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Biotin Status and Lipid Metabolism in Adult Obese Hypercholesterolemic Inbred Rats[1]

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Abstract. A statistically significant inverse association was generally found between plasma total lipid, cholesterol, or phospholipid and biotin status of 300-day-old male inbred BHE (IN-BHE) rats. Plasma, liver, and carcass lipid of both sexes generally had a significant direct association with liver lactate dehydrogenase activity; an inverse association in males resulted with improved biotin status. Elevated plasma lactate indicative of anaerobic glycolysis was found. It is proposed that an increased reductive environment – a consequence of accumulated NADH – could account for enhanced triglyceride synthesis and that this effect could explain the obesity in the IN-BHE rats. After the injection of 300 μg of biotin, plasma levels of lactate and pyruvate fell in male rats, indicating a stimulatory effect of biotin upon the oxidative pathways in these animals.

Obesity, with or without elevated blood lipid levels, was a frequent finding in nutrition studies of mature male rats from our laboratory strain (BHE) especially when sucrose was the dietary carbohydrate, and/or when low to moderate levels of dietary fat were used (29). Because the BHE rat became obese under dietary conditions which did not produce obesity in Wistar rats, the tendency was considered a genetic defect of the BHE rat along with other characteristics of the parent strain, the Osborne and Mendel rat (2, 24, 32, 33). If BHE rats with elevated blood lipids were inbred, many of the rats produced had elevated blood lipids, etc. (28, 29). However, some of the elevation in blood lipids was

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attributable to the spontaneous development of nephrosis, particularly in the males at about 300 days of age.

While studying the response of two inbred lines to diets with different protein levels, we found that typical symptoms of biotin deficiency such as 'spectacle' eye, accompanied by skin lesions, loss of weight, hair and appetite, developed in one line after about 6 weeks of feeding. The diet fed this line was semipurified with whole defatted egg and with 100 μg/kg biotin added. The other line, fed the same diet, did not develop the symptoms. Feeding additional biotin by mouth resulted in rapid disappearance of the symptoms within a few days. A third line of rats was subsequently developed from the BHE strain. Some characteristics of rats of this line have been described: early hyperinsulinemia (3), and later, elevated 'lipogenic' enzymes and blood and carcass lipids (4) with subsequent elevation in blood glucose (5). A study on the effects of feeding a diet low in biotin to young inbred-BHE (IN-BHE) rats of line 3 showed that they had lower plasma cholesterol levels, when given a biotin supplement, than did their unsupplemented or stock diet-fed littermates (30). Further studies were carried out with 300-day-old rats whose cholesterol levels were elevated not only so that more could be learned about the characteristics of these rats, but also so that it could be determined whether supplementation with biotin would reduce their blood lipids. In the biotin-supplemented group, an elevation of plasma immunoreactive insulin levels which accompanied an improvement in biotin status was reported (5). The present report presents the lipid data from a study of 300-day-old rats and the interrelationships among the lipids and some enzymes associated with lipogenesis. In addition, we have suggested some concepts to define the mechanism for development of obesity in these rats and the possible role of biotin status as measured by liver biotin content.

Methods

Experiment 1

In this experiment the effect of biotin on tissue lipids was studied. The rats used and the diets fed were as described in an earlier report (5). Both male and female BHE and line 3 IN-BHE rats were fed a commercial stock diet (Purina Laboratory Chow) unitl 300 days of age. At that time, groups of each sex and strain were killed as zero-time controls. Groups of littermates were either fed the stock diet or fed a semipurified diet for an additional 28-day period. The semipurified diet contained 30 % defattted whole egg, 58 % sucrose, 5 % stripped corn oil, 4 % Jones and Foster salt mix (22), 0.1 % inositol, 0.2 % choline chloride, 0.03 % *p*-aminobenzoic acid, and 0.05 % vitamin A powder (20,000 IU/g, retinyl acetate in modified carbohydrate carrier). The following vitamins were added (per kg diet): 2 mg each, ergocalciferol (500,000 IU/g), 2-methyl-1,4-naphthoquinone and folic acid; 5 mg each, thiamine and pyridoxine·HCl; 25 mg each, Ca pantothenate (d) and α-tocopherol; 10 mg riboflavin; 25 mg niacin, and 30 μg crystalline B_{12}. This diet contained 0.280 μg biotin/g. The stock diet contained 0.298 μg biotin/g. One group of each sex receiving the semipuri-

fied diet received also a daily oral supplement of 8 μg biotin in aqueous solution. All six groups were fed the diets *ad libitum*. Food intakes were measured and corrected for scattered food. 134 rats were studied.

At the end of the 28-day period, the rats were anesthetized with sodium amytal solution, the body cavity opened and heart blood removed into heparinized tubes. Care was taken to keep the blood cold at all times until it was frozen for lipid assay. Livers were quickly removed, weighed and frozen immediately after portions were removed for biotin and enzyme assay. The major portion of the liver was subsequently freeze-dried at −20 °C and extracted for lipid assay. Carcasses were frozen immediately after removal of gastrointestinal contents.

Livers and aliquots of plasma were initially extracted with chloroform:methanol, 2:1, v/v; the filtrate washed with water and subsequently evaporated under vaccum (Rinco apparatus) and the lipids extracted with warm petroleum ether. Total lipid was determined gravimetrically. Total cholesterol content of livers was measured colorimetrically by using the ferric chloride-acetic-sulfuric acid color reagent after saponification and extraction of cholesterol into ligroine (subsequently evaporated). Isopropanol extracts of plasma were assayed for total cholesterol in the AutoAnalyzer (Technicon) by using method N24A and the same color reagent as for the liver assays. The liver biotin content previously reported (5) was measured by the method of *Wright and Skeggs* (49). Assay of liver for lactate dehydrogenase activity (LDH, EC 1.1.1.27) was based on the enzymatic conversion of pyruvate to lactate (Sigma Chemicals). Assays of activities of glucose-6-phosphate dehydrogenase (G6PD, EC 1.1.1.49) and 6-phosphogluconate dehydrogenase (6PGD, EC 1.1.1.44) were based on the enzymatic production of NADPH.

