EFFECTS OF BIOTIN DEFICIENCY ON LIPOGENESIS AND CHOLESTEROGENESIS

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by

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To my wife

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ABSTRACT

Although acetyl CoA carboxylase (acetyl CoA:CO_2 ligase (ADP); EC 6.4.1.3) was the first to be recognized as a biotin enzyme, previous attempts to produce biotin deficiency effects on lipogenesis *in vivo* have not been successful. The effect of biotin deficiency on the lipid composition of liver, carcass and adipose tissue has been studied. Incorporation of acetate-1-^{14}C into liver lipids, liver acetyl CoA concentration and the *in vitro* activities of the mitochondrial and supernatant pathways of fatty acid synthesis were also investigated in both biotin deficient and pair-weighed control rats. Fat-free diets were used in these experiments. There was no significant difference in the total lipid content of liver between biotin deficient and control rats. However, deficient rat livers had significantly lower amounts of cholesterol esters and esterified fatty acids. The esterified fatty acid content of deficient carcasses was only 40% of the control level. In acetate-1-^{14}C incorporation experiments, specific activities of triglyceride, phospholipid and cholesterol ester fractions of the deficient rat liver were only 25% of those of controls. The increase in liver acetyl CoA concentration of deficient rats could not account for the large difference in the specific activities of the liver lipid components. Liver

acetyl CoA carboxylase activities of biotin deficient rats, per mg enzyme protein or per g fresh liver approximated 50% of that of controls. On the other hand, mitochondria from deficient rat livers had only 30% of the fatty acid synthetic activity of control mitochondria. The most significant effect of biotin deficiency was noted in the adipose tissue. The epididymal fat pad weight, total lipid, triglyceride, free fatty acid and phospholipid content of deficient rats were respectively 40, 35, 25, 20 and 25% of control values. These results indicate that the adipose tissue might quantitatively be more significant in lipogenesis and that biotin deficiency might have a more drastic effect on the metabolism of the adipose tissue than on liver.

ABBREVIATIONS

Tris	:	tris(hydroxymethyl)aminomethane
ATP	:	adenosine triphosphate
ADP	:	adenosine diphosphate
CoA	:	coenzyme A
PPO	:	2,5-diphenyloxazole
POPOP	:	1,4-bis-2(5-phenyloxazolyl)-benzene
TCA	:	trichloroacetic acid
NAD	:	nicotinamide adenine dinucleotide
NADH	:	nicotinamide adenine dinucleotide, reduced
NADP	:	nicotinamide adenine dinucleotide phosphate
NADPH	:	nicotinamide adenine dinucleotide phosphate, reduced
P_i	:	inorganic phosphate
EDTA	:	ethylenediamine tetra-acetate
GSH	:	glutathione
HMGCoA	:	β-hydroxy-β-methylglutaryl CoA

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A) Purpose

Although acetyl CoA carboxylase was the first to be recognized (1) as a biotin enzyme, previous attempts (2, 3) to study the effect of biotin deficiency on lipogenesis *in vivo* have not been entirely successful. Thus, Donaldson (2) found that the incorporation of acetate-1-^{14}C into the saponifiable fraction of liver lipids was not significantly decreased in biotin deficient chicks as compared to biotin treated controls. The incorporation into carcass saponifiable fraction of deficient chicks was lower than in controls. Puddu *et al.* (3) did not find any significant difference between normal and biotin deficient rats either in total lipid content or lipid composition of the liver. Suomalainen and Keranen (4) found that when baker's yeast was grown aerobically in absence of biotin there was a reduction in C_{18} fatty acids but an increase of fatty acids with 16 carbon atoms or less. There have been many contradictory reports regarding the participation of biotin in cholesterol synthesis (5-8). The effect of biotin deficiency on lipogenesis *in vivo* has been investigated. Its effect on the synthesis of various lipid components has also been studied.

B) General Approach

The effects of two deficient diets on cholesterol levels in serum, liver and carcass were first studied. Diet A was a high carbohydrate (66%) diet and diet B was a low carbohydrate (36%) diet. Both diets had the same fat content (5%). It was assumed that cholesterol synthesis on diet B would be contributed to a great extent by leucine of the dietary protein. Cholesterol synthesis from leucine is known to require a biotin enzyme, β-methylcrotonyl coenzyme A carboxylase (3-methylcrotonyl CoA:CO_2 ligase (ADP); EC 6.4.1.4). Hence, any reduction in cholesterol synthesis in biotin deficiency might be exaggerated by this altered dietary pattern where leucine rather than acetyl CoA would be the major precursor.

From this comparative study it was decided to use diet A in further work, with one modification; that the diet was made fat-free (diet C), since fat feeding has been reported to inhibit lipogenesis (9). This would mask any effects of biotin deficiency on lipid metabolism. Diet D, a low fat diet, otherwise identical to diet A, was also studied. This diet, which contained linoleic acid and corn oil, served as a comparison to diet C.

The total lipid and lipid component levels were determined in liver, carcass and epididymal fat pads to study

the effects of the deficiency.

Acetate-1-^{14}C incorporation into liver lipids was also studied. Changes in the rate of synthesis of lipids or their components should be reflected in the rate of incorporation of the label in short term incorporation studies.

Liver acetyl CoA and acetoacetate concentrations were determined in the biotin deficient rat and compared with the controls. This was undertaken to see if the acetyl CoA pool size could cause an isotope dilution effect in acetate-1-^{14}C incorporation.

The effects of the deficiency on the two major fatty acid synthesizing pathways in the liver were studied.

C) Organization of the Thesis

Three main sections, Literature Review, Experimental and Discussion comprise the body of the thesis. The Literature Review includes: the discovery of biotin, the metabolic effects of biotin deficiency, the metabolic role of biotin and the effects of biotin deficiency on lipogenesis and cholesterogenesis. The Experimental section is divided into two parts. The first part comprises the methods and is subdivided into: production of biotin deficiency, biochemical criterion of biotin deficiency, lipid distribution studies, acetate-1-^{14}C incorporation,

determination of acetyl CoA and acetoacetate, *in vitro* mitochondrial fatty acid synthesizing system and *in vitro* non mitochondrial fatty acid synthesizing system. The second part of the Experimental section presents the results of the above experiments. The Discussion tries to correlate both *in vivo* and *in vitro* results to explain the effects of biotin deficiency on fatty acid and cholesterol syntheses respectively.

SECTION II. LITERATURE REVIEW

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A) Discovery of Biotin

Since the year 1901 when Wildiers (10) first described the stimulating effects of small amounts of organic material on the growth of yeast, the name "bios" has been given to the substance or substances causing the increased growth of yeast. In later years, bios was shown to be multiple in nature and was fractionated into bios I, IIa, IIb, etc.

Bios IIb attracted the attention of Kögl (11) who announced the isolation from egg yolk of minute amounts of a crystalline compound possessing the greatest part of the yeast activity of the bios IIb fraction. This compound was called "biotin" by Kögl.

In 1933, Allison, Hoover and Burk (12) described the growth and respiration-promoting effects of extracts from various sources for Rhizobium trifolii, a legume nodule organism. The active agent was named "coenzyme R". In 1939, West and Wilson (13) pointed out the similarity between the two and Nilsson, Bjälfve and Burström (14) found that a sample of Kögl's crystalline biotin possessed coenzyme R activity. It appeared that coenzyme R was identical with biotin.

Boas in 1927 described the effects produced in rats when large amounts of dried egg white were added to the diet (15). On such a regime the animals gradually lost their hair.

Dermatitis, skin hemorrhages and loss of body weight occurred, a spasticity developed and death ultimately resulted. Parsons and associate (16) studied extensively the egg white injury factor and the distribution of the protective factor found by Boas. György investigated the chemical and physical properties of the protective factor, which he called "vitamin H", and early in 1940, György et al. (17) suggested the possible identity of vitamin H with biotin and coenzyme R.

Since these early discoveries of biotin, it has been found in animal and plant tissues also and occurs mainly in combined forms. One of these biotin complexes is biocytin (ε-N-biotinyl-L-lysine), isolated from yeast by Wright et al. (18). Another complex, whose structure has not been elucidated, is the "soluble bound biotin", extracted from the peptic digests of swine liver. Both of these complexes are degraded to biotin by an enzyme believed to be a peptidase (19). At least two distinct liver protein fractions containing biotin have been described; these biotin-containing proteins have been termed "biotoproteins" (20).

B) Metabolic Effects of Biotin Deficiency

Biotin is the simplest of the naturally occurring compounds that counteract the nutritional deficiency induced in animals (including man) by the feeding of raw egg white. The toxic material in egg is a protein (avidin) with which

biotin combines, in stoichiometric proportions, to form an avidin-biotin complex (21). This complex is not dissociable except by heat treatment or acid hydrolysis, nor is it split by the enzymes of the gastrointestinal tract of higher animals (22). Hence the feeding of avidin can result in a biotin deficiency caused by the formation of the nondigestible complex within the intestinal tract.

Biotin deficiency is not normally encountered in man or even in laboratory animals kept on apparently biotin-free diets. This is a reflection of the ability of intestinal bacteria to synthesize sufficient biotin to meet the requirements of the host organism. Consequently, biotin deficiency is usually induced by the administration of avidin (or raw egg white) or by elimination of intestinal bacteria by a bacteriostatic agent. The symptoms of this deficiency have been described earlier.

C) Metabolic Role of Biotin

Previously, biotin was thought to have a role of a coenzyme in certain carbon dioxide fixation reactions. Wakil et al. (1) found that purified preparations of acetyl CoA carboxylase contained biotin as a prosthetic group. Since then, five biotin enzymes have been characterized.

Acetyl CoA carboxylase, a biotin enzyme, is discussed further on in its role in fatty acid synthesis. The four

other biotin enzymes are: 1) β-methylcrotonyl CoA carboxylase (3-methylcrotonyl CoA:CO_2 ligase (ADP); EC 6.4.1.4), 2) propionyl CoA carboxylase (propionyl CoA:CO_2 ligase (ADP); EC 6.4.1.3), 3) methylmalonyl-oxalacetic transcarboxylase (methylmalonyl CoA:pyruvate carboxytransferase; EC 2.1.3.1), 4) pyruvate carboxylase (pyruvate:CO_2 ligase (ADP); EC 6.4.1.1).

1) β-Methylcrotonyl CoA carboxylase. In the metabolism of isovaleryl coenzyme A which is on the pathway of leucine degradation, the isopropyl portion of the molecule is converted as a unit to acetoacetate by fixation of carbon dioxide. The reaction catalyzed is as follows:

$$\beta\text{-Methylcrotonyl CoA} + HCO_3^- + ATP \rightleftharpoons \beta\text{-methylglutaconyl CoA} + ADP + P_i$$

Fischer (23) showed that mitochondria from biotin deficient rat livers failed to oxidize isovalerate, whereas normal mitochondria oxidized it to acetoacetate. Furthermore, biotin was shown to be an integral part of the enzyme and purified preparations contained as much as 1 mole of biotin per 344,000 g of protein (24, 25).

2) Propionyl CoA carboxylase. The following reaction is catalyzed by this enzyme:

$$\text{Propionyl CoA} + HCO_3^- + ATP \rightleftharpoons \text{methylmalonyl CoA} + ADP + P_i$$

Propionyl CoA carboxylase catalyzes the first step in the synthesis of succinyl CoA, since methylmalonyl CoA isomerizes to succinyl CoA. Lardy and Adler (26) showed that mitochondria from biotin deficient rats carboxylated propionate at a greatly reduced rate compared to mitochondria from normal animals. Kaziro et al. (27) found that the crystalline carboxylase had a molecular weight of 700,000 and contained 1 mole of biotin per 175,000 g of protein. Kosow and Lane (28) have shown that after eleven days on a biotin deficient diet there was a marked decrease of the carboxylase activity. Propionyl CoA carboxylase activity is a useful criterion for evaluating the biotin status of the animals as shown by Halenz and Lane (29).

