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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mine if the detoxification of benzoic acid as measured by the urinary excretion of hippuric acid could be used as a criterion of folic acid deficiency. Urinary formiminoglutamic acid (FIGLU) excretion, frequently used as an indication of folic acid deficiency, was determined for comparative purposes. The effect of vit. B_{12} was studied since a deficiency of this vitamin increases excretion of FIGLU (4,5) and thus indirectly affects folic acid metabolism.

Methods. Weanling albino rats were housed in wire-bottom cages (4 rats/cage) in a temperature controlled laboratory. The animals were fed and watered *ad libitum* and weighed twice each week. Feed consumption was recorded. The basal folic acid-deficient diet consisted of glucose monohydrate (Cerelose) 73.8%, extracted casein 20.0%, mineral mixture 4.0%(6), vitamin premix 1.0%, corn oil containing vit. A, D and E 1.0%, succinylsulfathiazole 1.0%, and choline chloride. The vitamin mixture supplied 50 mg of niacinamide, 50 mg calcium pantothenate, 10 mg thiamine, 10 mg riboflavin, 10 mg pyridoxine, 10 mg 5-acetoxy-2-methyl-4-naphthyl sodium phosphate, 0.2 mg biotin, 15,000 units vit. A (acetate), 2,000 A.O.A.C. chick units of vit. D, and 300 units mixed tocopherols per kg of diet. The casein was extracted with hot alcohol and further purified by dissolving in dilute sodium hydroxide and reprecipitation with acid by the method of Fox and Ludwig (7) to remove folic acid, and contained 0.04 μg/g by microbiological assay. Folic acid was added to the diet at a level of 2 mg per kg of diet or given intraperitoneally at a level of 1 mg 4 times a week. Vit. B_{12} was given in the diet at a level of 30 μg per kg of diet or 5 μg administered intraperitoneally 2 times per week. In the first experiment with 8 female rats per treatment, benzoic acid was fed at levels of 1, 2.5 and 5%, in the diet, vit. B_{12} was added to all diets at 30 μg per kg and folic acid, where indicated, was added at 2 mg per kg. Since the 2.5 and 5% levels of benzoic acid were highly toxic, causing death to all animals in less than one week, a second similar experiment was conducted in which 2% benzoic acid was fed and DL-serine was also added to determine whether this amino acid was limiting in the formation of glycine for detoxifying benzoic acid. Four male weanling rats were used per treatment in this experiment. Because of the observed effect of benzoic acid in the first two experiments on urinary excretion of FIGLU, a histidine metabolite known to be affected by both folic acid and vit. B_{12}, a third experiment was conducted. Four male weanling rats per treatment were fed diets similar to those fed in the first 2 experiments with the exceptions that 20.0% extracted(2) soybean protein (Drackett Assay Protein) (microbiological assay showed 0.05 μg folic acid/g) was substituted for extracted casein, 0.2% L-histidine and 1.0% of the folic acid "antagonist." X-CH_3-folic acid(8), were added at the expense of glucose monohydrate, and folic acid and vit. B_{12} were omitted. To eliminate variations in feed consumption the benzoic acid and serine were administered intraperitoneally.

Urine collections. When collected, the rats were placed in metabolism cages and 24-hour collections were made in 100 ml tubes containing 5.0 ml of 1.0 N HCl. The collected urines were diluted to 25.0 ml/rat with distilled H_2O and frozen until assayed.

Assay of urinary hippuric acid. This assay is based on the extraction of hippuric acid with ether, acid hydrolysis to benzoate and glycine, and determination of the glycine by the ninhydrin method(9). Ten ml of diluted urine or hippuric acid standard solution were placed in a glass stoppered 100 ml centrifuge tube after being acidified to pH 2.0-2.5 with H_2SO_4 or HCl. Fifty ml of anhydrous ethyl ether were added, and the tube carefully inverted 30 times. Subsequently, 25 ml of the ether layer were removed and evaporated to dryness. (Based on a 5:2 distribution coefficient for hippuric acid between H_2O and ether, the 25 ml of ether contained ⅓ of the hippuric acid present in the original 10 ml.) The ether residue was dissolved in 1-2 ml of 6 N HCl and autoclaved for 1½ hours at 120°C and 15 p.s.i to hydrolyze the hippuric acid to benzoate and glycine. The mixture was neutralized with NaOH and diluted with distilled H_2O to give a glycine concentration of 0.25 to 1.25 μmoles/ml.

Assay of urinary formiminoglutamic acid.

TABLE I. Urinary Excretion of Hippuric Acid.