Total carcass fat was determined gravimetrically on homogenates of whole carcass (minus liver) by acid hydrolysis and extraction with petroleum and ethyl ethers. Chloroform:methanol extracts of carcass homogenates were used for assay of total cholesterol and lipid phosphorus. For the cholesterol assay, extracts were evaporated and saponified with ethanolic KOH; the sterols were extracted into ligroine, evaporated, and color-developed as for livers and plasma. Lipid phosphorus was assayed in petroleum ether extracts of liver and in washed lipid extracts of plasma and carcass, by the method of *Sumner* (42). Carcass triglyceride was estimated as the difference between the total fat and the sum of total cholesterol plus phospholipid.

Experiment 2

A second experiment was conducted with the view of assessing the activity of the tricarboxylic acid cycle in line 3 IN-BHE rats. Male and female rats, 280 days old, and phenotypically obese, were fed the semipurified diet as in experiment 1, with or without the oral supplement of biotin for 28 days. Because the diet was not biotin-free, 20 mg/kg avidin (Nutritional Biochemicals; activity 2,000 U/g; purity greater than 99.5 %) was added to the diet of the rats that did not receive the supplement. At the end of the 4-week period, the rats were fasted overnight (16 h). The next morning each rat was injected intraperitoneally with 40 μCi $NaH^{14}CO_3$ in 0.89 % saline solution and, exactly 10 min later, it was injected with sodium amytal solution. Exactly 5 min later, blood was removed from the heart into cold heparinized tubes and kept cold unitl plasma was harvested and quick-frozen. Livers were removed immediately, blotted gently and promptly homogenized in cold acidified acetone (3 ml 1 *N* HCl/l). Lipids and organic acids were separated as follows: The filtrates were evaporated to about 0.5–1 ml each under N_2. The liquid was taken up with anhydrous sodium sulfate which was subsequently washed with warm petroleum ether for assay of the lipid counts and total lipid; then with absolute ethanol-acetone, and absolute ethanol for the organic acids; and finally with water to separate the labeled residual water-soluble com-

ponents. Appropriate aliquots were evaporated, and scintillation solution was added (toluene cocktail for the lipids and organic acids, and dioxane for the residual components with PPO + POPOP with or without naphthalene as the scintillator). The samples were counted in a liquid scintillation counter (Packard). For the assay of citrate, succinate and malate, total organic acids were concentrated and spotted on silica gel G thin-layer plates, and organic acids were separated by using a mixture of 15:1 diethyl ether:formic acid. Organic acids were compared with standards spotted on the plate, and adjacent bands were scraped into scintillation solution and counted. About 5 % of the injected radioactivity was accounted for in the liver. Assays for lipids were done as outlined in experiment 1.

Experiment 3

The purpose of this experiment was to ascertain the levels of lactate and pyruvate in the plasma of the line 3 rats, and the effect of biotin on these metabolites. The rats used in this experiment were not fasted. Care was taken to avoid infection by the techniques used. In part 1, apparently healthy 300-day-old line 3 IN-BHE male and female stock diet-fed rats were selected. The tail cleaned and the tip severed. Blood was then collected from the tail into heparinized tubes, the plasma harvested and aliquots immediately precipitated with 0.6 *N* perchloric acid. Blood, plasma, and all solutions were kept cold at all times. Only the results of lactate and pyruvate assays are reported here. In part 2, analyses of metabolites were conducted on a second group of approximately 280-day-old male rats treated identically as described above. The animals were tail bled and then divided into two groups so that the mean level of lactate was similar in each group. Then for 2 consecutive days, a dose of 150 μg biotin (total 300 μg biotin; kindly supplied by Hoffman-La Roche Ltd.) was injected intraperitoneally into one group and the same quantity of a sterile solution of 0.89 % sodium chloride was injected into the second group. On the morning following the second injection, tail blood was collected and the analyses for lactate and pyruvate were repeated; enzymatic methods were employed (31).

Statistical evaluation of the data was either by the χ^2 test, the t test or by regression analysis because of the large variation in data for some parameters and because we were interested in the interrelationships. The regression lines were calculated by the method of least squares.

Results

Experiment 1. Metabolic Characterization of IN-BHE Rats and Effects of Biotin Supplementation

Results of analyses of blood and tissue lipids and other constituents are reported here only for the 328-day-old IN-BHE rats. Similar results for the BHE and line 3 IN-BHE rats killed at zero-time are not included. No new findings were observed for BHE rats. For the IN-BHE rats, a comparison of the data for rats between 300- and 328-days old showed lower blood lipids and slightly higher tissue lipids for the younger animals than for the older ones.

