REFERENCES

- Anker, H. S. (1948). *J. biol. Chem.* **176**, 1337.
 Barker, S. B. & Summerson, W. H. (1941). *J. biol. Chem.* **138**, 535.
 Bartlett, G. R. & Barron, E. S. G. (1947). *J. biol. Chem.* **170**, 67.
 Black, S. & Hutchens, J. O. (1948). *Arch. Biochem.* **17**, 211.
 Bloch, K. (1948). *Cold Spr. Harb. Sym. quant. Biol.* **13**, 29.
 Brady, R. O. & Gurin, S. (1950). *Fed. Proc.* **9**, 155.
 Clifton, C. E. (1946). *Advanc. Enzymol.* **6**, 269.
 Coxon, R. V., Liébecq, C. & Peters, R. A. (1949). *Biochem. J.* **45**, 320.
 Dickens, F. & Greville, G. D. (1933). *Biochem. J.* **27**, 1479.
 Dickens, F. & Šimer, F. (1931). *Biochem. J.* **25**, 973.
 Dodds, E. C. & Greville, G. D. (1933). *Nature, Lond.*, **132**, 966.
 Dodds, E. C. & Greville, G. D. (1934). *Lancet*, **1**, 398.
 Folley, S. J. (1949). *Biol. Rev.* **24**, 316.
 Folley, S. J. & French, T. H. (1949a). *Biochem. J.* **45**, 117.
 Folley, S. J. & French, T. H. (1949b). *Biochem. J.* **45**, 270.
 Folley, S. J. & French, T. H. (1950). *Biochem. J.* **46**, 465.
 Friedemann, T. E. (1938). *J. biol. Chem.* **123**, 161.
 Friedemann, T. E. & Haugen, G. E. (1943). *J. biol. Chem.* **147**, 415.
 James, A. T. & Martin, A. J. P. (1951). *Biochem. J.* **48**, vii.
 Kalnitsky, G. & Barron, E. S. G. (1947). *J. biol. Chem.* **170**, 83.
 Knott, C. B. & Petersen, W. E. (1946). *J. Dairy Sci.* **29**, 115.
 Krebs, H. A. & Eggleston, L. V. (1940). *Biochem. J.* **34**, 442.
 Krebs, H. A. & Eggleston, L. V. (1945). *Biochem. J.* **39**, 408.
 Krebs, H. A. & Henseleit, K. (1932). *Hoppe-Seyl. Z.* **210**, 33.
 Lipmann, F. (1946). *Advanc. Enzymol.* **6**, 231.
 Lipschitz, M. A., Potter, V. R. & Elvehjem, C. A. (1938). *J. biol. Chem.* **123**, 267.
 Long, C. (1938). *Biochem. J.* **32**, 1711.
 Long, C. & Peters, R. A. (1939). *Biochem. J.* **33**, 759.
 Loomis, W. F. & Lipmann, F. (1948). *J. biol. Chem.* **173**, 807.
 Markham, R. (1942). *Biochem. J.* **36**, 790.
 Meyerhof, O. (1925). *Biochem. Z.* **162**, 43.
 Meyerhof, O. (1942). In *A Symposium on Respiratory Enzymes*. Madison: University of Wisconsin Press.
 Moore, R. O. & Nelson, W. L. (1951). *Fed. Proc.* **10**, 226.
 Nelson, N. (1944). *J. biol. Chem.* **153**, 375.
 Popják, G., French, T. H. & Folley, S. J. (1951). *Biochem. J.* **48**, 411.
 Potter, V. R. (1944). *Advanc. Enzymol.* **4**, 201.
 Smedley-MacLean, I. (1936). *Ergebn. Enzymforsch.* **5**, 285.
 Stadie, W. C. & Riggs, B. C. (1944). *J. biol. Chem.* **16**, 187.
 Turner, C. (1950). *Biochem. J.* **47**, xlix.
 Weil-Malherbe, H. & Bone, A. B. (1949). *Biochem. J.* **45**, 377.

Hippuric Acid Synthesis in the Rat

BY H. R. V. ARNSTEIN AND A. NEUBERGER
National Institute for Medical Research, Mill Hill, London, N.W. 7

(Received 16 February 1951)

The conjugation of benzoic acid with glycine in the mammalian body which was discovered by Wöhler more than 120 years ago, has been the subject of a large number of publications. Many workers have tried to establish the source of the glycine which is made available for this conjugation without obtaining a really satisfactory answer. Lehmann (1851) suggested that the 'nitrogenous constituent of hippuric acid which we may regard as fumaramide or as glycine, is undoubtedly derived from the animal albuminous substances'. This idea was further elaborated by Wiechowski (1906), who believed that glycine or glycine precursors are formed in large amounts during the normal metabolism of proteins. Since administration of big doses of benzoate may lead to the excretion of considerable quantities of hippuric acid and a corresponding diminution of urea output, Wiechowski (1906) concluded that at least a large part of the normal catabolism of protein to urea takes place through glycine. An alternative possibility, namely that administration of benzoate stimulates the break-

down of tissue proteins and thus makes glycine available, was first put forward by Ringer (1911) and further discussed by Delprat & Whipple (1921). The observation made by various workers that the total nitrogen of the urine increased somewhat after giving large doses of benzoate was quoted as evidence for this assumption. However, other workers calculated that the glycine content of tissue proteins was not large enough to account for the amounts of hippuric acid excreted in experiments in which benzoate was fed over long periods. It was therefore suggested that the glycine was derived from other amino-acids (Magnus-Levy, 1907) or synthesized from ammonia and acetic acid (Cohn, 1893, 1905) or glyoxylic acid (Friedmann & Tachau, 1911) respectively. It was shown later that addition to the diet of glycine or of proteins rich in glycine accelerates the rate of excretion of hippuric acid in the rabbit (Griffith & Lewis, 1923) and increases markedly the output of hippuric acid in the pig (Abderhalden & Strauss, 1914; Csonka, 1924) and to a lesser extent in the dog (Quick, 1926). A similar

effect of glycine ingestion was found in man, and it was suggested that the most important factor determining the extent of hippuric acid synthesis is 'the rate at which glycine is synthesized in the body' (Quick, 1931).

