groups, but that after irradiating rhodopsin, the retinene is combined with an amino group and not a sulphydryl group. A possible mechanism is as follows:

'active retinene' + opsin → retinene-*S*-opsin,
retinene-*S*-opsin → retinene-*N*-opsin (intramolecular). } (3)

Rhodopsin could be the compound with either a retinene-to-sulphur bond or a retinene-to-nitrogen bond. However, the fact that compounds of retinene and sulphydryl compounds absorb at short wavelengths (about 330 mμ.), while the compounds of retinene and a primary amine can absorb at long wavelengths (440 mμ.), and even at 500 mμ. under certain conditions (Collins, 1953), strongly suggests that rhodopsin contains a retinene-to-nitrogen bond.

The role of pyridoxal phosphate is not implicit in the foregoing. If it be assumed that it is acting as a coenzyme of transamination, its function may be to maintain an essential amino group, present in opsin. It is even conceivable that pyridoxal phosphate may be transformed into pyridoxamine phosphate, which may be part of the opsin molecule.

SUMMARY

1. The regeneration of rhodopsin has been studied with comminuted ox retina. The reaction mixture need only contain *all-trans*-vitamin-A alcohol and phosphate buffer (pH 7·4). The average amount after 2 hr. at 37° was 62 %, but the results were variable and ranged from 48 to 100 %.

2. Pyridoxal phosphate was found to increase the extent of regeneration.

3. The effect of light on the vitamin A or retinene used showed that vitamin A and retinene were equally effective and that prior exposure to light resulted in increased regeneration in both cases.

4. The implications of these results are discussed in connexion with current theories of the mechanism of the regeneration of rhodopsin.

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Comparative Detoxication

2. GLUCOSIDE FORMATION FROM PHENOLS IN LOCUSTS

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It has recently been shown that locusts, like mammals, can acetylate aromatic amino compounds (Myers & Smith, 1953*a*) and form substituted hippuric acids from aromatic acids (Friedler & Smith, 1953).

When phenols are absorbed by mammals, a considerable proportion of the dose is eliminated in the urine as ethereal sulphates and glucosiduronic acids (glucuronides) (Williams, 1947). With large doses of phenols glucosiduronic acid formation is usually predominant (Bray, Humphris, Thorpe, White & Wood, 1952). Glucosiduronic acids are also formed in birds (Sperber, 1947) and frogs can convert menthol into menthylglucosiduronic acid (Schmid, 1936).

Apart from a preliminary report on part of the present work (Myers & Smith, 1953*b*), the only reference to the conjugation of an ingested phenol

by an insect appears to be Zukel's (1944) histochemical detection of a 'leucothionol conjugate' in the malpighian tubules of cockroaches poisoned with phenothiazine. The chemical nature of this conjugate, however, was not established. In cockroaches, DDT (1:1:1-trichloro-2:2-di(*p*-chlorophenyl)ethane) is converted into a water-soluble metabolite of unknown constitution which may also be a conjugate of some kind (Butts, Chang, Christensen & Wang, 1953). The object of the present work was to find out whether glucosiduronic acids were formed from phenols in locusts.

It will be shown that the conjugation of *m*-aminophenol and quinol does occur in the locust to a limited extent, but that the conjugates are not the glucosiduronic acid derivatives into which these phenols are converted in vertebrates (Williams, 1943; Garton & Williams, 1949). It was not possible to isolate the conjugates and identify them by classical methods, but chromatographic and other evidence strongly suggests that they are the β-glucosides of the phenols administered.

MATERIALS AND METHODS

Locusts. The 5th-instar hoppers, or immature adults, of *Locusta migratoria* were obtained from the Anti-Locust Research Centre and kept as previously described (Myers & Smith, 1953*a*) on a diet of fresh grass.

Reference compounds. *m*-Aminophenyl-β-D-glucoside, m.p. 139–140°, $[\alpha]_D$ −67° in water (*c*, 1), was synthesized according to Latham, May & Mosettig (1950). Arbutin (*p*-hydroxyphenyl-β-D-glucoside), m.p. 199°, $[\alpha]_D$ −60° in water (*c*, 1), was purchased (British Drug Houses Ltd.). The glucosiduronic acids of 7- and 8-hydroxyquinoline and *m*-aminophenol were biosynthetic samples previously prepared in this laboratory (Smith, 1953; Robinson, Smith & Williams, 1953*a*; Williams, 1943). Quinolyl-8-sulphuric acid was prepared by the general method of Burkhardt & Lapworth (1926) using chlorosulphonic acid.