Average body and organ weights; lipids, and enzymes. It was previously reported that 328-day-old biotin-supplemented rats had significantly more biotin in their livers than did their unsupplemented littermates or the stock diet-fed rats (5). However, for the present report, a two- to threefold increase in biotin

Table I. Experiment 1. Food and biotin intakes; body and organ weights of 328-day-old IN-BHE rats (mean ± SE)

Diet[1]	Intakes, total in 4 weeks		Weights, g				
	food, g	biotin[2], μg	final body	change in body weight[3]	liver	kidneys (2)	heart
Males							
Stock diet	671 ± 19	200 ± 6	502 ± 12	+ 12 ± 2	21.3 ± 1.2	5.80 ± 0.71	1.43 ± 0.03
SP + biotin	444 ± 18	344 ± 7	477 ± 10	− 16 ± 5	19.1 ± 1.0	4.93 ± 0.80	1.40 ± 0.04
SP − biotin	482 ± 11	135 ± 3	495 ± 14	+ 1 ± 4	21.1 ± 1.5	5.15 ± 0.52	1.38 ± 0.02
Females							
Stock diet	492 ± 1.9	147 ± 6	336 ± 16	+ 2 ± 5	11.1 ± 0.85	2.52 ± 0.13	1.01 ± 0.03
SP + biotin	373 ± 13	326 ± 5	326 ± 10	+ 0.2 ± 4	11.1 ± 0.43	2.54 ± 0.10	1.00 ± 0.02
SP − biotin	393 ± 17	110 ± 5	322 ± 9	− 7 ± 4	10.9 ± 0.35	2.51 ± 0.09	1.02 ± 0.02

[1] SP = Semipurified diet.
[2] Biotin content of diets: stock diet, 0.298 μg/g; SP, 0.280 μg/g.
[3] Weight change in 4 weeks.

Table II. Experiment 1. Lipids in plasma, liver and carcass of 328-day-old IN-BHE rats (mean ± SE)

	Males			Females		
	stock diet	SP + biotin	SP − biotin	stock diet	SP + biotin	SP − biotin
Number of rats	11	13	12	9	15	13
Plasma, mg/100 ml						
Total lipid	1,656 ± 360[a1]	1,024 ± 153[b]	1,311 ± 191[a]	575 ± 117	577 ± 56	555 ± 66
Cholesterol	435 ± 88[a]	285 ± 37[b]	368 ± 56[a]	154 ± 24	155 ± 15	161 ± 19
Phospholipid[2]	358 ± 58[ab]	289 ± 45[a]	374 ± 47[b]	210 ± 32[a]	194 ± 20[a]	154 ± 12[b]
Ratio $\frac{\text{phospholipid}}{\text{cholesterol}}$	0.97 ± 0.13	0.99 ± 0.09	1.05 ± 0.13	1.38 ± 0.09[a]	1.34 ± 0.07[a]	1.14 ± 0.13[b]
Liver (wet basis)						
Total lipid, g/100 g	5.33 ± 0.15	5.45 ± 0.23	5.60 ± 0.27	6.12 ± 0.16[a]	6.95 ± 0.32[b]	6.56 ± 0.28[b]
Cholesterol, mg/g	2.78 ± 0.10[a]	3.53 ± 0.26[b]	3.63 ± 0.24[b]	2.67 ± 0.03[a]	3.00 ± 0.17[b]	2.89 ± 0.09[b]
Phospholipid, mg/g	31.7 ± 1.2[a]	29.3 ± 0.91[b]	29.0 ± 1.2[b]	33.5 ± 0.59[a]	30.5 ± 0.46[b]	30.8 ± 0.46[b]
Ratio $\frac{\text{phospholipid}}{\text{cholesterol}}$	11.6 ± 0.67[a]	8.8 ± 0.62[b]	8.3 ± 0.55[b]	12.5 ± 0.27[a]	10.5 ± 0.44[b]	10.8 ± 0.36[b]
Carcass (wet basis)						
Total lipid, g/100 g	15.0 ± 1.0	15.4 ± 0.84	16.7 ± 1.1	26.7 ± 1.2	29.2 ± 1.0	27.0 ± 1.4
Cholesterol, mg/g	1.80 ± 0.03	1.76 ± 0.04	1.76 ± 0.04	1.75 ± 0.04	1.80 ± 0.04	1.76 ± 0.04
Phospholipid, mg/g	6.46 ± 0.18	6.38 ± 0.27	6.55 ± 0.22	6.76 ± 0.22	6.88 ± 0.23	6.59 ± 0.27
Ratio $\frac{\text{phospholipid}}{\text{cholesterol}}$	3.61 ± 0.11	3.66 ± 0.16	3.75 ± 0.17	3.90 ± 0.19	3.86 ± 0.16	3.79 ± 0.23

[1] Values with the same superscript letter, for groups of males or females, are not significantly different.
[2] Lipid phosphorus × 25.

Table III. Experiment 1. Enzyme activity in livers of 328-day-old IN-BHE rats[1] (mean ± SE)

Diet	Per g liver			Per 100 g body weight		
	G6PD	6PGD	LDH	G6PD	6PGD	LDH
Males						
Stock diet	1.4 ± 0.11[a2]	1.7 ± 0.10[a]	294 ± 12	5.8 ± 0.5[a]	7.0 ± 0.5[a]	1,262 ± 97
SP + biotin	1.0 ± 0.13[b]	1.3 ± 0.20[b]	293 ± 19	4.2 ± 0.7[b]	5.5 ± 1.0[b]	1,160 ± 90
SP – biotin	1.0 ± 0.09[b]	1.2 ± 0.12[b]	296 ± 12	4.4 ± 0.4[b]	5.3 ± 0.6[b]	1,263 ± 98
Females						
Stock diet	3.5 ± 0.86	3.3 ± 0.52	259 ± 14[ab]	12.1 ± 3.3	11.2 ± 2.1	855 ± 68[ab]
SP + biotin	2.4 ± 0.18	3.1 ± 0.22	281 ± 12[a]	8.3 ± 0.7	10.8 ± 0.9	959 ± 43[a]
SP – biotin	2.6 ± 0.23	3.1 ± 0.25	236 ± 11[b]	8.7 ± 0.8	10.7 ± 0.9	798 ± 35[b]

[1] One unit of enzyme activity is defined as that amount of enzyme which produces 1 μmol of NADPH or NAD per minute under the conditions of the assay.