Supplement per kg diet			Avg body wt and No. of survivors ( ) (6-wk wt g)		μmoles hippuric acid/100 g body wt/day ( )% of ingested benzoate excreted			
Benzoic acid (g)	Folic acid (g)	Serine (g)			Day 18	Day 28	Day 39	Day 43
Exp 1:								
–	–	–	134(8)		27	20	17	23
–	2	–	146(8)		20	17	13	22
10	–	–	132(8)		570(58)	477(57)	567(67)	87*
"	2	–	155(7)		875(79)	434(70)	571(70)	58*
25	–	–	all dead†		—	—	—	—
"	2	–	"	"	—	—	—	—
50	–	–	"	"	—	—	—	—
"	2	–	"	"	—	—	—	—
Exp 2:			4-wk wt	10-wk wt	Day 43	Day 70		
–	–	–	110(4)	158(4)	16	22		
–	–	20	118(4)	168(4)	20	23		
–	5	–	125(4)	178(4)	18	20		
–	5	20	116(4)	168(4)	20	19		
10	–	–	112(4)	163(4)	193	234		
"	–	20	110(4)	146(4)	185	296		
"	5	–	120(4)	175(4)	111	208		
"	5	20	112(4)	174(4)	167	182		
20	–	–	50(3)	—(0)	—	—		
"	–	20	50(2)	—(0)	—	—		
"	5	–	58(2)	—(0)	—	—		
"	5	20	61(3)	—(0)	—	—		

* Benzoic acid removed from diets starting day 39.
† All animals dead in less than 7 days.

The assay of formiminoglutamic acid was conducted using the enzymatic system of Tabor and Wyngarden(10).

Results. The growth rates and hippuric acid excretion rates for Experiments 1 and 2 are given in Table I. Benzoic acid at a level of 10 g per kg produced no growth inhibition either with or without folic acid, while 20 g were toxic, inhibiting growth and killing all the animals by the sixth week. Higher levels of benzoic acid, 25 and 50 g per kg, were highly toxic, killing all animals before 1 week. Folic acid had no effect in reducing the toxicity of benzoic acid at the 20, 25, or 50 g levels. Supplementary serine similarly did not reduce toxicity as measured by growth and survival.

Urinary excretion of hippuric acid in the absence of supplemented benzoic acid ranged from 13 to 27 μmoles/100 g body weight/day in the first experiment and from 16 to 39 in the second with essentially no difference due to folic acid or serine. Supplementation with 10 g of benzoic acid increased hippuric acid excretion by 30-fold, or greater, in Exp. 1 and a 10-fold increase in Exp. 2. When expressed as a percentage of the ingested benzoic acid, excretion ranged from 57 to 67% in the absence of folic acid and from 70 to 79% in the presence of the vitamin. Though at days 18 and 28 the folic acid supplemented rats excreted *ca* 36 and 23%, respectively, more of the ingested benzoic acid than did the deficient animals, excretion was essentially the same by the 39th day, when the deficiency would be more pronounced. Removal of the benzoic acid supplement on the 39th day resulted, as expected, in a marked reduction of hippuric acid excretion by the 43rd day. However, excretion was still 2 to 4 times greater than from rats which had never received benzoic acid.

Table II shows urinary excretion of hippuric acid in rats with a dietary deficiency of folic acid and/or vit. B_{12} after the administration of a test dose of benzoic acid in Exp. 3. When folic acid was given to the rats, the percentage of the administered benzoic acid excreted as hippuric acid increased from 39 to 48 in the absence of vit. B_{12} and from 57 to 66 when the animals received vit. B_{12}, or an increase of 9 percentage points in each

TABLE II. Effect of Vitamin B_{12} and Folic Acid on the Excretion of Hippuric Acid. Experiment 3.

Vitamin supplementation		Avg body wt (9 wk)	μmoles hippuric acid/100 g body wt/day		
Vit B_{12}*	Folic acid†		4 day avg before test dose of benzoic acid	1 day after test dose of benzoic acid‡	% of benzoic acid excreted as hippuric acid
		(g)			
—	—	128(3)§	44	142(3)§	39
—	+	190(4)	33	153(4)	48
+	—	172(3)	36	178(2)	57
+	+	208(4)	32	196(4)	66

* B_{12}—intraperitoneal injection bi-weekly; 5 μg/rat.
† Folic acid—intraperitoneal injection 4 times/week; 1 mg/rat.
‡ 250 μmoles benzoic acid/100 g body wt by intraperitoneal injection, preceded by one hour by 500 μmoles DL-serine/100 g body wt on Day 66.
§ No. of rats.

case. Since it is known that vit. B_{12} is not required in the folic acid dependent conversion of serine to glycine(3), which in turn conjugates with benzoic acid to form hippuric acid, it was surprising to note that administration of vit. B_{12} to the doubly deficient animals increased excretion of the test dose from 39 to 57%, and increased it from 48 to 66% in the presence of folic acid. Thus, vit. B_{12} increased excretion by 18 percentage points whether folic acid was administered or not.