Administration of compounds. The phenols, dissolved in 0·01–0·02 ml. of water, were injected into the thorax at the root of the hind leg. An Agla micrometer syringe (Burroughs Wellcome and Co.) with a no. 27 needle was used.

Preparation of extracts. The dry excreta were ground roughly in a mortar to break up the pellets and allowed to stand for 15 min. in water with occasional stirring. It was usually convenient to use 1–5 ml. of water for the faeces from one locust, depending on the quantity excreted. For the quantitative experiments the mixture was centrifuged or filtered and portions of the clear solution were withdrawn for analysis. In the qualitative experiments the aqueous extracts were filtered under suction, evaporated to small bulk *in vacuo*; absolute ethanol was then added till precipitation of inorganic material and urates was complete. The filtered ethanolic solution was concentrated *in vacuo* before chromatography.

Paper chromatography. Downward chromatograms were run as previously described (e.g. Smith, Smithies & Williams, 1953). The solvents used were: *A*, butanol: acetic acid: water (4 : 1 : 5, v/v); *B*, butanol: benzene: ammonia solution (sp.gr. 0·880) (5:2:2, v/v); *C*, pyridine: ethyl acetate: water (1:2:2, v/v); *D*, butanol: water. In all cases the layer containing least water was used. R_F values were not exactly reproducible in the different tanks, but the general pattern and order of separation were consistent, and R_F values are quoted only to illustrate the degree of separation obtained between spots. All chromatographic identifications were made by comparison with an authentic sample run at the same time on the same paper, and were supported by colour reactions and spectrophotometric observations when sufficient material was available.

In general, when the crude faecal extracts, or extracts of normal faeces with added reference compounds, were chromatographed, the spots obtained had smaller R_F values than those given by pure solutions of reference compounds applied alongside. This appeared to be due to a slowing down of the solvent front as it passed the heavy deposit of irrelevant material in the spot of dried extract. It was necessary, therefore, to separate the metabolites on a preliminary chromatogram, to elute areas on the paper giving the appropriate colour tests and to re-chromatograph the eluates. These eluates, which contained less irrelevant material, yielded satisfactory chromatograms with no distortion of the front and were suitable for comparison with the reference compounds.

Paper ionophoresis. An apparatus essentially the same as that of Durrum (1950) was used, the whole assembly being set up on the grid of a large desiccator containing water to maintain humidity. A strip of Whatman no. 3 paper, 8 × 30 cm., was supported over a glass rail and dipped into the troughs containing the electrodes. The paper was first dipped into the buffer solution (Britton & Robinson, 1931), freed of excess of fluid by blotting with a sheet of dry filter paper, and hung over the rail dipping into the buffer in the troughs. Spots of the solutions under examination were applied to the centre of the paper and a potential of 350 v was applied to the electrodes from dry cells. A current of 6–10 mA was drawn. After 2 hr. the paper was removed and allowed to dry before being sprayed with reagents to reveal the spots.

Detecting reagents. Reducing sugars were detected by the naphthoresorcinol reagent (Partridge, 1948) or by *p*-anisidine hydrochloride (Hough, Jones & Wadman, 1950). Aromatic amino compounds were located by diazotizing and coupling. A spray of 1% (w/v) aqueous HNO_2 followed 5 min. later by one of 1% (w/v) β-naphthol in N-NaOH gave red colours. Quinol and its glucoside, arbutin, were detected by a spray of 0·1% (w/v) tetra-azotized di-*o*-anisidine in 0·5 N-$NaHCO_3$, which gave brown-purple colours.

Glucosiduronic acid was measured by Paul's (1951) modification of the naphthoresorcinol reaction and *m*-aminophenol by the method used by Smith & Williams (1949) for aniline.

Absorption spectra were determined in a Unicam S.P. 500 Spectrophotometer.