3) Methylmalonyl-oxalacetic transcarboxylase. The enzyme catalyzes the following reaction:

Methylmalonyl CoA + pyruvate $\rightleftharpoons$ propionyl CoA + oxalacetate

The role of biotin in the transcarboxylase reaction was established by Swick and Wood (30). An unusual feature of the methylmalonyl-oxalacetic transcarboxylase is that it catalyzes a transformation which involves compounds from different pathways, so shuttling carboxyl groups from one metabolic pathway to another for synthetic processes. This enzyme though is essentially a bacterial enzyme.

4) Pyruvate carboxylase. Utter and Keech (31) showed

conclusively the evidence for the occurrence in avian liver of an enzyme system which catalyzes the following reaction:

$$\text{Pyruvate} + HCO_3^- + ATP \rightleftharpoons \text{oxalacetate} + ADP + P_i$$

Keech and Utter (32) showed that avidin completely inhibited pyruvate carboxylase and Utter and Keech (31) also showed that it contained substantial amounts of biotin.

Thus biotin plays a direct role in five metabolic reactions, not as a cofactor but as an integral part of the enzyme catalyzing these reactions. However, the effects of biotin deficiency are felt in very many reactions in the intact organism. Biotin has been implicated in protein synthesis (33 - 36). Biotin has also been suggested to have an effect on carbohydrate metabolism. Dakshinamurti and Mistry (37) have shown that biotin deficiency results in increased incorporation of glucose-6-^{14}C and glucose-1-^{14}C into $^{14}CO_2$ as compared to pair-fed normals. Dakshinamurti and Cheah-Tan (38) also showed that glucose phosphorylation was decreased in biotin deficient rats when compared to pair-fed and pair-weighed controls.

Many effects of biotin deficiency have been noted, some direct and some indirect. In many cases the role of biotin is still obscure.

D) Role of Biotin in Fatty Acid Synthesis

The data from earlier studies concerning the participation of biotin in the synthesis of fatty acids were contradictory. Okey et al. (39) suggested that biotin was necessary for the synthesis and storage of fatty acids when they observed that the total fatty acid content of rat liver triples when the animals received a diet containing an excess of cholesterol, and that this did not occur when the cholesterol was administered to rats deficient in biotin. It should be noted that in this study, fatty acid values were total content per organ, and that the controls were fed ad libitum and consequently had larger livers. The differences would not have been so significant on a fatty acid content per unit weight of the organ as was done by Guggenheim and Olson (5) who disagreed with the above results. They showed that the total fatty acid level was more or less identical in the liver, heart and blood of biotin deficient rats in comparison with both pair-fed and ad libitum control groups. In this same paper, Guggenheim and Olson reported that the rate of incorporation of acetate-1-^{14}C into fatty acids revealed a normal capacity of biotin deficient rats to synthesize fatty acids.

Curran (40) reported similar findings to those of Guggenheim and Olson (5). He reported that in biotin

deficient rats the rate of fatty acid synthesis was slightly increased, but that this was due to the inanition accompanying biotin deficiency and not biotin deficiency itself. These observations as they pertain to fatty acid synthesis are indirect as in this work the distribution of deuterium oxide was the criterion used. Also the dose of administered deuterated water was not mentioned.

Wakil et al. (1) in studies with highly purified pigeon liver extracts showed that biotin was involved in a new enzymatic system for the synthesis of long chain fatty acids from acetyl CoA. Wakil et al. (41) isolated two main enzyme fractions, R_{1g} and R_{2g}, from pigeon liver supernatant. Fraction R_{1g} contained between 200 and 250 mμg of biotin per mg of protein or about 1 mole of biotin per 1×10^6 g of protein. This was the highest ratio between biotin and protein yet reported (1). The enzyme was later identified as acetyl CoA carboxylase (acetyl CoA:CO_2 ligase (ADP); EC 6.4.1.2). Support for the implication of biotin in this synthesis reaction came from the fact that the conversion of acetyl CoA to palmitate was inhibited in the presence of avidin and this inhibition was relieved by a supplement of biotin (1).

Bortz et al. (9) reported a feedback inhibition at the acetyl CoA carboxylase step brought about by fat feeding. They showed that after administration of 2 ml corn oil by

stomach tube the *in vitro* conversion of acetate to fatty acids was depressed as early as two hours after enteral administration to rats previously fed a fat-free, high carbohydrate diet. The depression was most pronounced at four hours after administration of the corn oil. They also reported that the conversion of malonate to fatty acids *in vitro* was not depressed by fat feeding.

Attempts (5, 40) to produce biotin effects on lipogenesis *in vivo* have not been successful, perhaps because the diets used contained 5-10% fat which itself would depress lipogenesis.

Donaldson (2), using a fat-free diet, showed that biotin deficiency in chicks resulted in increased incorporation of acetate-1-^{14}C into respiratory CO_2 and decreased incorporation into carcass fatty acids. He also showed that the deficiency had no effect on incorporation of malonate-2-^{14}C into respiratory CO_2 or liver and carcass lipids, and that the proportions of palmitate to stearate in the carcass were increased by biotin deficiency. The mechanism responsible for the decrease in stearate in biotin deficiency is still not known.

Puddu *et al.* (3), using a biotin deficient diet containing 5% fat, reported that no significant difference was found either in total lipid content or in lipid composition in the liver of the deficient rats when compared

to the biotin treated controls. They also reported that the rate of *in vitro* incorporation of acetate-1-^{14}C into total liver lipids by liver slices was significantly decreased in biotin deficiency. They also showed that the deficiency resulted in significant changes in the percentage fatty acid composition, namely more palmitoleic (16:1) and linoleic (18:2) and less stearic (18:0), were found in total liver lipids when compared to the biotin treated control. Also, the ratios of arachidonic (20:4) to linoleic (18:2) and saturated to unsaturated fatty acid were significantly decreased in total liver lipids in the biotin deficient rats. Puddu *et al*. (3) suggested that in biotin deficiency there may be an alteration in the synthesis of unsaturated fatty acids. Donaldson (42) more recently suggested that this increase in unsaturated fatty acid was due mainly to a derepression of a biotin insensitive pathway for monoene synthesis that was independent of saturated fatty acid synthesis and that it resulted in increased palmitoleate (19:1) concentrations.

E) Role of Biotin in Cholesterol Metabolism

The results suggesting a possible participation of biotin are contradictory. Curran (40), on the basis of rate of incorporation of deuterium oxide in cholesterol, concluded that biotin was not implicated. Guggenheim and Olson (5)

showed that the cholesterol content of the liver and adrenals was about the same in biotin deficient rats and in pair-fed controls, and also that in vivo incorporation of acetate-1-^{14}C into cholesterol of the liver and adrenals of the deficient rats was reduced in comparison with the pair-fed controls. It is difficult to analyze these results in the absence of details regarding the amount of the label injected. Gram and Okey (6) concluded that these results showed an inhibition of cholesterol synthesis from labelled acetate-2-^{14}C in the deficient rat compared with the pair-weighed control, as in all cases the specific activities of the liver lipids and the free and total cholesterol were the highest in the underfed controls, as was the percentage of the injected acetate-2-^{14}C found in the liver lipids.

Okey et al. (39) suggested that biotin deficiency played a secondary role in cholesterol storage. They showed that rats on a dried egg white diet, high in cholesterol, supplemented with ample biotin yielded liver cholesterol values 6-8 times as high as those rats fed similar cholesterol-free diets. Most of the increase in cholesterol was in the esterified fraction. Biotin deficient rats fed the egg white diets high in cholesterol failed to store excess liver cholesterol esters, even when they maintained nearly normal food intakes. Barnes et al. (7) confirmed this observation under the same experimental conditions, but

the interference with cholesterol storage was not observed in rats made mildly deficient by preventing coprophagy. The findings of Barnes et al. (7) are not valid since these rats on coprophagy-prevented biotin-free diets were three times the weight of deficient rats produced by the avidin diet. These latter rats were not biotin deficient. The intestinal flora would produce sufficient biotin for the animals' daily need.

Hypercholesterolaemia has been reported in biotin deficiency by Scott (8); this could also be due to a defect in the storage or catabolism of cholesterol.

Siperstein and Fagan (43) have shown that cholesterol feeding inhibits cholesterol synthesis. They reported that the inhibition occurred between β-hydroxy-β-methylglutaryl CoA and mevalonic acid, since mevalonate synthesis was markedly suppressed by cholesterol feeding (5% cholesterol in diet), while the synthesis of β-hydroxy-β-methylglutaryl CoA was unaffected. This implies a feedback system acting on β-hydroxy-β-methylglutaryl CoA reductase (mevalonate:NADP oxidoreductase (acylating CoA); EC 1.1.1.34). This inhibition was not evident by addition of cholesterol in vitro and Siperstein and Fagan (43) suggest a lipoprotein-cholesterol complex may be involved in the direct feedback inhibition.

In the current study, cholesterol was omitted from the

diet to avoid the feedback inhibition effect which might mask the effects of biotin deficiency.

SECTION III. EXPERIMENTAL

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A) Methods

a) Production of biotin deficiency

Male albino rats of the Holtzmann strain weighing 48 to 52 g were used to produce biotin deficiency. Four diets were used in the course of this work and their compositions are given in Table I. Both diets A and B contained 5% fat and were high and low respectively in carbohydrate content. Diet C was a fat-free high carbohydrate diet, otherwise similar in composition to diet A. Diet D was a low fat diet with a supplement of linoleic acid.

The rats were kept on these diets from 5 to 8 weeks and weighed weekly. The control rats were injected intraperitoneally with 100 μg biotin per 100 g body weight per week and used approximately one week after the last injection.

It is usual to compare a deficient group of rats with a pair-fed group of controls so that the food consumption on these two treatments can be equalized. However when this was done, the growth rate of the deficient animals was lower than that of the controls. Therefore it could not be determined whether the effect seen was due to the biotin deficiency or the accompanying inanition. The controls used in this work were pair-weighed, that is they received biotin supplementation, and their food intake was carefully adjusted such that the weights of these controls were in the same

Table I

Composition of experimental diets.

Components	Diet A	Diet B	Diet C	Diet D
Egg white	25.0	25.0	25.0	25.0
Vitamin-free casein	-	30.0	-	-
Dextrose	64.85	-	69.85	66.85
Starch	-	34.85	-	-
Corn oil	5.0	5.0	-	2.0
Linoleic acid	-	-	-	1.0
Minerals[+]	4.0	4.0	4.0	4.0
Vitamin mixture*	1.0	1.0	1.0	1.0
Choline chloride	0.15	0.15	0.15	0.15

[+]Mineral Mix #446 (General Biochemicals)
*The vitamin mixture provided, per kg of diet, the following in mg quantities: α-tocopherol, 110; ascorbic acid, 992; inositol, 110; menadione, 50; p-aminobenzoic acid, 110; niacin, 100; riboflavin, 22; pyridoxine HCl, 22; thiamine HCl, 102; Ca pantothenate, 66; folic acid, 2; vitamin B_{12} (triturate in 0.1% mannitol), 30; vitamins A and D_2 concentrate, 4; dextrose, 6.59 g.

range as the deficient ones. All controls in this work were pair-weighed.

b) Biochemical criterion of biotin deficiency

Liver propionyl CoA carboxylase activities were determined on deficient, positive control and normal rats. Rats on the biotin deficient diet for about 5 weeks in which the outward symptoms of biotin deficiency were evident were used in the deficient group. The positive controls were biotin deficient animals which received one intraperitoneal injection of biotin 3 days before use in the experiment. Normal animals received biotin throughout the 5 week experimental period.

