media under the influence of penicillin. High concentrations of NaCl increased slightly and high concentrations of phosphate increased markedly the number of strains which produced L forms. The behavior of the descendants of individual coccal chains of a strain which produced L forms was variable. In some strains the majority of the cocci present in a single colony lost the ability to produce L forms.

It is of interest that in several species such as *Bacteroides* and the hemophylic bacteria, which are the usual inhabitants of the human body, pleomorphism and spontaneous production of L forms is more frequent among the strains isolated from pathological processes than among those isolated from their usual habitat. Similarly, the strain of *alpha*-hemolytic streptococcus which produced L forms spontaneously originated from an abscess of long duration, and was characterized by extreme pleomorphism. This relation cannot be due entirely to the influence of antibiotics, because some of these observations were made before they came into use.

Summary. High concentrations of potassium and sodium phosphates incorporated in the media markedly increased the number of strains of *alpha*-hemolytic streptococci which produced L-type growth when exposed to penicillin. High concentrations of NaCl produced this effect only slightly. Using the phosphate media, strains of *alpha*-hemolytic streptococci which produced L forms were found in about one-fourth of the throat cultures examined. One unusual strain of *alpha*-hemolytic streptococcus produced L forms spontaneously without exposure to penicillin.

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Endogenous Formation of Hippuric Acid.* (22134)

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(Introduced by Emil L. Smith.)

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Occurrence of hippuric acid in mammalian urine has been known since its isolation by Liebig(1) in 1829. It has usually been supposed to arise by conjugation of benzoic acid of dietary origin with glycine in the body, and a great part of biochemical investigations of hippuric acid formation have been concerned with glycine metabolism. Recently, presence of hippuric acid in urine of fasting humans was reported and a possible endogenous origin was suggested for it(2). Shortly thereafter, Schreier *et al.*(3) observed a dilution in specific activity of radioactive benzoic acid in fasting rats, and suggested that an endogenous formation of benzoic acid could explain their results. In addition, they cited other observations, unsupported by experimental data, which were in agreement with this idea. Our investigation indicates that in humans and in the white rat there is a small amount of hippuric acid produced endogenously and that in the rat the aromatic ring of phenylalanine can, to a slight extent, serve as a precursor for the benzoic acid moiety. A major proportion of hippuric acid present in urine of humans and of rats receiving a natural diet is, however, derived from dietary precursors.

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Methods. Albino rats (Sprague-Dawley), weighing 300 to 400 g, were fed either Purina

chow or lactalbumin-dextrin diet which has been described earlier(4). Each animal was allowed 15 g of food daily and given water *ad libitum;* fasting animals were allowed water. For experiments in which a laboratory diet was used with humans, the basal diet contained lactalbumin, sucrose, a vegetable oil (Wesson oil), vitamins and minerals(5). The amounts allowed in g/kg/day were lactalbumin, 1; sucrose, 0.8; fat, 0.8; mineral salts (No. 2, General Biochemicals, Inc.), 0.2, and vitamins. Rat urine was collected with toluene as a preservative; 24-hour samples were acidified to pH 2, extracted twice with 3 volumes of ethyl acetate, and the ethyl acetate was concentrated *in vacuo*. Final volume was adjusted to contain about 0.2 mg of hippuric acid ml of ethyl acetate. Human urine was collected and frozen immediately; 24-hour samples were assayed for creatinine and organic acids of an aliquot were extracted by the standard procedure reported previously(6). Hippuric acid concentration of the ethyl acetate extracts was estimated by modification of procedure described by Gaffney *et al.*(7). Aliquots which contained between 2 and 10 μg of hippuric acid were placed on Whatman No. 1 filter paper along with 1 to 10 μg amounts of authentic hippuric acid, and the chromatograms developed with isopropyl alcohol-aqu. ammonia-H_2O (8:1:1) for 16 hours at 25°C. Hippuric acid (R_f = 0.59) was visualized by spraying with *p*-dimethylaminobenzaldehyde Ac_2O NaOAc reagent(4) followed by either heating at 100°C for 5 minutes or letting the sprayed chromatograms stand overnight; the latter procedure results in a better background on chromatograms. The amount of hippuric acid was estimated by comparing intensity of color of unknowns and standards as seen with a Kodak cold light X-ray illuminator. Recovery experiments showed that the procedure for rat urine gave a quantitative recovery, and that procedure used for human urine gave approximately 90% recovery of hippuric acid. Accuracy of estimation is within ±20%, which is adequate for purposes of these experiments.

