enzyme has both transglucosidase and glucosidase activities.

2. The transglucosidase synthesizes glucosides of several *iso*alloxazine derivatives, many of which are antivitamins. Certain specificity requirements have been defined for the glucose donor and for the flavin acceptor.

3. The quantitative characteristics of the formation of riboflavinyl glucoside from riboflavin and maltose have been investigated, and the inhibition of this reaction by α-D-glucose 1-phosphate has been shown to be competitive.

4. The solubility of riboflavinyl glucoside in water is much greater than the solubility of riboflavin, and it is suggested that this may be of importance in the transport of riboflavin.

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Comparative Detoxication

3. HIPPURIC ACID FORMATION IN ADULT LOCUSTS

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It has been shown that the detoxication of phenols in the locust is accomplished by a conjugation mechanism different from that used by vertebrates, glucosides rather than glucuronides being formed (Myers & Smith, 1954). It was of interest, therefore, to consider the fate of aromatic acids in this insect, since even among vertebrates much variation is found in the method of conjugation of these acids, which may be condensed with glucuronic acid, glycine, ornithine, or glutamine in different species (Williams, 1947). Previous work has given very little indication of the existence of a conjugation mechanism in insects for the detoxication of aromatic acids though the reported presence of hippuric acid in some insects suggested that this might be possible. This identification of hippuric acid, in the faeces of various caterpillars (Davy, 1854; van der Hoeven, 1850), and in the fat body of *Dytiscus* (Sirodt, 1858) rests on microscopical observation of crystal form and is uncertain.

The presence of some conjugated benzoic acid such as hippuric acid in the excreta of locusts, which feed on grass, might be expected since this compound is a typical urinary constituent of vertebrate herbivores. However, Brown (1937) was unable to find hippuric acid in the excreta of another Acridid, the grasshopper, *Melanoplus bivittatus*.

We have studied this detoxication mechanism by observing the metabolism of some aromatic acids whose glycine conjugates can readily be detected in the excreta, and it will be shown that the locust, like most animals, conjugates the aromatic acids with glycine to form hippuric acids.

EXPERIMENTAL

Reference compounds. *p*-Nitrohippuric acid, m.p. 135°, and dibenzoylornithine (ornithuric acid), m.p. 185°, were prepared by the Schotten-Baumann reaction using the appropriate amino acids and aroyl chlorides. *o*-Hydroxyhippuric acid (salicyluric acid), m.p. 166°, was prepared from salicylic azide according to Bondi (1907). Hippuric acid, m.p. 186°, and *p*-aminohippuric acid, m.p. 199°, were com-

mercial products. The diphenylmethyl ester of hippuric acid was prepared from diphenyldiazomethane and hippuric acid (cf. Elvidge, Linstead, Sims & Orkin, 1950). *Diphenylmethyl hippurate* formed narrow plates, m.p. 123–124°. (Found: C, 76·1; H, 5·0. $C_{22}H_{19}NO_5$ requires C, 76·5; H, 5·6%.)

Locusts. Adult *Locusta migratoria*, obtained from the Anti-Locust Research Centre, London, were maintained on a liberal diet of fresh grass. Compounds were administered mixed with crushed biscuit or by injection with an Agla micrometer syringe (Burroughs Wellcome Ltd.). In quantitative experiments separate insects were kept in 500 ml. beakers on a restricted grass diet (cf. Myers & Smith, 1953, 1954).

Preparation of extracts. The droppings were roughly ground to break up the pellets and boiled with water (about 10 ml./g. of faeces). This extract was filtered with suction and the filtrate evaporated to dryness at reduced pressure. The residue was dissolved in hot 80% ethanol (10 ml./g.), filtered and the filtrate concentrated to small bulk. This concentrate was then submitted to a preliminary separation on a large-scale paper chromatogram (cf. Myers & Smith, 1954) and any metabolites were located by suitable colour tests. Elution of appropriate sections of this chromatogram gave extracts suitable for chromatographic or spectrophotometric characterization.

Paper chromatography. This was carried out as described by Smith, Smithies & Williams (1953) and the R_F values obtained are quoted in Table 1. Unless stated otherwise, extracts were compared with reference compounds in all of the solvents in this table. The preparation and chromatography of the dinitrophenyl derivatives of the amino acids were carried out as described by Krol (1952) except that Whatman no. 1 paper, prepared by soaking in the buffer and drying, was used instead of a Celite column. The dried, buffered papers were then used in the normal way.