More recently it was found that administration of benzoate produces in man a decrease of the content of free glycine in the plasma (Christensen, Cooper, Johnson & Lynch, 1947; de Vries & Alexander, 1948) and in the guinea pig a very marked reduction in the free glycine of liver, but not of muscle (Christensen, Streicher & Elbinger, 1948). These findings suggest that stores of free glycine in liver, kidney and plasma are primarily concerned in the conjugation. Moreover, the high isotope content of the hippuric acid obtained on simultaneous administration of ^{15}N -labelled glycine and sodium benzoate (Rittenberg & Schoenheimer, 1939; Waelsch & Rittenberg, 1941, 1942) supports such a conclusion. But the amounts of free glycine present in liver and plasma are sufficient only for the conjugation of very small amounts of benzoate, and the larger part of the glycine required under the usual conditions must ultimately be formed from other glycine stores, by synthesis or from the hydrolysis of proteins and other substances.

The question of the source of glycine used for hippuric acid formation has recently become important in the interpretation of metabolic experiments in which isotopically labelled compounds are used. Various workers (Shemin, 1946; Arnstein & Neuberger, 1949, 1951) have fed simultaneously benzoate and labelled potential precursors of glycine, isolated hippuric acid and determined its isotope content. It is clear that, if benzoate stimulates glycine synthesis or protein breakdown, the isotope content of the hippuric acid cannot be quantitatively related to normal glycine metabolism without further information.

In the present paper an attempt was made to obtain further information on this conjugation by varying the doses of labelled glycine and of benzoate, by varying the time interval between the administration of the two substances and relating the observed isotope values to these changes.

EXPERIMENTAL

Animals and diets. In all experiments but one, male and female adult albino rats of the Wistar strain supplied by Dr K. Coward, Pharmaceutical Society, were used. The body weights of the animals ranged from 220 to 460 g. For a week before the beginning of the experiment and during the experiment a low-protein diet of the following composition was fed: casein, 3; yeast, 5; salt mixture (Hubbell, Mendel & Wakeman, 1937), 4; maize starch, 80; mixture of 4 parts of cod liver oil and of 1 part of arachis oil, 8%.

Administration of glycine and sodium benzoate. Glycine labelled with ^{15}N (31.0 atom % excess) and glycine labelled

with ^{14}C in the methylene group having a specific activity of $1 \mu\text{c./mg.}$ were mixed with weighed amounts of non-isotopic glycine. The glycine mixture was dissolved in water and the solution made up to a suitable volume. Sodium benzoate, A.R., was dissolved in water to a final concentration of 5 or 10% (w/v) depending on the dose. In one set of experiments the dose of glycine was kept low and constant while the dose of benzoate was varied within wide limits. In another experiment the dose of glycine was varied while the dose of benzoate was kept constant and in a third type of experiment doses of both glycine and benzoate were kept constant and the time interval between administration of the former and of the latter was varied. Both glycine and benzoate were, except in one experiment (p. 157), administered intraperitoneally by injection.

Collection of hippuric acid and determination of isotope content. The rats were kept during the experiments in metabolism cages and the urine was collected for 24 hr. after injection of the benzoate. The hippuric acid was isolated as described (Arnstein & Neuberger, 1951) and recrystallized from water to constant melting point. In several experiments the specific radioactivity was determined after successive recrystallizations from water. No significant change was found after one recrystallization.

The excess ^{15}N in the hippuric acid was measured in the mass spectrometer (Sprinson & Rittenberg, 1949). The specific radioactivities were determined with a bell-shaped helium-filled Geiger-Müller counter having a thin mica window. Background count was 11–13 counts/min. The measurements were made on solid samples spread evenly on disks of identical geometry and having 1 sq.cm. area. Each sample contained at least 25 mg./sq.cm. and the number of counts is therefore linearly proportional to activity. No correction for self-absorption was made. In one experiment some of the glycine used for the injections was converted to hippuric acid *in vitro* by the Schotten-Baumann method and the specific activity of the latter determined. This produced a satisfactory check on the calculation of dilution factors which were obtained by comparison of the specific activities of the injected glycine and of the isolated hippuric acid. Results are expressed as counts/min.

Nitrogen balance. In one experiment a group of seven female albino rats of body weight ranging from 245 to 260 g. was put for 2 weeks on the low-protein diet fed *ad lib.* Food consumption was measured accurately and the total N of the urine was determined. The rats were kept in metabolism cages and the urine collected over periods of 24 hr. The cages, wire screens and funnels were washed down with water, the combined washings and urine were filtered and the volume made up to 100 ml. N was determined by the micro-Kjeldahl method on 1 ml. samples. The rats were then given subcutaneously varying doses of sodium benzoate together with a constant amount of ^{15}N -labelled glycine. Daily food consumption and urinary N excretion were measured for 1 week after injection. Hippuric acid was isolated and its ^{15}N content determined.

