EuS (saponification equiv. 270.1, m.p. 39.8°, *C*-methyl 8.06 %) recently isolated from butterfat (Hansen & Shorland, 1951). A melting-point determination made on an equimolecular mixture of fractions FL2SLSS from ox suet (m.p. 40.5°) and EuS from butterfat (m.p. 39.8°) gave a value of 40.0°. This evidence is consistent with the two fractions being identical.

In order to confirm the purity of this fraction and to identify conclusively other branched-chain acids whose presence has been indicated by examination of the fractions described in Table 1, further quantities of acetone-soluble 'liquids' from ox fat are in the process of investigation.

Although a comprehensive search for branched-chain acids has not been made on the total fatty acid constituents of ox fat, this investigation indicates that their presence in the C_{18} unsaturated acid fraction alone is of the order of 1 % of the total fatty acids.

SUMMARY

A C_{17} methyl branched-chain saturated fatty acid isomeric with *n*-heptadecanoic acid has been shown to be present in the fat extracted from ox suet.

We are much indebted to Mr M. Fieldes of the Soil Bureau, Department of Scientific and Industrial Research, Wellington, for X-ray diffraction analysis.

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The Fate of Certain Organic Acids and Amides in the Rabbit

13. CHLORO- AND FLUORO-BENZOIC ACIDS AND AMIDES

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The primary object of this investigation was to determine the effect of chloro- and fluoro-groups upon the stability *in vivo* of the carbamyl group as it occurs in benzamide. When it was found that all the chloro- and fluoro-benzamides were readily converted into the corresponding acids by the rabbit, there was the added interest of comparing the proportion of the dose excreted conjugated with glucuronic acid when an amide was administered, with that excreted when the corresponding acid was given. It had been noted previously (Bray, Neale & Thorpe, 1946) that a greater proportion of a dose was excreted as benzoylglucuronide when benzoic acid itself was given, than when benzamide was administered. (Benzamide was readily hydrolysed *in vivo* to benzoic acid.) This was subsequently explained as a result of a study of the kinetics of this reaction (Bray, Thorpe & White, 1951).

The metabolism of chloro- or fluoro-benzamides does not appear to have been studied previously, but some investigations of the fate of the acids have

been reported. Graebe & Schultzen (1867) isolated a compound which was probably the calcium salt of *m*-chlorohippuric acid from human *m*-chlorobenzoic acid urine. Novello, Miriam & Sherwin (1926) failed to detect glycine conjugation of the chlorobenzoic acids in the rabbit, and found that only the para isomer was excreted as a hippuric acid derivative by the dog. Schübel & Manger (1929) observed that 10–70 % of doses of *p*-chlorobenzoic acid were excreted unchanged by the rabbit. Quick (1932) found that all three isomers were conjugated with both glycine and glucuronic acid in the dog, glycine conjugation occurring to a much smaller extent with the ortho isomer than with the others; the extent of glucuronic acid conjugation was similar for all three. Only *o*-chlorobenzoic acid was excreted unconjugated to an appreciable extent. Hildebrandt (1903) had also found that the rabbit excreted this acid unchanged, and investigated the formation of chlorobenzoic and hippuric acids from chlorotoluenes. *p*-Chlorohippuric acid was isolated as a

metabolite of *p*-chlorotoluene in the dog. Although pure glycine conjugates were not isolated from the urine after administration of the other isomers, products were obtained which gave *o*- and *m*-chlorobenzoic acids on hydrolysis. In the rabbit, only chlorobenzoic acids were isolated as metabolites of the chlorotoluenes, no glycine conjugation being detected. As far as we are aware, the only recorded account of an investigation of the metabolism of fluorobenzoic acids is that of Coppola (1883), who showed that the dog conjugated all three isomers with glycine.

In the present investigation, the nature of the chief metabolites of the fluoro- and chloro-benzoic acids and their amides has been established by observing the effect of their administration upon the excretion of ether-soluble acid, ester glucuronide, glycine conjugate and ethereal sulphate by rabbits. A new method for the determination of hippuric acids in urine has been worked out.

MATERIALS AND METHODS

Materials. The chlorobenzoic acids were purchased (British Drug Houses Ltd.; Hopkin & Williams Ltd.). The chlorobenzamides were prepared from the corresponding acids via the acid chlorides. *o*- and *p*-Fluorobenzoic acids were prepared from the corresponding ethyl or methyl aminobenzoates by the diazotization fluorination method (Schiemann & Winkelmüller, 1933; Dippy & Williams, 1934). *m*-Fluorobenzoic acid was prepared by the same fluorination method from *m*-aminobenzoic acid. The fluoro-benzamides were prepared from the acids via the acid chlorides.