[2] Values with the same superscript letter, within groups of males and females, are not significantly different.

Table IV. Regression coefficients from linear regression analysis relating various plasma, liver and carcass components in 328-day-old male and female IN-BHE rats

X and Y variables	Males			Females		
	stock diet	SP + biotin	SP − biotin	stock diet	SP + biotin	SP − biotin
X_1 plasma lipid, mg/100 ml[1] vs.						
Y_1 plasma cholesterol, mg/100 ml	+ 0.231***[2]	+ 0.219***	+ 0.286**	+ 0.189***	+ 0.203***	+ 0.169***
Y_2 liver LDH, units[3]	0.239***	+ 0.388*	+ 0.425***	+ 0.396**	+ 0.344†	+ 0.216
X_2 plasma cholesterol, mg/100 ml vs.						
Y_3 liver cholesterol, mg	+ 14.6***	+ 2.3	+ 4.4*	+ 10.1***	+ 2.0	+ 5.1†
Y_4 liver phospholipid, mg/g	−0.010*	−0.018***	−0.014*	−0.006	−0.024***	−0.002
Y_5 liver G6PD, units	−0.001	+ 0.012*	+ 0.002	+ 0.120***	+ 0.036***	+ 0.004
Y_6 liver 6PGD, units	+ 0.001	+ 0.018**	+ 0.004	+ 0.077***	+ 0.034*	+ 0.009
Y_7 liver LDH, units	+ 0.877**	+ 0.120†	+ 1.348***	+ 2.10***	+ 1.20	+ 0.274
X_3 liver lipid, g vs.						
Y_8 plasma lipid, mg/100 ml	+ 3.94***	+ 2.43***	+ 1.44*	+ 2.088***	+ 97	+ 786†
Y_9 plasma cholesterol, mg/100 ml	+ 757**	+ 465*	+ 361*	+ 354**	+ 400	+ 113
Y_{10} plasma phospholipid, mg/100 ml	+ 191	+ 529*	+ 287†	+ 485**	+ 150	−22
Y_{11} liver cholesterol, mg	+ 63***	+ 89***	+ 89***	+ 37***	+ 49***	+ 41***
Y_{12} liver cholesterol, mg/g	+ 1.07**	+ 1.89	+ 1.34†	−0.21	+ 3.28***	+ 1.97***
Y_{13} liver phospholipid, mg/g	−7.6	−13.3**	−2.5	+ 0.08	−1.53	−2.24
Y_{14} liver P/C ratio[4]	−6.4**	−8.7*	−3.3†	+ 1.2	−8.9***	−7.7***
Y_{15} liver G6PD, units	+ 0.16	+ 3.76	−0.04	−57***	+ 7.9†	+ 11.5*
Y_{16} liver 6PGD, units	+ 1.7	+ 5.4	+ 3.3	+ 25.6	+ 5.5	+ 3.0
Y_{17} liver LDH, units	+ 1,056***	+ 1,422***	+ 986***	+ 713*	+ 735***	+ 571**

X_4 carcass lipid, g vs.						
Y_{18} plasma lipid, mg/100 ml	+ 0.44[1]	+ 3.14	+ 12.47	+ 12.51***	+ 7.78***	+ 9.15***
Y_{19} plasma cholesterol, mg/100 ml	−2.90[1]	−0.01	+ 2.39	+ 2.20*	+ 1.64**	+ 1.47†
Y_{20} plasma phospholipid, mg/100 ml	−1.72[1]	+ 1.28	+ 2.34	+ 2.80*	+ 2.24**	+ 0.16
Y_{21} liver lipid, g	+ 0.005	+ 0.002†	+ 0.010***	+ 0.006***	+ 0.003	+ 0.004**
Y_{22} liver cholesterol, mg	+ 0.14	+ 0.19	+ 0.69*	+ 0.24***	+ 0.009	+ 0.22***
Y_{23} liver cholesterol, mg/g	−0.005	+ 0.003	+ 0.003	−0.001	−0.006	+ 0.007†
Y_{24} liver phospholipid, mg/g	−0.05	−0.07†	−0.013	−0.0005	−0.023	−0.017
Y_{25} liver G6PD, units	+ 0.05*	−0.02	+ 0.012	+ 0.34**	+ 0.09***	+ 0.052
Y_{26} liver 6PGD, units	+ 0.02	−0.05	+ 0.049†	+ 0.16	+ 0.052	−0.014
Y_{27} liver LDH, units	+ 6.85	+ 7.11†	+ 10.25**	+ 5.28†	+ 2.27	+ 2.33
Y_{28} carcass cholesterol, g	+ 0.025**	+ 0.0018	+ 0.0026**	+ 0.0016†	+ 0.0019*	+ 0.0016*
Y_{29} carcass cholesterol, mg/g	−0.0005	−0.0004	−0.0002	−0.0064***	−0.0024	−0.0023
Y_{30} carcass phospholipid, g	+ 0.08	+ 0.015†	−0.002	+ 0.011***	+ 0.005	+ 0.001
Y_{31} carcass phospholipid, mg/g	−0.003	+ 0.004	−0.026***	−0.007	−0.016	−0.025*
X_5 carcass cholesterol, g vs.						
Y_{32} plasma cholesterol, mg/100 ml	+ 2,112	+ 1,191	+ 1,228†	+ 1,184***	+ 155	+ 287
Y_{33} liver cholesterol, mg	+ 27.0***	+ 23.5*	+ 17.5*	+ 44.4***	+ 6.0	+ 10.6*

[1] For males, regression coefficients all in g/100 ml.