RESULTS

Nitrogen balance and hippuric acid formation

In the first experiment, the details of which are given in Table 1, the effect of benzoate, administered subcutaneously, on nitrogen balance was determined. During the first period, before the injection of the benzoate, the average daily food

intake amounted to 14.0 g./rat and the average urinary N excretion was 78 mg./rat/day. After injection of the benzoate, food intake fell sharply in all animals. It dropped about 8 g./rat/day for the first 3 days in the four rats which had received 0.10 or 0.30 mmol. benzoate/100 g. and reached the normal level again 6 days after injection. With doses of 0.5 or 0.75 mmol. benzoate/100 g. food intake decreased during the first 3 or 4 days to about 5 and 3 g./rat/day respectively and increased to about 10 g./day at the end of 1 week. The daily urinary N did not increase in the two rats which had received the lowest dose of benzoate, but rose to about 100 mg. the day after the injection in the two rats given 0.30 mmol. benzoate/100 g. With the three rats which received the larger doses of benzoate the urinary N increased the day after the injection to between 120 and 150 mg./day. Normal levels of N excretion were again obtained 5 days after the injection. All animals showed a loss of weight. Injection of benzoate thus produced in all rats a marked negative N balance and the loss of nitrogen from the body was greater than could be accounted for by the glycine required for the conjugation with benzoate.

Griffith (1929) has shown that continuous feeding of a diet containing 35 % casein and varying amounts of benzoate to rats produces loss of weight and reduction of food intake. The effects found in our experiments were apparently more severe than those observed by Griffith; this may be due to the very low protein content of our diet. However, the subcutaneous administration of benzoate used in this first experiment produced lesions on the site of the injection and thus local inflammatory reactions may have enhanced the systemic toxic effect of benzoate. In subsequent experiments we therefore used the intraperitoneal route.

The effect of the dose of benzoate on the isotope content of hippuric acid

Simultaneous administration of benzoate and glycine. Increase of the dose of the benzoate reduced markedly the ^{15}N content of hippuric acid as shown in Table 1, which also demonstrates the uniformity of response of different animals to identical doses of glycine and benzoate. Thus the differences in isotope content of hippuric acid excreted by animals which had received similar treatment rarely exceeded 15 %. In a second experiment a mixture of ^{14}C - and ^{15}N -labelled glycine was given, the lowest dose of benzoate was reduced to 0.05 mmol./100 g. and the substances were injected intraperitoneally. In this and following experiments the urines of animals receiving identical doses of benzoate/unit body weight were combined. A comparison of the results of the two experiments (Tables 1 and 2) shows that the dilution factors and even more the dilution coefficients, which take the dose of glycine/unit body weight into account, were very similar for identical doses of benzoate. Thus the dilution coefficient was about four to five times greater with the highest doses of benzoate than with the lowest dose investigated. Reduction of the dose of benzoate from 0.1 to 0.05 mmol./100 g. produced a marked change in isotope content and it is likely, therefore, that a further decrease of the amount of benzoate administered would have further increased the isotope content of the hippuric acid. All the results obtained with simultaneous administration of variable amounts of benzoate and more or less constant amounts of glycine have been collected in Fig. 1. The relationship between benzoate concentration and isotope content of hippuric acid appears to be neither strictly linear

nor exponential and extrapolation to zero benzoate concentration would therefore be hazardous. It would seem, however, that the corrected dilution coefficient approaches a value of about 0.1 mmol./100 g. body weight or less, as the amount of benzoate administered becomes very small.

Fig. 1. The effect of dose of benzoate on the isotope content of hippuric acid (expressed as corrected dilution coefficient (c.d.c.), see Table 1) on simultaneous administration of labelled glycine and sodium benzoate. Some of the experimental points are taken from Tables 1 and 2.

Effect of administration of benzoate at various times after that of glycine. When differing doses of benzoate were given to rats which had received 3 hr. earlier identical doses of glycine/unit body weight, the isotope content of hippuric acid still varied with the benzoate concentration (Table 3), but not quite as much as in the earlier experiments (Tables 1 and 2) in which glycine and benzoate were given simultaneously. When, however, the benzoate was injected 6 hr. after glycine had been given, the isotope content of the hippuric acid was independent of the dose of benzoate (Table 3). A similar result was obtained, when the interval between the administration of the two substances was increased to 24 hr.

In this experiment 0.044 mmol. glycine (containing 31.0 atom % excess ^{15}N)/100 g. body weight was injected into twelve rats. Amounts of benzoate similar to those used in the earlier experiments (0.1–0.6 mmol./100 g. body weight) were injected 24 hr. later and the hippuric acid isolated. The mean ^{15}N contents of the hippuric acid samples were 0.118, 0.120, 0.142, 0.122 and 0.128 atom % excess. Thus there was no evidence for a variation of ^{15}N content with the amount of benzoate administered.

In another experiment (Table 4) the interval was increased to 67.5 hr. The individual variations appear to be somewhat greater than in the earlier experiments, but again there was no evidence for a diminution of isotope content with increasing amounts of benzoate administered.