As has been mentioned, propionyl CoA carboxylase is a biotin enzyme and its level of activity in the tissues serves as a good biochemical criterion for the biotin status of the animals as shown by Halenz and Lane (29).

This enzyme was partially purified and assayed by the method described by Halenz and Lane (29).

i) Partial purification

Acetone powder. One modification was made in that the whole liver was used to obtain the acetone powder instead of using the mitochondrial pellet. The livers from normal, positive control and deficient rats were disintegrated directly in 10 volumes of acetone at -15^{o} C in

a Waring blendor for 3 minutes, and filtered on a Büchner funnel. The cake formed was retreated with acetone as described above, filtered and the cake was powdered with a mortar and pestle. The powder was allowed to dry to remove the residual acetone. All operations were carried out in a cold room at 0-3° C. The acetone powders, when not immediately used, were stored at -20° C. It has been reported by Halenz and Lane (29) that the acetone powder stored in this way retained its original propionyl CoA carboxylase activity for at least 3 months.

Extraction. The acetone powder was extracted with 20 volumes of 0.0025 M Tris, pH 7.2 for 30 minutes with occasional stirring, centrifuging at 12,800 x g for 10 minutes. The supernatant was used for the propionyl CoA carboxylase assay.

ii) Assay procedure

Reagents.

- Tris buffer - $KH^{14}CO_3$ mixture; 0.222 M Tris, pH 8.5, and 0.0334 M $KH^{14}CO_3$, specific activity 0.037 μc per μmole.
- ATP - $MgCl_2$; each present at 0.02 M.
- Glutathione; 0.025 M solution.
- Trichloroacetic acid - sucrose - detergent mixture; prepared immediately before use by mixing equal volumes of 20% trichloroacetic acid, 10% sucrose and 0.5% Sparkleen detergent (Fisher Scientific Co.).

- Enzyme; dilutions of enzyme made with 0.005 M Tris, pH 7.3.
- Propionyl-S-CoA; 0.005 M, prepared by the method of Simon and Shemin (44). Thirty-five mg of coenzyme A (Sigma) were dissolved in 30 ml of ice cold water. To this solution were added 3 mg of propionic anhydride followed by sodium bicarbonate until the pH was 7-7.5. The mixture was kept in an ice bath and shaken frequently. The reaction was complete after 30 minutes. The pH of the solution was brought to 3.0, nitrogen was bubbled through the solution for 10 minutes to remove excess bicarbonate, then the pH was adjusted to 6.0.

The reaction mixture included 100 μmoles of Tris, 15 μmoles of $KH^{14}CO_3$ (0.45 ml of Tris buffer - $KH^{14}CO_3$ mixture), 4 μmoles ATP and $MgCl_2$ (0.2 ml ATP - $MgCl_2$ mixture), 5 μmoles glutathione, 1 μmole propionyl-S-CoA, and enzyme (3 to 11 mg of protein per incubation mixture). The final volume was 1.5 ml. Propionyl-S-CoA was omitted in control tubes. The reaction was initiated by the addition of enzyme after which the tubes were incubated for 20 minutes at 37° C and terminated by the addition of 0.6 ml of the trichloro-acetic acid - sucrose - detergent mixture. The tubes were centrifuged when it was found necessary and a 0.6 ml aliquot of the reaction mixture was pipetted into a liquid scintillation vial. The vials were dried under an infrared

lamp for 30 minutes and the radioactivity was measured in a Packard liquid scintillation counter, model 3000, at optimal settings for ^{14}C counting using a toluene - ethanol scintillator prepared by mixing 378 ml of absolute ethanol with 600 ml toluene (spectrophotometric grade) containing 0.4% PPO (2,5-diphenyloxazole) and 0.0015% POPOP (1,4-bis-2(5-phenyloxazolyl)-benzene). The specific activity of the enzyme was described as μmoles CO_2 fixed per hour per mg protein.

Protein was estimated by the method of Lowry et al. (45).

i) Assay procedure

Reagents.

- Reagent A; 2% sodium carbonate in 0.1 N NaOH.
- Reagent B; 1% cupric sulphate in 2% sodium tartrate.
- Reagent C; 50 ml reagent A with 1 ml reagent B.
- Reagent D; phenol reagent (Folin and Ciocalteu) (Canadian Laboratory Supply Co.) diluted with water (1:2, v/v).

One ml samples containing 10 to 200 μg of protein were added to test tubes. To this were added 5 ml of reagent C, mixed and the tubes were permitted to stand for 10 minutes. To the solution was added 0.5 ml of reagent D, mixed and again the tubes were permitted to stand for 30 minutes. The absorbance of the samples was measured at 650 mμ on a Beckman DU spectrophotometer.

The blank was carried through the same procedure as the samples but water was used instead of the protein sample. Crystalline bovine serum albumin was used as a standard for the calibration curve.

c) Lipid distribution studies

1) Lipid extraction

i) Solvents

Acetone and diethyl ether were redistilled before use. Petroleum ether was also redistilled, and the fraction with a boiling range of 30-60^{o} C was used.

ii) Isolation of tissues

The deficients were all taken from the plateau region of the deficiency and only pair-weighed rats were used. The animals were sacrificed by decapitation, the blood was collected, and the liver and epididymal fat pads were removed. The liver, epididymal fat pads and the remaining carcass minus the head were either used immediately or quickly frozen in liquid nitrogen and kept at -20^{o} C until used.

The blood, once collected, was allowed to clot for half an hour, centrifuged in a clinical centrifuge and the serum was pipetted off and used immediately.

iii) Extraction of total lipids

The serum and various tissues were extracted according to the procedure of Enteman (46) as follows:

Serum. An 0.5 ml aliquot of serum was placed in a centrifuge tube with 4.5 ml isopropyl alcohol. The mixture was shaken for 10 minutes, then centrifuged and the supernatant used directly for cholesterol and cholesterol ester determinations.

Liver and epididymal fat pads. The tissue was homogenized in a Waring blendor for 2 minutes with a 10:1 solvent:tissue of alcohol:ether (3:1, v/v). The mixture was kept in a water bath at 60° C for 6 hours under N_2. The mixture was then decanted through fat-free filters. The tissue was re-extracted with 6 portions of diethyl ether. The combined filtrates were then evaporated to near dryness at 60° C under reduced pressure. Nitrogen was then flushed through to remove the remaining solvent. The lipid residue was then re-extracted with petroleum ether and dried by adding anhydrous sodium sulphate and letting stand for one hour. The mixture was then filtered and brought to volume with petroleum ether.

Carcass. The carcass was sliced into small pieces and passed through a meat grinder. The tissue was then extracted with 10 volumes ethanol:ether (3:1, v/v) for 18 hours under nitrogen. The extract was then filtered, evaporated, re-extracted and dried over anhydrous sodium sulphate, and finally filtered and made up to volume in exactly the same way as was described for extraction of liver

and epididymal fat pads.

iv) Saponification

For total cholesterol estimations the carcass was saponified using the method described by Russell et al. (47).

The carcass was cut into small pieces and placed in a large round-bottomed flask. For each gram of carcass were added 2 ml of 25% alcoholic potassium hydroxide (50% ethanol). The mixture was refluxed for 2 hours, then cooled to room temperature and filtered through glass wool into a graduated cylinder. The flask was rinsed twice with 10 ml of water bringing to a boil, and twice with 20 ml absolute ethanol again bringing to a boil. The residual bones were weighed and subtracted from the carcass weight.

A 25 ml aliquot of this dark brown liquid was extracted twice with 20 ml petroleum ether. The extract was then washed four times with 10 ml water and dried over anhydrous sodium sulphate. The extract was then filtered and evaporated under reduced pressure at 60^{o} C as described previously and redissolved to volume with 95% ethanol. One ml aliquots were used to determine total cholesterol.

2) Assay of lipid components

Cholesterol, cholesterol esters, phospholipids, esterified fatty acids and free fatty acids were determined in the total extracted lipid. Total lipid was determined

gravimetrically.

i) Cholesterol and cholesterol esters

Aliquots of the lipid extract were evaporated under reduced pressure at 60° C, redissolved and made up to volume in ethanol. Aliquots of the ethanol extract were then used to determine total and free cholesterol by the method of Ferro and Ham (48) as follows:

Reagents.

- Alcoholic potassium hydroxide; 5% potassium hydroxide in 95% ethanol.
- Acetone; redistilled.
- Aluminum chloride solution; 30% aluminum chloride in water.
- Digitonin solution; 1% digitonin in 50% ethanol.
- Acid anhydride; acetic anhydride with glacial acetic acid (3:2, v/v).
- Colour development mixture; acid anhydride with concentrated sulphuric acid (10:1, v/v).

Total cholesterol. One ml of the alcohol extract was placed in a test tube with 0.5 ml of alcoholic potassium hydroxide. The solution was mixed and placed in a water bath at 37° C for 30 minutes. To this was added 1 ml of digitonin solution and one drop of aluminum chloride solution. The solution was mixed and the tubes were permitted to stand for 30 minutes. The tubes were then centrifuged at 3000 rpm for 10 minutes, and the supernatant was removed with a Pasteur

pipette. The precipitate was washed with 2 ml of acetone, centrifuged and drained. The white precipitate was suspended in 0.2 ml of water and 6 ml of colour developing mixture was added. The colour appeared 90 ± 30 seconds after the last addition and remained stable for one minute. Absorbance was measured at 640 mμ with water as the blank.

Free cholesterol. Two ml of the alcohol extract were placed in a test tube and the cholesterol was precipitated by the addition of 1 ml of digitonin solution. The same procedure as in total cholesterol was used after precipitation as described above. A standard curve was calibrated using chromatographically pure cholesterol.

ii) Phospholipids

The phospholipids were precipitated and assayed by the method of Bloor (49) as follows:

Reagents.

- Magnesium chloride solution; saturated magnesium chloride in 95% ethanol, solution kept at 0-5^{o} C.
- Acetone; redistilled.
- Moist ether; redistilled, diethyl ether and water were mixed vigorously for 5 minutes in a separatory funnel such that the ether was saturated with water.
- Potassium iodide; 10% solution.
- Potassium dichromate; 1 N solution.
- Sodium thiosulphate; 0.1 N sodium thiosulphate was

standardized against potassium iodate. Accurately weighed quantities of pure potassium iodate were dissolved in 25 ml of water, then 2 g of potassium iodide (iodate-free) and 10 ml of 1 N hydrochloric acid were added. The solution was titrated with standard thiosulphate solution with constant stirring. When the colour was yellow, 2 ml of starch solution were added and the titration was continued until the colour changed from blue to colourless.

- Starch solution; 1% starch in water.
- Silver reagent; pure concentrated sulphuric acid containing silver dichromate was prepared by the method of Nicloux (50) as follows: to 5 g of silver nitrate dissolved in 25 ml of water in a 100 ml centrifuge tube were added 5 g potassium dichromate dissolved in about 50 ml of water. The precipitated silver dichromate was separated by centrifugation, washed twice by centrifugation with water and the cake precipitate was dissolved without drying in 500 ml pure concentrated sulphuric acid.