RESULTS

Attempted detection of glucosiduronide formation

Quantitative estimations

The excreta of locusts feeding on grass contained a considerable amount of reducing carbohydrate material and gave a strong qualitative naphthoresorcinol reaction for uronic acids. Faecal pellets

had an apparent glucosiduronic acid content of 6 mg./g. which was the same as that obtained on a sample of air-dried grass. Separate examination of the gritty, malpighian excretion, hand picked from excreta, showed that this too gave a naphthoresorcinol colour which corresponded to 13 mg. glucosiduronic acid/g. These amounts were such that any small increase of glucosiduronic acid arising from doses of test compounds would not be detectable. Since the carbohydrate material was not soluble in ether, attempts were made to detect the ether-soluble glucosiduronic acids of borneol and (−)-menthol which are known to be formed in many of the higher animals (Williams, 1947).

Aqueous extracts of locust excreta were acidified with 2N-H_2SO_4 and continuously extracted with ether for 2 hr. The residue after evaporation of the ether was dissolved in 0·1N-NaOH and its glucosiduronic acid content determined. Excreta from locusts eating 1 g. of grass daily gave only 0·1 mg. of apparently ether-soluble glucosiduronic acid/locust/day; 90% of (+)-*iso*menthylglucosiduronic acid (Williams, 1940) added to faeces could be accounted for in recovery experiments. When, however, 2 mg. of borneol or (−)-menthol in concentrated ethanolic solution were injected into the locusts, no rise in the excretion of ether-soluble glucosiduronic acids was found. It appeared therefore that little, if any, of these two compounds was conjugated with glucosiduronic acid.

Qualitative experiments

Compounds selected for preliminary experiments were *m*-aminophenol and 7- and 8-hydroxyquinolines, since the quinolyl glucosiduronic acids are readily detected by their fluorescence in ultraviolet light, and *m*-aminophenylglucosiduronic acid gives an intense red colour with the diazo reagents. These colour reactions enable small amounts of the glucosiduronic acids to be easily located on paper chromatograms.

7-Hydroxyquinoline. Two locusts were injected in the thorax with 1 mg. of the compound dissolved in 0·01 ml. of water containing the minimum amount of hydrochloric acid for complete solution (pH about 5) and the faeces were collected during the next 24 hr. The excreta were ground with 2 ml. of 2% (w/v) aqueous sodium bicarbonate and filtered. The filtrate was neutralized with dilute hydrochloric acid and ethanol was added to precipitate inorganic salts. After filtration, the ethanol was evaporated to small bulk and the solution chromatographed on paper using solvent mixture *B* and reference spots of authentic quinolyl-7-glucosiduronic acid and 7-hydroxyquinoline. No spots were found on this chromatogram corresponding with the R_F or fluorescence of the glucosiduronic acid, though two other, faster-moving, unidentified spots (R_F 0·1 and 0·2) were present which fluoresced blue in ultraviolet light.

8-Hydroxyquinoline. A similar experiment with 8-hydroxyquinoline yielded a chromatogram on which two spots were present. One, which moved faster (R_F 0·26) than quinolyl-8-glucosiduronic acid (R_F 0·07), fluoresced blue in alkaline and acid solutions and was not identified; the other corresponded approximately (R_F 0·36) to quinolyl-8-sulphuric acid (R_F 0·33) and had a similar blue fluorescence in ultraviolet light.

m-*Aminophenol.* A similar experiment with *m*-aminophenol yielded an extract which was chromatographed on paper in solvent mixture *A*. In addition to a spot corresponding to *m*-aminophenol, there was present a diazotizable substance which had an R_F greater than that of *m*-aminophenylglucosiduronic acid (Table 2). No spot was found which corresponded to that of the glucosiduronic acid though some diazotizable material was left at the starting point.

Separation of the m-*aminophenol conjugate*

Fifty immature adult locusts were each injected on two successive days with 1 mg. of *m*-aminophenol dissolved in 0·01 ml. of water containing the minimum amount of hydrochloric acid for solution (pH about 4). The faeces were collected on the third day, ground in a mortar and extracted twice with 10 ml. of boiling water. The filtrate from the grass residues was concentrated *in vacuo* to small bulk and absolute ethanol was added to precipitate inorganic salts. The pink precipitate was removed and the yellow filtrate concentrated and applied from edge to edge near the end of a sheet of Whatman no. 1 paper as a zone 2 × 20 cm. The paper was irrigated overnight with the top layer of solvent mixture *A* and, after drying, a strip was cut from one edge and sprayed with the diazotizing reagents. Apart from some residual reacting material at the origin, two main bands were present. Unchanged *m*-aminophenol, R_F 0·7, was eluted chromatographically with 0·1N-HCl and its identity confirmed by its ultraviolet absorption (Table 1) and behaviour on paper chromatograms (Table 2). The metabolite having R_F 0·3 was eluted chromatographically with water and purified on another large-scale paper chromatogram in solvent mixture *B*. The diazotizable zone was eluted from the dried paper with water, and after hydrolysis of a small portion with N-HCl for 1 hr. at 100°, it was estimated that this fraction contained 5 mg. of combined *m*-aminophenol. Thus about 5% of the dose could be separated as this conjugate. When run on paper chromatograms it had the same R_F values as authentic *m*-aminophenyl-β-glucoside. A solution of the conjugate gave $[\alpha]_D^{20} - 86° \pm 6°$ in water (*c*, 0·174, estimated from *m*-aminophenol formed on