Diet and dosage. The rabbits used were does of 2–3 kg. which were maintained throughout on the diet of pellets and water, previously described (Bray, Ryman & Thorpe, 1947). The compounds under investigation were administered by stomach tube, the acids as solutions of their sodium salts, and the amides as suspensions in water. All the twelve compounds appeared to be non-toxic for the doses (1 g. throughout) used.

Estimation of metabolites. Ether-soluble acid, ester glucuronide and ethereal sulphate were estimated in the experimental urines by methods already described (Bray, Thorpe & Wood, 1949a).

Estimation of hippuric acids in urine. Owing to the laborious nature of the method used previously (Bray *et al.* 1946; Bray *et al.* 1947), a method more suitable for routine estimation of many samples has been devised. This is based upon liberation of glycine from the hippuric acids by hydrolysis, and its subsequent colorimetric estimation by a ninhydrin reaction (cf. Milton & Waters, 1949). The urine (25 ml.) is acidified with H_2SO_4 (2 ml., 4N) and continuously extracted with ether for 6 hr. After evaporation of ether from the extract, the solid residue is hydrolysed by boiling under reflux with HCl (10 ml., 6N) for 1.5 hr. After cooling, the hydrolysate is adjusted to approximately pH 6.8 by addition of K_2CO_3 (70% w/v), using bromothymol blue (one drop) as indicator. Glycine is estimated in this solution diluted so as to contain between 1 and 7 mg. glycine/100 ml.

Estimation of glycine

The following reagents are used:

Buffer pH 7.0 containing glycine. KH_2PO_4 (3.62 g.) and $\text{Na}_2\text{HPO}_4 \cdot 2\text{H}_2\text{O}$ (7.11 g.) in water (1 l.). Glycine (50 mg./l.) added when required for use. The solution is not stable overnight.

Ninhydrin solution. 1% (w/v) in water.

Glycine standard. 90 mg./l. in water, made up immediately before use.

The dilute solution (2 ml.) from the hydrolysis (above) is mixed with the buffer (2 ml.) and ninhydrin solution (1 ml.) in a Pyrex test tube. Tube and contents are heated in a boiling-water bath for 1 hr. using a 'cold finger' to reduce evaporation. After cooling, the purple solution is made up to 25 ml. and the colour intensity estimated in a Spekker photoabsorptiometer using Chance green filter OG 1. A calibration curve was constructed using the glycine standard, appropriately diluted and heated, with ninhydrin alongside the solutions in which glycine was being estimated. The curve is linear over the range 0–180 $\mu\text{g.}/2\text{ ml.}$, an extinction (drum reading) of 0.1 corresponding to about 22 $\mu\text{g.}$ Control experiments using hippuric acid in water gave recoveries of 99%, s.d. $\pm 3\%$. Similar recoveries were obtained with substituted hippuric acids. In experiments in which hippuric acid was added to the urine of normal rabbits the average recovery was 97%, s.d. ± 6.7 .

RESULTS

Excretion of normal metabolites

The average daily excretion of ether-soluble acid (expressed as hippuric acid) by the rabbits used in this investigation ranged from 630 to 688 mg. The average percentage by which individual daily values differed from the corresponding weekly averages used as baselines, for purposes of calculation, was ± 11 . The corresponding values for reducing material (expressed as glucuronic acid) were 204–228 mg. and $\pm 7\%$, for ethereal sulphate 38–42 mg. SO_3 and $\pm 13\%$ (5 mg. SO_3), and for conjugated glycine (expressed as hippuric acid) 206–309 mg. and $\pm 11\%$. Of the material in the ether-soluble acid which was responsible for the ninhydrin reaction, 7–8% was present in unconjugated form, i.e. it reacted without preliminary hydrolysis.

Excretion products of chloro- and fluoro-benzoic acids and amides

Table 1 shows the increases in excretion of ether-soluble acid, ester glucuronide and ethereal sulphate observed after administration of the chloro- and fluoro-benzoic acids and amides. All the compounds administered were excreted mainly as ether-soluble acid, which consisted of a mixture of the chloro- or fluoro-benzoic acid with its glycine and glucuronic acid conjugates. For the amides, the differences between the totals accounted for and the dose administered are probably not due, to any appreciable extent, to the excretion of unchanged amides.

