Liver wt}}{\text{Body wt}} \times 100$	% Liver lipid	% 16:1 in fatty acids	Substrate converted to total lipid [nmol h^{-1} (g liver)$^{-1}$]	
						Acetate	Glucose
0	Absent	20	2·57	5·30	5·33	95·2	17·7
			**	n.s.	**	*	n.s.
	Present	16	4·48	4·67	10·67	129·4	18·4
18	Absent	27	1·98	4·73	2·00	11·6	14·3
			**	**	**	**	*
	Present	13	3·78	7·40	8·87	32·0	27·2

[A] FLKS was considered to be present when the liver weight/body weight ratio was greater than the mean value for the non-affected chicks by at least two standard deviations.
* $0·01 < P < 0·05$.   ** $0·001 < P < 0·01$.   n.s., Not significant ($P > 0·05$).

As the fasting period was increased, lipogenesis decreased when labelled acetate was used as the substrate; no difference was observed when [U-^{14}C]glucose was used as substrate (Table 1). However, after 6- and 12-h fasting periods, it was difficult to interpret the results because of overlapping factors in the supply of precursors for fatty acid synthesis. After fasting, less substrate (glucose) for lipogenesis would normally be available, whereas in the FLKS chicks increased substrate (pyruvate) for lipogenesis would be available due to impaired gluconeogenesis.

In the chick either glucose or other carbohydrates which are metabolized in a similar way to glucose serve as an energy source and also provide the carbon for fatty acid synthesis. When the birds are fasted or placed under stress, glycogen

ieverses are depleted to provide glucose for necessary metabolic processes. However, since pyruvate carboxylase and hence gluconeogenesis is reduced in FLKS, excess glucose metabolites (e.g. pyruvate, lactate, and acetyl-CoA) accumulate unless utilized by an alternate pathway. One way these metabolites can be removed from the system is by the synthesis of fatty acid via the citrate cleavage pathway. In chicks which have not been fasted and are not under stress, sufficient oxaloacetate can normally be generated within the mitochondria to allow the citrate cleavage pathway to function adequately. This is indicated by the similar incorporation of glucose into total lipid in both groups of non-fasted chicks (Table 3). However, chicks which have been fasted for 18 h and are severely affected with FLKS (i.e. low blood glucose levels) attempted to normalize blood glucose levels. This can be achieved by using substrates such as amino acids which enter the tricarboxylic acid cycle and are able to bypass the pyruvate carboxylase step in gluconeogenesis. A significant portion of this oxaloacetate may be condensed with intramitochondrial acetyl-CoA, which is in excess, to form citrate. The citrate enters the citrate cleavage pathway and is then available for lipid synthesis. The citrate cleavage pathway may serve as an alternate pathway for gluconeogenesis. Besides providing oxaloacetate for gluconeogenesis, this pathway transfers excess acetyl-CoA from the mitochondria to the cytoplasm where it can be removed through fatty acid synthesis. Increased lipogenesis is a consequence of the transfer. This interpretation of the results offers an explanation for the increased rate of lipogenesis from glucose in the fasted chicks afflicted with FLKS. In contrast with these results, Evans et al. (1975) have observed a decline in hepatic lipogenesis from [U-^{14}C]glucose when non-fasted chicks affected with FLKS were compared with non-affected chicks. However, in their study the composition of the two diets was quite different for each treatment.

Lipogenesis increased in chicks affected with FLKS (Table 3). In the biotin-deficient chick, where acetyl-CoA is in excess due to reduced activity of pyruvate carboxylase, the metabolic machinery, through hyperfunctional hepatomegaly, is presumably built up to remove this metabolite. This is shown in Tables 1 and 2 where the activities of malate dehydrogenase (decarboxylating) (NADP$^+$) and acetyl-CoA carboxylase increased in the FLKS chicks. The metabolic machinery is therefore present for increased fatty acid synthesis. This is further demonstrated by the increased rate of in vitro lipogenesis in both non-fasted and fasted chicks afflicted with FLKS when acetate, a substrate which bypasses the citrate cleavage pathway, serves as substrate for lipogenesis.

There is a great difference in the potential flux between the gluconeogenic and lipogenic pathways, i.e. pyruvate carboxylase is considerably more active than acetyl-CoA carboxylase and even then the activity of the latter is at least twenty times the rate required for lipogenesis as measured in vitro from glucose (Table 1). So the small loss of oxaloacetate to lipogenesis is negligible compared with the demand for gluconeogenesis.

