transamination reaction in the body. The activities of the arterial transaminases did not change appreciably. Concentrations of most of the free amino acids increased in the serum and diminished in the aorta of hypercholesteremic-atherosclerotic chickens. Chemical changes accompany the morphological alterations during the progress of atherosclerosis.

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Effects of Biotin Deficiency on Pyruvate Metabolism.* (30684)

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The studies of Lynen *et al*(1) and Wakil *et al*(2) have demonstrated that biotin serves as a co-enzyme for several enzymatic carboxylation reactions. These specifically include the formation of methylmalonyl CoA from propionyl CoA and CO_2(3), malonyl CoA from acetyl CoA and CO_2(4) and conversion of methylcrotonyl CoA and CO_2 to β-methyl-glutaconyl CoA(5). Terroine(6) has reported that in acute biotin deficiency there is an increase in blood pyruvic acid levels. Such changes may result from decreased pyruvate utilization. In the metabolism of pyruvate to lactate or acetyl CoA biotin is presumably not involved; however, Keech and Utter(7,8) have recently described the properties of a pyruvic carboxylase which converts pyruvate and CO_2 into oxaloacetate in chicken liver preparations. Possible involvement of biotin in glucose metabolism has been suggested(9), and recently we have reported(10) reduced incorporation of $^{14}CO_2$ into glucose in biotin-deficient rats. The conversion of pyruvate to phosphoenolpyruvate (PEP) is a crucial step in the synthesis of glycogen or glucose from precursors of pyruvate in liver. The formation of phosphoenolpyruvate could occur through the reversal of the glycolytic reaction catalyzed by pyruvate kinase, but the physiological significance has been questioned on energetic grounds(11). The fact that phosphoenolpyruvate could be formed from oxaloacetate by phosphoenolpyruvate carboxykinase(12) would suggest that PEP might be formed by a route which bypasses pyruvate kinase. In addition oxaloacetate can be formed by carboxylation of pyruvate by pyruvate carboxylase(7,8). These reactions are as follows:

$$\text{I Pyruvate} + CO_2 + \text{ATP} \xleftarrow[\text{Pyruvate carboxylase}]{\text{Acetyl CoA, } Mg^{++}} \text{oxaloacetate} + \text{ADP} + P_i$$

$$\text{II Oxaloacetate} + \text{ITP (or GTP)} \xleftarrow[\text{PEP carboxykinase}]{Mn^{++}} \text{Phosphoenol pyruvate} + CO_2 + \text{IDP (or GDP)} + P_i$$

It is reported(7,8) that pyruvic carboxylase is a biotin-containing enzyme, and that this reaction may become rate limiting in glucose formation from lactate or pyruvate in biotin-deficient animals. To test this hypothesis liver slices from normal and biotin-deficient rats were incubated with pyruvate-2-^{14}C and

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TABLE I. Incorporation of Pyruvate-2-^{14}C into Glucose, Glycogen and Its Oxidation by Liver Slices Incubated for 90 Minutes from Normal and Biotin Deficient Animals.*

Animal preparation	No. of weeks on diet	Glucose		Glycogen		CO_2
		μmoles/g	C.P.M./g	μmoles/g	C.P.M./g	C.P.M./g
Normal†	8	88 ± 8.2	6880 ± 900	92 ± 6.8	880 ± 86	26,000 ± 3200
Biotin deficient‡ supplemented with biotin	8	92 ± 8.6	6430 ± 930	90 ± 7.2	1020 ± 80	24,600 ± 2300
Biotin deficient	4	103 ± 9.6	5400 ± 600	80 ± 7.3	620 ± 56	24,000 ± 1800
" "	6	96 ± 8.5	3600 ± 350	78 ± 8.0	410 ± 45	18,000 ± 1600
" "	8	83 ± 7.6	2800 ± 300	75 ± 7.6	360 ± 38	12,800 ± 1400
" " §	8	98 ± 11.2	6060 ± 600	79 ± 6.8	460 ± 30	20,200 ± 2100

* Approximately 0.5 g of liver slices from normal and biotin deficient animals were incubated in 6 ml of Ringer-bicarbonate medium containing pyruvate-2-^{14}C (2-2.5 × 10^5 cpm) at a concentration of 1 mg/ml. Each figure represents average of 4 values with standard error.

† Received casein diet.

