

the eliminated protein can be recovered in the liver but very little in the lungs.

2. Iodoproteins injected intravenously into specifically immunized rabbits are eliminated from the blood at the same rate as in normal animals, but immunized rabbits with good antisera to 2-dichloroethyl sulphone-treated proteins eliminate these proteins from their blood more rapidly than do normal animals.

3. With both antigens the deposition in the liver and lungs is greater in immune animals than in controls.

4. One hour after injection of iodoprotein at least one-third of the radioactivity in the blood is no longer protein-bound.

5. The deiodination of iodoproteins in the tissues renders them unreliable for studies *in vivo*, even for comparatively short-term experiments.

We are most grateful to Professor A. Wormall for his constant advice and encouragement. We are indebted to the Medical Research Council and the Central Research Fund of the University of London for grants (to Professor A. Wormall) which have partly covered the costs of these investigations. We are also thankful to Mrs A. E. Almond for assistance with many of the experiments described here, and to Miss A. Routledge for technical help throughout these investigations.

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The Formation of Hippuric Acid

THE INFLUENCE OF BENZOATE ADMINISTRATION ON TISSUE GLYCINE LEVELS

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(Received 20 July 1956)

The conjugation of benzoic acid with glycine has been extensively studied by many workers. One aspect of the problem that has received much attention is the origin of the glycine used for conjugation, but the exact source nevertheless remains uncertain. In the present investigation the problem has been approached by studying the concentration of certain tissue metabolites under various experimental conditions; this paper deals with the influence of the administration of benzoate upon the concentration of tissue glycine. The aim was to disturb the normal precursor \rightarrow glycine reaction as little as possible, since the administration of relatively large amounts of suspected glycine precursors may cause unusual metabolic pathways to be followed. Few studies comparable with the present

one appear to have been carried out. Christensen, Cooper, Johnson & Lynch (1947) and Vries & Alexander (1948) found that the administration of benzoate produced a fall in the concentration of free glycine in the blood of man, and Christensen, Streicher & Elbinger (1948) observed a similar decrease in the blood and liver of the guinea pig, although there was only a small change in the concentration of glycine in the kidney and no detectable change in muscle.

In the present investigation the influence of the administration of benzoate on the free glycine of various rat tissues and on the protein glycine of liver has been studied. Experiments in which blood glycine was investigated were also repeated on the rabbit since consecutive blood samples could be obtained from a single animal. In most of the experiments with the rabbit, urine samples were

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also collected in order to study the kinetics of the conjugation reaction with respect to the conjugated moiety (cf. Bray, Thorpe & White, 1951).

METHODS

Animals and diet. Female albino rats (body wt. usually 130–170 g.) and female rabbits (2.5–3.3 kg. body wt.) were used. The animals were maintained on a normal diet of cubes or pellets except that, to eliminate any effects of dietary glycine, they were restricted to a 'protein-free' diet during the 48 hr. before an experiment. This diet for rats consisted of wheat starch 90%, arachis oil 6.4% and salt mixture (Hubbell, Mendel & Wakeman, 1937) 3.6%, and that for rabbits contained wheat starch 89%, paper pulp 9% and salt mixture 2%; the starch and salt mixture were made into a smooth paste with water and the paper pulp was added and mixed with the paste. The mixture was baked at 100° until hard and then broken up into small lumps. These diets contained less than 1 mg. of N/g. A ration (10 g. for the rat and 50 g. for the rabbit) of the diet was given 48 and 16 hr. before the experiment began. Rats and rabbits were kept in wire cages with wide-mesh floors.

Dosage. Sodium benzoate was administered by intraperitoneal injection of an aqueous solution at a dose level of approximately 400 μ moles/100 g. body wt., unless otherwise stated. The volume injected was generally 1 ml. for a rat and 10 ml. for a rabbit.

Preparation of tissues for analysis

Blood. Samples were obtained from rats by cardiac puncture under ether anaesthesia; coagulation was prevented by collecting blood in a syringe rinsed with citrate and discharging it into 1% (w/v) sodium citrate in 0.9% (w/v) saline (0.2 ml./ml. of blood). In the rabbit, blood was collected from a marginal vein of the ear; coagulation was prevented either by the use of oxalate or, more frequently, by collecting the blood directly into tungstic acid mixture (equal parts of 0.66N-H₂SO₄ and 10% (w/v) Na₂WO₄·2H₂O).

Other tissues. Rats were killed either by a sharp blow on the head or, if blood samples were required, by severing the aorta after cardiac puncture. The required organs were rapidly dissected out, washed with water, dried with filter paper and placed in tared beakers immersed in a freezing mixture of solid carbon dioxide and ethanol. All tissues were in the freezing mixture within 2 min. of the death of the animal. The individual tissues from a particular group of rats were pooled. The contents of each beaker were allowed to thaw slightly and then minced very finely with scissors and well mixed. A sample (usually 1.5–3 g.) was then frozen solid and kept in this condition until worked up (up to 5 hr.).