The *results* (Table I) show that a major portion of hippuric acid excreted by rats receiving a chow diet arises from some precursor in the diet; no preformed hippuric acid could be detected in the chow. When animals were given lactalbumin diet, hippuric acid excretion fell during the first few days to 3 to 4 mg/kg/day and then remained constant. Fasting or inclusion of 2% sulfasuxidine in the diet to modify intestinal microorganisms did not lead to significant decrease in hippuric acid excretion nor did oral administration or intraperitoneal injection of phenylalanine lead to increased excretion.

TABLE I. Excretion of Hippuric Acid by the Rat.

Regimen	No. of animals	Days studied	Avg daily hippuric acid excreted (mg/kg body wt/day)
Laboratory chow	6	4	22.5 ± 6.1
Laboratory diet			
Day 1	6	1	14.0 ± 5.0
2	6	1	8.8 ± 4.0
3	6	1	5.6 ± 2.5
4	6	1	5.0 ± 1.0
5	3	1	4.3 ± .3
6	3	1	3.9 ± .6
Fasting			
Day 7	3	1	4.4 ± 1.4
8	3	1	3.6 ± .2
9	3	1	3.5 ± .1
10	3	1	3.6 ± .5
11	3	1	3.6 ± .4
12	3	1	3.6 ± .3
13	3	1	3.4 ± .3
Synthetic diet + 2% sulfasuxidine—5th and 6th days	3	2	3.1 ± .3
Synthetic diet + 2% sulfasuxidine + 200 mg L-phenylalanine intraper.	3	3	3.3 ± .2

The results obtained with humans (Table II) were similar to those with rats. A much higher excretion of hippuric acid was observed in individuals eating a natural diet than in the same persons after they had eaten a synthetic diet similar to that fed the rats. Excretion of hippuric acid decreased rapidly as soon as synthetic diet was commenced, and then remained relatively constant, with different individuals excreting different amounts, ranging from 1 to about 3 mg/kg body weight/day—values similar to those observed with rats.

Some caution is mandatory when a color reaction is applied to the analysis of materials

TABLE II. Hippuric Acid Excretion in the Human Receiving a Laboratory Diet.

	Hippuric acid excretion (mg/kg/day)					
	Natural diet	Consecutive days of laboratory diet 1	2	3	4	5
MA (♂)	14.3	4.4	.7	.5	.7	.7
VP (♂)	7.1	2.3	1.6	1.3	1.5	1.7
PW (♀)	58.0	23.0	3.3	2.4	2.4	2.0

of biological origin. To assure that the material estimated with the reagent of Gaffney *et al.*(7) actually was hippuric acid, the final 24-hour urine sample of one person receiving the synthetic diet for 5 days was extracted in the usual manner, and hippuric acid was isolated from the ethyl acetate extract (200 ml, containing an estimated 100 mg of hippuric acid) with the use of a modified counter-current distribution procedure. Thirty-eight mg of impure hippuric acid, obtained in the first crop, was recrystallized (Norit) from 0.6 ml of hot water to yield 16 mg of white material. The isolated compound was identical with authentic hippuric acid in all its physical properties. The demonstration that hippuric acid actually could be isolated from urine of an individual receiving a well-defined diet supports the conclusion that the material estimated as hippuric acid in this work actually was hippuric acid and not another chromogen with similar chromatographic behavior.

Preparation of 3-C^{14}-L-phenylalanine. 77.9 mg of 3-C^{14}-DL-phenylalanine hydrochloride (Tracerlab, Inc., 3.84 Mc/mg) and 227.1 mg of inert DL-phenylalanine were mixed to provide 291.0 mg of DL-phenylalanine (1 Mc/mg). This material was resolved by modification of the method of Wretlind(8). 115 mg of L-phenylalanine, $\alpha]_D^{24} = -34°$ (c,l,H_2O) ($-35°$)(8), and 101 mg of D-phenylalanine, $\alpha]_D^{24} = +33°$ (c,l,H_2O) were obtained.