Table 1. *Ultraviolet absorption values for* m-*aminophenol and its glucoside*

	In 0·1 N-HCl			In 0·1 N-NaOH		
	$\lambda_{max.}$ (mμ.)	$E_{1\,cm.}$	$\epsilon_{max.}$	$\lambda_{max.}$ (mμ.)	$E_{1\,cm.}$	$\epsilon_{max.}$
m-Aminophenyl glucoside						
From locust excreta	265	0·79*	—	283	1·28*	—
Reference sample	266	—	770	283	—	1600
				230–235		5800
m-Aminophenol						
From glucoside of locust excreta	270	1·79*	—	290	2·31*	—
Unchanged, from locust excreta	270	0·52	—	291	0·70	—
Reference sample	270	—	1800	291	—	2900

* Values obtained at equivalent concentrations.

Table 2. R_P *values of some phenols and their conjugates*

(Solvent mixtures: *A*, butanol:acetic acid:water (4:1:5, v/v); *B*, butanol:benzene:ammonia, sp.gr. 0·880 (5:2:2, v/v); *C*, pyridine:ethyl acetate:water (1:2:2, v/v); *D*, butanol:water. Run on Whatman no. 4 paper until fronts had moved about 12 in.)

	R_F value in solvent mixture			
	A	*B*	*C*	*D*
7-Quinolinol	—	0·75	—	—
Quinolyl-7-glucosiduronic acid	—	0·03	—	—
8-Quinolinol	—	0·82	—	—
Quinolyl-8-glucosiduronic acid	—	0·07	—	—
Quinolyl-8-sulphuric acid	—	0·33	—	—
m-Aminophenol	0·85	0·75	0·95	0·75
m-Aminophenylglucoside	0·43	0·17	0·80	0·15
m-Aminophenylglucosiduronic acid	0·21	0·0	0·70	0·0
Quinol	0·95	0·90	1·0	0·85
p-Hydroxyphenyl glucoside	0·20	0·43	0·92	0·30

Table 3. *Ionophoresis on paper of* m-*aminophenol and its conjugates*

(The signs −, +, 0 indicate movement towards cathode, towards anode or no movement, respectively, under the conditions described in the text.)

pH	Excreted conjugate	*m*-Aminophenyl-glucosiduronic acid	*m*-Aminophenyl-sulphate	*m*-Aminophenyl-glucoside	*m*-Aminophenol
1·0	−	−	+	−	−
2·5	−	−	+	−	−
2·9	−	0	+	−	−
3·1	−	+	+	−	−
3·6	−	+	+	−	−
6·0	−	+	+	−	−
8·0	−	+	+	−	−
12·0	−	+	+	−	+

hydrolysis and assuming that the conjugate is *m*-aminophenylglucoside).

The conjugate was obtained also from the excreta of 5th-instar hoppers and immature adults after oral administration and from hoppers to which *m*-aminophenol had been applied topically in acetone solution.

Paper ionophoresis. The directions of movement of the metabolite and reference compounds in buffers of different pH is shown in Table 3. In 2 hr. the displacement of the spots was of the order of 1–1·5 cm., except in the case of the ethereal sulphate which separated rapidly from the other spots at all pH values to a distance of 4–5 cm. Some extracts of locust faeces prepared as described above also contained a trace of a diazotizable substance which moved to the anode like the ethereal sulphate. The nature of this compound, which was not present in all experiments, is being investigated. The behaviour of the major metabolite was similar to that of *m*-aminophenyl-β-glucoside in that it never acquired a negative charge in the pH range 1–12 and therefore contained no acid group. The small migration to the cathode in alkaline solution which occurred to the same extent with conjugate or glucoside, and also with *m*-aminophenol except at pH 12, was presumably due to electro-osmotic effects, since it was found that a spot of glucose was similarly displaced a small way towards the cathode. In the acid region, where the amino group was positively

charged, migration to the cathode was in the expected direction.