The ratio of ^{14}C and ^{15}N content in the isolated hippuric acid compared with that of the injected glycine. In many of the experiments glycine labelled with ^{14}C and ^{15}N was used and both isotopes were determined in the hippuric acid. The probable error of the 'isotope ratio', i.e. the quotient of the dilution factor for ^{14}C over that for ^{15}N , is a function of the mean errors of four separate estimations. The isotope content of the administered glycine was estimated with an

Table 1. ^{15}N contents of hippuric acid samples excreted by rats which had been injected subcutaneously with ^{15}N -labelled glycine (31.0 atom % excess) and varying doses of sodium benzoate at the same time

(The animals were female albino rats of body weight between 225 and 260 g.; they received 10 mg. isotopic glycine/100 g. body weight. The corrected dilution factor (C.D.F.) is defined as $C_0/C - 1$, where C_0 is the isotope content of administered glycine and C the isotope content of the excreted hippuric acid. The corrected dilution coefficient (C.D.C.) is defined as $(C_0/C - 1)Q$, where Q is the administered dose of glycine in mmol./100 g. body weight. This form of representing results is considered in the Discussion, p. 159.)

Rat no.	Dose of benzoate (mmol./100 g. body wt.)	^{15}N content of hippuric acid (atom % excess)	C.D.F.	C.D.C. (mmol./100 g. body wt.)
101	0.1	4.380	6.1	0.32
102	0.1	5.035	5.1	0.27
103	0.3	2.612	10.8	0.57
104	0.3	2.830	10.0	0.53
105	0.5	2.280	12.6	0.67
106	0.5	2.115	13.6	0.72
107	0.75	1.409	20.7	1.10

Table 2. Isotope contents of hippuric acid samples excreted by rats which had been injected intraperitoneally with doubly labelled glycine and sodium benzoate simultaneously

(The glycine contained 15.5 atom % excess ^{15}N and gave 866.5 counts/min. Each animal received 0.033 mmol. glycine/100 g. body weight. Where small doses of benzoate were given, the urines of rats which had received identical amounts of benzoate/unit body weight were combined as indicated in the table. For definition of corrected dilution factor (C.D.F.) and corrected dilution coefficient (C.D.C.) see Table 1. Isotope ratio is the quotient of the ratio of the radioactivity of the injected glycine to that of the excreted bound glycine (both expressed as counts/min.) by the ratio of the ^{15}N content (atom % excess) of the injected glycine to that of the excreted hippuric acid. The radioactivity of the excreted glycine was obtained by multiplying the observed radioactivity of the hippuric acid by the ratio of molecular weight of hippuric acid to that of glycine.)

Rats no.	Dose of benzoate (mmol./100 g. body wt.)	Hippuric acid		C.D.F. from results with		C.D.C. (mmol./100 g. body weight) from results with		Isotope ratio
		^{14}C content (counts/min.)	^{15}N content (atom % excess)	^{14}C	^{15}N	^{14}C	^{15}N	
1, 2, 3	0.05	138	2.467	5.2	5.3	0.172	0.175	0.98
4, 5, 6	0.10	95.6	1.737	8.1	7.9	0.267	0.260	1.03
7, 8	0.15	79.8	1.476	9.9	9.5	0.327	0.314	1.04
9, 10	0.20	73.2	1.278	10.8	11.1	0.356	0.366	0.97
11	0.30	52.2	0.890	15.6	16.4	0.515	0.541	0.95
12	0.60	35.5	0.716	23.4	20.7	0.772	0.683	1.13

* Calculated for glycine.

Table 3. Effect of changes in the dose of benzoate on the isotope contents of hippuric acid excreted by rats which had received doubly labelled glycine and, 3 or 6 hr. later, sodium benzoate

(Each animal received 0.044 mmol. glycine/100 g. body weight; the glycine contained 15.62 atom % excess ^{15}N and had radioactivity equivalent to 7460 counts/min. of hippuric acid. For definition of C.D.F. and C.D.C. see Table 1 and for definition of isotope ratio see Table 2.)

		Hippuric acid		C.D.F. from results with		C.D.C. (mmol./100 g. body weight) from results with		Isotope ratio
Rats no.	Dose of benzoate (mmol./100 g. body wt.)	¹⁴ C content (counts/min.)	¹⁵ N content (atom % excess)	¹⁴ C	¹⁵ N	¹⁴ C	¹⁵ N	
Benzoate given 3 hr. after glycine								
37, 38, 39	0.05	163.7	0.357	44.6	42.7	1.96	1.88	1.04
40, 41, 42	0.10	190.3	0.363	38.2	41.2	1.68	1.81	0.95
43, 44	0.15	130.5	0.305	56.2	50.2	2.47	2.21	1.11
45, 46	0.20	115.8	0.289	63.4	53.6	2.81	2.33	1.19
47	0.30	105.9	0.253	69.4	60.7	3.05	2.67	1.14
48	0.60	72.6	0.189	101.7	81.6	4.47	3.59	1.24
Benzoate given 6 hr. after glycine								
49, 50, 51	0.05	—	0.153	—	101	—	4.44	—
52, 53, 54	0.10	63.3	0.158	117	97	5.15	4.27	1.20
55, 56	0.15	60.6	0.149	123	104	5.41	4.58	1.18
57, 58	0.20	62.1	0.151	119	102	5.24	4.49	1.16
59	0.30	56.1	0.145	132	107	5.81	4.71	1.23
60	0.60	56.9	0.140	130	111	5.72	4.88	1.17

— means value not available.

Table 4. *Effect of changes of the dose of benzoate on the isotope content of hippuric acid excreted by rats which had received doubly labelled glycine and 67.5 hr. later sodium benzoate*

(Each animal received 0.044 mmol. glycine/100 g. body weight. The glycine contained 25.2 atom % excess ^{15}N and had radioactivity equivalent to 161,000 counts/min. counted as hippuric acid. The samples of hippuric acid excreted contained between 0.045 and 0.063 atom % excess ^{15}N with a mean value of 0.053 atom % excess and corresponding c.d.f. value of 475. For definition of c.d.f. and c.d.c. see Table 1.)