Detection of acids. The acids were conveniently located on the papers by illumination with ultraviolet light from a Hanovia Chromatolite lamp which emits mainly radiation of 254 mμ. wavelength. In this light, salicylic acid and salicyluric acid fluoresced blue, *p*-aminobenzoic and *p*-aminohippuric acids feebly purple. *p*-Nitrobenzoic and *p*-nitrohippuric acids strongly quenched the fluorescence of the paper and appeared as dark spots. Benzoic, hippuric and ornithuric acids could also be detected by a weak quenching effect. These three acids were also located as blue spots when the paper, after drying at room temperature for 24 hr., was sprayed with 2% (w/v) starch solution containing 5% (w/v) each of KIO_3 and KI. Aromatic amino compounds were located by diazotization and coupling with β-naphthol. Glycine was detected by the *o*-phthalaldehyde reagent (Sandstrom & Lillevik, 1941) which gave a green colour, and other amino acids by heating the paper at 100° after spraying with 0·1% ninhydrin in butanol.

Ultraviolet absorption spectra. These were measured in a Unicam S.P. 500 Spectrophotometer.

Estimation of amino compounds in faeces. The droppings from each locust were broken up with a glass rod under 2 ml. of 2 N-NH_3 in a 10 ml. centrifuge tube and mixed with 3 ml. of ethanol. After centrifuging down the debris, 1 ml. of the clear supernatant liquid was evaporated in a current of warm air on Whatman no. 4 paper to give a spot about 2 cm. diameter, 10 cm. from the end of the paper. The paper was irrigated with a saturated solution of water in ethyl methyl ketone for 4–5 hr. and dried. The zone of the chromatogram containing *p*-aminohippuric acid was located by spraying a reference spot with the diazo reagents and corresponding strips of paper cut out and eluted chromatographically with 2–3 ml. of 0·1 N-NH_3. *p*-Aminohippuric acid was estimated in this eluate by the colorimetric method of Bratton & Marshall (1939) using *N*-1-naphthylethylenediamine as a coupling agent. A standard curve was constructed using *p*-aminohippuric acid, and colour intensities were measured in a Spekker Absorptiometer with an Ilford 604 filter (maximum transmission, 520 mμ.).

Recoveries of *p*-aminohippuric acid (50–500 μg.) added to locust faeces ranged from 91 to 97% when estimated according to this technique.

Estimation of diazotizable amines were also made on the ethanolic extract before chromatography. This value represented the sum of the unacetylated aromatic amino acids excreted, since within the limits of the method *p*-aminobenzoic and *p*-aminohippuric acids gave equivalent colour intensities.

Metabolic experiments

Benzoic acid. Seventy adult locusts were allowed to feed for a week on a mixture of wet biscuit (cream crackers) which contained 5% (w/w) of benzoic acid. The faeces collected

Table 1. *R_F values of some acids and their conjugates*

Whatman no. 4 paper, run till front had moved about 30 cm.

Solvent system:

I Benzene:*n*-butanol:ammonia, sp.gr. 0·88 (2:5:2, v/v) (upper phase).
II *n*-Butanol:acetic acid:water (4:1:5, v/v) (upper phase).
III Benzene:acetic acid:water (1:1:2, v/v) (upper phase).
IV *n*-Butanol saturated with water.
V Ethyl methyl ketone saturated with water.

Acid	R_F values				
	I	II	III	IV	V
Benzoic	0·30	0·90	0·95	0·95	0·95
Hippuric	0·20	0·85	0·15	0·70	0·35
Ornithuric	0·60	0·95	0·30	0·90	0·50
p-Nitrobenzoic	0·30	0·95	0·80	0·70	0·50
p-Nitrohippuric	0·20	0·85	0·15	0·40	0·20
p-Aminobenzoic	0·05	0·95	0·80	0·70	0·85
p-Aminohippuric	0·05	0·65	0·05	0·10	0·10
Salicylic	0·40	0·90	0·90	0·60	0·45
Salicyluric	0·05	0·80	0·50	0·35	0·35

over this period were extracted with water and then ethanol, and at this stage drops of the ethanolic solution were chromatographed in the solvents shown in Table 1. The extract gave intense spots corresponding to hippuric acid and benzoic acid, but no evidence of the presence of ornithuric acid was found.