Ultraviolet absorption spectra. Spectra of *m*-aminophenyl-β-D-glucoside are plotted in Fig. 1 together with spectra of arbitrary dilutions of a concentrate of the conjugate. On hydrolysis of the solutions of the conjugate by N-HCl for 20 min. at 100°, the absorption maxima shifted to wavelengths characteristic of *m*-aminophenol (Table 1).

Acid hydrolysis of the conjugate. A solution (2 ml.) of the metabolite containing the equivalent of 3 mg. of *m*-aminophenol was made 0·5N with HCl and heated at 100° for 30 min. Spots of the hydrolysate were chromatographed in solvent mixture *A* with reference spots of *m*-aminophenol, glucosiduronic acid, glucurone, glucose, fructose, xylose, arabinose, rhamnose and 6-deoxyglucose. *m*-Aminophenol was detected but no unhydrolysed conjugate was present. An intense spot corresponding to glucose was found together with a weak spot corresponding to xylose. Weak xylose and glucose spots were, however, also found in control extracts made from the excreta of locusts which had not received *m*-aminophenol. Treatment of the bulk of the hydrolysate with phenylhydrazine acetate yielded yellow crystals having the form of glucosazone, but insufficient in amount for further identification.

Enzymic hydrolysis. A drop of a solution of the conjugate containing the equivalent of about 50 μg. of *m*-aminophenol was incubated with 1 mg. of emulsin (British Drug Houses Ltd.) for 2 hr. at 37°.

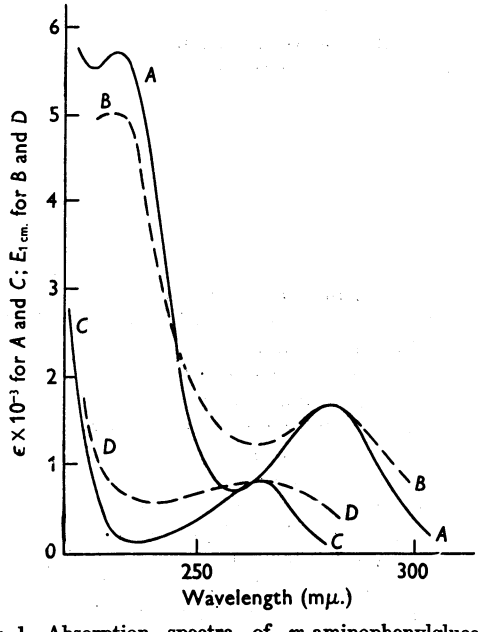

Fig. 1. Absorption spectra of *m*-aminophenylglucoside (*A*, in 0·1N-NaOH, *C*, in 0·1N-HCl) and of extract from locust faeces (*B*, in 0·1N-NaOH, *D* in 0·1N-HCl).

Chromatography in four solvents showed that complete hydrolysis to *m*-aminophenol had occurred. *m*-Aminophenylglucosiduronide was not hydrolysed by this treatment. On the other hand, locust crop fluid, which readily hydrolysed *m*-aminophenylglucosiduronic acid (cf. Robinson *et al.* 1953*a*), had little effect on the conjugate, and after 24 hr. incubation and subsequent chromatography most of the diazotizable material was still present as conjugate.

The conjugation of quinol in the locust

Twenty immature adult locusts each received 0·3 mg. of quinol dissolved in 0·005 ml. of water by injection into the thorax. The dose was repeated the next day and 2 days' excreta were collected and extracted with water and ethanol as described for *m*-aminophenol. The extract was separated on a large-scale paper chromatogram as above, using solvent mixture *B* and Whatman no. 4 paper in a 5 hr. run. In addition to unchanged quinol, moving with the solvent front, and material remaining at the point of application, substances with R_F 0·1, and 0·2 were detected. Corresponding zones were eluted with water and examined chromatographically. The substance with R_F 0·1 appeared to be a conjugate as it yielded quinol on hydrolysis and it is still under investigation. The substance with R_F 0·2 was chromatographically identical with *p*-hydroxyphenyl-β-D-glucoside in the four solvents used (Table 2).