The sample, mixed with tungstate mixture (1.5–2 ml./g. of tissue) and 15–20 ml. of water, was comminuted in a macerator (Measuring and Scientific Equipment Ltd.) for 3–5 min. at 14 000 rev./min. (Histological examination of a kidney preparation, made under these conditions except that tungstate mixture was replaced by water, showed that no intact cells remained.) The suspension was diluted with water to 50 ml. and the protein removed by centrifuging. The tungstic acid filtrate was stored overnight in the refrigerator until assayed.

The protein fraction separated by centrifuging was suspended successively twice in 5% (w/v) trichloroacetic acid

(10 ml.), twice in acetone (15 ml.) and once in ether (15 ml.); the suspension was centrifuged and the supernatant was discarded at each stage (cf. Winnick, Friedberg & Greenberg, 1948). The protein was air-dried at room temperature.

Analytical methods

Free glycine. The tungstic acid filtrate described above was assayed by the method of Alexander, Landwehr & Seligman (1945), as modified (modification A) by Christensen, Riggs & Ray (1951); this depends on the deamination of glycine by ninhydrin to form formaldehyde, which is determined with chromotropic acid (1:8-dihydroxynaphthalene-3:6-disulphonic acid). A Spekker photoelectric absorptiometer was used with Ilford 605 filters. Samples of filtrate containing 1.5–3 μ g. of glycine/4 ml. were used.

The term 'free' glycine refers to that glycine which appears to be free when determined by customary analytical procedures.

Total non-protein glycine. This was determined in the hydrolysed tungstic acid filtrate. Traces of protein, if present, were first removed by acidification (0.1 ml. of 6N-HCl/10 ml. of filtrate), heating for 1 min. in a boiling-water bath and filtering off the coagulated protein. A sample of the filtrate (5 ml.) was hydrolysed for 36 hr. with conc. HCl (2.5 ml.) in a boiling-water bath and then evaporated to dryness. Water was added to the residue and again removed by evaporation. Water (5 ml.) was added to this residue and a sample of the solution (4 ml. for blood, 1 ml. for tissues) assayed as above. Control experiments with blood and liver in which deproteinized filtrates were compared with diffusates prepared by a method similar to that of Hamilton & Archibald (1944) gave results agreeing within 0.2 mg. of glycine N/100 g. (cf. Christensen & Lynch, 1946).

Hippuric acid glycine. A sample of the tungstic acid filtrate (5 ml. for blood, 10 ml. for other tissues) was further acidified with 1 ml. of 2N-H₂SO₄ and continuously extracted with redistilled peroxide-free ether for 6 hr. The solvent was removed from the extract and 6N-HCl (2 ml.) was added to the residue, which was then refluxed on a sand bath for 2 hr. The acid was removed by evaporation to dryness. The residue was dissolved in 5 ml. of water and 3 ml. was taken for glycine assay.

Protein glycine. The air-dried protein fraction described above was hydrolysed by heating with 6N-HCl (9 ml.) in a boiling-water bath for about 36 hr. The hydrolysate was diluted to 50 ml. with water and the 'humin' filtered off. The samples (0.1 ml.) used for assay did not contain sufficient HCl to alter significantly the pH of the buffer used in the glycine determination.

Peptide non-protein glycine. This term is used to express the difference between total non-protein glycine and free glycine plus, if the amount is significant, hippuric acid. This is probably mainly glutathione (Arnstein, 1954).

Analysis of urine

The rate of excretion of hippuric acid by the rat was determined on urine samples collected after the administration of benzoate. A correction was applied for the excretion under 'base-line' conditions determined under similar conditions on the previous day. To stimulate the flow of urine, the rats were given water (2.5–5 ml., depending on body wt.) by stomach tube at three equal intervals during the period of urine collection, which was usually for 4.5–5 hr. after benzoate administration. Conjugation of the

larger doses (about 400 μ moles/100 g. body wt.) used is not complete in this period. Hippuric acid was estimated by the method of Bray, Clowes, Thorpe, White & Wood (1952). In most experiments with rabbits, urine was collected as described by Bray *et al.* (1951) and the urinary ether-soluble acid (Bray, Neale & Thorpe, 1946), after correction for free benzoic acid, was taken as a measure of the excretion of hippuric acid.

Plan of experiments

Rats. In general, six groups of two rats of similar weight were used. Those in groups A and B, injected with water, served as controls and those in groups C, D, E and F were injected with sodium benzoate. The latter were killed at suitable time intervals after injection (up to 5 hr.). Rats in group A were killed at the same time as those in group C, and those in group B at the same time as those in group F. The mean values from the two control groups were taken as the zero-time values for each experiment. In some experiments, however, rats of a control group were killed at the same times as those in each experimental group.

Rabbits. Two blood samples were collected before the administration of benzoate and others at suitable intervals after administration. Control experiments showed that no significant variation in the concentration of free glycine occurred during the period of observation in the absence of benzoate. In most experiments urine was also collected.