[2] *** $p < 0.001$; ** $p < 0.01$; * $p < 0.05$; † $p < 0.10$.

[3] Units per 100 g final body weight.

[4] Liver $\frac{\text{phospholipid}}{\text{cholesterol}}$ ratio.

intakes of male and female rats during the 28-day period had statistically insignificant effects on *mean* food intakes or body or organ weights (table I).

The mean total lipid in the plasma was lower in the supplemented males (those fed biotin by mouth) than in unsupplemented or stock diet-fed males (table II), but the level of supplementation in this experiment was not sufficient to reduce the mean levels of any of the blood lipids to normal levels (mean total plasma lipids in mg/100 ml in 300-day-old males and females, the parent strain of IN-BHE rats, were 545 and 554 mg/100 ml, respectively; these levels may be considered normal levels for the BHE strain). The median total plasma lipid, mg/100 ml, levels were for the IN-BHE males, stock diet-fed, 1,137; + biotin, 862; and −biotin, 1,218; median values for the females were 475, 482 and 487, respectively. Females of this age had more carcass fat than the males reflecting the earlier onset of nephrosis in males than in females, but the mean plasma lipids were markedly lower for females than for the males and the total plasma lipid levels of females were not influenced by the present level of biotin supplementation. For the supplemented males, there was a significantly lower level of plasma cholesterol and phospholipid but only for the phospholipid content of the plasma did the females show a difference and this difference was in the opposite direction from that in the males. Mean liver cholesterol did not vary with biotin intake. For all diets and tissues, phospholipid:cholesterol ratios were higher in females than in males although the differences were not always statistically significant.

Mean enzyme levels in the liver of males showed little variation with biotin supplementation; levels of G6PD and 6PGD were significantly higher and LDH was slightly but not significantly lower in females than in males (table III). Levels of LDH in biotin-supplemented females were higher than in their unsupplemented littermates.

Interrelationships between lipids in plasma, liver, and carcass; liver enzymes, and biotin status. In the following discussion, biotin status, which was previously reported (5), was measured by the liver biotin content in μg/g liver. In table IV are the slopes of the regression lines and their significance relating lipids and enzymes. As expected, in all groups, plasma cholesterol (Y_1) was significantly elevated when total plasma lipid was elevated. Similar results (not shown) were obtained for plasma phospholipid versus total lipid or cholesterol. Also, total liver cholesterol (Y_{11}) was significantly elevated when total liver lipid was elevated. These data are included to show that with respect to these parameters, the line 3 rats are similar to nonobese strains. Four of the six groups of rats with the largest amounts of liver lipid (Y_{21}) also had the largest amounts of carcass lipid. Although mean liver lipid (table II) was slightly elevated (average 5.33–6.95 %), it was not considered excessive. In all groups, liver cholesterol (Y_{33}) was elevated when carcass cholesterol was elevated. The relationship was statistically significant except for the supplemented females. Only the unsupplemented males ($p <$

0.10) and the stock diet-fed females showed a direct relationship between their plasma (Y_{32}) and carcass cholesterol levels. A direct relationship was observed between plasma and liver cholesterol (Y_3) of stock diet-fed and unsupplemented groups ($p < 0.10$ for females) of both sexes. A tendency toward a smaller amount of plasma cholesterol per unit of liver cholesterol was found for supplemented males and females. There was a significant direct relationship between carcass cholesterol and carcass triglyceride in rats of both sexes fed most diets. (Values not shown because results were the same as for carcass cholesterol vs. carcass lipid.) There was generally less phospholipid (Y_{13}) in the liver as total liver lipid increased, which could account for the significant decrease observed in the phospholipid:cholesterol ratio (Y_{14}) in the livers of the groups except the stock diet-fed females (SP-biotin males $p < 0.10$). Specific amounts (mg/g) of liver phospholipid (Y_4) were reduced in all groups (reductions were statistically significant except for unsupplemented and stock diet-fed females) as plasma cholesterol levels rose. Grams of carcass cholesterol (Y_{28}) were significantly elevated when carcass lipid was elevated except for supplemented males (stock diet-fed females $p < 0.10$). Changes in carcass phospholipid mg/g (Y_{31}) and the phospholipid:cholesterol ratio were unrelated to changes in carcass lipid in nearly all groups.

Total plasma lipid, cholesterol, and phospholipid of males tended to be lower when biotin status was improved (table V). The change was greater in the supplemented rats than in the unsupplemented ones. The association between liver biotin level and total liver lipid (Y_5) or plasma lipid (Y_1) or plasma phospholipid levels (Y_3) however, was statistically significant for the stock diet-fed males. Liver weight and lipid (Y_4, Y_5) tended to be lower when biotin status was improved in males. Liver cholesterol (Y_6) was lower when biotin status was higher in the stock diet-fed rats.

Total carcass lipid (Y_{10}) of 5 of the 6 groups of rats was inversely related to biotin status but results were not quite statistically significant. IN-BHE males had larger amounts of body fat at an earlier age than did the females, but some lost both weight and fat as their blood glucose levels rose between 250 and 300 days of age. There tended to be an inverse relationship between total carcass cholesterol (Y_{13}), total carcass phospholipid, both g and mg/g (Y_{14}, Y_{15}), and liver biotin content for nearly all groups of males except for the unsupplemented rats.

