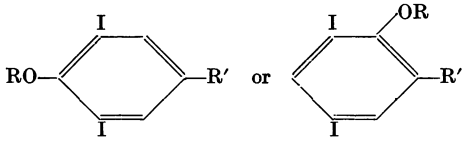
given in our experiments is so large that a mere inhibition of endogenous thyroxine production would probably have little effect on the results. The mode of action has not, however, been completely defined by our present method of testing, which was adopted as a screening method in order to select suitable substances for further biological study. The fact that the compounds when given alone are without effect on oxygen consumption is difficult to explain, but it is well known that such a depression of metabolism is not easy to achieve in mice even with drugs of the thiouracil type.

Whatever the mechanism of the effect observed, it appears to require a fairly high degree of specificity in molecular structure, and the structures of successful compounds bear at least a superficial resemblance to that of thyroxine. Thus activity in this series of compounds is confined to those substances which contain either the group



and it is possible that the diiodophenol part of the esters discussed may compete with the same part of the thyroxine molecule for a position on some receptor surface.

We have been privileged to read the manuscript of a paper by Barker, Kiely, Dirks, Klitgaard, Wang & Wawzonek (1950) which is in general agreement with this hypothesis. These workers describe the antithyroxine effect of a series of iodophenoxyacetic acids in normal and thyroidectomized mice.

SUMMARY

1. The effect of a series of *n*-alkyl esters of 3:5-diiodo-4-hydroxybenzoic acid on the oxygen consumption of thyroxine-treated mice has been studied.

2. The methyl, ethyl, propyl and butyl esters exhibited a strong antithyroxine effect, the butyl compound being the most active. The activity was almost as great by mouth as by the subcutaneous route. The higher esters were less potent, and the acid itself showed only weak activity.

3. Variations in halogen substitution or elimination of the 4-hydroxyl group in the *n*-butyl ester completely abolished the thyroxine-inhibitory effect. *n*-Butyl 3:5-diiodosalicylate showed slight antithyroxine activity. Sodium iodide was inactive.

4. Evidence is presented in support of the view that a specific competitive inhibition of thyroxine is involved.

It is a pleasure to acknowledge the continued assistance given to this research by Glaxo Laboratories Ltd. The toxicity tests on *n*-butyl 3:5-diiodo-4-hydroxybenzoate were carried out by them.

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Metabolism of Derivatives of Toluene

6. TOLUNITRILES, BENZONITRILE AND SOME RELATED COMPOUNDS

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We have suggested previously (see Thorpe, 1950) that methyl and carbamyl groups in aromatic compounds may be termed 'potential centres for conjugation', since under suitable conditions they are converted by the animal to carboxyl and the resulting acids conjugated with glycine or glucuronic acid. We are investigating the behaviour in the rabbit of other potential centres which might be converted to carboxyl, e.g. —CH_2OH, —CHO, —CN, and this present report is concerned with the nitrile group as it occurs in benzonitrile and the tolunitriles. This group might be hydrolysed to carbamyl or to carboxyl. No previous investigation of the fate of the tolunitriles appears to have been carried out.

Benzonitrile was found by Giacosa (1883) to be converted to benzoic acid to a small extent by the dog, while Baumann (1883) stated that its administration to dogs increased the excretion of ethereal sulphate. The latter also isolated small amounts of *o*- and *p*-hydroxybenzoic acids from benzonitrile urine. Adeline, Cerecedo & Sherwin (1926) were unable to detect any metabolites of benzonitrile in the rabbit. Smith & Williams (1950) found that benzonitrile was eliminated very slowly by the rabbit, about 60 % being excreted as *O*-conjugates; they claimed that small amounts were probably converted to benzoic acid and a mercapturic acid. *m*- And *p*-cyanophenols were isolated from the ethereal sulphate fraction of the urine. In the experiments now reported the effect of these four compounds on the excretion by rabbits of ether-soluble acid, ester and ether glucuronides and ethereal sulphate has been determined and several metabolites of the tolunitriles have been isolated. The fate of some of these metabolites has also been examined.