RESULTS

Effect of administration of benzoate on the free-glycine level of blood and tissues

The concentration of free glycine in rat liver fell after the administration of benzoate and a steady low level about 50% of the initial level was established within 0.5–1.5 hr. of dosage (Table 1). More detailed results from two typical experiments are shown in Fig. 1. The relative magnitude of the change appeared to be independent of the size of the dose within the range (170–470 μ moles/100 g. body wt.) studied. The correlation coefficient, r , was 0.01, ($n=23$). The steady low glycine level was of short duration in curve B of Fig. 1 on account of the very

small dose level used; it was calculated that excretion of hippuric acid was complete in 4 hr. (curve F). In the experiments at higher dose levels excretion of hippuric acid was not complete until after about 8 hr.

Administration of benzoate to the rat produced a fall in blood free glycine, which, as shown in Table 1, appears to be significant. The relative fall (to about 80% of the original level) was smaller than in liver and was unrelated to dose level ($r=0.09$, $n=18$). Similar results were obtained when consecutive blood samples were taken from a

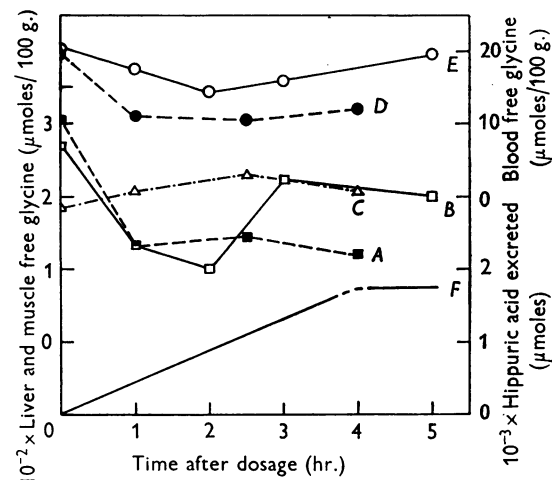


Fig. 1. Effect of intraperitoneal injection of benzoate upon the concentration of free glycine in rat liver, muscle and blood. Doses (given in parentheses) are in μ moles/100 g. body wt. A (■), Liver (350); B (□), liver (170); C (△), muscle (380); D (●), blood in Expt. A; E (○), blood in Expt. B; F, excretion curve for hippuric acid for Expt. B. F was calculated with $k_p=0.08$ hr.⁻¹ and $v_h=36$ μ moles/100 g. body wt./hr. (see Bray *et al.* 1952). Each experimental point represents the result of analysis of the pooled tissue from two rats killed at the time indicated.

Table 1. Free-glycine concentration of rat tissues when steady low level has been attained after injection of sodium benzoate

Mean dose level 400 μ moles/100 g. body wt. in 1 ml. of solution. Control rats were injected with water (1 ml.). Individual values were obtained from determinations on pooled material from two or three rats, except for blood, where some values were obtained from only one rat. Values are means \pm s.d. with ranges in parentheses. P is the probability that there is no significant change in concentration after administration of benzoate.

Tissue	Control (g_1)			After injection of benzoate (g_2)			P
	No. of expts.	Total no. of rats	(μ moles/100 g. of tissue)	No. of expts.	Total no. of rats	(μ moles/100 g. of tissue)	
Liver	33	69	268 \pm 42 (190–346)	21	45	144 \pm 46 (88–252)	< 0.001
Blood	29	52	20 \pm 4 (11–26)	22	38	16 \pm 6 (11–33)	< 0.01
Kidney	6	14	582 \pm 33 (540–614)	4	10	637 \pm 119 (514–795)	> 0.1
Muscle	9	21	199 \pm 33 (155–262)	10	23	215 \pm 49 (164–342)	> 0.1
Intestine	3	9	158 \pm 14 (144–169)	3	9	180 \pm 16 (163–194)	> 0.1

Table 2. Effect of administration of sodium benzoate on the concentration of free glycine in rabbit blood

Doses were given by oral (Or) or intraperitoneal (Ip) routes.

Expt. no.	Days on protein-free diet before expt.	Dose level ($\mu\text{moles}/100\text{ g. body wt.}$)	Route of injection	Blood free glycine ($\mu\text{moles}/100\text{ ml.}$)			Rate of excretion, v_h ($\mu\text{moles}/\text{hr.}/100\text{ g.}$)
				Initial	Mean new level after dose	New level as % of initial level	
1	0	420	Or	137	63	46	—
2	2	250	Ip	53	24	45	22
3	2	180*	Or	56	22	40	—
4	2	430	Ip	81	23	28	—
5	9	390	Or	116	33	28	24
6	9	370	Or	121	16	13	27

* Benzamide was administered instead of benzoate.