Precipitation. An 8 ml aliquot from the lipid extract was reduced by evaporation to a volume of 2 ml in a centrifuge tube. To this were added 7 ml acetone and three drops of cold magnesium chloride solution. The mixture was well-stirred with a small glass rod and the tubes were left to stand for 15 minutes. The mixture was then centrifuged at about 1500 rpm for 5 minutes. At the end of the

centrifugation, the acetone solution was poured off the phospholipid, which now adhered to the bottom and walls of the tube. The precipitate was rinsed once with acetone and it was redissolved in 5 ml of moist ether. Thirty minutes were allowed for solubilization, and traces of undissolved residues were removed by centrifugation for 3 minutes at 1500 rpm. The ether was transferred quantitatively to a digestion flask. The tubes were rinsed twice with 1 ml moist ether and the washings were added to the main body of the ether. The ether was evaporated to dryness under an atmosphere of nitrogen.

To the residue in the digestion flask were added 5 ml of silver reagent and 3 ml of 1 N potassium dichromate. A control containing all the reagents mentioned above but omitting the phospholipid was prepared and run along with the samples under the same conditions. The mixture was well shaken, loosely stoppered and placed in a water bath at 88-90^{o} C for one hour. At the end of this period the flasks were removed and 75 ml distilled water were added. After cooling the excess dichromate was measured by titration.

Titration. To the flasks were added 10 ml 10% potassium iodide. Then without stirring 0.1 N sodium thiosulphate was run in from a burette, and the flasks were mixed gently by rotation. Near the end point, one ml of 1% starch solution was added and the titration carried to the light

green end point. Phospholipids were calibrated with chromatographically pure L-α-lecithin (dipalmitoyl).

iii) Esterified fatty acids

They were determined by the method of Stern and Shapiro (51). The determinations were carried out on the supernatant fraction obtained after phospholipid precipitation. The supernatant was evaporated to dryness at 60° C under nitrogen and redissolved in alcohol:ether (3:1, v/v). This assay determines all fatty acid esters such as triglycerides, diglycerides and monoglycerides, but gives no reaction with cholesterol esters or free fatty acids.

Reagents.

- Hydrochloric acid; 4 N solution.
- Ferric chloride solution; 0.37 M ferric chloride in 0.1 N hydrochloric acid.
- Hydroxylamine - sodium hydroxide solution; 2 M hydroxylamine hydrochloride mixed with 3.5 N sodium hydroxide in a 1:1 ratio, volume to volume.

A 3 ml aliquot of the alcohol:ether extract containing 0.3 to 0.8 mg of the esterified fatty acid was placed in a test tube. One ml hydroxylamine - sodium hydroxide solution was added. The solution was mixed and the tubes were permitted to stand for 20 minutes at room temperature. To this solution was then added 0.6 ml 4 N hydrochloric acid. The solution was mixed and 0.5 ml of the ferric chloride

solution was added and the whole solution was mixed. The reagent blank was set at zero at 520 mμ and the sample's absorbance was measured. Chromatographically pure trimyristin was used as a standard.

Values were expressed as mgs non phospholipid esterified fatty acids when determinations were made on the total lipid extract after phospholipid removal, and were expressed as mgs triglycerides when determinations were made on the pooled triglyceride fractions after separation of the lipid components on florisil (Floridin Co.).

iv) Free fatty acids

They were determined by the method of Davis (52). The determinations were carried out on the supernatant of the total lipid extract after phospholipid removal. The supernatant was evaporated to dryness at 60° C under nitrogen, redissolved in petroleum ether, acidified with dilute sulphuric acid, washed with water and dried over anhydrous sodium sulphate.

Reagents.

- Nile blue; 0.1% Nile blue in 95% ethanol.
- Sodium hydroxide; 0.02 N sodium hydroxide standardized against potassium biphthalate.

The samples from the petroleum ether were evaporated to dryness at 60° C under nitrogen and redissolved in 5 ml of 95% ethanol. One drop of 0.1% Nile blue was added and

nitrogen was bubbled through the solution for 10 minutes. The solution was then titrated with 0.02 N sodium hydroxide to a just pink end point. Chromatographically pure stearic acid was used as a standard.

v) Hydrocarbons

These were determined by the method of Rothblat et al. (53). This assay could only be used when the hydrocarbons were separated from other lipid components on a florisil column because of the wide specificity of the determination.

Reagents.

- Formaldehyde solution; 36-38% formaldehyde solution.
- concentrated sulphuric acid.
- Glacial acetic acid.

Aliquots of the extract containing the hydrocarbons were placed in tubes and reduced to dryness under a stream of nitrogen, thorough drying being necessary since even traces of solvent interfere with colour development. One ml of concentrated sulphuric acid was added to the tubes and they were placed in a water bath maintained at 70° C for 5 minutes. To this solution was slowly added 0.5 ml formaldehyde solution and the tubes were shaken to ensure thorough mixing. The tubes were placed in a boiling water bath for 10 minutes. Immediately upon removal 2.5 ml of glacial acetic acid were added to bring the final volume to 4.0 ml. The colour was

measured at 400 mμ in a Beckman DU spectrophotometer. A blank containing no hydrocarbon was run simultaneously. Chromatographically pure squalene was used as a standard.

d) Acetate-1-^{14}C incorporation

1) Administration of the labelled compound

The in vivo incorporation of acetate-1-^{14}C into liver lipids was studied.

One deficient and one pair-weighed control were run simultaneously. Both rats were starved for 6 hours, from 7:00 A.M. to 1:00 P.M. The rats were then fed for one hour, the deficient being fed ad libitum and the control limited quantities to approximately the same amount normally eaten by the deficient rats. The rats were then injected intraperitoneally with 5 μcuries of acetate-1-^{14}C (specific activity 2.0 mcurie/mM) and placed in metabolic cages where the CO_2 was collected for 2 hours in 2.5 N sodium hydroxide. The rats were then sacrificed by decapitation and the livers were perfused with ice cold 0.9% sodium chloride. The livers were removed, weighed and frozen in liquid nitrogen. In these incorporation studies, tissues were either used immediately or kept frozen at -20° C for one day and then used.

The lipids were extracted as described previously. The assays for the different lipid classes were exactly the

same but could not be carried out on the extract itself, as had been done previously, since radioactive determination requires separation of the components such that specific activities could be calculated.

2) Separation of lipids on florisil column

The lipid classes were separated on a florisil (Floridin Co.) column as described by Carroll (54). This adsorbent consists of hard, porous, white granules whose composition is reported as follows: magnesium oxide, 15.5 ± 0.5%; silicon dioxide, 84.0 ± 0.5%; and sodium sulphate, 0.5% average (1.0% maximum). The florisil used was of 60 - 100 mesh.

In all experiments a 12 g column was used, measuring 1.2 cm x 15 cm. The florisil was deactivated by the addition of 7 ml water to 100 g of florisil. The mixture was then shaken for one hour and was permitted to stand 24 hours before use.

The columns were packed using hexane to transfer the florisil to the column. Once in the column, the solvent was drained from the column until the surface of the liquid reached the top of the packed column. The lipids to be separated were placed on top of the column in a small volume of hexane. The lipids were prepared by taking an aliquot from the petroleum ether extract, evaporating under nitrogen and redissolving in a minimum volume of hexane. The surface

of the liquid was again allowed to drop to the head of the column. The various classes of lipids were eluted off the column at a flow rate of 40 ml/hour in 9 ml fractions. The order of eluting solvents as well as the lipid class eluted in the different fractions are given in Table II. The percentage recovery for each eluted class was determined using chromatographically pure squalene, cholesterol palmitate, trimyristin, cholesterol and stearic acid. The phospholipids remained on the column such that a separate column was used for each separation.

After calibration of the column as seen in Fig. 1, the various lipids were separated. The fractions for each lipid class were pooled. The pooled fractions were evaporated to dryness at 60^{o} C under nitrogen and each fraction was redissolved in the appropriate solvent for assay.

3) Separation of phospholipids on silicic acid column

As mentioned, the phospholipids were not eluted from florisil with the solvents listed in Table II. Carroll (54) made an attempt to separate neutral lipids from phospholipids using a florisil column but showed that this could not be done quantitatively.

To separate the phospholipids quantitatively from the other lipids, a column containing 5 g silicic acid and 2.5 g of Hyflo super-cel (Johns-Manville) was used as described by Hanahan et al. (55). All columns used measured 1.2 cm x 15 cm.

Table II

Separation of lipid components on florisil.

Eluent	Eluting Solvent	Volume (mls)	Recovery (%)	Fractions
Hydrocarbons	hexane	20	94	1-3
Cholesterol esters	5% ether in hexane	50	105	4-7
Triglycerides	15% ether in hexane	75	100	8-14
Cholesterol	25% ether in hexane	60	103	15-21
Diglycerides*	50% ether in hexane	60	-	-
Monoglycerides*	2% methanol in ether	75	-	-
Free fatty acids	4% acetic acid in ether	75	101	34-42

*as reported by Carroll (33)

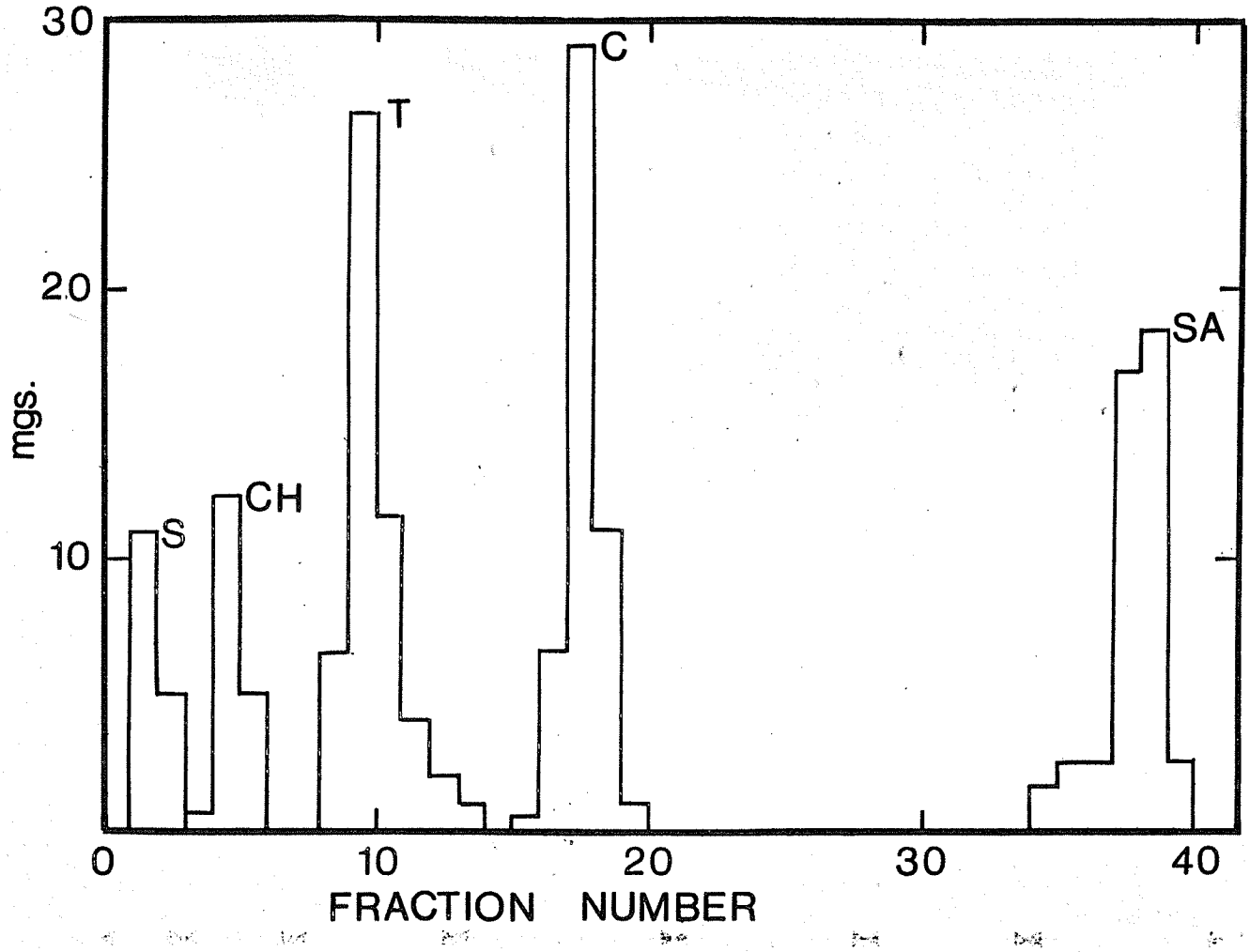

FIGURE 1. Separation of lipid classes on 12 g florisil column.