Rats no.	Dose of benzoate (mmol./100 g. body wt.)	Radioactivity of hippuric acid (counts/min.)*	C.D.F.	C.D.C. (mmol./100 g. body wt.)
31, 32, 33	0.10	238.5	675	29.7
29, 30	0.15	229.5	700	30.8
27, 28	0.20	354.0	454	20.0
26	0.30	269.0	597	26.3
25	0.60	344.0	466	20.5

* Calculated as glycine.

Table 5. *Effect of the dose of glycine on the isotope content of hippuric acid excreted by rats after simultaneous administration of ^{14}C -labelled glycine and sodium benzoate*

(Fractions of approx. 0.1 ml. of solution of labelled glycine (10.2 mg.; activity: 663,100 counts/min.) were weighed out; these were mixed with appropriate amounts of an aqueous solution of non-labelled glycine or of water. Fractions of the diluted solutions were then injected intraperitoneally into four groups of rats (three in each group) so as to give the doses of total glycine/100 g. body weight stated in the table. A solution of sodium benzoate (0.05 mmol./100 g. body weight) was injected at the same time. For definitions of corrected dilution factor (c.d.f.) and corrected dilution coefficient (c.d.c.) see Table 1.)

Rats no.	Glycine given		Activity of hippuric acid (counts/min.)	C.D.F.	C.D.C. (mmol./100 g. body wt.)
	Amount ($\mu\text{mol.}/100\text{ g.}$ body wt.)	Activity (counts/min.)			
76, 77, 78	44.4	1,041	87.8	3.97	0.166
73, 74, 75	11.16	3,585	99.6	14.08	0.157
70, 71, 72	2.84	14,705	108.4	56.0	0.159
67, 68, 69	0.061	663,100	96.7	2873	0.178

Table 6. *Effect of change in the time interval between the administration of glycine and that of benzoate on the isotope content of hippuric acid excreted by rats*

(‘Black and white’ rats weighing about 250 g. were each given intraperitoneally $10\mu\text{C. CH}_3^{14}\text{COONa}$ and 0.4 mmol. glycine (31.0 atom % excess ^{15}N)/100 g. body weight. After intervals given in the table the animals received sodium benzoate (0.42 mmol./100 g. body weight) and ethyl DL- α -amino-phenylbutyrate (0.3 mmol./100 g. body weight). For definition of corrected dilution factor (c.d.f.) and corrected dilution coefficient (c.d.c.) see Table 1. The activities of the acetamido acid are reported by Arnstein & Neuberger, 1951.)

Rat no.	Time between injection of glycine and of benzoate (hr.)	^{15}N content of hippuric acid (atom % excess)	C.D.F.	C.D.C. (mmol./100 g. body wt.)
201	0	10.59	1.93	0.77
202	0	7.29	3.12	1.25
203	3	3.35	8.22	3.29
204	3	3.43	8.05	3.22
205	7	1.88	15.5	6.2
207	12	1.11	27.0	10.8
208	12	1.26	23.5	9.4
211	24	0.84	35.9	14.4

accuracy of ± 1.5 – 2.5% , but for the hippuric acid the error is somewhat larger, especially for samples of low isotope content, and may be as high as $\pm 6.0\%$. It is believed that the values of the isotope ratio in Table 2 do not differ significantly from 1.0, but some of the isotope ratios in Table 3 appeared to be somewhat outside the probable limits of error. Some of the glycine used for the injection was

therefore converted to hippuric acid *in vitro* and its ^{15}N and ^{14}C content estimated. Similarly, most of the hippuric acid samples were recrystallized, counted again and the ^{15}N analysis repeated. The results differed only slightly from those obtained initially. The values in Table 3 are almost consistently above 1.0 with a mean of about 1.15. This may be due to an undetected analytical error or it may indicate

a breakdown of part of the glycine followed by a synthesis in which the nitrogen is re-incorporated to a greater extent than the methylene carbon atom.

The effect of the amount of glycine administered on the isotope content of the hippuric acid. In one experiment the amount of glycine administered was varied between 44.4 and 0.061 mmol./100 g. body weight, whilst the dose of benzoate was kept constant at 0.05 mmol. The results (Table 5) show that, whilst the dilution factor varies within wide limits, the dilution coefficient remains remarkably constant. However, with very high doses of glycine, such as those employed in another experiment (Table 6), the corrected dilution coefficient seems to be about 30% higher than was found with small amounts of glycine using similar intervals and benzoate doses (Tables 1-3).

Fig. 2. The effect of the length of the interval between administration of labelled glycine and that of benzoate to rats on the isotope content of excreted hippuric acid. Curve A refers to low benzoate doses (0.05 mmol./100 g. body weight), whilst curve B refers to high benzoate doses (0.5 to 0.6 mmol./100 g. body weight). C.D.C. is defined in Table 1.