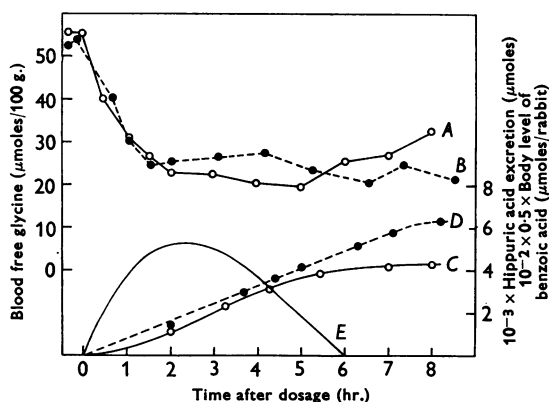


Fig. 2. Effect of administration of benzamide and benzoic acid upon the concentration of free glycine in rabbit blood. Doses (given in parentheses) are in $\mu\text{moles}/100\text{ g. body wt.}$. A, Blood free glycine (benzamide, 180); B, blood free glycine (benzoate, 250). The corresponding excretion of hippuric acid is shown in curves C and D respectively. Curve E shows the calculated body level of benzoate in the benzamide experiment (k_g , 0.08 hr.^{-1} ; k_b , 0.32 hr.^{-1} ; v_h , $750\text{ }\mu\text{moles}/\text{rabbit}/\text{hr.}$). Rabbits had been kept on a protein-free diet for 2 days before the experiment.

rabbit which had received benzoate by intraperitoneal injection (Fig. 2, B). The experiment was not continued long enough to determine whether the beginning of the return to the original free-glycine level coincided with completion of conjugation of the benzoate but Fig. 1 (E and F) and the results of the experiment with benzamide described below (Fig. 2, A) suggest that this is likely. The results from six experiments, including the one with benzamide, are shown in Table 2. The initial values for blood free glycine are all within the range found in rabbits which had had 2 days of 'protein-free' diet ($53\text{--}157\text{ }\mu\text{moles}/100\text{ g. body wt.}$ in 12 expts., mean $91 \pm 30\text{ s.d.}$). There appears to be no correlation between the extent of the fall in the level of

blood free glycine and the dose administered (and, presumably, the body level of benzoic acid). The rate of conjugation of benzoic acid as judged by the rate of excretion of hippuric acid in three experiments is directly related to the percentage fall in the level of blood free glycine. While this is in the expected direction the significance of the difference in the rates of excretion observed is doubtful. The new level of blood free glycine took longer to establish (about 1.5–3 hr.) than in the rat (about 1 hr.). The conclusion that the fall in concentration is independent of the dose (body) level of benzoate is supported by the results of the experiment in which a small dose of benzamide was administered (Fig. 2, A, C, E). Benzamide forms benzoate relatively slowly *in vivo* (Bray *et al.* 1951), so that lower body levels of benzoate, which persist for a longer time, can be obtained by the administration of benzamide than by giving small doses of benzoate which are very rapidly eliminated. The fall in the level of blood free glycine was similar to that obtained with benzoate (Fig. 2, B) and independent of the body level of benzoate until very low body levels were reached, when the concentration of blood free glycine began to rise again. The establishment of a low level of blood free glycine and its independence of body level down to very low body levels of benzoate is to be expected from the fact that the conjugation reaction follows the kinetics of a zero-order reaction down to very low body levels of benzoate, when the kinetics change to those of a first-order reaction (Bray *et al.* 1951).

The transition period after dosing with benzoate was studied more closely in the rabbit. The concentration of blood free glycine fell approximately exponentially (Fig. 3) to reach the low constant level. The data of Christensen *et al.* (1947) and Vries & Alexander (1948) show that the free-glycine concentration of human plasma changes in the same way as that of rabbit blood after the administration of benzoate.

No significant change in the concentration of free glycine in rat muscle, kidney or small intestine was found after the administration of benzoate (Fig. 1, C; Table 1).

Effect of administration of benzoate on other forms of glycine in rat tissues

Preliminary results, summarized in Table 3, indicated that only insignificant changes in the concentration of peptide non-protein glycine

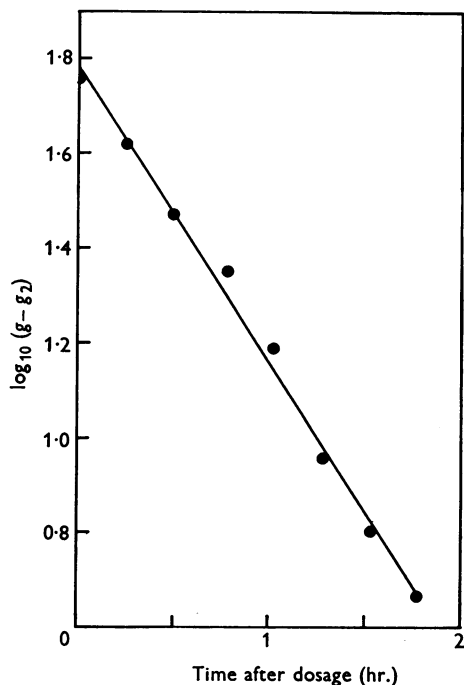


Fig. 3. Effect of intraperitoneal injection of benzoate (430 μ moles/100 g. body wt.) upon concentration of blood free glycine in the rabbit; g is the value for free glycine at a given time and g_2 that for the mean low level finally reached (23 μ moles/100 ml.).