‡ Received biotin deficient diet and were injected with biotin.

§ These animals were fed biotin deficient diet for 8 weeks and then given 50 γ of biotin intraperitoneally twice daily and were killed 48 hours after administration of biotin for *in vitro* studies.

recovery of labeled carbon in glucose, glycogen and CO_2 determined. In addition activities of pyruvate carboxylase and PEP carboxykinase have been assayed under similar conditions.

Experimental procedures. Animals: Production of biotin deficiency. Weanling male rats of the Sprague-Dawley strain weighing 35-40 g were divided into 3 groups of 36 animals each and were fed *ad libitum* on normal, biotin-deficient diet with 30% egg white(13) purchased from General Biochemicals Corp. (Chagrin Falls, Ohio; Bulletin D15, p. 8, biotin-deficient rat test diet) and biotin-deficient diet supplemented with biotin (20 γ of biotin per rat was administered intraperitoneally, twice weekly). Normal animals received complete diet of the following composition: Casein, 18.0%; sucrose, 72.0%; salts, 446(14), 4%; corn oil, 4%. The ration was supplemented with vit A (20,000 U.S.P. units per kg), vit D (2,000 units per kg) and vit E (100 mg per kg). All the B vitamins were added at adequate levels.† The animals were housed individually in wire bottom cages and maintained for 8 to 10 weeks. The rats were weighed twice weekly. Reduction in growth rates as reported previously(10) along with usual signs of deficiency, *e.g.*, loss of weight, spectacled eye, dermatitis and alopecia, were used as criteria for production of the vitamin deficiency. The normal animals weighed 180 ± 15, 210 ± 18, and 230 ± 20 g at the end of 4, 6, and 8 weeks. Corresponding weights for biotin-deficient animals were 130 ± 12, 120 ± 8, and 116 ± 11 g, and those for biotin-deficient diet supplemented with biotin were 168 ± 12, 187 ± 20, and 210 ± 25 g.

Studies with liver slices. Liver slices prepared as described previously(9) from normal and biotin-deficient animals were incubated in Ringer-bicarbonate medium equilibrated with 95% O_2, 5% CO_2. Pyruvate-2-^{14}C (specific activity 2.72 mc/mM) was diluted with non-radioactive K pyruvate and added to the medium to give an initial concentration of 10 mmoles. Approximately 0.5 g of liver slices weighed on a Roller Smith Torsion balance was incubated in 6 ml of medium containing from 2.0 to 2.5 × 10^5 cpm of labeled substrate. After 90 minutes incubation, tissues were analyzed for glycogen and medium for gluocse and CO_2. Glucose and glycogen were isolated as phenylglucosazone and CO_2 as $BaCO_3$ for radioactivity assay(15). The results of this study are given in Table I.

Studies on stability and extraction of pyruvate carboxylase from normal and biotin-deficient animals. Livers from normal and biotin deficient animals were homogenized in

† Following B vitamins were added: 25.0 mg thiamine hydrochloride; 10.0 mg riboflavin; 40.0 mg calcium pantothenate; 100.0 mg nicotinic acid; 6.0 mg pyridoxine hydrochloride; 0.6 mg biotin; 0.4 mg folic acid; 1.0 mg 2 methyl-1-4-naphthoquinone and 50 γ of vit. B_{12} per kg of diet.

TABLE II. Pyruvate Carboxylase Activity in Normal and Biotin Deficient Animals (6 Weeks) Under Various Conditions of Preparations.*

Preparation	Temperature prepared	Normal (μmoles CO_2 fixed/g/hr)	Biotin deficient (μmoles CO_2 fixed/g/hr)
Mitochondria	Room	1.8 ± 0.12	1.2 ± 0.11
Supernatant	"	12.3 ± 1.3	5.6 ± 0.66
Mitochondria	2°C	13.5 ± 1.5	6.9 ± 0.88
Supernatant	"	1.2 ± 0.13	0.88 ± 0.07

* Mitochondria and supernatant equivalent to 50 mg of fresh liver were incubated as described in Experimental Procedures. Each figure is average of 4 values with standard error.