S - squalene
CH - cholesterol hexadecanoic acid
T - trimyristin
C - cholesterol
SA - stearic acid

The columns were packed and the sample applied as described previously.

The column was first eluted with 100 ml chloroform and then with 50 ml methanol. Neutral lipid and phospholipid were recovered quantitatively in the chloroform and methanol fractions respectively. Chromatographically pure L-α-lecithin (dipalmitoyl) was used as the reference phospholipid. The methanol fraction containing the phospholipids was evaporated to near dryness at 60° C under nitrogen and made up to volume with methanol. Aliquots were taken and the phospholipids were determined as described previously.

4) Determination of radioactivity

Once the lipids had been separated on the columns and the fractions pooled, evaporated, redissolved and made up to volume in their appropriate solvents, an aliquot of the sample to be measured was placed in a liquid scintillation vial and the solvent evaporated under nitrogen. Fifteen ml of the scintillator prepared by mixing 378 ml absolute ethanol with 600 ml toluene (spectrophotometric grade) containing 0.4% PPO (2,5-diphenyloxazole) and 0.0015% POPOP (1,4-bis-2(5-phenyloxazolyl)-benzene), were added to each vial. The samples were read in a Packard, model 3000, liquid scintillation spectrophotometer at the optimal settings for ^{14}C counting.

The radioactive carbon dioxide was determined using

the method of Bhagavan et al. (56). Scintillation vials were loosely filled with Cab-o-sil, a thixotropic gel. To this were added 15 ml of the scintillator solution mentioned previously and 0.5 ml aliquots of the sodium hydroxide containing the radioactive CO_2. The samples were shaken vigorously and tapped gently to remove entrapped air bubbles, and counted as before in a Packard liquid scintillation spectrophotometer. Counting efficiency was determined by the internal standardization method.

e) Determination of acetyl CoA and acetoacetate

1) Acetyl CoA

The acetyl CoA was estimated by the fluorimetric method of Herrera and Freinkel (57) using an internal standard.

Reagents.

- Potassium phosphate buffer; 0.05 M solution (pH 6.8).
- Potassium phosphate buffer; 1.00 M solution (pH 6.8).
- Potassium hydroxide; 1.6 N solution.
- Perchloric acid; 6% solution.
- Tris (Cl^-) buffer; 5 mM solution (pH 8.0).
- Sodium malate; 2 mM solution.
- Nicotinamide adenine dinucleotide; 1 mM solution.
- Malic dehydrogenase (Sigma).
- Citrate synthase (Sigma).

- Acetyl CoA (90% pure from Sigma); 0.1 mM solution.

i) Preparation of powdered liver extract

The rat was sacrificed by decapitation, and the livers were excised and immersed in liquid nitrogen within 30 seconds. The tissues were powdered with a mortar and pestle continuously cooled with liquid nitrogen. The powder was then transferred to an ice cold preweighed beaker containing 3 volumes of 6% perchloric acid. The liver weight was then calculated and the contents of the beaker were homogenized in a ground glass homogenizer. The homogenate was centrifuged at 100,000 x g for 15 minutes at 4^{o} C. The supernatant was removed and neutralized with 1.6 N potassium hydroxide to a pH of 6.8 and sufficient 1 M potassium phosphate buffer (pH 6.8) was added to make the final solution 0.05 M with respect to the buffer. The solution was left to settle for 30 minutes and the precipitated perchlorate was removed by centrifugation at 800 x g for 10 minutes. All operations were carried out at $0-3^{o}$ C and the extracts were used immediately for acetyl CoA estimation.

ii) Procedure

The following reaction mixture, in a final volume of 3 ml, was introduced into each cuvette: 0.2 μmole of NAD, 0.2 μmole of sodium malate, 0.5 μmole of Tris (Cl^{-}) buffer (pH 6.8), 100 μg of malic dehydrogenase, and the extract from 50-150 mg of fresh powdered liver. Cuvettes

were maintained at 34° C in a Turner model 110 fluorimeter. The activating light was at 360 mμ and the emitted light was estimated at 420 mμ and above. The blank contained the same reagents but no citrate synthase was added. This had no effect on the fluorescence. After the malic dehydrogenase reaction had come to equilibrium, 50 μg citrate synthase was added and the increase in fluorescence was noted. Once a new plateau had been established, an internal standard was employed to calibrate the change in fluorescence as follows: to each cuvette were added 25 μl of a standard solution of acetyl CoA (approximately 2.5 mμmoles) and the increase in fluorescence was noted. This was repeated a second time and the increase in fluorescence was again noted.

iii) Acetyl CoA standardization

The concentration of the acetyl CoA in standard solution was estimated by the method of Stadtman (58) before each acetyl CoA determination.

Reagents.

- Potassium arsenate; 0.5 M solution (pH 7.0).
- Phosphotransacetylase; 500 units/ml (Boeringher).

Procedure. The following reaction mixture, in a final volume of 1.0 ml, was introduced into each cuvette: 0.05 ml of potassium arsenate (0.5 M), test solution containing 0.02 to 0.1 μmoles acetyl CoA. The blank contained all but the acetyl CoA. The optical density was

read at 232 mμ. To each cuvette was added 0.01 ml phosphotransacetylase and the decrease in optical density was determined at 232 mμ. The difference in molar extinction coefficient of acetyl CoA and its hydrolysis products (ΔE_{232}) was given as 4.5×10^6 cm^2/mole. Therefore, under the above conditions the change in optical density due to 0.1 μmoles acetyl CoA was 0.45 at 232 mμ.

2) Acetoacetate

The acetoacetate was estimated by the colorimetric method described by Walker (59) as follows:

Reagents.

- Trichloroacetic acid; 3% solution.
- Trichloroacetic acid; 10% solution.
- ρ-Nitroaniline; 0.05% solution in 0.05 N HCl.
- Diazo reagent; prepared immediately before use by adding 3.0 ml $NaNO_2$ (0.5% solution) to 20.0 ml ρ-nitroaniline solution. The colourless solution of the diazonium salt was chilled in an ice bath and 7.0 ml of 0.2 M sodium acetate were added.
- Sodium acetate; 0.2 M solution.
- Sodium acetate - acetic acid buffer; 1 M solution (pH 5.2).
- Hydrochloric acid; 5 N solution.
- Ethyl acetate.
- Acetoacetic acid; obtained as methyl ester of acetoacetate (Sigma) and hydrolyzed. The acetoacetate solution was

standardized manometrically.

i) Preparation of samples for analysis

Blood. Blood was collected from the decapitated animals in siliconized test tubes. Aliquots were placed in centrifuge tubes containing 5 volumes of 3% TCA solution. The contents were mixed and centrifuged in the cold. The clear supernatant was used for analysis.

Liver. The liver was frozen in liquid nitrogen and powdered as described previously in the acetyl CoA determination. The powdered liver was homogenized in a Waring blendor with 3 volumes of cold 10% TCA solution. The tissue suspension was cleared by centrifugation. The supernatant was used for analysis.

ii) Procedure

When this method was applied to estimation of acetoacetate levels in TCA extracts of tissues, some colour was contributed by other compounds. This was compensated for by having an appropriate blank. Aliquots of the TCA extracts of tissue were heated in boiling water for 5 minutes. This treatment destroyed acetoacetate completely, whereas the material responsible for the residual increase in colour remained unchanged. Acetoacetate levels in tissue extracts were therefore estimated by difference from the readings obtained using fresh and heated extracts.

To 0.5 ml of standard acetoacetate solution or tissue

extract in glass-stoppered tube, were added 0.5 ml sodium acetate - acetic acid buffer (pH 5.2) and 3.0 ml freshly prepared diazo reagent. After allowing to stand for 30 minutes at room temperature, the reaction was stopped by addition of 1.0 ml 5 N HCl and the product was extracted by shaking with 4.0 ml ethyl acetate. Water was used instead of standard acetoacetate solution and tissue extract for the reagent blank. The colour was determined at 440 mμ. A calibration curve was determined with standard acetoacetate.

f) In vitro mitochondrial fatty acid synthesizing system

1) Isolation of the mitochondrial pellet

The mitochondria were isolated by a procedure described by Langdon (60). The livers were immediately placed in ice cold 0.25 M sucrose. They were then squeezed between filter papers to remove excess moisture, weighed, minced into small pieces with scissors and homogenized in 1.5 volumes of 0.25 M sucrose with a mortar and Teflon pestle. After the first complete pass of the pestle, the pestle was passed five more times. The homogenate was then centrifuged at 600 x g for 10 minutes to remove whole cells, cell debris and nuclei. The supernatant was removed with a Pasteur pipette and the mitochondria were centrifuged down at 10,000 x g for 10 minutes. The white fatty material was removed from the top of the supernatant with cotton batting, the

supernatant was removed and discarded. The sediment was washed twice by dispersion in fresh cold 0.25 M sucrose and recentrifuged at 10,000 x g for 10 minutes. All operations were carried out in a cold room at 0-3° C.

2) Assay system

The system of Wakil et al. (61) was used to study the in vitro synthesis of fatty acids by mitochondria. The fatty acids synthesized were isolated by the method of Wakil et al. (41).

The reaction mixture contained 34.5 mμmoles of acetyl-CoA-1-^{14}C (61,000 cpm), 2 μmoles ATP, 0.25 μmoles NADPH, 0.25 μmoles NADH, 3.0 mg mitochondrial pellet, 50 μmoles phosphate buffer (pH 6.5) and water in a final volume of 0.5 ml. The reaction was initiated by the addition of the mitochondrial protein and was incubated anaerobically at 38° C for one hour.

At the end of the incubation time, the reaction was stopped by the addition of 0.5 ml 10% alcoholic KOH and the tubes were kept in a boiling water bath for 30 minutes. At the end of this time, the saponification was complete and 0.5 ml 2 N HCl was added to bring the mixture to pH 2-3. The tubes were extracted twice with 4 ml fractions of petroleum ether (45-60° C) and the tubes were shaken for 3 minutes at each extraction. The pooled petroleum ether extracts were then washed with two 4 ml fractions of water and dried over

anhydrous sodium sulphate for 2 hours. The petroleum ether extracts were transferred to liquid scintillation vials and the solvent was evaporated off at 60° C under nitrogen. Fifteen ml of the scintillator (0.3% PPO in toluene) was added to the vials and counted in a Packard liquid scintillation spectrophotometer. Counting efficiency was determined by the internal standardization method.