occurred in the chosen rat tissues after the administration of benzoate. Since it is likely that the liver protein would be that which would be degraded if the glycine of protein were required for conjugation, the effect of the administration of benzoate upon the concentration of liver-protein glycine in the rat was investigated. The results showed that no significant change was produced by benzoate up to 4 hr. after injection. The mean control value (8 expts.) was 7560 ± 713 (s.d.) μ moles/100 g. of liver (316 ± 19 μ moles/100 g. body wt.), and the corresponding value after the administration of benzoate (9 expts.) was 7130 ± 927 (287 ± 33). If liver-protein glycine were the main source of glycine used for conjugation a fall in concentration in the liver should be detectable several hours after dosage, owing to the relatively low turnover rate of liver mixed protein; e.g. Shemin & Rittenberg (1944) reported that about 10% is replaced daily. Arnstein & Neuberger (1951) reported that they were unable to find a significant reduction in the total protein N of rat liver after the administration of benzoate.

Rate of conjugation of benzoate with glycine in the rat

The rate of excretion of hippuric acid in the rat seems to be approximately equal to the rate of conjugation of benzoate with glycine, since little hippuric acid could be detected in the tissues, except kidney, after the administration of benzoate. The rate of excretion of hippuric acid was approximately constant (cf. rabbit, Bray *et al.* 1951). The mean value from six experiments was 36 ± 6 (s.d.) μ moles/100 g. body wt./hr. (range 24–43). The rate did not appear to vary markedly with body weights of 112–194 g. These values were obtained with rats dosed with water, but control experiments showed that the rate of urine flow did not have a marked effect upon the rate of excretion of hippuric acid. Sheppeck & Griffith (1936) were also unable to find any relation between the rate of excretion of hippuric acid and the volume of urine excreted in the rat.

Table 3. *Effect of the intraperitoneal injection of sodium benzoate on the concentration of peptide non-protein glycine in tissues of the rat*

Each determination was carried out on pooled material from three rats. Tissues were examined 3 hr. after injection of 1 ml. of sodium benzoate (dose 340–400 μ moles/100 g. body wt.). Control rats were injected with water (1 ml.). P is the probability that there is no significant change in concentration after administration of benzoate.

Tissue	Control		After benzoate		P
	No. of expts.	Mean \pm s.d. (range) (μ moles of glycine/100 g.)	No. of expts.	Mean \pm s.d. (range) (μ moles of glycine/100 g.)	
Liver	3	778 ± 157 (600–906)	3	856 ± 200 (671–1071)	>0.1
Blood	2	91 ± 16 (82, 100)	2	88 ± 32 (66, 110)	>0.1
Kidney	3	357 ± 146 (193–478)	3	492 ± 93 (414–600)	>0.1
Muscle	3	514 ± 64 (464–578)	3	528 ± 14 (421–685)	>0.1
Intestine	3	404 ± 50 (364–457)	2	278 ± 7 (270, 286)	>0.05

DISCUSSION

The information summarized in Table 1 suggests that liver is the most important site of hippuric acid synthesis in the rat. The kidney of the rat can also effect synthesis of hippuric acid *in vitro* but there appears to be some disagreement as to the relative activities of the two organs in this respect (Borsook & Dubnoff, 1940; Cohen & McGilvery, 1946). The only tissue other than liver in which a significant decrease of free glycine was observed was blood. Since there is no evidence that hippuric acid can be synthesized by blood, it may be concluded that the decrease in blood free glycine is a consequence of either transport of glycine into the liver or retention of glycine by the liver.

From the steady levels of liver free glycine, before and after administration of benzoate, the turnover rate of liver free glycine can be calculated if it be assumed that (a) the rate at which free glycine is introduced into the liver is constant, (b) the rates of the reactions which remove glycine are proportional to the level of liver free glycine and (c) the liver is metabolically homogeneous (see Henriques, Henriques & Neuberger, 1955). The justification for (a) and (b) is examined later; (c), though probably incorrect, is commonly assumed in investigations similar to the present one.

If the first two assumptions are incorrect, the value of the turnover rate may, in theory, be either greater or less than the calculation indicates, but in practice errors are most likely to result in an underestimate of the turnover rate. The following values should therefore be regarded as minimal.

It is shown in the Appendix that on the above assumptions the turnover rate, v , is given by the equation

$$v = v_h g_1 / (g_1 - g_2),$$

where v_h is the rate of formation of hippuric acid, and g_1 and g_2 are the steady levels of liver free glycine before and after administration of benzoate. Substitution of the values of g_1 and g_2 recorded in Table 1, and the mean value found for v_h (36 μ moles/100 g. body wt./hr.), yields a value for v of 78 μ moles/100 g. body wt./hr. Since the livers used averaged 4% of the weight of the rats, and their glycine level was 268 μ moles/100 g. of liver (Table 1) it follows that the level of liver free glycine (in the absence of benzoate) is 10.7 μ moles/100 g. body wt., so that the relative turnover is $78/10.7 = 7.3 \text{ hr.}^{-1}$, i.e. the half-life period is approximately 6 min.