TABLE III. Pyruvate Carboxylase and PEP Carboxykinase Activity in Cell-free Preparations from Livers of Normal and Biotin Deficient Animals.*

Animal preparation	No. of weeks on diet	Pyruvate carboxylase (μmoles CO_2 fixed/g/hr)	PEP carboxykinase (μmoles CO_2 fixed/g/hr)
Normal†	8	12.8 ± 1.10	3.4 ± 0.42
Biotin deficient‡ supplemented with biotin	8	12.2 ± 1.3	3.2 ± 0.4
Biotin deficient	4	9.6 ± 0.85	3.3 ± 0.35
" "	6	6.0 ± 0.73	2.8 ± 0.32
" "	8	3.4 ± 0.41	1.9 ± 0.23
" " §	8	9.8 ± 0.80	2.4 ± 0.32

* 0.5 ml of supernatant obtained after centrifugation at 105,000 × g was incubated with the necessary cofactors as described previously. Each figure is average of 4 values with standard error.

† Received casein diet.

‡ Received biotin deficient diet and were injected with biotin.

§ These animals were fed biotin deficient diet for 8 weeks and then given 50 γ of biotin intraperitoneally twice daily and were killed 48 hr after administration of biotin for *in vitro* studies.

0.25 M sucrose (1 g liver/2.5 ml 0.25 M sucrose) at room temperature and at 2°C and centrifuged at 600 × *g* to remove nuclei. After removal of the nuclei the homogenate was centrifuged at 10,000 × *g* for 15 minutes to obtain mitochondria fraction, and after removal of mitochondria was again centrifuged at 105,000 × *g* to obtain the supernatant fraction. The mitochondria and supernatant fractions were then assayed(7,8,16) for pyruvate carboxylase activity with 20 μmoles of K pyruvate, 50 μmoles of $NaHCO_3$ (5.0 μc), 3.3 μmoles $MgCl_2$, 1.25 μmoles ATP, 0.38 μmole Acetyl CoA and 50 μmoles of Tris HCl pH 7.4 in a total volume of 1 ml. The results are given in Tables II and III.

Studies on PEP carboxykinase activity in normal and biotin-deficient animals. PEP carboxykinase activity was measured by the method of Utter and Kurahashi(17). The livers from normal and biotin deficient animals were homogenized in 0.154 M KCl (1 g liver/2.5 ml) and centrifuged at 105,000 × *g* for 60 minutes. The supernatant fraction obtained was incubated with 50 μmoles of $NaHCO_3$ (5.0 μc); 20 μmoles of oxaloacetate, 2 μmoles of $MgCl_2$ and 2 μmoles inosine triphosphate. At the end of a 10-minute incubation period, solutions were deproteinized with 0.5 ml of 10% trichloroacetic acid and protein removed by centrifugation. The supernatant fluid was gassed for 10 minutes with CO_2 to remove any excess of $^{14}CO_2$ and an aliquot was counted in an anthracene packed cuvette in a Packard Scintillation Counter. The results of the study are given in Table III.

Studies on pyruvate utilization by liver homogenates and 105,000 × g supernatant from normal and biotin-deficient animals. The liver homogenates and 105,000 × *g* supernatant fractions were prepared as described previously and were incubated with non-radioactive pyruvate for thirty minutes. At the end of incubation, unused pyruvate was measured by the procedure of Tonhazy *et al*(18). The results are given in Table IV.

TABLE IV. Utilization of Pyruvate by Liver Homogenates and 105,000 × g Supernatant.

Animal preparation	No. of weeks on diet	Homogenate (μmoles/g/10 min)	Supernatant (μmoles/g/10 min)
Normal*	8	38.3 ± 3.6	21.5 ± 2.8
Biotin deficient† supplemented with biotin	8	42.6 ± 4.2	24.8 ± 3.0
Biotin deficient	4	31.6 ± 3.0	15.8 ± 1.2
" "	6	26.2 ± 2.5	12.8 ± 1.6
" "	8	21.3 ± 2.1	10.6 ± 0.9

* Received casein diet.
† Received biotin deficient diet and were injected with biotin.