Protein was estimated by the method of Lowry et al. (45) as described earlier.

g) In vitro non mitochondrial fatty acid synthesizing system

1) Isolation and partial purification of acetyl CoA carboxylase

The acetyl CoA carboxylase was isolated, partially purified and assayed by the method of Chang et al. (62). The livers were homogenized in 3 volumes of a medium (pH 7.2 at 25° C) containing 0.15 M KCl, 0.05 M Tris (Cl^-) and 0.1 mM EDTA (ethylenediamine tetra-acetic acid). The homogenate was centrifuged at 105,000 x g for 60 minutes. The high speed supernatant (cytosol) was then placed on top of a 2.5 cm x 45 cm Sephadex G-25 column. The column was packed as previously described with Sephadex G-25 (medium) which had been permitted to swell overnight in 4 volumes of the homogenizing medium. The column was eluted with the

homogenizing medium in 4 ml fractions at a flow rate of 50 ml per hour. The cytosol was eluted off the column after the void volume had passed through. Fractions containing the gel-filtered cytosol were then pooled and used for determination of acetyl CoA carboxylase activity.

After elution of the cytosol, the column was washed with 3-4 bed volumes (500 ml) of the homogenizing medium and used again. All operations were carried out at 0-5° C.

2) Assay system

Preincubation. The gel-filtered cytosol was preincubated at 37° C for 30 minutes in a medium (final pH 7.0, at 37° C) containing 60 mM Tris (Cl^-) buffer, 3 mM glutathione (GSH), 8 mM $MgCl_2$, 0.1 mM EDTA, bovine serum albumin (0.6 mg per ml) and 5 mM citrate (K^+). In experiments to determine the effects of avidin and d-biotin on acetyl CoA carboxylase, the gel-filtered cytosol was preincubated as above but the medium contained in addition either 1 unit of avidin (specific activity 2150 units/g) or 0.04 and 0.004 μmoles d-biotin per mg protein of the gel-filtered cytosol. In some experiments with avidin, 1 unit of avidin was incubated at 0° C for 15 minutes with 0.04 μmoles d-biotin in the same preincubation medium without the gel-filtered cytosol. The treated avidin was then incubated in the same way as before with the gel-filtered cytosol.

Assay. The assay medium contained 60 mM Tris

(Cl^-) buffer, 2 mM ATP, 8 mM $MgCl_2$, 10 mM $NaH^{14}CO_3$ (specific activity 0.39 μc per μmole), 0.2 mM acetyl CoA, 3 mM GSH, 0.1 mM EDTA, 0.6 mg/ml bovine serum albumin and 5 mM citrate. Carboxylation was initiated by the addition of an aliquot of the preincubated enzyme containing 1 mg of protein. The final assay volume was 1.0 ml and the reaction was carried out for 5 minutes at 37° C and stopped by the addition of 0.2 ml 6 N HCl. Protein was estimated by the method of Lowry et al. (45) as described previously using crystalline bovine serum albumin as standard.

Incorporation of ^{14}C activity into malonyl CoA was determined by the method of Gregolin et al. (63) as follows: a 150 litre aliquot of the reaction mixture was placed in a liquid scintillation vial and was taken to dryness under a heat lamp at 85° C for 30 minutes. To this was added 0.1 ml water and 15 ml of a toluene - ethanol scintillator prepared as described previously. The acid stable radioactivity was measured in a Packard liquid scintillation spectrophotometer. Counting efficiency was determined by the internal standardization method.

B) Results

a) Production of biotin deficiency

Fig. 2 demonstrates a typical growth rate of normal and biotin deficient rats. During the first four weeks of the deficiency the body weights steadily increased. In the fifth week their body weights levelled off and in the following weeks showed a steady decline. These three regions of the growth rate were called pre-plateau, plateau and post-plateau respectively.

The plateau region of the deficiency was typified by a dermatitis and alopecia near the mouth and fore limbs of the animals, as well as the appearance of "spectacle eyes". The post-plateau region was marked by severe dermatitis and alopecia as well as the appearance of a "spastic gait". The post-plateau stage lasted for usually a week or two before death ensued.

b) Biochemical criterion of biotin deficiency

The liver propionyl CoA carboxylase activities of normal, deficient and positive control rats are shown in Table III. This enzyme activity in biotin deficient rat liver was about one tenth of that in normal livers. The response of liver propionyl CoA carboxylase activity to biotin administration was a rapid and dramatic four- to five-fold

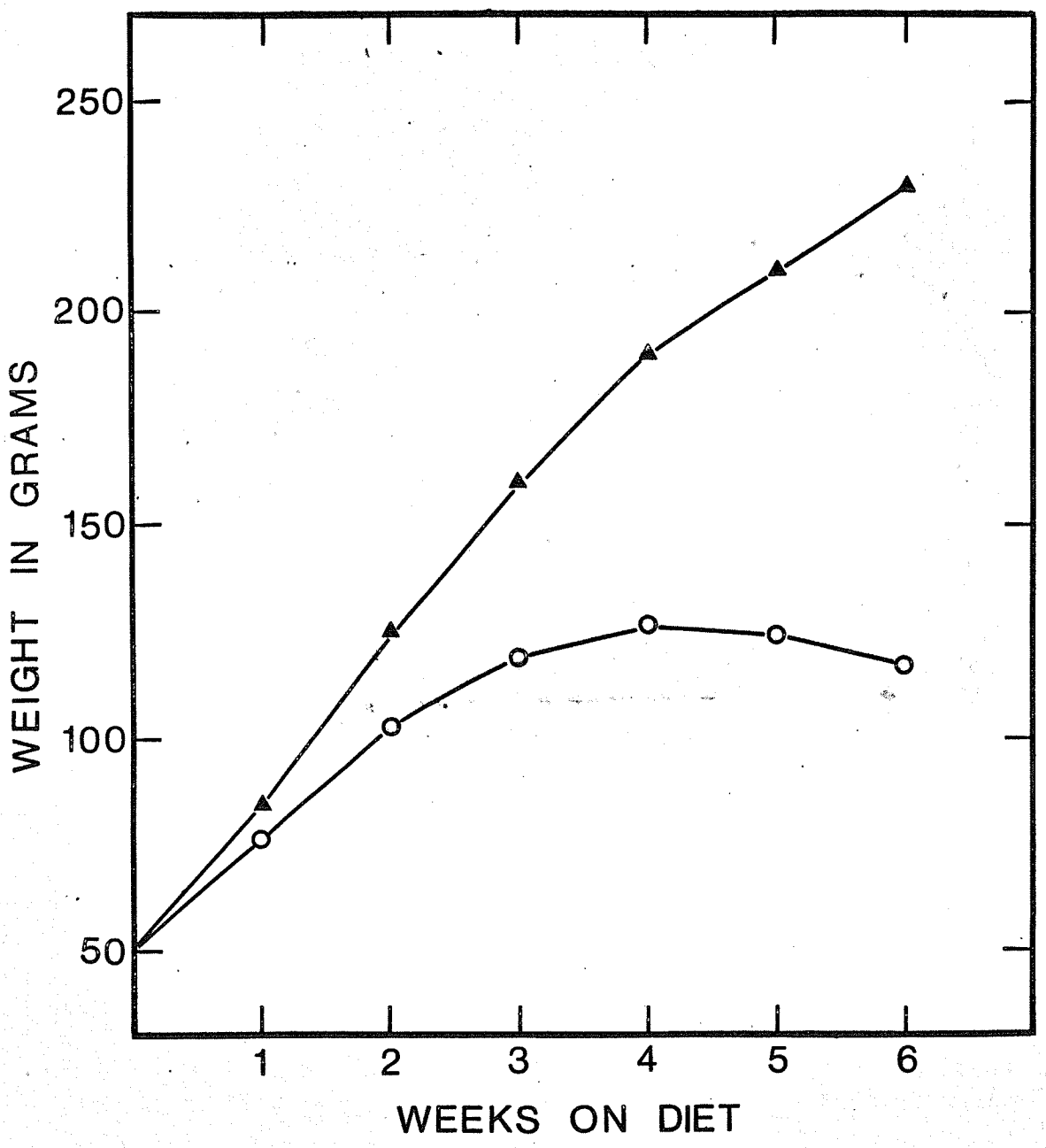

FIGURE 2. Growth curve of normal ▲——▲, and biotin deficient rats o——o. The animals were fed ad libitum.

Table III

Propionyl CoA dependent $H^{14}CO_3^-$ fixation in rat liver.

% dextrose in avidin diet	μmoles $H^{14}CO_3^-$ fixed/hour/mg protein*			Ratio of
	Normal[1]	Biotin deficient	Positive[2] control	Positive control / Biotin deficient
66	0.104 ± 0.008	0.008 ± 0.002	0.035 ± 0.006	4.4
36	0.100 ± 0.011	0.012 ± 0.003	0.058 ± 0.008	4.8

*values are means ± standard deviations of triplicate assays on acetone powder of whole liver from 3 rats in each group

[1]received 100 μg biotin each week

[2]deficient rat which received one 100 μg dose of biotin 2-3 days before the experiment

increase as early as 3 days after biotin administration. This was seen in both groups on the high and low carbohydrate diets. The activity of propionyl CoA carboxylase was definitely reduced after five weeks on the biotin deficient diet and was generally used as a criterion of the biotin status of the animals of any group.

c) Effect of biotin deficiency on serum and tissue cholesterol

The cholesterol contents in blood, liver and carcass of biotin deficient and control rats are given in Table IV. Carcass weights in the experiments reported represented the weight of the animal less that of the head, liver and epididymal fat pads. There was no significant difference in serum cholesterol between biotin deficient and control rats, although Scott (8) reported hypercholesterolaemia in a biotin deficient human infant. Inclusion of linoleic acid in the diet reduced serum cholesterol. There was no significant difference in liver total cholesterol between biotin deficient and control groups on any of the diets studied although there were differences among the various dietary treatments. Liver and carcass levels of cholesterol on the fat-free diet were significantly ($p<0.05$) higher than those on the 5% corn oil diets. Similar differences in the tissue levels of cholesterol in mice fed different levels of

Table IV

Effect of biotin deficiency on serum, liver and carcass total cholesterol.

	Diet A	Diet B	Diet C	Diet D
Serum (mg/ml)[1]				
+ biotin	0.62 ± 0.15	0.71 ± 0.14	-	0.41 ± 0.12
- biotin	0.60 ± 0.11	0.58 ± 0.05	-	0.46 ± 0.08
Liver (mg/g)[1]				
+ biotin	2.07 ± 0.43	1.93 ± 0.27	2.52 ± 0.28	2.42 ± 0.23
- biotin	1.92 ± 0.29	2.03 ± 0.34	2.47 ± 0.22	2.43 ± 0.21
Carcass (mg/g)[1]				
+ biotin	1.07 ± 0.23	1.13 ± 0.15	1.80 ± 0.28	1.59 ± 0.15
- biotin	1.55* ± 0.02	1.58* ± 0.18	2.30* ± 0.14	1.88** ± 0.25

Diet A - high carbohydrate, 5% fat
Diet B - high protein, 5% fat
Diet C - high carbohydrate, fat-free
Diet D - high carbohydrate, 2% corn oil plus 1% linoleic acid

*$p<0.01$ with respect to the control (+ biotin)
**$p<0.05$ with respect to the control (+ biotin)
[1]values are means ± standard deviation obtained with 6 animals in each group

corn oil in the diet have been reported by Jansen et al. (64). These cholesterol data are contrary to earlier findings (5-7) of a decrease in cholesterol synthesis in biotin deficiency but are in agreement with the results of Fletcher and Myant (65) who found that in cell-free preparations of rat liver, avidin did not inhibit incorporation of labelled acetate into cholesterol. In our studies, the carcass cholesterol content of all the biotin deficient groups was significantly ($p<0.01$) elevated over the corresponding control levels. The capacity of extrahepatic tissues to synthesize cholesterol was demonstrated by Dietschy and Siperstein (66). From experiments in which hepatic cholesterol synthesis was suppressed, Jansen et al. (64) found that in both the rat and the mice about 70-75% of the cholesterol newly synthesized from carbohydrate was made in extrahepatic tissues. In terms of absolute amounts, carcass cholesterol content was about 10 to 20 times greater than liver cholesterol. Thus the extent of the increase in carcass cholesterol of the biotin deficient rat is quite striking.

d) Effect of biotin deficiency on the composition of liver, adipose tissue and carcass lipids

Masoro (67) showed that the in vitro conversion of acetate into liver fatty acid was decreased with as little

as 2.5% corn oil in the diet. Later, Bortz et al. (9) identified the site of inhibition as the carboxylation of acetyl CoA. Earlier work (3,5,6) did not reveal any effect of biotin deficiency on liver fatty acid synthesis probably because of the high level (5-12%) of fat in the diets used by them. Donaldson (2), in studying the effects of biotin deficiency in chicks, used a fat-free diet and found that the percentage fatty acid content of carcass was depressed by biotin deficiency. The possibility of imposing an essential fatty acid deficiency on top of biotin deficiency had to be considered in using a fat-free biotin deficient diet. Aaes-Jørgenson (68) found that it took 9 to 12 weeks of feeding a fat-free diet to weanling rats for symptoms of essential fatty acid deficiency to appear. The rats used in our experiments were larger (45-50 g) when placed on the fat-free diet and only 5-6 weeks were required for biotin deficiency to develop. During this time the biotin-treated controls were gaining in weight to the same extent as fat-fed rats. Their coats as well as tails appeared quite normal. All this suggested that in using the fat-free avidin diet, the effects of biotin deficiency were primarily studied.