Although *mean* enzyme levels did not show significant changes with biotin supplementation, G6PD, 6PGD and LDH tended to be directly related to liver fat. Positive correlations of LDH activity (Y_2, Y_{17}, Y_{27}) with plasma, liver, and carcass lipids are seen in table IV. The slopes of the lines for most groups are statistically significant. Results for G6PD (Y_{25}) and 6PGD (Y_{26}) were variable when carcass lipid was considered but direct relationships were most often seen in groups of females. All three liver enzymes for all groups of males and G6PD

Table V. Regression coefficients from linear regression analysis relating various plasma, liver and carcass components (Y variables) to liver biotin, μg/g (X variable)

Y variables	Males			Females		
	stock diet	SP + biotin	SP − biotin	stock diet	SP + biotin	SP − biotin
Plasma						
Y_1 total lipid, mg/100 ml	−7,933***[1]	−1,638†	−1,617	+ 1,418	−45	−811
Y_2 cholesterol, mg/100 ml	−1,826***	−540*	−403	+ 146	−139†	−190
Y_3 phospholipid, mg/100 ml	−848*	−434	−431	+ 66	−134	−116
Liver						
Y_4 wet weight, g	−24.2***	−16.2*	−29.4†	+ 7.5	−5.5*	−4.3
Y_5 lipid, g	−1.5***	−0.19	−1.7*	+ 0.6	−0.3	−0.09
Y_6 cholesterol, mg/g	−1.9**	+ 3.5†	−2.90	−0.94***	−0.93	+ 0.61
Y_7 phospholipid mg/g	+ 15.6†	+ 12.0†	+ 23.3†	−1.4	+ 1.4	+ 7.3†
Y_8 G6PD, units[2]	−4.6	−11.4	−16.1***	+ 19.8	−3.9*	−0.03
Y_8 6PGD, units[2]	−2.4	−5.7	−5.2	+ 12.9	−1.1	−2.5
Y_9 LDH, units[2]	−2,216***	−1,339*	−2,014*	+ 2.5	−279	+ 99
Carcass						
Y_{10} lipid, g	−19.9	−20.1	−100.1	+ 27.8	−12.5†	−43.9
Y_{11} triglyceride, g						
Y_{12} cholesterol mg/g	−0.439*	−0.286	+ 0.170	+ 0.035	+ 0.220	+ 0.723†
Y_{13} cholesterol, g	−0.264†	−0.256†	−0.233	+ 0.123	−0.086	+ 0.155
Y_{14} phospholipid, mg/g	−0.79	−3.84†	+ 2.15	−3.88	−0.87	+ 0.14
Y_{15} phospholipid, g	−0.69	−2.19*	−0.12	−0.58	−0.86*	−0.12

[1] *** $p < 0.001$; ** $p < 0.01$; * $p < 0.05$; † $p < 0.10$.
[2] Units per 100 g final body weight.

for biotin-supplemented females tended to have an inverse relationship with liver biotin content (table V). The lower LDH activity (Y_9) seen with improvement in biotin status of males was statistically significant. Although the regression coefficients for biotin-supplemented females were not significant, the mean LDH values for biotin-supplemented females were elevated (table III). This finding agrees with other reports of elevated liver LDH in obese rats (6) and mice (38).

Experiment 2. Assessment of Krebs Cycle Activity in Obese Rats

The data in tables VI and VII show the results of the attempts to assess the level of Krebs cycle activity in phenotypically obese rats. Although the unsupplemented males had slightly more fat and cholesterol in their livers than supplemented rats (table VI), the results with respect to the effect of biotin supplementation on Krebs cycle activity were not definitive (table VII), probably because of the selection of a single time measurement of the labeling in the organic acids. The major result of this experiment was the finding of a highly labeled lipid fraction in the livers of these rats, with the highest amount of labeling found in livers of females. This labeling was thought to be primarily in the glycerol moiety of the neutral lipids and other glyceride fragments. *Chang et al.* (7) reported that BHE rats given uniformly labeled glucose incorporated a larger percentage of the label into the glycerol moiety of the neutral lipids of liver than did Wistar rats.

Experiment 3. Plasma Lactate and Pyruvate Levels; Effects of Biotin

In experiment 3, we assessed the redox state of the tissue (19, 45) indirectly, by the assay of liver LDH activity, and directly, by the assay of plasma levels of lactate and pyruvate. Results of these assays of plasma lactate and pyruvate levels in selected groups of 300-day-old stock diet-fed rats of both sexes showed that both lactate and lactate:pyruvate ratios were elevated in these rats (table VIII). (17 mg/100 ml lactate is the level found in Wistar rats of the same age; *Marshall et al.*, unpublished results, 1972). Females had higher pyruvate levels than males. However, 48 h after stock diet-fed IN-BHE rats were given two injections of biotin, 150 μg each, significant decreases in plasma lactate and pyruvate were observed (table VIII). Since the levels of both lactate and pyruvate fell, no change in the lactate:pyruvate ratios as a result of biotin administration was observed. However, during this same period, a slight increase in the plasma lactate of the saline-injected rats was observed.

Discussion

Biotin is a component of several key enzymes in mammalian systems. The biotin enzyme pyruvate carboxylase plays a key role in gluconeogenesis and

Table VI. Experiment 2. Food and biotin intakes, body weight and liver composition of 300-day-old IN-BHE obese rats

Diet	Intakes, total in 4 weeks		Weights			Liver, wet basis	
	food g	biotin μg	initial body g	final body[1] g	Liver final body[2] × 100	total lipid %	cholesterol mg/g
Males							
SP + biotin	455 ± 18[3]	351 ± 5[4]	528 ± 12	506 ± 18	3.3 ± 0.14	3.87 ± 0.62	4.59 ± 0.87
SP − biotin (+ avidin)	481 ± 13	131 ± 4	530 ± 17	519 ± 16	3.2 ± 0.14	4.38 ± 0.41	5.38 ± 0.50
Females							
SP + biotin	457 ± 14	352 ± 4[4]	357 ± 10	367 ± 10	2.6 ± 0.13	5.20 ± 0.92	3.37 ± 0.24
SP − biotin (+ avidin)	482 ± 24	129 ± 6	385 ± 13	371 ± 13	2.7 ± 0.17	5.18 ± 0.49	3.37 ± 0.20

[1] Weight prior to overnight fast.
[2] Starved weights.
[3] Mean ± SE; 5 rats per group.
[4] Includes oral supplement of biotin 8 μg/rat/day.