The decrease in isotope content with time of the glycine available for hippuric acid synthesis. If the doses of benzoate and of glycine administered are kept constant and the interval between injection of glycine and of benzoate is varied, the isotope content of the hippuric acid may be expected to yield information about the turnover rate of that 'glycine fraction' which is used in hippuric acid formation. During an investigation on the biosynthesis of glycine from acetate (Arnstein & Neuberger, 1949, 1951) ^{15}N -labelled glycine (0.4 mmol./100 g. body weight) was injected into a group of rats, followed after differing intervals by administration of benzoate. The marked diminution in isotope content which occurred in the first 7 hr. was followed by a slower decrease in the succeeding 17 hr. (Table 6). A similar curve was obtained by plotting against time the values found in the earlier experiments (Tables 1-4) with high benzoate concentrations (Fig. 2, curve B). If experiments with low benzoate concentrations (0.05 mmol./100 g. body wt.) are compared, the difference between the sharp fall in the first few hours after administration of glycine and the relatively slow decrease occurring later becomes even more marked (Fig. 2, curve A). Thus, the isotope content of the glycine used for hippuric acid formation diminished to about one twenty-fifth during the first 6 hr., whilst at the end of the following 60 hr. the further decrease was only to about one-fifth of the 6 hr. value.

DISCUSSION

The significance of dilution factors

The quantitative interpretation of experiments in which labelled compounds are fed to an animal presents difficulties which are largely absent in work on isolated tissue homogenates or enzyme systems. The complexity of the various catabolic and anabolic reactions and our lack of knowledge of the rates of absorption and penetration into cells of the substances under consideration make it necessary to use as a first approach a highly simplified model which is admittedly inadequate, but which can be made more elaborate in the light of further observations.

If an isotopically labelled substance A such as glycine of label content C_q is fed in an amount Q (in mmol./100 g. body weight) and A, or a metabolic derivative thereof, is isolated with a label content C, then the ratio C_q/C is called the dilution factor (Bloch & Rittenberg, 1945). In order to give a physical significance to this factor we assume that the injected or fed glycine mixes very quickly after administration with a fraction or the whole of the free glycine present in the body. This 'first glycine pool' is assumed to be homogeneous with respect to glycine and is considered to be an isolated system, i.e. the rate of diffusion of glycine from one part of the pool to another is assumed to be infinitely fast compared with any other reaction glycine may undergo, whilst diffusion into any other part of the body is assumed to be infinitely slow. It is also postulated that the labelled glycine is quantitatively transferred to this pool and that the rate of this transfer is fast; in other words, absorption is supposed to be quantitative and almost instantaneous. Under such conditions the following equation must hold:

$$\frac{x+Q}{Q} = \frac{C_q}{C}, \quad (1)$$

where x is the amount of glycine (in mmol./100 g.) present in the 'first glycine pool'. From this we get

$$\frac{x}{Q} = \frac{C_q}{C} - 1. \quad (2)$$

In some of the present experiments the dilution factors are small and the use of $C_q/C - 1$, which is named the corrected dilution factor (C.D.F.), is preferable (see Bloch & Rittenberg, 1945). The dilution factor gives only incomplete information about metabolic interrelationships, since the dose of the administered, labelled material and the weight of the animal are not taken into account. Bloch & Rittenberg (1945) have therefore used the product of the dilution factor and of the dose (in mmol./100 g. rat/day) to calculate acetate formation in the rat. We shall use x as defined by equation (1) for a similar purpose, and call it the corrected dilution

coefficient (c.d.c.). The labelled glycine in our experiments was given over an extremely short time and c.d.c. has therefore the dimensions mmol./100 g. of rat.

In this investigation the glycine in the pool after mixing was not isolated as such, but the benzoate which was given was supposed to combine specifically with this free-glycine fraction. For this sampling method to be valid three further conditions have to be fulfilled. The rate of absorption of the benzoate has to be fast relative to the turnover rate of the glycine pool. The conjugation of glycine with the benzoate has to be slow compared with the rate of diffusion of glycine within the pool, i.e. the mixing of the glycine within the 'first pool' has to be complete, before hippuric acid formation takes place. But, on the other hand, this conjugation has to be fast compared with any metabolic reaction of glycine or with the diffusion of glycine to any part of the body outside the pool. If these conditions are met, the isotope content of the excreted hippuric acid is a direct measure of *C* for glycine.

This highly simplified picture yields convenient formulae for the presentation of results of metabolic experiments in which isotopes are used. The assumptions made are, at the best, crude approximations, and it will have to be considered in the light of other evidence how far such an expression as c.d.c. represents an identifiable fraction of the body glycine.

The effect of dose of benzoate on the isotope content of hippuric acid

When the interval between the injection of the labelled glycine and that of benzoate was 6 hr. or more, the isotope content of the hippuric acid was found to be independent of the dose of benzoate. This finding strongly suggests that the administration of benzoate to the rat does not directly increase the rate of synthesis of glycine. If such a stimulation of glycine synthesis were to occur, it should be proportional to the amount of benzoate given and thus lead to a progressive diminution of isotope content of the hippurate with increasing dose of benzoate. If glycine synthesis from non-nitrogenous precursors were to take place, we should also expect a marked change in the ratio of ^{14}C to ^{15}N in the excreted hippuric acid as compared with that of the administered glycine. In fact, the 'isotope ratio' never differed greatly from 1.0, and such increases of 'isotope ratio' as did occur were not correlated with the dose of benzoate. The possibility that administration of benzoate produces an increase in the rate of liberation of glycine from proteins cannot be completely excluded. The relatively small increase in nitrogen excretion on giving benzoate observed by ourselves and other workers suggests that proteolysis is not markedly raised. Such increases of urinary

nitrogen excretion as were observed occurred mainly 1–2 days after the administration of benzoate and are probably associated with the regeneration of the free glycine level in the tissues. Our results show that, if benzoate brings about increased protein breakdown, the additional glycine thus made available must have the same isotope content 6 hr. after the injection of the labelled glycine as the free glycine which is primarily utilized for conjugation of benzoate. Preliminary experiments in this laboratory indicate that the total protein nitrogen of rat liver is not significantly reduced by injection of 0.3 mmol. benzoate/100 g. of rat, and it is therefore believed that proteolysis is not markedly increased during hippuric acid formation.