Table 1. *Excretion of ether-soluble acid, ester glucuronide and ethereal sulphate after administration of chloro- and fluoro-benzoic acids and amides to rabbits*

(Dose 1 g. Ranges given in parentheses and number of experiments indicated by superior figures.)

Compound	Average percentage of dose excreted as		
	Ether-soluble* acid	Ester glucuronide	Ethereal sulphate
<i>o</i> -Chlorobenzoic acid	87 (69-96) ⁵	7 (0-11) ⁵	0 ³
<i>o</i> -Chlorobenzamide	71 (65-75) ⁵	11 (8-18) ⁵	0 ²
<i>m</i> -Chlorobenzoic acid	100 (87-109) ⁴	23 (12-31) ⁴	0 ²
<i>m</i> -Chlorobenzamide	70 (56-89) ⁵	1 (0-4) ⁵	0 ²
<i>p</i> -Chlorobenzoic acid	69 (65-83) ⁵	16 (3-33) ⁵	0 ³
<i>p</i> -Chlorobenzamide	84 (58-108) ⁹	5 (0-9) ⁹	0 ³
<i>o</i> -Fluorobenzoic acid	93 (87-97) ⁴	7 (2-10) ⁴	0 ²
<i>o</i> -Fluorobenzamide	76 (61-89) ⁴	3 (0-9) ⁵	2 (0-3) ³
<i>m</i> -Fluorobenzoic acid	95 (92-97) ⁴	10 (5-12) ⁴	0 ²
<i>m</i> -Fluorobenzamide	76 (70-82) ⁴	4 (2-6) ⁴	0 ²
<i>p</i> -Fluorobenzoic acid	85 (72-99) ⁵	11 (7-14) ⁵	2 (0-5) ³
<i>p</i> -Fluorobenzamide	79 (70-96) ⁴	3 (0-7) ⁴	0 ²

* Substituted benzoic and hippuric acids + substituted benzoylglucuronides.

Table 2. *Excretion of glycine conjugates and unconjugated acid and the ratio of the former to the glucuronide excreted after administration of chloro- and fluoro-benzoic acids and amides to rabbits*

(Dose 1 g. Two experiments for each compound. Values for each experiment given in parentheses after average value.)

Compound	Average percentage of dose excreted as				<i>R</i> †
	Ether-soluble acid	Ester glucuronide	Glycine conjugate	Uncon- jugated acid*	
<i>o</i> -Chlorobenzoic acid	84 (76, 92)	19 (19, 18)	5 (4, 6)	60	0.3
<i>o</i> -Chlorobenzamide	95 (86, 103)	14 (14, 14)	17 (16, 18)	64	1.2
<i>m</i> -Chlorobenzoic acid	75 (74, 76)	23 (22, 23)	51 (46, 56)	1	2.2
<i>m</i> -Chlorobenzamide	94 (87, 101)	17 (17, 17)	74 (73, 75)	3	4.4
<i>p</i> -Chlorobenzoic acid	91 (81, 100)	21 (17, 25)	63 (67, 59)	7	3.0
<i>p</i> -Chlorobenzamide	95 (89, 120)	4 (0, 7)	40 (31, 49)	51	10.0
<i>o</i> -Fluorobenzoic acid	86 (74, 102)	9 (0, 18)	43 (45, 41)	34	4.8
<i>o</i> -Fluorobenzamide	83 (61, 104)	6 (5, 6)	60 (62, 57)	17	10.0
<i>m</i> -Fluorobenzoic acid‡	99 (98, 100)	6 (2, 10)	24 (26, 22)	69	4.0
<i>m</i> -Fluorobenzamide	93 (85, 101)	9 (6, 12)	74 (62, 85)	10	8.2
<i>p</i> -Fluorobenzoic acid	87 (95, 79)	9 (8, 9)	43 (38, 48)	35	4.8
<i>p</i> -Fluorobenzamide	103 (85, 121)	6 (5, 6)	93 (88, 97)	4	15.5

* Ether-soluble acid - (ester glucuronide + glycine conjugate) from average values.

† *R* = the ratio glycine conjugate/ester glucuronide from average values. Values of *R* for benzoic acid and benzamide are 2.8 and 15.7 respectively (unpublished results).

‡ Dose 0.5 and 0.75 g.