Table VII. Experiment 2. Radioactivity in livers of 300-day-old obese IN-BHE rats after administration of $NaH^{14}CO_3$

Diet	Percent of recovered dose, dpm			Per g liver, dpm × 10^4			Per mg liver fat dpm	Ratio, dpm citrate / succinate
	lipid	organic acids	residue	lipid	organic acids	residue		
Males								
SP + biotin	78.3 ± 5.7[1]	20.5 ± 5.6	1.2 ± 0.4	2.42 ± 0.56	0.51 ± 0.14	0.03 ± 0.01	632 ± 119	4.3 ± 2.5
SP − biotin (+ avidin)	79.9 ± 4.1	18.1 ± 4.1	2.0 ± 0.6	2.82 ± 0.52	0.60 ± 0.15	0.06 ± 0.01	645 ± 104	0.7 ± 0.1
Females								
SP + biotin	91.0 ± 4.7	5.4 ± 1.8	1.7 ± 0.5	3.82 ± 0.34	0.31 ± 0.18	0.07 ± 0.02	805 ± 126	1.5 ± 0.43 (4)
SP − biotin (+ avidin)	92.9 ± 2.5	7.3 ± 4.2	1.7 ± 0.7	4.30 ± 0.17	0.24 ± 0.08	0.07 ± 0.03	797 ± 56	1.4 ± 0.56

[1] Mean ± SE; 5 rats per group except where indicated in parentheses.

Table VIII. Experiment 3. Plasma lactate, pyruvate, and lactate:pyruvate ratios of stock diet-fed line 3 IN-BHE rats, and these metabolites in male rats before and 48 h after injection of 300 μg biotin[1] (mean ± SE)

	Stock diet (300-day-old rats)			Males (280 days old)					
				injected with biotin[2]			injected with saline[3]		
	males		females	before		after	before		after
Number of rats	13		13	8		8	10		10
Lactate, mg/100 ml	31.9 ± 2.4		38.7 ± 2.9	37.4 ± 3.3		26.5 ± 2.2	35.4 ± 3.4		38.6 ± 3.1
		$p < 0.10$			$p < 0.025$			$p < 0.10$	
Pyruvate, mg/100 ml	0.76 ± 0.04		1.38 ± 0.07	0.97 ± 0.11		0.69 ± 0.06	0.88 ± 0.09		1.12 ± 0.11
		$p < 0.001$			$p < 0.05$			NS	
L/P ratio	44.6 ± 3.7		28.4 ± 1.9	40.6 ± 3.9		40.7 ± 5.1	43.2 ± 4.5		36.4 ± 3.6
		$p < 0.01$			NS			NS	

[1] Tail blood from fed live rats.
[2] Rats injected intraperitoneally with 0.4 ml of solution containing 150 μg biotin each 24-hour period.
[3] Rats injected intraperitoneally with 0.4 ml of sterile physiological saline solution each 24-hour period.

requires acetyl CoA for activation. The biotin enzyme propionyl CoA carboxylase takes part in the carboxylation of propionate → methylmalonyl CoA → succinate, a four carbon dicarboxylic acid which also plays a role in oxidative reactions. The propionyl CoA pathway has been proposed by *Dupont and Mathias* (15) to be a route for the oxidation of unsaturated fatty acids. A recent role for biotin in protein synthesis at the RNA level was demonstrated by *Dakshinamurti and Litvak* (10). Earlier work had shown reduced incorporation of labeled methionine into protein in biotin-deficient rat liver. Many workers have been concerned with the well-known role of biotin in the synthesis of fatty acids by a pathway that involves the carboxylation of acetyl CoA to malonyl CoA by the biotin enzyme, acetyl CoA carboxylase. Elevated synthesis of cholesterol in carcasses, and accumulation of acetoacetate in livers of young biotin-deficient rats was also reported (9). Relatively little attention has been given to the reports of reduced oxidative phosphorylation and ATP synthesis in biotin-deficient rats (11).

Symptoms such as skin lesions, lassitude and anorexia have been seen in man and in rats when given a biotin-deficient diet or a diet containing raw egg white, but reproducible results have been difficult to achieve (1). Hypercholesterolemia in human beings, one of the most controversial findings, was reported in a classical study of biotin deficiency in man in 1942 (43). However, we now know that some of the apparent contradictions in the reports of responses to a biotin-deficient diet were related to the kind and amount of fat in the diet; the length of feeding time; the nutritional status of the animal (whether fasted or fed); whether the studies were done *in vivo* or *in vitro;* the source of tissue, liver, or adipose; level of substrate; rat strain; and sex.