The bulk of the material from the elution was hydrolysed and chromatographed overnight in the same way as the conjugate of *m*-aminophenol. Quinol and glucose with a trace of xylose were detected.

Toxicity of quinol and arbutin. Groups of four adult locusts which had been maintained on a liberal diet of fresh grass were injected in the thorax with varying amounts of quinol in 5% (w/v) aqueous solutions. All locusts in the group injected with 0·5 mg. were dead within 24 hr.; those receiving 0·1 mg. showed no ill effects, while half of those receiving 0·3 mg. were dead in 24 hr. and all showed signs of distress on injection. Similar groups which were injected with increasing volumes of a 10% (w/v) aqueous solution of arbutin, up to 8 mg. arbutin/locust, showed no ill effects and sustained no deaths within 24 hr. Arbutin (4 mg.) administered orally as previously described (Myers & Smith, 1953*a*) had no apparent effect on the locusts.

DISCUSSION

The above results show that the locust eliminates roughly 5% of a 1 mg. dose of *m*-aminophenol in a conjugated form. This conjugate does not behave on paper chromatograms like *m*-aminophenylglucosiduronic acid and electrophoretic experiments show that the molecule has no acid function. Moreover, on hydrolysis, no glucosiduronic acid nor

glucurone was detected, but only glucose. There was the possibility that the sugar part of the molecule was a glucose oligosaccharide, but the formation of a similar conjugate from quinol with the same chromatographic behaviour as *p*-hydroxyphenyl-β-glucoside supports the idea that the metabolites are in fact simple glucosides. That these have the β-configuration is indicated by the ready hydrolysis by emulsin and the optical rotation. This conjugation is, moreover, a genuine detoxication since, in our locusts, injection of arbutin equivalent to 3 mg. of quinol had no apparent effect while one-fifth of this amount of free quinol was rapidly fatal.

The formation of glucosides is well known in plants where the natural glycosides have been thought to be waste products of the plants' metabolism detoxified by conjugation with various sugars. Foreign organic compounds, e.g. chloral, which can give rise to glucosiduronates in animals, form glucosides or gentiobiosides in plants (see Pigman & Goepp, 1948).

The glucosidic detoxication is, however, unusual in the animal kingdom though some observations have been made of the presence of glucosides as normal constituents of insects. Hollande (1913) considered that the pigmented granules in the larvae of the beetle *Cionus olens* were a glucoside which was probably the anthocyanin of the food deposited unchanged in the fat body. Carminic acid, from the cochineal bug, is a sugar derivative but the nature of the sugar is uncertain (Dimroth & Kammerer, 1920). The pigments of aphids can be obtained as water-soluble precursors, protoaphins, which have been shown to be the glucosides of the aphins (Brown, Ekstrand, Johnson, Macdonald & Todd, 1952). In the light of the present work it seems possible that the protoaphins may be detoxication products of the phenolic aphins and that the glucose detoxication mechanism may not be confined to the locust.

On the other hand, some insects are said to be capable of digesting and metabolizing glucosides in their food and salicin (*o*-hydroxymethylphenyl-glucoside) is excreted as salicyl aldehyde or salicylic acid by a variety of insects (Wain, 1943; Hollande, 1909). The locust has a very active glucosiduronic acid-splitting enzyme in its crop fluid (Robinson *et al.* 1953*a*) and this factor may have influenced the use of glucose rather than glucosiduronic acid for detoxication. If any of this enzyme passes into the mid gut, where the malpighian tubes open just anterior to the ileoventricular sphincter, it might hydrolyse any glucosiduronic acid excreted by the tubules. Elimination of glucosiduronic acids would not, therefore, be an efficient mode of detoxication in this insect.

It is also of interest that glucosides are neutral products. Highly ionized products, such as glucosiduronic acids, ethereal sulphates, or hippuric acids, are characteristic of vertebrate detoxication (cf. Robinson, Smith & Williams, 1953*b*).

SUMMARY

1. The conjugation of hydroxy compounds by *Locusta migratoria* has been investigated by paper chromatographic and other methods.

2. No glucosiduronic acid conjugates were produced by (−)-menthol, borneol, 7- or 8-hydroxyquinoline, or *m*-aminophenol.

3. *m*-Aminophenol and quinol were converted, in part, into compounds having the properties of the corresponding β-D-glucoside.

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