MATERIALS AND METHODS

Materials

m-Tolunitrile, *m*- and *p*-cyanophenols and *m*- and *p*-cyanobenzoic acids were prepared by the Sandmeyer reaction, starting from the corresponding amino compounds. *o*-Cyanobenzoic acid could not be obtained in this way, but was prepared from phthalimide by the method of Braun & Tcherniac (1907) in such small yield as to render it impracticable to accumulate sufficient for dosing. The acid is unstable and forms phthalamic acid on standing in water at 37° for 24 hr. *o*-Cyanophenol was prepared by the action of acetic anhydride upon salicylaldoxime (Bone, 1893). Terephthalamic acid was obtained by the action of H_2O_2 on *p*-cyanobenzoic acid (cf. the preparation of *o*-toluamide from *o*-tolunitrile, Noller, 1933). Phthalamic acid was prepared by the action of KOH on phthalimide at room temperature (Aschan, 1886). All melting points agreed with those given in the literature. Attempts to prepare *o*-cyanobenzyl alcohol by hydrolysis of *o*-cyanobenzyl chloride were unsuccessful.

p-*Cyanohippuric acid* was obtained by a Sandmeyer reaction upon *p*-aminohippuric acid. It formed colourless leaflets, m.p. 191°. (Found: C, 59·2; H, 4·0; N, 13·5; equiv. (by titration) 208. $C_{10}H_8O_3N_2$ requires C, 58·8; H, 3·9; N, 13·7 %; equiv. 204.)

Diet and dosage

The rabbits used, does weighing 2–3 kg., were maintained throughout on the standard diet of rabbit pellets previously described (Bray, Ryman & Thorpe, 1947). The nitriles were administered by stomach tube as emulsions in warm water and the acids as solutions of their Na salts. *m*- and *p*-tolunitriles could be given in doses of 1 g. without toxic effects, but the maximum safe dose of *o*-tolunitrile was 0·7 g. Benzonitrile was more toxic and doses of 0·7 g. caused in some animals considerable toxic symptoms, including anorexia, serous discharge from the eyes, and, in some cases, the excretion of large amounts of reducing material, starting 3–4 days after dosage. Smith & Williams (1950), who gave benzonitrile in olive oil, did not record these effects. It is possible that the toxicity was diminished by delaying absorption, although in a few experiments in which we administered benzonitrile in olive oil we observed definite toxic effects in some rabbits, a dose of 1 g. in olive oil proving fatal to one rabbit. Many rabbits survived a 1 g. dose in water. *p*-Cyanobenzoic, *p*-cyanohippuric, terephthalamic and phthalamic acids caused no obvious toxic effects in doses of 1 g.

Methods of analysis

Ether-soluble acid, ester and ether type glucuronides and ethereal sulphate were estimated as previously described (see Bray *et al.* 1947; Bray, Thorpe & White, 1950*b*). The paper-chromatographic technique was essentially that described in an earlier paper (Bray, Thorpe & White, 1950*a*).

RESULTS

Normal excretion of metabolites

The average daily excretion of ethereal sulphate by the rabbits used in this investigation ranged from 28 to 35 mg. SO_3. The average by which the normal individual daily values differed from the corresponding weekly averages used as 'baseline' for purposes of calculation was ± 8 % (± 3 mg. SO_3). The average daily excretion by individual rabbits of reducing material ranged from 235 to 354 mg. (calculated as glucuronic acid). The average by which the daily individual normal values differed from the weekly averages was ± 6 %. The corresponding values for ether-soluble acid were 623–855 mg. (calculated as hippuric acid) and ± 4 %.

Quantitative experiments with tolunitriles

The results obtained are summarized in Table 1. *m*- And *p*-tolunitriles are excreted mainly as ether-soluble acid, the nature of which is discussed in a later section. The increase in excretion of ester glucuronide with both these isomers and of ethereal sulphate with *m*-tolunitrile is of doubtful significance since the actual increases observed were of the same order as normal baseline fluctuations. About half of the *o*-tolunitrile administered was accounted for by the quantitative results. The only acidic metabolite isolated was phthalic acid. Phthalide, equivalent to about 20 % of the dose, was, however, isolated from hydrolysed *o*-tolunitrile urine, and since phthalide is readily formed from *o*-hydroxymethylbenzoic acid in acid solution, some of the latter compound may also have been present. (We have isolated *o*-hydroxymethylbenzoic acid from acidified urines after giving either phthalide or *o*-hydroxymethylbenzoic acid.) The values obtained for ester glucuronide excretion from *o*-tolunitrile are not regarded as very reliable since the administration of this compound sometimes caused the excretion of large amounts of reducing material. The smaller amounts found here may be due to this rather than to the elimination of an actual metabolite. The phenols responsible for the increased excretion of ethereal sulphate have not yet been identified.

Table 1. *Excretion by the rabbit of metabolites of tolunitriles, expressed as percentage of the dose*

Metabolite	No. of experiments	Range	Average
	o-Tolunitrile		
Ether-soluble acid	7*	5–30	21
Ester glucuronide	7*	0–5	2
Ether glucuronide	4†	8–18	14
Ethereal sulphate	3*	12	12
	m-Tolunitrile		
Ether-soluble acid	7*	78–109	92
Ester glucuronide	7*	0–5	3
Ether glucuronide	3*	0	0
Ethereal sulphate	3*	0–2	1
	p-Tolunitrile		
Ether-soluble acid	16	62–113	86
Ester glucuronide	16	0–7	2
Ether glucuronide	11*	0	0
Ethereal sulphate	3	0	0

* One experiment in this group was made on the pooled urines of ten rabbits.