Urinary excretion of FIGLU, Table III, showed a normal range of between 0 and 2 μmoles/100 g body weight/day in the presence of folic acid regardless of benzoic acid or serine supplementation. In the absence of the vitamin FIGLU excretion increased to a range of 23.6 to 45.6 μmoles/100 g body weight/day. On the other hand, supplementation of the deficient rats with benzoic acid reduced the excretion of FIGLU by *ca* 50%. Serine supplementation with or without added benzoic acid resulted in a slight increase in FIGLU excretion. Removal of the benzoic acid from the diet, Exp. 1, resulted in a sharp increase in FIGLU excretion to a level comparable to the previously unsupplemented animals.

TABLE III. Urinary Excretion of Formiminoglutamic Acid.

Supplement per kg diet			μmoles FIGLU/100 g body wt/day		
Benzoic acid	Folic acid	Serine	Day 28	Day 39	Day 43
(g)	(g)	(g)			
Exp 1:					
–	–	–	31.3	45.6	35.4
–	2	–	.2	.9	1.0
10	–	–	12.0	24.4	41.1*
"	2	–	.5	.9	1.1*
Exp 2:					
–	–	–	34.0	23.6	32.8
–	–	20	50.0	43.2	47.9
–	5	–	.2	1.3	1.7
–	"	20	0	1.6	1.9
10	–	–	22.6	14.8	14.4
"	–	20	27.7	24.4	27.5
"	5	–	.1	1.9	2.2
"	"	20	.5	1.6	1.9

* Benzoic acid removed from diets on Day 39.

Discussion. The toxicity produced by benzoic acid in these experiments is similar to that reported by Griffith(11) who observed high mortality and reduced growth with 3.5% sodium benzoate and none with 2.5%. Although these levels appear higher than those reported here, they were fed in a high fat diet with restricted feed intake. The highest nontoxic dose of 2.5% sodium benzoate was equal to 0.8 mmole/day/100 g body weight, which corresponds to the nontoxic level of 0.8 mmole/day on the 1% benzoic acid level with *ad libitum* feeding reported here. The 2% level of benzoic acid caused marked growth depression and approximately 50% mortality at 4 weeks.

The toxicity of benzoic acid was not increased in these experiments on the folic acid-deficient diets. If all glycine were formed exclusively from serine, as the data of Arnstein and Keglevic(3) suggest, then glycine pro-

duction on a folic acid-deficient diet would be reduced to a level where an additional demand for glycine in hippuric acid production should induce a glycine deficiency. This is based on the estimate of Arnstein and Stankovic(14) that the rate of glycine synthesis is 2.7 mmoles/day/100 g body weight on an adequate diet, and 1.65 mmoles/day on a folic acid deficiency diet. The glycine requirement of the growing rat has been estimated by Arnstein and Neuberger(15) to be at 1.5 to 2.0 mmoles/day/100 g body weight. This estimate of glycine requirement and synthetic ability is in accord with the data of Griffith(11) showing that up to 0.8 mmole of benzoic acid per 100 g body weight per day may be detoxified without evidence of glycine deficiency. When higher levels of sodium benzoate (1.1 mmoles/day) are fed, toxicity develops which can be prevented by feeding supplementary glycine(11). This shows that one of the first causes of benzoic acid toxicity is induction of glycine deficiency. If the production of glycine is 1.6 mmoles on a folic acid-deficient diet as estimated by Arnstein and Stankovic(14), the removal of 0.57 mmole for hippuric acid (Exp. 1) would leave *ca* 1.0 mmole/day for growth which should result in a marked glycine deficiency. The marked excretion of FIGLU observed here in the animals which did not receive folic acid, indicates that they had become deficient in this vitamin as measured by this criterion. Since 0.6 mmole of benzoate per day is the maximum amount that can be conjugated with glycine without producing glycine deficiency on a normal diet, it would be logical to expect that a further reduction of glycine production by folic acid deficiency would precipitate severe glycine deficiency and growth depression. The absence of any effect of folic acid deficiency on the toxicity of benzoic acid in these experiments indicates either that the deficiency was not severe enough to impair glycine formation, or that glycine may also be synthesized by a non-folic acid dependent pathway from glyoxylate. The latter pathway is possible in the presence of dietary glyoxylate since glyoxylate is as effective as glycine in preventing the toxicity of benzoate(11), and labeled glyoxylate is incorporated into hippuric acid(13). Glycolaldehyde and glyoxylate may be derived from ethanolamine which is formed by decarboxylation of serine(16). Thus, a pathway for the synthesis of glycine from serine without involvement of folic acid may be visualized. The failure of folic acid deficiency to affect the tolerance of the rat to benzoic acid suggests that this non-folic acid pathway may be an important factor in the economy of glycine production.