On evaporation, the ethanolic solution deposited a dark brown crystalline solid which was recrystallized several times from water (charcoal) to give colourless plates (15 mg.) of hippuric acid, m.p. and mixed m.p. 185°. (Found: C, 60·3; H, 5·5. Calc. for $C_9H_9NO_3$, C, 60·3; H, 5·1%.) These depressed the melting point of ornithuric acid. The diphenyl methyl ester of hippuric acid prepared from 5 mg. of this material had m.p. and mixed m.p. 123°.

Similar experiments with injected benzoic acid also yielded faecal extracts in which hippuric acid could be detected after paper chromatography.

No hippuric acid was found in similar extracts of the faeces of stock locusts maintained on a grass diet with no added benzoic acid.

p-*Nitrobenzoic acid.* Twelve immature adult locusts were each injected with 1 mg. of the sodium salt of the acid and the faeces collected for the next 24 hr. The crude ethanolic extract was separated in a large-scale chromatogram on no. 4 Whatman paper with benzene:acetic acid:water (1:1:2, v/v) till the front had moved 35 cm. Strips cut from the edge of the paper were then examined for metabolites under ultraviolet light and with the diazo reagents. Four bands were present, of which *A* (R_F about 0·2) and *B* (R_F about 0·9) quenched the fluorescence of the paper in ultraviolet light and two, *C* (R_F 0·05) and *D* (R_F 0·6), gave a positive diazo reaction. These areas of the paper were eluted with water and small portions of the eluates run on new chromatograms alongside reference spots in the three solvent mixtures I, II, III (Table 1). The eluate of zone *A* had the same R_F values as *p*-nitrohippuric acid, and that of zone *B* the same as *p*-nitrobenzoic acid. The extract of zone *D* was identified chromatographically with *p*-aminobenzoic acid and *C* with *p*-aminohippuric acid. From the relative intensity of the reactions it appeared that *p*-nitrobenzoic acid and *p*-aminobenzoic acid were present in larger amounts than the other two metabolites.

The bulk of the eluate of zone *A* was rechromatographed in butanol:acetic acid:water (4:1:5, v/v) and the appropriate zone eluted with water; its ultraviolet spectra in acid $\lambda_{max.}$ 268 mμ. and alkali $\lambda_{max.}$ 271 mμ., were found to be identical with those of authentic *p*-nitrohippuric acid over a range of 260–350 mμ. (Fig. 1). A strip of paper of the same size was cut from the chromatogram and eluted with water for use as a blank in the spectrophotometer.

p-*Aminobenzoic acid*

Qualitative experiments. Ten mature adult locusts, previously fed on fresh grass, were injected with 0·5 mg. each of sodium *p*-aminobenzoate, kept without food for 48 hr. and the droppings collected over this period. The faeces were made alkaline with a few drops of conc. NH_3 and extracted in the usual way. The ethanolic solution was chromatographed in butanol:water on Whatman no. 4 paper till the front had run about 30 cm. A strip cut from the edge of the sheet after diazotization and coupling showed two coloured zones. A large one, R_F 0·5–0·8, was shown by paper chromatography after elution with water to consist of unchanged *p*-aminobenzoic acid. The slow moving band

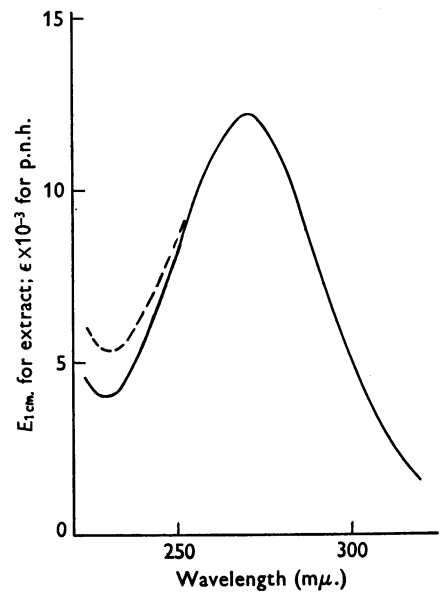

Fig. 1. Ultraviolet absorption spectra in 0·1 N-NaOH of an arbitrary dilution of an extract of locust faeces, – – –, and of *p*-nitrohippuric acid (p.n.h.), ——; $\lambda_{max.}$ 271, $\epsilon_{max.}$ 12200.