The effects of the deficiency on lipid composition of various tissues are given in Tables Va and Vb. Determinations of the lipid components of the liver and carcass (Table Va) were carried out on the lipid extracts themselves as

Table Va

Tissue lipid composition of biotin deficient and control rats on the fat-free diet[1].

	Liver (per g fresh liver)		Carcass (per g carcass)	
	+ biotin*	- biotin*	+ biotin*	- biotin*
Organ weight (g)	5.66 ± 0.39	5.25 ± 0.82	-	-
Total lipids (mg)	46 ± 14	39 ± 9	-	-
Cholesterol, free (mg)	2.17 ± 0.27	2.46 ± 0.38	1.80 ± 0.28	2.30 ± 0.14***
Cholesterol, esterified (mg)	0.58 ± 0.21	0.20 ± 0.20***	0.19 ± 0.15	0.15 ± 0.15
Phospholipids[2] (mg)	20.85 ± 7.04	26.34 ± 5.00	9.6 ± 1.5	9.1 ± 1.2
Non phospholipid esterified fatty acids (mg)	13.9 ± 3.9	9.7 ± 3.2**	41.4 ± 12.4	16.4 ± 5.1***

[1]determinations carried out on the lipid extract
[2]precipitated from lipid extract with $MgCl_2$ and acetone
*values are means ± standard deviation with 6 rats in each group
**$p<0.05$ with respect to the control (+ biotin)
***$p<0.01$ with respect to the control (+ biotin)

Table Vb

Tissue lipid composition of biotin deficient and control rats on the fat-free diet[1].

	Liver (per g fresh liver)		Epididymal fat pad (per two fat pads)	
	+ biotin*	- biotin*	+ biotin*	- biotin*
Organ weight (g)	5.66 ± 0.39	5.25 ± 0.82	0.896 ± 0.163	0.370 ± 0.117
Total lipids (mg)	46 ± 14	39 ± 9	486 ± 116	150 ± 77
Cholesterol, free (mg)	1.77 ± 0.29	1.73 ± 0.16	1.01 ± 0.75	0.39 ± 0.21
Cholesterol, esterified (mg)	0.85 ± 0.46	0.18 ± 0.05***	0.62 ± 0.08	0.14 ± 0.04
Triglycerides (mg)	6.3 ± 3.6	4.0 ± 1.1	432 ± 118	119 ± 59***
Phospholipids[2] (mg)	53.0 ± 13.6	58.4 ± 13.1	201 ± 106	55.0 ± 23.0**

[1]determinations were carried out on the pooled fractions after chromatographic separation as described in the Methods
[2]separated on silicic acid - Hyflo super-cel column
*values are means ± standard deviation with 6 rats in each group
**$p<0.05$ with respect to the control (+ biotin)
***$p<0.01$ with respect to the control (+ biotin)

described in the Methods. Determinations of the lipid components of liver and epididymal fat pads (Table Vb) were done on the pooled fractions after separation of the different lipid fractions by chromatography as described in the Methods.

There was no significant difference in liver weight or total liver lipid between the biotin deficient and pair-weighed control group. However, deficient rat liver had significantly ($p<0.05$) lower amounts of esterified cholesterol and non phospholipid esterified fatty acids than controls. The esterified fatty acid content of deficient carcasses was only 40% of the control level. Carcass cholesterol in the deficient group, as described before, was elevated. The epididymal fat pad weight, total lipid, triglyceride and phospholipid content of deficient rats were respectively 40, 35, 25 and 25% of control values (Tables Va and Vb). There was good correlation between the two methods for determination of lipid components in the lipid extract except in the case of phospholipids. Values obtained for phospholipids by precipitation with magnesium chloride and acetone are in good agreement with the results of Bloor (49). Separation on silicic acid - Hyflo super-cel columns seemed to affect the assay for phospholipids. The overestimation of phospholipid by this method may be due to elution of silicic acid from the column by the solvent.

e) Acetate-1-^{14}C incorporation into liver lipids

Of the injected dose of labelled acetate, 64.5% was recovered as expired CO_2 in the biotin deficient group. Only 41.5% of the injected dose appeared as expired CO_2 in the control group. Similar differences reported by Donaldson (2) might be due to the reduced capacity of biotin deficient rats to fix CO_2 *in vivo* (56). The incorporation of acetate into various lipid fractions in deficient and control rats is presented in Table VI. The specific activities of esterified cholesterol, triglyceride and phospholipid fractions of control rat livers were 4 to 9 times greater than those of similar fractions from deficient liver. The differences in the specific activities of esterified cholesterol presumably were due to the fatty acid portion since the specific activities of the free cholesterol fractions of both deficient and control rats were about the same. The phospholipid fraction of control rat livers had a higher specific activity than that from the deficient rat liver. This again might reflect the lability of the fatty acid moiety of phospholipids and the effect of incorporation of, or exchange with, newly synthesized fatty acids. The lower specific activities of various lipid fractions of the biotin deficient rats might also arise if the labelled acetate were diluted in a large pool of liver acetyl CoA in

Table VI

Acetate-1-^{14}C incorporation into liver lipids.

	Control (+ biotin)*	Biotin Deficient (- biotin)*
Animal weight (g)	134.8 ± 5.5	133.7 ± 3.3
Liver weight (g)	5.31 ± 0.39	5.32 ± 0.31
Percentage of injected dose as expired CO_2	41.5 ± 1.3	64.4 ± 5.2
Total liver lipid (mg)	234 ± 86	200 ± 32
Specific activity (dpm/mg)	2220 ± 494	905 ± 104
Cholesterol, free (mg/g)	1.85 ± 0.37	1.64 ± 0.13
Specific activity (dpm/mg)	778 ± 290	747 ± 223
Cholesterol, esterified (mg/g)	0.72 ± 0.50	0.20 ± 0.07
Specific activity (dpm/mg)	6190 ± 459	1638 ± 475
Triglyceride (mg/g)	3.73 ± 0.91	3.70 ± 0.40
Specific activity (dpm/mg)	2850 ± 232	557 ± 50
Phospholipid (mg/g)	52.6 ± 20.3	59.0 ± 5.3
Specific activity (dpm/mg)	828 ± 35	353 ± 31
Free fatty acids (mg/g)	1.06 ± 0.92	1.89 ± 1.74
Specific activity (dpm/mg)	1655 ± 128	644 ± 197

*values are means ± standard deviation with 3 rats in each group

these animals. As seen in Table VII, acetyl CoA concentrations of deficient rat livers are significantly larger than those in control livers. However, this increase would dilute the injected trace dose of acetate by only 28% and therefore cannot explain the large difference in the specific activities of the fatty acids of deficient and control livers. The observations of Numa et al. (69) in the normal rat along with the increased acetyl CoA levels in the biotin deficient rat observed here would suggest that in both biotin deficient and control rats the initial step of activation of acetate may not be rate limiting in fatty acid synthesis. The difference in specific activities of lipid fractions would thus reflect the different rates of fatty acid synthesis in the deficient and control rats.

f) Acetoacetate levels in deficient and control rats

The data are presented in Table VIII. The liver acetoacetate concentration in the biotin deficient group was twice that of controls and the blood level was increased nearly ten-fold. The increase in liver acetoacetate may be significant in disposing of acetyl CoA accumulation in the liver of the biotin deficient rat.

g) Liver acetyl CoA carboxylase

The effect of biotin deficiency on in vitro acetyl

Table VII

Liver acetyl CoA concentration.

Biotin Status	Weight (g)	mμmoles/g
Control (+ biotin)	133 ± 5* (6)**	32 ± 2*
Biotin Deficient (- biotin)	127 ± 8 (6)	43 ± 9

*mean ± standard deviation
**number of rats used for estimation of the mean
[1]$p<0.01$ with respect to the control (+ biotin)

Table VIII

Blood and liver acetoacetate concentration.

Biotin Status	Number of Rats	Weight	Acetoacetate*	
			mg/g fresh liver (x 10^3)	mg/100 ml blood
Control (+ biotin)	7	141 ± 9	26.8 ± 2.8	1.1 ± 0.1
Biotin Deficient (- biotin)	6	141 ± 11	41.0[1] ± 3.6	10.5 ± 0.85

*values are means ± standard deviation
[1]$p<0.01$ with respect to the control (+ biotin)

CoA carboxylase activity was studied. As seen in Table IX, the deficient rat liver had about 50-60% of the activity of the control whether expressed per mg protein or per g fresh liver. Malonyl CoA formation by both the control and deficient enzyme preparations was completely inhibited by preincubation with avidin and the avidin inhibition was quite specific (Table X). It seems that the various biotin enzymes are reduced to different extents during the progress of biotin deficiency. In similar states of deficiency in terms of body weight, time on avidin diet and outward symptoms of deficiency, the liver propionyl CoA carboxylase activity was reduced to one tenth of control levels as seen in Table III, whereas acetyl CoA carboxylase activity was still about one half the control levels.

h) In vitro liver mitochondrial fatty acid synthesis

On the assumption that in biotin deficiency mitochondrial fatty acid synthesis may play a compensatory role for the reduced non mitochondrial activity, acetyl CoA incorporation into fatty acids by mitochondria was investigated. The results given in Table XI show that acetyl CoA incorporation into fatty acids by deficient liver mitochondria was about one tenth of the control level. This decrease in incorporation is not likely to be due to a biotin dependent enzyme since Alexander et al. (70) reported that avidin does

Table IX

Acetyl CoA carboxylase activity in biotin deficient and control rats.

Biotin Status	mμmoles acetyl-CoA-1-^{14}C incorporated into malonyl CoA/minute/mg protein	mμmoles acetyl-CoA-1-^{14}C incorporated into malonyl CoA/minute/g wet wt liver
Control (+ biotin)	10.23 ± 0.54* (3)**	511 ± 112 (3)
Biotin Deficient (- biotin)	5.08 ± 0.58 (4)	290 ± 46 (4)
Statistical Significance p^1	<0.001	<0.05

*mean ± standard deviation
**number of rats used for estimation of the mean value
[1]probability based on Student's t-test

Table X

Effect of avidin on acetyl CoA carboxylase activity in biotin deficient and control rats.