NADPH-generating Enzymes

Listed in Table 2 are the activities of malate dehydrogenase (decarboxylating) (NADP$^+$), isocitrate dehydrogenase (NADP$^+$), glucose-6-phosphate dehydrogenase and phosphogluconate dehydrogenase (decarboxylating), each of which is a potential source of NADPH, an essential cofactor for lipogenesis. No significant differences

were observed in the activities of glucose-6-phosphate dehydrogenase and phospho-gluconate dehydrogenase (decarboxylating) in FLKS chicks in response to increased lipogenesis as measured by increased acetyl-CoA carboxylase activity. Other workers (Duncan and Common 1967; O'Hea and Leveille 1968; Madappally *et al.* 1971) have also reported that these pentose pathway dehydrogenases are not important in the chicken as sources of NADPH, since they have low activities and are not adaptable to changing lipogenesis rates. No concomitant increase in isocitrate dehydrogenase ($NADP^+$) activity with rate of lipogenesis was observed in the chicks showing symptoms of FLKS. Other authors, using rats (Young *et al.* 1964), pigs (Hood and Allen 1973a) and chickens (Madappally *et al.* 1971) which also metabolize glucose as a source of carbon for fatty acid synthesis, have also reported that iso-citrate dehydrogenase ($NADP^+$) is not an adaptive enzyme. Therefore isocitrate dehydrogenase ($NADP^+$) does not appear to be an important supply of NADPH for lipogenesis in the chick, as also reported by Goodridge (1968). Malate dehydrogenase (decarboxylating) ($NADP^+$) activities (Tables 1 and 2), however, are significantly higher in livers from chicks afflicted with FLKS, which implies that malate dehydrogenase (decarboxylating) ($NADP^+$) may serve an important role in the production of reducing equivalents for fatty acid synthesis in the chicken liver, as suggested by O'Hea and Leveille (1968).

Lipid Composition

The percentage liver lipid was similar between affected and non-affected chicks in the treatments with non-fasted chicks (Table 1). However, as the fasting period increased, lipid accumulation in the livers of severely affected chicks increased significantly and rapidly, whereas no change was observed in non-affected chicks. Elevated palmitoleic acid (16 : 1) levels were also observed in the livers of all affected groups of chicks, particularly those severely affected with FLKS (Table 1). After the removal of the food, 16 : 1 levels in non-affected chicks decreased, whereas 16 : 1 increased in all affected chicks. Elevated 16 : 1 levels in the liver probably provide the first indication that the chick is responding to a metabolic stress imposed by a low level of dietary biotin. This is reflected in increased fatty acid synthesis (Table 3) and increased desaturation (Pearson *et al.* 1976). Blood triacylglycerols were found to be elevated in fasted chicks which were severely afflicted with FLKS (Table 1). These elevated levels are related to increased lipid synthesis in the liver (Table 3) and a decreased lipoprotein lipase activity in adipose tissue (Evans and Bannister 1974). The elevated blood triacylglycerol levels and their impaired clearance from the plasma are probably important in the infiltration of lipid into the kidneys of severely affected chicks.

Conclusion

In a marginal deficiency of dietary biotin, the level of biotin in the liver has differing effects on the activities of two biotin-dependent enzymes, pyruvate carboxylase and acetyl-CoA carboxylase. The activity of the latter is in fact increased, but when the dietary deficiency of biotin produces biotin levels which are below $0 \cdot 8$ $\mu g/g$ of liver the activity of pyruvate carboxylase is probably insufficient to metabolize pyruvate via gluconeogenesis. In order to remove this metabolite and maintain homeostasis, the liver increases in size and the activities of enzymes involved

in alternate pathways for the removal of pyruvate increase. This is reflected in an accumulation of blood lactate, increased synthesis of fatty acids, particularly palmitic acid, and the accumulation of 16 : 1. These steps are accomplished by increased activities of at least the following enzymes: acetyl-CoA carboxylase, malate dehydrogenase (decarboxylating) ($NADP^+$) and the desaturase enzyme.

When the biotin level is below $0 \cdot 35$ $\mu g/g$ of liver and the chick is subjected to a stress, the mechanisms involved in hyperfunctional hepatomegaly are inadequate and finally they collapse, resulting in triacylglycerol accumulation in the liver and blood. The chick is unable to maintain blood glucose levels and death occurs, often only a few hours after the imposition of the stress. Thus the ultimate result of FLKS, i.e. the death of the chick, is the final step in a chain of events. It is dependent on the extent to which the above biochemical pathways are affected by two factors—firstly, the level of biotin, which is dependent not only on the level of available dietary biotin but also on the biotin level of the chick at hatching, which reflects the biotin status of the parent flock; and secondly, on the severity of the stress applied to the chick and the ability of the chick to withstand that stress. Thus within any apparently homogeneous group of chicks there is a tremendous variation in the extent to which biochemical pathways are affected. Nevertheless, a low level of dietary biotin and stress account for the malfunctions in carbohydrate and lipid metabolism which ultimately result in the death of a young chick from FLKS.

The biotin status and requirements of many species of animals, including man (*Nutrition Reviews* 1975), are unknown. The observed role of biotin and stress in the rapid onset of death in FLKS suggests that a biotin deficiency combined with the presence of stress may be involved in disorders which result in sudden and otherwise inexplicable death in animals of other species.

Acknowledgments

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