The finding of elevated levels of lactate in the plasma accompanied by elevated lipids in our older line 3 IN-BHE rats as well as elevated lactate:pyruvate ratios, even in the absence of overt symptoms of biotin deficiency, was not surprising in view of earlier studies of *Deodhar and Mistry* (13) who reported a large excretion of lactate in the urine of young biotin-deficient rats. *Oxman and Ball* (34) found the conversion of glucose to lactate in adipose tissue of biotin-deficient rats markedly elevated over that of controls (40 vs. 9 %). A significant reduction in lactate formation was achieved in their study in 58 h after a single injection of 300 μg biotin. These reports and our findings of lactate accumulation in rats in the present report, and of the rapid removal of lactate and pyruvate with biotin administration, indicate an influence of biotin on the redox state and suggest the possibility that the reduction of lipids may be mediated through the reoxidation of NADH with the subsequent reduction in lipid synthesis from glucose or through an increase in the oxidation of lipid through increased Krebs cycle activity following the carboxylation of pyruvate to oxaloacetate. A report of lowered levels of lactate and pyruvate in blood of human subjects after administration of biotin was attributed to increased oxidation via

the Krebs cycle (40). Results of the present study suggest that, in the obese hypercholesterolemic rat, elevated levels of lactate may be indicative of the hypoxic or anaerobic state – a condition shown by several workers to provide an environment favorable for elevated synthesis of lipid from glucose with dihydroxyacetone phosphate as the source of α-glycerol phosphate (glycerol) or with lactate as the source of 2-carbon units. Reduced oxidation of pyruvate and elevated levels of lactate have been seen in biotin deficiency, in hypoxia, and in obesity, in rodents and in human beings (18, 20, 35, 41, 48). An increase in reducing equivalents as a result of the accumulation of NADH (and elevated NADH/NAD ratio) could account for the obesity in the line 3 IN-BHE rats. The recent report of *Woods and Krebs* (48) showed that in the living animal, livers of well-fed rats produced lactate (and lipid) from glycogen or glucose at relatively high rates under anaerobic conditions.

Whereat (47) suggested that the redox state of the NADH/NAD couple is the controlling factor for the synthesis of lipids in the heart and arteries. *Rous* (36) suggested also that NADH supplies much of the hydrogen for lipid synthesis in the living animal. *Filipovic and Buddecke* (17) demonstrated that an increased NADH/NAD ratio as a result of hypoxia led to a marked increase in lipid synthesis in calf arteries. Although the pathways of fat synthesis in these studies were primarily located in microsomes and mitochondria, they have not been studied extensively in intact mammalian systems. *Shreeve et al.* (39) had previously shown that in one strain of obese mice, more fatty acids were synthesized from lactate 2-^{14}C or 2-^{3}H and glycerol 1,3-^{14}C or 2-^{3}H than from glucose and that in this obese strain, the *liver* played a more important role in lipogenesis than did the carcass. *Lagunas et al.* (25) demonstrated that an increase in the oxidized state of the mitochondria of rat liver, after administration of a single dose of nicotinamide, resulted in an inhibition of lipogenesis and a decrease in α-glycerol phosphate in the living animal.

An increase in total LDH was found in hypoxia in studies of rat skin autografts (26). Preliminary, but more direct evidence of the influence of biotin on the redox state was seen in our recent study (*Marshall et al.*, unpublished results) of lactate dehydrogenase isoenzymes in plasma of our line 3 IN-BHE rats. Administration of biotin to stock diet-fed 300-day-old rats resulted in a change in the isoenzyme patterns from M-type to H-type LDH in some of the rats. Further work is being done to confirm these results. M type LDH subunits have been associated with the hypoxic state (8). Varying the ambient oxygen was shown to influence the type of LDH activity (27).

In one phase of the present study previously reported, an improvement in biotin status in male rats was associated with an increase in plasma immunoreactive insulin, but blood glucose levels were not reduced (5). It was recently shown by others that insulin stimulates the pyruvate dehydrogenase reaction independently of its effect on glucose transport (12, 46). The question whether the

reduction in blood lipids in the study reported here was a direct result of the improvement in the insulin status or an indirect result of the improvement in biotin status *per se* has not been answered. We believe, however, that evidence from results of the present study indicating an effect of biotin on the redox state, and results from other laboratories on the effect of biotin on oxidative phosphorylation and on the oxidation of pyruvate suggest a direct effect of biotin on lipid oxidation. This effect of biotin could be mediated through the reoxidation of NADH, as a result of an increase in oxidation of glucose through the Krebs cycle, and thereby exert a regulatory effect on lipid metabolism. The control of lipogenesis by the redox state of the nicotinamide nucleotides and by the availability of H^+ has been suggested (16, 25). Implications for human coronary artery disease may be derived from these studies. *Schrade et al.* (37) reported that lactate and pyruvate in plasma of hyperlipidemic patients with arteriosclerosis were elevated over levels found in normal subjects.

It is interesting to speculate that a diminished tissue respiration in the obese rat (accompanied perhaps by a defective insulin response), results in adaptation to the anaerobic state and a concomitant increase in lipid synthesis; the conversion of the anaerobic to the aerobic state is dependent upon the exogenous administration of extra biotin. The line 3 IN-BHE rat or its parent BHE strain could serve as excellent models for the elucidation of this dependency.

After the completion of this study another report appeared on the effect of biotin on oxidation of lipid (44). Also, *Katz and Wals* (23) reported recently that *in vivo* blood lactate of fed rats under certain conditions may serve as an important source of fatty acids. *Dokusova and Klimov* (14) reported that injection of biotin into cholesterol-fed rabbits led to a substantial reduction of the amount of cholesterol and β-lipoproteins in blood plasma and in the amount of cholesterol in the liver when compared with the amounts in animals given cholesterol without the biotin. They proposed that the lowered amounts of cholesterol in plasma and aortas were associated with an increased oxidation of cholesterol to bile acids via the splitting off of propionic acid and subsequent oxidation of propionate by the carboxylation of propionate to succinate under the influence of biotin. Further, the work of *Ivanov* (21) showed statistically significant increases (78–500 %) in tissue oxygen absorption of biotin-treated hypercholesteremic rats.

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