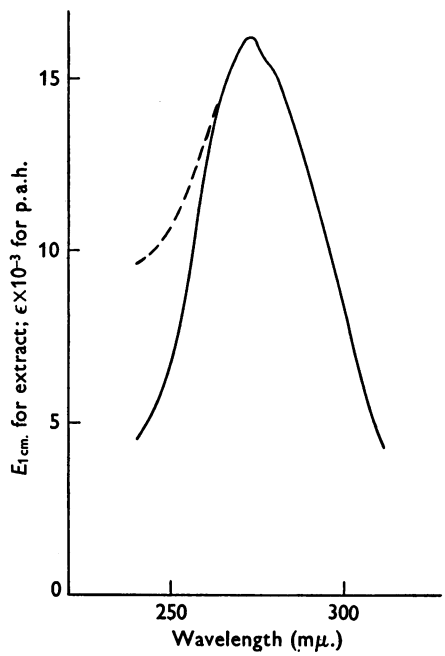

Fig. 2. Ultraviolet absorption spectra in 0·1 N-NaOH of an arbitrary dilution of an extract of locust faeces, – – –, and of *p*-aminohippuric acid (p.a.h.), ——; $\lambda_{max.}$ 273, $\epsilon_{max.}$ 16200.

Table 2. *Formation of* p-*aminohippuric acid* (*PAH*) *from* p-*aminobenzoic acid* (*PAB*) *by individual locusts*

Temp. (°)	PAB injected (μg.)	Period of collection of faeces (hr.)	PAB + PAH in faeces		PAH in faeces	
			μg.	% dose	μg.	% dose
35	310	72	290	94	118	28
			300	97	78	18
			190	61	50	12
			255	82	62	14
			230	74	62	14
	375	24	356	95	48	9
			250	67	32	6
			338	90	66	12
			213	57	74	14
25	310	72	240	78	90	21
			265	86	148	34
			170	55	75	18
			255	82	55	13
			300	97	96	22
			265	86	78	18
	375	24	200	53	24	5
			250	67	50	9
			375	100	24	5

(R_F 0–0·15) was eluted with 0·1 N-NH_3 and further purified by chromatography on paper with butanol:acetic acid:water (4:1:5, v/v). The diazo-reacting zone on this chromatogram was eluted with water and shown to have the same chromatographic properties as *p*-aminohippuric acid in all the solvents used (Table 1).

Its ultraviolet absorption spectrum in 0·1 N-NaOH (Fig. 2), had a band at 273 mμ. In acid no specific absorption was found in this region.

The conjugate was hydrolysed with N-H_2SO_4 at 100° and the presence of *p*-aminobenzoic acid was demonstrated on paper chromatograms. Chromatograms of the hydrolysate run in butanol:acetic acid:water (4:1:5, v/v) showed a spot corresponding with the position of a reference spot of glycine (R_F 0·21) which reacted with ninhydrin and gave the green colour characteristic of glycine when sprayed with the phthalaldehyde reagent. Another portion of the hydrolysate was evaporated to dryness *in vacuo* and converted into the dinitrophenyl derivative which was then chromatographed on buffered paper in water saturated chloroform:butanol (93:7, v/v) (cf. Krol, 1952). The dinitrophenyl derivative from the extracts ran at the same speed as the authentic glycine derivative (R_F 0·26) and was readily distinguished from the ornithine (R_F 0·76) and alanine (R_F 0·65) derivatives.

Quantitative experiments. Some figures for the excretion of *p*-aminohippuric acid are quoted in Table 2. In other experiments it was observed that most of the injected *p*-aminobenzoic acid was eliminated in the first 24 hr. after dosing, though traces of diazotizable amines were detectable in the faeces up to 2 weeks later. After the initial excretion of *p*-aminobenzoic acid the proportion of glycine conjugate in each day's excretion rose. In ten immature adults which each received 800 μg. *p*-aminobenzoic acid, the combined faeces of the second and third days after dosing contained 21–34 μg. (mean 26 μg.) of *p*-aminohippuric acid and only 22–26 μg. (mean 23 μg.) of *p*-aminobenzoic acid. Since some reports suggest that detoxication mechanisms in insects may be affected by temperature (e.g. Sternburg & Kearns, 1952; Butts, Chang, Christensen & Wang, 1953), the conjugation of *p*-aminobenzoic acid was measured at 25 and 35° on locusts from the same hatching. No significant difference at the two temperatures could be observed (Table 2).