Biotin Status	Experimental Condition (per mg protein)	mμmoles acetyl CoA incorporated into fatty acids/minute/ mg protein
Control (+ biotin)	-	12.54
	+ 1 unit avidin	0.00
	+ 1 unit avidin treated with 10 μg biotin	10.54
Biotin Deficient (- biotin)	-	5.60
	+ 1 unit avidin	0.23
	+ 1 μg biotin	5.04
	+ 10 μg biotin	5.78

Table XI

Liver mitochondrial fatty acid synthetic activity.

Biotin Status	mμmoles acetyl CoA incorporated into fatty acids/hour/mg protein
Control (+ biotin)	0.338 ± 0.18* (6)**
Biotin Deficient (- biotin)	0.120^{+} ± 0.04 (6)

*mean ± standard deviation
**number of rats used for estimation of the mean value
$^{+}p<0.05$ with respect to the control (+ biotin)

not inhibit acetyl CoA utilization by mitochondria. Mitochondrial acetyl CoA incorporation into fatty acids, in any event, could not be a major pathway of fatty acid synthesis in view of its very low activity as compared to the supernatant pathway.

SECTION IV. DISCUSSION

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If leucine degradation contributed significantly to cholesterol synthesis (71), this would be reflected in decreased cholesterol content of the tissues of the biotin deficient rat since β-methylcrotonyl CoA carboxylase (3-methylcrotonyl CoA:CO_2 ligase (ADP); EC 6.4.1.4) in the pathway of leucine catabolism is a biotin enzyme (72). The high protein diet fed to one of the experimental groups contained the equivalent of 10% leucine in the diet, and yet there was no decrease in cholesterogenesis in the biotin deficient rat. On the other hand, there was quite a marked increase in the carcass cholesterol in the deficient rat. The pathways of cholesterol, fatty acid and acetoacetate synthesis are described in Fig. 3. With in vitro systems using subcellular fractions cholesterol synthesis takes place in microsomes, whereas fatty acid synthesis takes place in the supernatant. However, in the intact cell the synthesis of these compounds probably is interdependent. The malonyl CoA pathway is the major one in fatty acid synthesis (73). Combined activities of β-hydroxy-β-methyl-glutaryl CoA condensing and cleavage enzymes are responsible for acetoacetate formation (74). Two different pathways for cholesterol synthesis have been proposed. Rudney (75) suggested that HMGCoA was an intermediate in cholesterol synthesis. Brodie et al. (76) reported that malonyl CoA

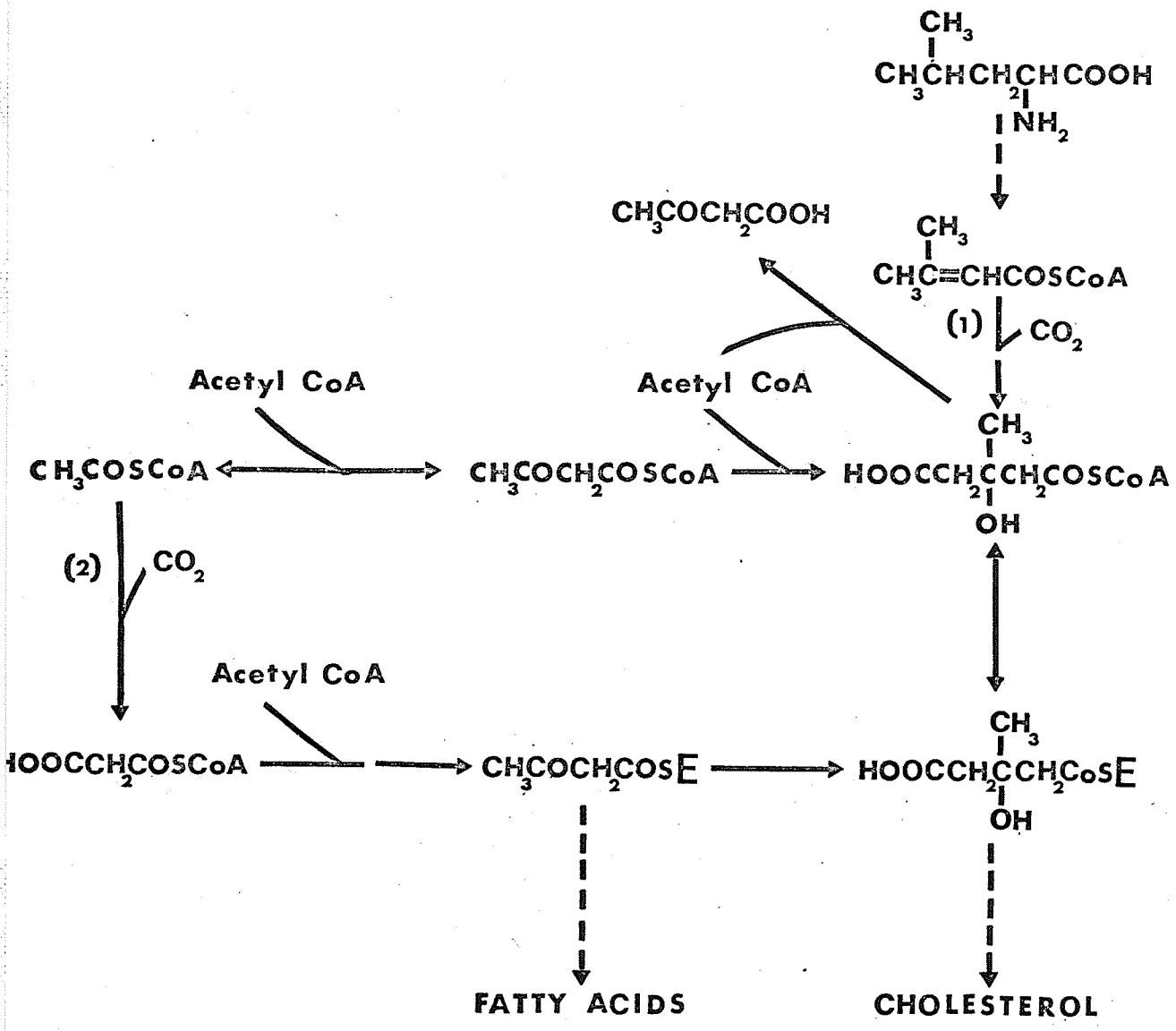

FIGURE 3. Pathways of synthesis of cholesterol, fatty acid and acetoacetate.

(carrier protein) was an intermediate in the cholesterol synthetic pathway. Ichihara et al. (77) showed that malonyl-CoA-2-^{14}C was incorporated more efficiently than acetate-1-^{14}C into cholesterol by dispersed rat liver cells. The relative physiological significance of the two pathways of cholesterogenesis is not clear. If both fatty acid and cholesterol syntheses shared a common initial step through the formation of malonyl CoA, factors such as deficiency of biotin or citrate (78), which markedly affect the activity of acetyl CoA carboxylase, should have similar effects on their syntheses. Ichihara et al. (77) found that when they removed citrate from their complete incubation medium, labelled acetate incorporation into fatty acids was reduced to 9%, whereas the incorporation into cholesterol was still 43% of that in the presence of citrate. Foster and Bloom (79) reported that citrate augmented fatty acid synthesis and dispersed cholesterol synthesis by homogenates and slices of normal rat liver. These observations as well as the results found here would suggest that the malonyl CoA pathway is not the major pathway of cholesterol synthesis. In biotin deficient rats there was a decrease in the total esterified fatty acid fraction of the liver although this was much less than was seen in the adipose tissue. On the other hand, levels of cholesterol in the carcass as well as acetoacetate in blood and liver showed a significant

increase. Bloomfield and Bloch (80) found that addition of biotin to the medium of biotin deficient yeast stimulated fatty acid synthesis and depressed cholesterol synthesis. In diabetic animals too, the formation of cholesterol is either normal or enhanced (81), and acetoacetate synthesis is enhanced whereas fatty acid synthesis is depressed (82). Direct reciprocity *in vitro* between fatty acid and ketone body formation has been shown by Wieland and Weiss (83). In the diabetic or biotin deficient animal the increase in cholesterol and acetoacetate may represent an overflow for the excess acetyl CoA when acetyl CoA carboxylase is depressed. Alteration of the lipogenic pattern reported here is another example where parallel defects in both diabetes and biotin deficiency are evident. Similar defects in glucose utilization have been pointed out by Dakshinamurti and Cheah-Tan (38) as well as by Mistry *et al*. (84).

A comparison of specific activities of liver lipids two hours after injection of acetate-1-^{14}C was assumed to provide a comparison of rates of synthesis rather than degradation. Balnave and Wood (86) have shown that acetate-2-^{14}C injected intraperitoneally into chicks reached maximal incorporation in all of the liver lipid components between 1.5 and 2.5 hours after similar administration of the label. Gram and Okey (6) injected acetate-2-^{14}C

intraperitoneally into biotin deficient and control rats and studied the incorporation of the label into expired CO_2. The peak of the specific radioactivity of CO_2 was seen at approximately one hour in both biotin deficient and control rats.

The increased excretion of labelled CO_2 by the biotin deficient rat after acetate-1-^{14}C administration, when compared to the control, was similar to the observations of Donaldson (2) and Gram and Okey (6). Dakshinamurti and coworkers (56) have also shown increased incorporation of glucose-1-^{14}C and glucose-6-^{14}C into $^{14}CO_2$ in biotin deficient rats. The increased acetyl CoA levels in the biotin deficient rat suggest that the activation of acetate was not impaired in the deficient animal. In vitro acetyl CoA carboxylase activity suggests that acetyl CoA utilization via lipogenesis was decreased in the biotin deficient animal and yet acetate-1-^{14}C incorporation into $^{14}CO_2$ was increased. This would suggest that oxidation of the acetyl CoA through the tricarboxylic acid cycle was not impaired, but that the reutilization of this endogenous carbon dioxide was decreased. If the tricarboxylic cycle were impaired in biotin deficiency, a decrease of acetate-1-^{14}C incorporation into $^{14}CO_2$ would be expected. This was not the case. This decrease in carbon dioxide fixation in the biotin deficient animal could be explained on the basis of a

decrease in the activity of all the biotin dependent carboxylases.

Puddu et al. (3) have reported that there was no difference between the total lipid content of the liver in biotin deficient and control rats. This study confirms this fact. In view of the decreased acetate-1-^{14}C incorporation into liver lipids in vivo and the 50% decrease in the specific activity of acetyl CoA carboxylase in the biotin deficient liver, a decreased lipid content would be expected. The mitochondrial fatty acid synthesizing system certainly does not play a compensatory role for the reduced non mitochondrial pathway. The mitochondrial system has a lower lipogenic ability when compared to the non mitochondrial fatty acid synthesizing system. Also, the mitochondrial system was only 30% as effective in the biotin deficient animal when compared to the control. The liver acetyl CoA carboxylase activity of the biotin deficient rat, although appreciably reduced, retains sufficient synthetic ability to maintain near normal lipogenesis.

The large reduction in the weight of the adipose tissue as well as its fatty acid content is of great significance. This decrease in fatty acid synthesis by the adipose tissue might be reflected in the greatly reduced carcass fatty acid levels in the deficient rat. Favarger and Gerlach (85) showed that the brain intercapsular adipose

tissue of the mice synthesized more fatty acid than the liver. It seems probable that the adipose tissue might quantitatively be more significant than liver in fatty acid synthesis and that biotin deficiency might have a more drastic effect on the metabolism of the adipose tissue than on that of the liver.

SECTION V. BIBLIOGRAPHY

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