These conclusions apply only to the rat. In man, evidence has been obtained (Gray & Neuberger, 1950) which suggests that administration of benzoate may lead either to an increased synthesis of glycine or to a mobilization of the glycine bound in proteins.

When benzoate was administered, together with the labelled glycine or 3 hr. later, the isotope content of the hippuric acid fell with increasing doses of benzoate. This may be explained as follows: the investigations of various workers (Griffith & Lewis, 1923; Csonka, 1924; Bray, Thorpe & White, 1951) suggest that in rabbit and man the rate of hippuric acid synthesis is more or less independent of the dose of benzoate, and this presumably also applies to the rat. An increase of dose of benzoate is therefore equivalent to an extension of the time interval during which conjugation takes place. During the first 4–6 hr. after injection of labelled glycine the isotope content of the free-glycine fraction which is utilized for hippurate formation falls very sharply (Fig. 2), whilst later the rate of decrease with time is relatively small. It is therefore easy to understand why an increase of dose of benzoate produces a decrease in isotope content during the first few hours after glycine injection, but not later.

The source of glycine used for hippuric acid formation

For a more quantitative interpretation of the results we shall have to consider the amounts of free glycine and of precursors of free glycine present in various tissues of the rat, the rates of absorption and of transfer of glycine from one compartment of the body to another and the rates of some relevant metabolic reactions. The data on the amounts of free glycine in tissues (Todd & Talman, 1949; Awapara, 1949; Krueger, 1950) are not in very good agreement, but the following approximate figures (expressed in mg./100 g. body weight) may be assigned to the various tissues of the rat: blood, 0.25; kidney, 1.2–1.4; intestine, 3.0; liver, 1.2–2.9; muscle, 25–30. It is likely that the total free glycine of the internal organs is about 5–7 mg./100 g. body

weight, whilst the total free glycine in the whole rat is about 30–45 mg. When labelled glycine is injected, it will be taken up very quickly by the cells of the liver, kidney and intestine. This follows from the isotope experiments of Hultin (1950) on chicks, and those of Borsook, Deasy, Haagen-Smit, Keighley & Lowy (1950) on mice. It appears that muscle cells are not very permeable to glycine (Van Slyke & Meyer, 1913; Luck, 1928; Friedberg & Greenberg, 1947; Handler, Kamin & Harris, 1949; Kamin & Handler, 1951), and it is probable that equilibration of the labelled glycine with the intracellular glycine of muscle will be relatively slow.

In experiments in which labelled glycine and a very small amount of benzoate were injected to-

rate of conjugation of benzoate. This conclusion is supported by the observations that the isotope content of hippuric acid is not markedly affected by the dose of glycine in which a constant amount of isotope is administered, at least within wide limits (Table 5). The figure for the 'first glycine pool' is much lower than the total free glycine of the body and this finding is compatible with the assumption that with small amounts of benzoate conjugation is complete before any extensive mixing with the free intracellular glycine of tissues other than those of the internal organs has taken place.

The interpretation offered here postulates that the isotope content of the hippuric acid is a measure of the mean isotope content of the free glycine of the

Fig. 3. Diagrammatic representation of possible fate of glycine. Rates of reactions indicated as follows in decreasing order: VF , very fast; F , fast; MF , moderately fast; M , moderate (come to equilibrium within 4–5 hr. after injection); S , slow (not near equilibrium after 4–5 hr.).

gether (Table 2), a value for c.d.c. or 'first glycine pool' of about 0.17 mmol./100 g. or 10 mg./100 g. of rat was obtained. It is possible that a lower value might have been found, if it had been possible to reduce further the dose of benzoate. In any case the figure of 10 mg. is not very much higher than the value of the estimated free glycine content of the internal organs which is approx. 5–7 mg. With any finite amount of benzoate, conjugation will require a period of time during which the labelled glycine will be diluted owing to incorporation into proteins, the occurrence of other reversible and irreversible reactions and also due to some penetration into cells of tissues other than those of internal organs. It is thus not surprising that even the lowest value for c.d.c. is higher than the amount of free glycine in internal organs. It is suggested that the rate of mixing of labelled glycine with the glycine of liver and that of other organs with similar permeability characteristics is relatively fast compared with the

liver over a time interval, the length of which is a function of the dose of benzoate. The time curve (Fig. 2) is thus a rough representation of the changes of the isotope content of free glycine of rat liver after intraperitoneal injection of labelled glycine. This curve indicates that during the first 4–5 hr. after injection the isotope content falls sharply. This rapid decrease must be caused by relatively fast reactions such as formation of glutathione and serine, incorporation of glycine into proteins with high turnover rates and diffusion of glycine into muscle and possibly brain cells. It would also appear that the 'half-life' of the free liver glycine is extremely short. After about 6 hr. the total free glycine in the body, the serine and the glycine bound in glutathione and in some protein or peptide fractions are likely to be in approximate equilibrium with respect to their isotope contents. The much slower rate of dilution observed in the later part of the curve (Fig. 2) will represent mainly the re-

latively low turnover rates of the bulk of the proteins of the internal organs and of muscle and other proteins of the body. Diminution of isotope content will also occur owing to synthesis of glycine which will affect both parts of the curve.