Salicylic acid. Twelve immature adult locusts were injected with 0·5 mg. each of sodium salicylate in aqueous solution (0·01 ml.). Faeces collected 24 hr. later were extracted with water and ethanol. This extract was separated on a large-scale paper chromatogram in solvent 1 on Whatman no. 4 paper. In addition to zones corresponding with salicylic acid and salicyluric acid a third unidentified material fluorescing in ultraviolet light with R_F about 0·2 was present. The zone corresponding to salicyluric acid was eluted with water, and the presence of this compound confirmed by comparison with authentic material on paper chromatograms run in the solvents in Table 1. Insufficient material was available for spectroscopic characterization and the degree of conjugation of salicylic acid appeared to be smaller than the other acids studied.

Metabolism of hippuric acids. Three groups, each of six immature adult locusts were injected with 1 mg. each of the sodium salts of hippuric acid, *p*-aminohippuric acid and *p*-nitrohippuric acid. Faeces from each group were collected after 24 hr. and extracted as described above. Paper chromatography of these extracts showed in each case that the glycine conjugate was excreted unhydrolysed. In the case of *p*-nitrohippuric acid a very small trace of *p*-aminohippuric acid was found in the extracts of the faeces.

DISCUSSION

Each of the acids studied was excreted to some extent as a glycine conjugate, and no evidence was found of any other conjugation of the carboxyl group. As we have previously observed with the acetylation mechanism (Myers & Smith, 1953), the capacity of the locust for detoxication by conjugation is low. With *p*-aminobenzoic acid only about one-fifth of the administered material was recovered as the conjugated form, and this conjugation was

not appreciably affected by temperature. Most of the material given was eliminated in the first few days after dosing, but small amounts continued to be excreted for several weeks. Even with the much smaller quantities eliminated on these later days, about half was still unconjugated.

The reduction of the nitro group in *p*-nitrobenzoic acid which was observed indicates the presence of another possible detoxication mechanism in locusts, since reduction of nitro groups often lowers toxicity (cf. Smith *et al.* 1953). This reduction is not unexpected since Dennel (1949) has observed that insect blood has a low redox potential.

The formation of hippuric acids in the locust is interesting in connexion with the comparative biochemistry of nitrogen metabolism. Locusts differ from the common experimental animals in that uric acid is a major end-product of their nitrogen metabolism (Chauvin, 1941). The hen, which also eliminates most of its nitrogen as uric acid, uses ornithine for the detoxication of aromatic acids, and it is sometimes suggested (Quick, 1948; Baldwin, 1948) that other animals with uricotelic metabolism, such as reptiles, may also form ornithuric acids. The only reptile which appears to have been studied is a turtle, which produced hippuric acids (Komori & Sendyu, 1926), but it was not stated whether this turtle had a ureotelic or uricotelic metabolism (cf. Baldwin, 1952). The present work suggests that there may be no general connexion between uric acid production and the use of ornithine as a conjugating agent.

SUMMARY

1. The metabolism of benzoic, *p*-nitrobenzoic, *p*-aminobenzoic and salicylic acids has been studied in the adult locust.

2. The glycine conjugates of these acids have been identified in the excreta by spectroscopic and paper chromatographic techniques.

3. Quantitative measurements showed that about 20% of *p*-aminobenzoic acid was converted into *p*-aminohippuric acid.

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Some Observations on Volatile Fatty Acids in the Sheep's Rumen

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A number of investigations of the volatile fatty acids (VFA's) of the rumen have been reported since the subject was reviewed by Elsden & Phillipson (1948). Particular interest has focused on rumen VFA's other than acetic, propionic and butyric acids. McClymont (1951) made a detailed study of the higher VFA's in the rumen of an ox fed various diets, and Gray, Pilgrim, Rodda & Weller (1952) used a combination of liquid/liquid partition chromatography and chemical methods to demonstrate the presence of formic, *iso*butyric and hexanoic acids, a valeric acid isomer and possibly heptanoic acid in the sheep rumen. The analysis of mixtures of VFA's has been greatly simplified, however, by the development of the method of James & Martin (1952) using gas/liquid partition