Extraction of *o*-chloro- and *p*-fluoro-benzamide urines as collected (pH about 8.0) gave an amount of unchanged amide corresponding to less than 1 % of the dose fed: no amide was obtained by similar treatment of *p*-chlorobenzamide urine. There was no significant increase in the excretion of ethereal sulphate. About 2 years after these results had been obtained, the method for estimation of glycine conjugates was worked out. Two experiments were then performed with each compound in which the glycine conjugate as well as ether-soluble acid and glucuronide was estimated. This permitted the calculation of the ratio (glycine conjugate)/(ester glucuronide), *R*, and also of the amount of acid excreted unconjugated. These results are given in Table 2.

Nature of ether-soluble acid

The ether-soluble material extracted from metabolic urines, under the conditions used for the determination of ether-soluble acid, reduced alkaline cupric reagents and gave a positive naphthoresorcinol reaction, showing that ester glucuronides of the chloro- and fluoro-benzoic acids are soluble in ether. These could not, however, be isolated from the extracts or obtained in crystalline form via the usual lead procedure (cf. Bray *et al.* 1947).

Fractionation of the ether-soluble material by refluxing with successive small amounts of toluene (cf. Bray *et al.* 1947) led to the isolation, in every case, of the toluene-soluble halogen-substituted benzoic

Table 3. *Properties of chloro- and fluoro-hippuric acids isolated from metabolic urines*

Compound	Percentage of N		Melting point	
	Found	Calc.	Found	Literature
<i>o</i> -Chlorohippuric acid*	6.8	6.6	177-179°	176°†
<i>m</i> -Chlorohippuric acid	6.8	6.6	142°	143-144°†
<i>p</i> -Chlorohippuric acid	6.7	6.6	142-144°	143°†‡
<i>o</i> -Fluorohippuric acid	7.1	7.1	147°	121-121.5°§
<i>m</i> -Fluorohippuric acid	7.0	7.1	153°	152-153°§
<i>p</i> -Fluorohippuric acid	7.2	7.1	171°	161-161.5°§

* Found: Cl, 16.5; calc. 16.6%.

† Novello *et al.* (1926).

‡ Hildebrandt (1903).

§ Coppola (1883).

acid, and except from *o*-fluorobenzoic acid urine, of the toluene-insoluble hippuric acid derivative. The properties of the hippuric acid derivatives are given in Table 3.

DISCUSSION

The results given in Tables 1 and 2 suggest that all the six amides studied are almost completely hydrolysed in the rabbit. This finding may be compared with the results previously obtained *in vitro*, which showed that the percentages of *o*-, *m*- and *p*-chloro- and *o*-, *m*- and *p*-fluoro-benzamides hydrolysed by rabbit-liver extracts in 5 hr. were 16, 42, 61, 59, 45, 46, respectively (Bray *et al.* 1950). From analogy with earlier comparisons (Bray *et al.* 1949*a*) it might have been expected that all but the first would be readily hydrolysed *in vivo*. The percentage hydrolysis *in vivo*, however, depends on the rate of excretion of the amide by the kidney, as well as on the rate of hydrolysis by the enzyme system. If administered amides are excreted only slowly, then they will be almost completely hydrolysed *in vivo* as long as the rate of hydrolysis is much greater than the rate of excretion unchanged. It appears that the chloro- and fluoro-benzamides are excreted unchanged very slowly, since the variations observed in hydrolysis *in vitro* are not revealed in the metabolic fate of those amides.

Similar considerations suggest that the proportion of the dose of an acid (or amide) excreted in conjugated form depends on the rate of excretion of the unconjugated acid and on the efficiency of the two conjugating enzyme systems. However, the ratio (*R*) of the amount of acid or amide excreted as the glycine conjugate to that excreted as the glucuronide will give the relative average rates of production of the two conjugates, whatever the rate of excretion of the free acid. It can be seen from Table 2 that in each case *R* is greater for an administered amide than for the corresponding acid. In a previous study of the metabolism of benzoic acid and its precursors (Bray *et al.* 1951) this variation in *R* was found to be a simple consequence of the kinetics of the conjugations. The rate of conjugation of benzoic acid with glucuronic acid is proportional to the body level of benzoic acid, whereas the rate of formation of hippuric acid is constant, so that *R* depends both on the dose level and on the rate of