Whilst accurate information about the rates of relevant reactions is lacking, the present results are compatible with the interpretations discussed above and which are diagrammatically represented in Fig. 3.

SUMMARY

1. Hippuric acid formation by the rat has been studied by injecting graded doses of sodium benzoate and ^{15}N - and $[\alpha\text{-}^{14}\text{C}]$ -labelled glycine at different time intervals and isolating the excreted hippuric acid. With doubly labelled glycine no marked change in the $^{15}\text{N}/^{14}\text{C}$ ratio was found.

2. With simultaneous administration of benzoate and labelled glycine, increase of the benzoate dose

diminished markedly the isotope content of the excreted hippuric acid.

3. Injection of varying doses of benzoate 3 hr. after the glycine resulted in only slight variations of the isotope content of the hippuric acid; with time intervals of 6 hr. or longer there was no variation.

4. Within wide limits the quantity of glycine in which a given amount of isotope was administered had little or no effect on the isotope content of the hippuric acid.

5. It is concluded that the amount of glycine immediately available for conjugation with small doses of benzoate is about 10 mg./100 g. body weight. This 'first glycine pool' is in relatively slow equilibrium with a larger 'pool' of glycine and glycine precursors, which participate in hippuric acid formation only when larger doses of benzoate are given.

We wish to thank Dr R. Bentley and Mr G. Dickinson for the ^{15}N analyses, and Miss B. Wiltshire for technical assistance.

REFERENCES

- Abderhalden, E. & Strauss, H. (1914). *Hoppe-Seyl. Z.* **91**, 81.
 Arnstein, H. R. V. & Neuberger, A. (1949). *Biochem. J.* **45**, iii.
 Arnstein, H. R. V. & Neuberger, A. (1951). To be published.
 Awapara, J. (1949). *J. biol. Chem.* **178**, 113.
 Bloch, K. & Rittenberg, D. (1945). *J. biol. Chem.* **159**, 45.
 Borsook, H., Deasy, C. L., Haagen-Smit, A. J., Keighley, G. & Lowy, P. H. (1950). *J. biol. Chem.* **187**, 839.
 Bray, H. G., Thorpe, W. V. & White, K. (1951). *Biochem. J.* **48**, 88.
 Christensen, H. N., Cooper, P. F., Johnson, R. D. & Lynch, E. L. (1947). *J. biol. Chem.* **168**, 191.
 Christensen, H. N., Streicher, J. A. & Elbinger, R. L. (1948). *J. biol. Chem.* **172**, 515.
 Cohn, R. (1893). *Hoppe-Seyl. Z.* **17**, 274.
 Cohn, R. (1905). *Arch. exp. Path. Pharmac.* **53**, 435.
 Csonka, F. A. (1924). *J. biol. Chem.* **60**, 545.
 Delprat, G. D. & Whipple, G. H. (1921). *J. biol. Chem.* **49**, 229.
 Friedberg, E. F. & Greenberg, D. M. (1947). *J. biol. Chem.* **168**, 411.
 Friedmann, E. & Tachau, H. (1911). *Biochem. Z.* **35**, 88.
 Gray, C. H. & Neuberger, A. (1950). *Biochem. J.* **47**, 81.
 Griffith, W. H. (1929). *J. biol. Chem.* **82**, 415.
 Griffith, W. H. & Lewis, H. B. (1923). *J. biol. Chem.* **57**, 1.
 Handler, P., Kamin, H. & Harris, J. S. (1949). *J. biol. Chem.* **179**, 283.
 Hubbell, R. B., Mendel, L. B. & Wakeman, A. J. (1937). *J. Nutrit.* **14**, 273.
 Hultin, T. (1950). *Exp. Cell Res.* **1**, 376.
 Kamin, H. & Handler, P. (1951). *J. biol. Chem.* **188**, 193.
 Krueger, R. (1950). *Helv. chim. Acta*, **33**, 429.
 Lehmann, C. G. (1851). *Physiological Chemistry*, vol. 1, p. 198, translated by G. E. Day. London: Harrison and Son.
 Luck, J. M. (1928). *J. biol. Chem.* **77**, 1.
 Magnus-Levy, A. (1907). *Biochem. Z.* **6**, 523.
 Quick, A. J. (1926). *J. biol. Chem.* **67**, 477.
 Quick, A. J. (1931). *J. biol. Chem.* **92**, 65.
 Rittenberg, D. & Schoenheimer, R. (1939). *J. biol. Chem.* **127**, 329.
 Ringer, A. I. (1911). *J. biol. Chem.* **10**, 327.
 Shemin, D. (1946). *J. biol. Chem.* **162**, 297.
 Sprinson, D. & Rittenberg, D. (1949). *J. biol. Chem.* **180**, 707.
 Todd, W. R. & Talman, E. (1949). *Arch. Biochem.* **22**, 386.
 Van Slyke, D. D. & Meyer, G. M. (1913). *J. biol. Chem.* **16**, 197.
 de Vries, A. & Alexander, B. (1948). *J. clin. Invest.* **27**, 655.
 Waelsch, H. & Rittenberg, D. (1941). *J. biol. Chem.* **139**, 761.
 Waelsch, H. & Rittenberg, D. (1942). *J. biol. Chem.* **144**, 53.
 Wiechowksi, W. (1906). *Beitr. chem. Physiol. Path.* **6**, 204.