Assumptions made in calculating turnover rates

Input of glycine. The validity of the above calculations depends to some extent on the correctness of the assumptions made. There is evidence which suggests that administration of benzoate does not change the rate of introduction of free glycine into

the liver. The results of Arnstein & Neuberger (1953) show that the gross rate of synthesis of glycine from serine and other sources did not vary with the dietary supply of glycine. There is evidence (unpublished results) that after administration of benzoate the liver-serine level remains constant. It is likely, therefore, that the gross rate at which serine is converted into glycine is also constant. (The net rate of conversion, which depends also on glycine concentration, is believed to vary.) The fact that the growth of young rats is arrested by the incorporation of benzoate into a low-glycine diet, and restored by the addition of glycine (Griffith, 1929, 1930; White, 1941) suggests that any increase in the rate of synthesis of glycine caused by a more specific effect of benzoate must be limited in extent, if it occurs at all. Other evidence that benzoate does not cause an appreciable increase in the production of glycine, by synthesis or by liberation from protein or other compounds, is provided by the work of Arnstein & Neuberger (1951), who found that administration of labelled glycine to the rat resulted in a rapid equilibration with the 'first glycine pool', after which the content of labelled glycine in the liver, as excreted in hippuric acid, decreased slowly. The rate of this decrease corresponded to the introduction of unlabelled glycine to the liver by synthesis and liberation at a rate of only 20 μ moles/100 g. body wt./hr. in the presence of benzoate. Finally, no evidence has been found that benzoate results in the depletion of glycine in peptides or liver proteins (cf. p. 578). Arnstein & Neuberger (1951) reported that they were unable to find a significant reduction in the total protein nitrogen of rat liver after the administration of benzoate.

It is shown in the present paper that after administration of benzoate, the level of blood free glycine decreases. If the rat resembles the rabbit in having no effective permeability barrier to the transport of free glycine between liver and blood, the transfer of free glycine to the liver will decrease by approximately 20% (Table 1). Since exchange of free glycine between liver and blood accounts for about 20–30% of the turnover of liver free glycine, neglect of the decrease in the level of blood free glycine would cause an error of approximately 5%.

Output of glycine. It has been assumed that the processes by which free glycine is lost from liver vary in rate with the glycine level. This is known to be so for some reactions, e.g. serine formation by way of formaldehyde (Arnstein & Neuberger, 1953), benzoate conjugation (Bray *et al.* 1951), and also for transport processes, since the free-glycine content of liver would otherwise be unable to rise on administration of glycine or the blood free glycine to fall on administration of benzoate. The fact that on administration of benzoate a new steady level of

free glycine is attained, with a value about one-half that of the original level, shows that the extra loss of glycine due to hippuric acid formation is checked by the lowering of the glycine level, and hence that an appreciable proportion of the processes by which glycine is removed from the pool of free glycine in the liver vary in rate with glycine level. It is shown in the Appendix that, provided an appreciable fraction of the glycine is removed by processes with first-order kinetics, the errors in the deduction of turnover rate due to the operation of reactions of zero order with respect to glycine are not large and would result in the turnover rate being underestimated. It is unlikely that an appreciable proportion of the output is by way of processes which follow kinetics of orders greater than one with respect to glycine, and hence that the calculated turnover rate is too large.

The source of glycine for hippuric acid conjugation

It is now possible to consider the magnitude of the rates of some reactions supplying and removing glycine, and of the glycine stores available. It is known that the conjugating moiety in hippuric acid synthesis is free glycine, and that the benzoate is 'activated' before conjugation (cf. Schachter & Taggart, 1953). The liver free glycine is about $10.7 \mu\text{moles}/100 \text{ g. body wt.}$, while the rate of hippuric acid synthesis is $36 \mu\text{moles}/100 \text{ g.}$ It is evident that the pool of liver free glycine existing at the time of benzoate administration can supply glycine for only a small fraction of the usual doses (e.g. $50\text{--}600 \mu\text{moles}/100 \text{ g. body wt.}$).

It is likely that plasma, and therefore liver, can withdraw free glycine from the remaining viscera quite readily. From the values for the concentration of tissue free glycine in Table 1 and those for other organs given by Krueger (1950), and from organ weights given by Donaldson (1924), it can be calculated that there are about $35 \mu\text{moles}$ of free glycine/ 100 g. body wt. in the viscera and $86 \mu\text{moles}$ of muscle. [On the basis of higher values for tissue free glycine Arnstein & Neuberger (1951) calculated $70\text{--}90 \mu\text{moles}$ for viscera.] Muscle has relatively slight permeability (Henriques *et al.* 1955). Hence there is a store of approximately $35 \mu\text{moles}$ of free glycine/ 100 g. body wt. available for conjugation, equivalent to only a small dose of benzoate. Arnstein & Neuberger (1951) calculated the size of a 'first glycine pool' in the rat to be $170 \mu\text{moles}/100 \text{ g. body wt.}$, but this is a maximum value, and the size is probably much less. (See Fig. 1, Arnstein & Neuberger, 1951.)