production of benzoic acid from a precursor. Since *R* is greater for the substituted amides than for the acids, it seems possible that the kinetics of the hydrolysis and conjugation reactions are similar to those of the unsubstituted acids and amides. We have previously reported that for benzoic, phenylacetic and *m*- and *p*-toluic acids and certain of their precursors (Bray *et al.* 1946; Bray, Thorpe & Wood, 1949*b*; Bray, Humphris & Thorpe, 1949) a greater proportion of glucuronide is excreted after administration of the free acid than after the precursor. Since after administration of these compounds the free acid is excreted to only a small extent and the value of *R* is greater for the amide than for the acid, the absolute amount of glucuronide produced is greater for the acid than for the amide. This will not necessarily be so when the unconjugated acid is readily excreted, as for the halogen-substituted benzoic acids, and in the absence of information on the kinetics of excretion of the unconjugated acids by the kidney it can only be concluded that *R* will be greater for amides than for acids, where the metabolic reactions follow kinetics of the same type as those found for benzoic acid and its precursors.

SUMMARY

1. The metabolism of the chloro- and fluoro-benzoic acids and amides in the rabbit has been studied.
2. The acids are excreted unconjugated and conjugated with glucuronic acid and glycine to varying extents.
3. The amides are all almost completely hydrolysed, and the resulting acids are excreted partly free and partly conjugated. Traces of unchanged amide equivalent to less than 1% of the dose were isolated from *o*-chloro- and *p*-fluoro-benzamide urines.
4. The results obtained are discussed with reference to the kinetics of the reactions involved.
5. The substituted hippuric acids were isolated from the metabolic urines.
6. A method for the estimation of glycine in its conjugates in urine is described.

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The Effect of Heat on the Microbiological and Anti-anaemic Properties of Human Gastric Juice mixed with Vitamin B₁₂

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Ternberg & Eakin (1949) found in normal human gastric juice a substance which combined with vitamin B₁₂ to make the vitamin unavailable as a growth factor in microbiological assays. The gastric juice of normal people was reported to contain more of the substance than the juice of patients with pernicious anaemia in relapse. It was suggested that this substance was the intrinsic factor of Castle. This suggestion aroused much interest, for, if confirmed, it could provide a relatively simple method for detecting intrinsic factor. Clinical observations by Hall *et al.* (1950) indicated that the complex formed between the intrinsic factor, extracted from hog stomach or duodenum, and vitamin B₁₂, was more stable to heat than the intrinsic factor alone. Ross (1950) found that substances having similar growth-promoting activity to that of vitamin B₁₂ for *Euglena gracilis* were released on heating human serum. Heating at 100° for 30 min. released more activity than heating at 70° for the same time, so that serum apparently contains a complex of vitamin B₁₂ which is fairly stable to heat.

During experiments to see whether certain proteins other than the substance in gastric juice could bind vitamin B₁₂, i.e. make it unavailable to bacteria, indications were obtained that the factor in gastric juice was more stable to heat in neutral than in acid solution. In view of present interest in the role of intrinsic factor in the metabolism of vitamin B₁₂, it seemed worth while to investigate

the phenomenon further, using both microbiological and clinical methods.

EXPERIMENTAL AND RESULTS

The effect of acidity on the stability of the vitamin B₁₂-binding factor to heat

Treatment of gastric juice. The samples of gastric juice were residues from fractional test meals, obtained through the kindness of the Biochemistry Department of this hospital. Only samples containing free acid were used. The juice was filtered and stored in sterile flasks at 0° for at least 24 hr. After this treatment there was no evidence of bacterial growth when 0.5 ml. portions of the juice were added aseptically to broth and incubated for several days at 37°. The juice was neutralized just before use by adding sterile 2*N*-NaOH drop by drop, and the pH was checked on a meter to between 7.2 and 7.6.

Microbiological assays. Vitamin B₁₂ was assayed with *Lactobacillus leichmanii* in a medium containing acid-hydrolysed casein with the usual supplements. A solution of crystalline vitamin B₁₂ (10 µg./ml., 'Cobione', Merck) was diluted for use as standard, and for addition to the gastric juice.

Sterile vitamin B₁₂ solution was added to gastric juice in sterile graduated test tubes to give concentrations of 0.25 µg. or 0.5 µg./100 ml. of juice. After receiving the treatment indicated in Tables 1-3, the solutions were diluted with sterile water to give apparent vitamin B₁₂ concentrations of 0.5 mµg./ml., and 1 ml. portions were added to the culture medium for assay. The amount of bacterial growth was read turbidimetrically after incubating at 37° for 18 hr.