Results. The results from Table I indicate that rats on biotin-deficient diet for 4 weeks show no difference in utilization of pyruvate-$2^{14}C$ to $^{14}CO_2$. These results are in agreement with earlier studies in which rats fed biotin-deficient diet for 4 to 5 weeks were used. It was observed that oxidation of C^{14} glucose to $^{14}CO_2$ was unaltered in biotin-deficient animals (4 weeks on biotin-deficient diet) as compared to normal. However, there was a significant decrease in $C^{14}O_2$ incorporated into glucose *in vivo* but no effect *in vitro* in early periods of biotin deficiency. The present studies from Table I further indicate a progressive decrease in conversion of pyruvate-$2^{14}C$ into glucose in biotin-deficient animals with increasing duration of the vitamin deficiency (6 to 8 weeks). Similarly, a decrease in oxidation of pyruvate to CO_2 was also observed. A small decrease in glycogen activity was also observed. However, levels of medium glucose or liver slice glycogen were unaltered significantly in biotin deficiency.

Table II summarizes pyruvate carboxylase activity observed in mitochondrial and supernatant fractions of livers of normal and biotin-deficient rats. When liver homogenates were made at room temperature, most of the pyruvate carboxylase activity was present in 105,000 × *g* supernatant fraction. On the other hand, when homogenates were prepared at 2°C, this enzymatic activity was present in the mitochondrial fraction. Pyruvate carboxylase activity was quite stable at room temperature, and no loss of activity was observed when preparations were allowed to stand at room temperature for 30 minutes, but this activity was lost on freezing the supernatant fraction.

Pyruvate carboxylase and PEP carboxykinase activities observed in biotin-deficient animals at various stages of deficiency are summarized in Table III. Pyruvate carboxylase activity markedly decreased with severity of biotin deficiency whereas only a small but significant change was observed in PEP carboxykinase activity in the corresponding period as compared to normal. Furthermore, 48 hours after *in vitro* administration of biotin to biotin-deficient animals pyruvate carboxylase activity was almost restored to normal levels whereas PEP carboxykinase activity was only slightly altered. The results in Table IV also show a decreased utilization of pyruvate in liver homogenates of biotin-deficient animals as compared to normal liver preparations. In addition, homogenates were more effective in pyruvate utilization as compared to supernatant preparations.

Discussion. The decreased conversion of pyruvate-$2^{14}C$ into glucose in biotin-deficient animals may be due to the decrease in pyruvate carboxylase activity in biotin-deficient animals. This decrease in enzymatic activity will probably lead to decrease in oxaloacetate formation and its conversion to phosphoenolpyruvate by PEP carboxykinase. Both of the enzymes are decreased under these conditions. The increase in blood pyruvate observed by Terroine(6) may be explained on the basis of the present results which show a decreased utilization of pyruvate in biotin-deficient animals.

Pyruvate carboxylase which is a mitochondrial enzyme can be easily extracted from this fraction at room temperature. It is probable that this enzyme is leached out of the mitochondria at room temperature. That the decrease observed in pyruvate carboxylase is a true decrease and not due to reduction in

the enzymatic activity in the extraction may be seen from Table II which shows very little activity left in mitochondrial preparations in both deficient and normal preparations. Koscow and Lane(19) have observed a reduction in the biotin containing enzymes in biotin deficiency. The present results on pyruvate carboxylase are similar to those reported by these workers on other biotin containing enzymes(19).

Summary. Biotin-deficient animals show a reduction in incorporation of pyruvate-2^{14}C into glucose with progressive onset of the deficiency. The oxidation and utilization of pyruvate is reduced by 50 per cent. The pyruvate carboxylase activity was greatly decreased whereas PEP carboxykinase activity was found to be slightly decreased in biotin deficiency. *In vitro* administration of biotin to biotin-deficient animals almost restored the pyruvate carboxylase activity and PEP carboxykinase activity was only slightly altered. In addition, it is observed that pyruvate carboxylase can be extracted from the mitochondria by homogenizing at room temperature and is quite stable under these conditions.

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Effect of Folic Acid and Vitamin B_{12} on Excretion of Hippuric Acid And Formiminoglutamic Acid. (30685)

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It is well established that a folic acid derivative is required for the conversion of serine to glycine(1,2), and there is evidence that the glycine in the body is derived from serine (3) which, in turn, is derived from glucose. Since glycine is required for the formation of hippuric acid in the detoxification of benzoic acid, it seemed possible that the formation of hippuric acid would be decreased by a folic acid deficiency. Similarly, the toxicity of benzoic acid might be expected to be increased by folic acid deficiency because of reduced ability to form glycine for detoxification of benzoic acid. This should be especially true on a casein diet which is low in glycine.

The present study was initiated to deter-

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