Isolation of labelled hippuric acid from rat urine. Hippuric acid was extracted from 24-hour samples of urine with ethyl acetate as described above, and a small aliquot used for estimating total quantity present. Ethyl acetate solution was then extracted 4 times with small portions of saturated $NaHCO_3$ solution and 30 mg of carrier hippuric acid was added to the combined bicarbonate extracts. The solution was cooled to 0°, acidified to pH 2 by the addition of 6 N HCl, and allowed to stand overnight in a refrigerator. The hippuric acid was recrystallized from hot water. Hippuric acid, obtained by the above procedure, was hydrolyzed by heating with 2 ml of 6 N HCl at 110° in a sealed tube for 12 hours. The acidic solution was extracted 3 times with 5 ml portions of ether, the combined ether extracts were evaporated to dryness, and benzoic acid was purified by sublimation at 120°; m.p., 122°. For counting, aliquots of urine, of the ethyl acetate extract, and ethyl acetate solutions of hippuric and benzoic acids were placed on aluminum discs (1-inch diameter). Radioactivity was measured on duplicate samples with an internal gas flow counter having a geometry of about 50%. Sufficient counts were measured to allow a counting error of ±5% and the counts were corrected for background and self-absorption. The results of tracer experiments with 3-C^{14}-L-phenylalanine and uniformly labelled C^{14}-glucose are given in Table III.

Discussion. The experiments reported here serve to establish 2 points: first, there is a small endogenous production of benzoic acid in rat and human, and second, this benzoic acid probably is formed in some indirect and, as yet unknown, manner from phenylalanine.

By far the greater proportion of the hippuric acid excreted by rats and humans who are ingesting a natural diet has its origin in dietary precursors—either combined benzoic acid present in plant materials or, in the case of humans, additional small amounts of sodium benzoate, which is added to some foods as a preservative. After the institution of well-defined laboratory diet, amount of hippuric acid excreted in urine of both species decreased rapidly over 2 to 3 days to a fraction of the amount observed originally. The amount, on a body weight basis, of this endogenous hippuric acid excreted was comparable in human and rat.

In the earlier report of presence of hippuric acid in urine of a fasting human(2), the amount of hippuric acid excreted was somewhat greater than that observed here. This may have resulted because of too short a

TABLE III. Results of Tracer Experiments with Rats.

Compound administered by intraper. inj. ⟶	C^{14}-Glucose; one portion		3-C^{14}-L-Phenylalanine; 3 portions at 6 hr intervals		3-C^{14}-L-Phenylalanine; one portion		
	Fasting		Laboratory diet		Fasting		
Days studied ⟶	2nd	3rd	6th	7th	2nd	3rd	4th
Amt of compound given (mg)	65.5		1.		1.		
C^{14} given (cts/min. × 10^{-6})	.95		.90		1.		
C^{14} in 24 hr urine (cts/min.)	7530	990	4610	1760	8790	4370	3000
Proportion of C^{14} excreted in urine (%)	.793	.103	.512	.195	.879	.437	.30
C^{14} in EtOAc extract (cts/min.)	870	380	1760	—	7970	3330	2980
Proportion of urinary C^{14} in EtOAc (%)	11.5	38.5	38.2	—	90.6	76.2	99.4
Hippuric acid excreted (mg)	1.0	1.	1.	1.	1.	1.	1.
Hippuric acid isolated (30 mg carrier added) (mg)	22.	—	23.7	—	24.	—	—
Sp. act. of isolated hippuric acid (cts/min./mg)	0	—	17.5	6.9	20.1	9.5	7.
Sp. act. of benzoic acid from isolated hippuric acid (cts/min./mg)	—	—	14.8	—	17.4	—	—
C^{14} in benzoic acid portion of hippuric acid (cts/min.)	0	—	459	—	540	—	—
Proportion of C^{14} excreted in benzoic acid moiety of hippuric acid (%)	0	—	0.051	—	0.054	—	—

period of fasting before urine was collected; the data of Table I show that a constant low excretion of hippuric acid was not observed until the third day of ingestion of laboratory diet. Presumably, this length of time is required for material of dietary origin present in intestinal tract to be cleared from the body. A similar finding was made in connection with excretion of *m*-hydroxy-hippuric acid, which is also derived in large part from some dietary precursor(6).