The turnover rate of rat-liver free glycine ($78 \mu\text{moles}/100 \text{ g. body wt./hr.}$) is made up of several components. The input due to transport from plasma can be calculated (assuming no permeability barrier, a liver 4% of the body weight,

a blood flow of $50\text{--}100 \text{ ml./}100 \text{ g. of liver/hr.}$, and a haematocrit value of 45%) to be $13\text{--}26 \mu\text{moles}/100 \text{ g. body wt./hr.}$, corresponding to 17–33% of the turnover rate. In the presence of benzoate, values of $10\text{--}20 \mu\text{moles}$ are obtained. The contributions of peptide and proteins to the turnover rate are small. Thus by applying the 'replacement times' found for the rabbit (Henriques *et al.* 1955), it can be calculated that liver peptides (assumed to have the replacement time found for glutathione) contribute approx. $2 \mu\text{moles}/100 \text{ g. body wt./hr.}$, and liver proteins approx. $7 \mu\text{moles}$. Plasma proteins synthesized in the liver require about $4 \mu\text{moles}/100 \text{ g. body wt./hr.}$, but presumably do not return this directly.

The rate at which glycine is synthesized in the liver is not known, but since glycine is synthesized chiefly via serine (Arnstein & Koglević, 1956; Arnstein & Stanković, 1956) it presumably represents the difference between the rate of production of glycine from serine and of serine from glycine. The rapid equilibration of isotopically labelled glycine and serine suggests that these rates are high, and probably account for most of the remaining fraction of the liver turnover rate.

On the basis of the above considerations, the most probable sources of glycine for hippuric acid synthesis are as follows. In the first few minutes after administration of benzoate, part of the existing free glycine in the liver, and to a lesser extent in the blood, is consumed. Thereafter, the pool of liver free glycine is replenished from normal sources, i.e. by synthesis, by transport via blood, and by liberation of bound glycine. The level of liver free glycine falls until the rate of removal of glycine equals the rate of introduction and a new steady level is attained. The rate of removal of glycine by the normal processes of peptide and protein synthesis, degradation and transport is diminished. The relative extent to which the various removal processes are affected will depend on the reaction kinetics; thus the processes following first-order kinetics would be equally affected, whereas those following zero-order kinetics with respect to glycine would not be affected at all. No evidence has been found for the preferential depletion of the glycine of any constituent or of any tissue except liver and blood, so that it is likely that most of the removal processes are reduced to a comparable extent. The fall in blood free glycine which is due to a decline in the efflux of liver free glycine will lower the diffusion rate into all other organs, and, in effect, make the whole organism a source of glycine for conjugation. The relative contribution of these organs will depend on their permeability, mass and turnover rate.

Since only liver and blood are significantly depleted of free glycine, the replacement of any free

glycine lost from other tissues to liver must be readily accomplished, and the net rate of glycine transfer to liver must be a small fraction of the turnover rate of the free glycine of these tissues. The total rate of glycine synthesis, which Arnstein & Neuberger (1953) found to be 104 μ moles/100 g. body wt./hr. in immature rats and somewhat less (70 μ moles) in two adult rats, is evidently adequate for this purpose in the rats used in the present study. The inhibition by benzoate of the growth of young rats suggests, however, that there is only a small margin above sufficiency. This is borne out by a calculation made by Arnstein & Neuberger (1953), which shows that the requirement of glycine for synthetic purposes is almost as great as the rate of synthesis.

This discussion applies only to rats on a glycine-poor diet. When ample amounts of dietary glycine are available, hippuric acid synthesis will become simply one of the mechanisms for disposing of surplus glycine, comparable, for instance, to the degradation of glycine to formaldehyde (Arnstein & Neuberger, 1953).

APPENDIX

The relationship between the turnover rate for liver free glycine and the change in steady levels of free glycine can be deduced as follows.

Assuming that the input of free glycine to the liver, v_1 , is constant, and the output is proportional to the glycine level, g , then

$$dg/dt = v_1 - kg, \quad (1)$$

where k is a constant.

Let g_1 and k_1 be the values of g and k in the steady state in the absence of benzoate. Then

$$dg/dt = 0 \quad \text{and} \quad g_1 = v_1/k_1. \quad (2)$$

After the administration of benzoate, the output of glycine is increased to $(k_1 + k_2)g$, where k_2 is the velocity constant for hippuric acid formation. The free glycine level falls to a new steady value, g_2 , when

$$dg/dt = 0, \quad g_2 = v_1/(k_1 + k_2). \quad (3)$$

From (2) and (3), the turnover rate v is given by

$$v = v_1 = k_1 g_1 = (k_1 + k_2) g_2 = k_2 g_2 g_1 / (g_1 - g_2) = v_h g_1 / (g_1 - g_2), \quad (4)$$

where v_h is the constant rate of formation of hippuric acid. The relative turnover is v/g (see Reiner, 1953) and the half-life period is $0.7 g/v$.