It will be noted that addition of 2.0% serine did not decrease the toxicity of benzoic acid (Exp. 2). Since supplementary glycine has been reported to decrease the toxicity of benzoic acid(11), the inactivity of serine indicates that the formation of glycine from serine or other intermediate is limiting rather than the formation of serine itself.

Folic acid deficiency failed to have any marked effect on the rate of hippuric acid synthesis in Exp. 1 and 2 where *ad libitum* feeding was used. The lowered excretion of hippuric acid observed in Exp. 2, as compared to Exp. 1, cannot be explained solely on the basis of variations in the intake of benzoic acid, as feed consumption in both cases was approximately the same. This difference is unexplainable other than to note that there was a sex difference in that females were used in Exp. 1 and males in Exp. 2.

In the third experiment, Table II, where benzoic acid was given by injection at 0.25 mmole/100 g body weight, there was *ca* 20% increase in hippuric acid excretion in the presence of folic acid. However, supplementation with vit. B_{12}, either with or without folic acid, increased hippuric acid excretion by *ca* 40%. Although there is no known direct role of vit. B_{12} in either the conversion of serine to glycine or in the formation of hippuric acid, the effect of vit. B_{12} is not completely unexpected in view of the role of vit. B_{12} in indirectly affecting folic acid metabolism such as the effect of vit. B_{12} deficiency in increasing FIGLU excretion(4,5).

More unexpected was the observation that FIGLU excretion was decreased in the folic acid-deficient animals when benzoic acid was added to the diet. It has been shown that FIGLU excretion is increased in folic acid de-

ficiency and to a lesser degree in vit. B_{12} deficiency(4,5). In folic acid deficiency the functional form of the vitamin necessary to accept the formimino group of FIGLU is absent or not available, and thus FIGLU is excreted in the urine. It may be reasoned that an increased demand for glycine would increase the folic acid requirement for glycine synthesis and thus decrease that available for FIGLU metabolism, resulting in an increased excretion of FIGLU. The decreased urinary FIGLU with benzoic acid supplementation remains unexplained.

The increase in FIGLU excretion, due to the serine supplementation in the absence of folic acid and with or without the presence of benzoic acid, has also been observed in this laboratory as well as by Brown *et al*(17).

Summary. The effects of folic acid and vit. B_{12} on the toxicity of benzoic acid and on excretion of hippuric acid were studied. Benzoic acid at levels of 2 to 5% produced high mortality and the toxicity was not decreased by folic acid or serine supplementation. Detoxification of benzoic acid was not affected or only slightly reduced in folic acid deficiency. Further supplementation of the benzoic acid diets with DL-serine had no effect on hippuric acid excretion with or without folic acid. Vitamin B_{12} deficiency resulted in a greater reduction of hippuric acid excretion than did folic acid deficiency when the rats were given a test dose of benzoic acid by injection. The inclusion of benzoic acid in the diet decreased urinary excretion of FIGLU in folic acid-deficient rats. On the other hand, serine supplementation increased FIGLU excretion in the vitamin-deficient animals with or without added benzoic acid.

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Cerebrospinal Fluid Binding Capacity for Cyano- and Hydroxocobalamin. (30686)

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In 1960 it was demonstrated that hydroxocobalamin, when injected into dogs, resulted in a higher serum level of vit. B_{12} than did cyanocobalamin(11). Several authors soon reported that this applied also to humans(3, 5,8,9). Chosy, Killander and Schilling(1) found that the *in vitro* binding capacity in serum is considerably higher for hydroxocobalamin than for cyanocobalamin, while in the gastric juice it is approximately equal. Glass *et al*(4) demonstrated a greater retention of injected hydroxo- than of cyanocobalamin in muscles.

Recently, Hertz, Kristensen and Hoff-Jør-