The tracer experiments with C^{14}-labelled phenylalanine, designed to test the possibility that benzoic aicd might arise directly from phenylalanine by removal of the 2 terminal carbon atoms of the side chain, clearly showed that, although phenylalanine may definitely serve as precursor to a very limited extent, it can hardly be a direct precursor. The very small amount of radioactivity of administered 3-C^{14}-phenylalanine appearing in hippuric acid excreted was present in the benzoic acid portion of the hippuric acid rather than in the glycine portion, in which it might have occurred as the result of glycine formation from acetoacetate or acetate formed by complete breakdown of phenylalanine. The possibility that *de novo* synthesis of a small amount of C^{14}-containing benzoic acid from acetate might have been carried out by intestinal microorganisms was considered and was tested by administration of uniformly labelled C^{14}-glucose, which has been shown to serve as precursor for microbial synthesis of aromatic rings. Hippuric acid excreted after administration of labelled glucose did not contain a detectable amount of C^{14}.

Thus, the observation of a significant production of endogenous benzoic acid in the rat, which was indicated by the dilution of administered C^{14}-benzoic acid in the work of Schreier *et al.*(3), has been confirmed in the present experiments. In addition, it has been shown that phenylalanine serves but poorly as precursor, and is probably first converted to some unknown intermediate which gives rise to benzoic acid. Utilization of 3-C^{14} rather than ring-labelled phenylalanine leaves open the possibility that phenylalanine might have served as precursor for C^{14}-benzoic acid,

which then underwent decarboxylation and recarboxylation, losing the label in the process. In addition to absence of known metabolic transformations of this type, however, lack of incorporation of C^{14} from C^{14}-labelled glucose into the hippuric acid renders this possibility unlikely.

Summary. 1. Humans and rats receiving a purified laboratory diet excrete hippuric acid, 1 to 3 mg/kg/day. This amount appears to be formed endogenously and its excretion in the rat is not affected by fasting, by inclusion of sulfasuxidine in the diet, or by injection of phenylalanine. 2. 0.05% of the radioactivity of 3-C^{14}-L-phenylalanine injected into a rat was excreted in 24 hours in the form of hippuric acid. Further experiments suggested that endogenous hippuric acid could not be derived from phenylalanine in any direct manner.

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Studies on Thrombin Formation. (22135)

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Treatment of plasma with barium sulfate has been reported to remove the following clotting factors: Prothrombin, the serum prothrombin accelerator system*(1), plasma thromboplastin component*(2) and plasma thromboplastin*(3). However, antihemophilic factor* (AHF) and plasma-accelerator-globulin* (PacG) are supposedly not removed by the adsorbent(4). The present study is concerned with the influence of AHF and of PAcG on the rate of thrombin formation in a reaction mixture containing calcium ions and platelets as well as factors removed from bovine plasma by barium sulfate (plasma eluate). Concomitant investigations were made on the influence of a barium sulfate eluate from rabbit serum (serum eluate) on the rate of thrombin formation.

Materials. Bovine plasma: Oxalated bovine blood (9 parts of blood and 1 part of 3.7% potassium oxalate in 1% oxalic acid) was collected at the slaughter house and centrifuged at 4°C. *Veronal buffer*: A stock solution was made by dissolving 19.428 g sodium acetate • $3H_2O$ and 29.428 g sodium diethylbarbiturate in one l of distilled water. Veronal buffer of pH 7.2 was used throughout and consisted of 100 ml of the stock solution, 40 ml of 8.5% sodium chloride, 100 ml of 0.1 M hydrochloric acid and 260 ml of distilled water. *Bovine platelets* were obtained from oxalated bovine plasma by differential centrifugation according to the procedure described by Seegers and his associates(5). After the final washing with 0.9% NaCl, the platelets were dried with acetone and then pulverized in a mortar. Suspensions of the dried platelet material were made in veronal buffer. *Bovine plasma eluate*: Oxalated bovine plasma was stirred at room temperature for 10 minutes with $BaSO_4$ (50 mg/ml), centrifuged at 4°C and the supernatant plasma decanted. The $BaSO_4$ was washed successively with 0.9% NaCl, 0.1 M sodium oxal-

* For a different terminology of these factors reference is made to Appendix, The Coagulation of Blood. Method of Study. Edited by L. M. Tocantins, Grune and Stratton, N. Y. City, 1955.