If on administration of benzoate the input of free glycine is decreased, the turnover rate calculated from equation (4) will be too low. Thus if glycine input is decreased to αv_1 , equation (3) becomes

$$g_2 = \alpha v_1 / (k_1 + k_2),$$

$$\text{and} \quad v = k_1 g_1 = v_h g_1 / (\alpha g_1 - g_2).$$

Similarly, if the decline in output of free glycine is less than proportional to the glycine level, equation (4) gives too low a value for v . Suppose that only a fraction, β , of the output is due to a set of reactions of first order with respect to glycine, and the remainder is due to zero-order reactions. Equation (1) then becomes

$$dg/dt = v_1 - kg - (1 - \beta) v_1 = \beta v_1 - kg,$$

$$\text{and hence} \quad v = v_h g_1 / \beta (g_1 - g_2).$$

The course of the transition between the two glycine levels may be derived as follows. After the administration of benzoate,

$$dg/dt = v_1 - (k_1 + k_2)g.$$

$$\text{Integrating,} \quad g = C e^{-kt} + v_1 / (k_1 + k_2).$$

The integration constant, C , may be evaluated, since when $t = 0$, $g = g_1 = v_1/k_1$. Hence

$$C = v_1/k_1 - v_1/(k_1 + k_2) = g_1 - g_2,$$

and

$$g - g_2 = (g_1 - g_2) e^{-(k_1 + k_2)t}.$$

A plot of $\log_{10} (g - g_2)$ and t should be linear, with a slope of $-(k_1 + k_2)/2.3$ (see Fig. 3).

SUMMARY

1. The influence of the administration of benzoate upon the concentration of glycine in various tissues of the rat and the blood of the rabbit has been investigated. The administration of benzoate resulted in a fall in the concentration of free glycine in liver and blood but no significant change could be detected in kidney, intestine and skeletal muscle. No change in the concentration of liver-protein glycine could be observed.

2. After the administration of benzoate, the concentration of free glycine in liver and blood fell rapidly and reached an approximately steady low level which was maintained for as long as conjugation continued. The change in concentration was independent of the body level of benzoate until very low levels of benzoate were reached.

3. The significance of the results obtained is discussed with reference to the source of the glycine utilized for conjugation, and a value for the turnover rate of free glycine in the liver has been calculated. It is suggested that, in the rat under the experimental conditions described, a substantial proportion of the glycine used for conjugation is provided via the free glycine of the liver.

We are indebted to Dr W. V. Thorpe and Dr H. G. Bray for their interest and discussion of this work and to Mr P. B. Wood, Mrs B. G. Taylor and Miss D. A. Clarke for technical assistance.

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The Isolation and Identification of Horse-Dandruff Allergen

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(Received 16 July 1956)

Although much work has been carried out on the characterization of the agents which elicit the symptoms of human hypersensitivity, a chemically pure allergen has not yet been isolated. This can be attributed to various difficulties encountered in fractionating the complex mixtures obtained by extraction of allergenic materials, as well as to the lack of accurate methods for assaying biological activity. Consequently, it is not known whether the activity of extracts is confined to a single constituent. Until this is established it is impossible to ascertain whether there is any underlying chemical structure peculiar to all types of allergen molecules.

Extracts of inhalant-type allergens, such as pollens, house dust and animal danders, have been found to contain multiple proteins, pigment and a wide range of substances of low molecular weight. From such starting materials, numerous attempts have been made to isolate pure constituents (see, for instance, the reviews of Augustin, 1952, 1955). In all cases, the active fractions (often several were isolated from one system) proved to be of protein nature, although most showed an unusual stability to heat treatment and some appeared even to resist proteolysis. The part played by prosthetic groups in the biological reactions of these materials is still

uncertain. Most active fractions of plant origin have been found to contain 'bound' carbohydrate and, in some cases, pigment, but removal of these 'conjugated' substances often failed to reduce skin reactivity.

The work described here was undertaken with a view to identifying the allergen (or allergens) of horse dandruff and to obtaining a pure preparation which might prove useful in biochemical studies of the allergic reaction. Only the mildest of chemical procedures have been adopted in an attempt to preserve the native structure of the sensitizing substance. The drastic treatment applied to many allergenic extracts in the past has probably contributed to the difficulty of isolating pure constituents. In many cases the sole aim appears to have been the preparation of skin-reactive materials regardless of the deleterious action of the reagents employed (strong acid, strong alkali, organic solvents, etc.). Although such treatments sometimes apparently did not decrease the potency of the allergen, as indicated by prick testing of patients, they would tend to alter its chemical structure. This is particularly undesirable where the preparation of the allergen is required for immunochemical studies.