† Determination by naphthoresorcinol method (Hanson, Mills & Williams, 1944) gave on two other urines values of 22 and 29%.

Qualitative experiments

o-Tolunitrile. The following is typical of several experiments made with urines from rabbits dosed with *o*-tolunitrile. Continuous extraction with ether of *o*-tolunitrile urine as collected (pH 7·8–8·0) gave only urea. Extraction of the residual urine, after acidification with HCl to pH 2–3, gave a mobile brown syrup which on chilling partially crystallized. Trituration with ethanol (96%) yielded a yellow powder which after recrystallization from the same solvent gave colourless crystals, m.p. 192° (with sublimation). On fusion with resorcinol and conc. H_2SO_4 these gave a product which in alkaline solution showed the characteristic fluorescence of fluorescein. The identity of this compound with phthalic acid was confirmed by comparison (mixed m.p.) with an authentic sample. (Found: C, 57·6; H, 3·2. Calc. for $C_8H_6O_4$: C, 57·8; H, 3·6%.) Yield 280 mg. from the 24 hr. urine of ten rabbits, each of which had received 0·7 g. *o*-tolunitrile, i.e. 3% of the dose. The material soluble in ethanol (above) consisted almost entirely of benzoic acid (150 mg.). The urine remaining from the last ether extraction was hydrolysed by boiling for 1 hr. with conc. HCl (0·2 vol.). Extraction with ether then gave a syrupy product from which phthalide, m.p. 70°, identical (mixed m.p.) with an authentic sample, was isolated. Yield 0·7 g., i.e. 21% of dose. The mother liquors from the purification of phthalide yielded a further small amount of phthalic acid (50 mg.). In other experiments phthalide was obtained from extracts of acidified urine without hydrolysis.

As described below, phthalamic acid gives rise in the rabbit to phthalic anhydride, and since the former may theoretically be an intermediate in the conversion of *o*-tolunitrile to phthalic acid, the ether-soluble material extracted from *o*-tolunitrile urine as collected was examined for the presence of phthalic anhydride. (Phthalic acid is not extracted under these conditions.) Although this could not be isolated, the extract gave a strongly positive fluorescein test. An extract of normal rabbit urine obtained under the same conditions gives only a very feeble fluorescence, if any. It is, therefore, probable that a small amount of phthalic anhydride was present.

The quantitative results in Table 1 show that *o*-tolunitrile causes a comparatively large increase in ethereal sulphate and ether glucuronide excretion, but examination of ether extracts of acidified and hydrolysed urines failed to yield any phenolic metabolite. Paper chromatograms (see Bray *et al.* 1950*a*; Bray, Lake, Thorpe & White, 1950) also failed to reveal any phenols other than those known to be present in normal urine. As we have not yet found a solvent mixture suitable for use with very fast moving phenols (e.g. phenol, oresols, xylenols) the metabolite in question may belong to this class. Another possibility is that *o*-tolunitrile increases the output of normal urinary phenols. Preliminary evidence of such an increase was obtained after administration of *o*-toluamide to rabbits (unpublished results), when examination of the urine by paper chromatography suggested the presence of significantly increased amounts of *m*- and *p*-hydroxybenzoic and *p*-hydroxyphenylacetic acids. An attempt to isolate the glucuronide by the usual procedure via the Pb salt (cf. Bray *et al.* 1947) gave a syrup which was partly soluble in ether. The ether-soluble part, which could not be crystallized, was hydrolysed by boiling with HCl (5 N for 1 hr.). Only phenols occurring in normal urine could be detected on paper chromatograms of the ether extract of the hydrolysate and a very small amount of *p*-hydroxybenzoic acid was isolated. Examination of the hydrolysate of the ether-insoluble part showed the presence of *m*- and *p*-hydroxybenzoic acid and also three spots on the paper chromatogram which were not matched by any from normal urine. Two of these had R_F 1·0 in benzene-formic acid, and gave green and orange colours with diazotized *p*-nitraniline; the third spot had R_F about 0·05 (4 hr.) and gave a pale-blue colour. None of these has been identified.

Phthalamic acid. The 24 hr. urine of five rabbits which had each received 1 g. phthalamic acid was extracted at pH 8·3 and from the extract phthalic anhydride (m.p. 130°) was isolated. Yield 300 mg. (7% of the dose). The urine was then acidified (pH 2–3) and re-extracted to give a crystalline extract from which phthalic acid (3·4 g., m.p. 191°) was obtained. Yield, 68% of the dose. Control experiments showed that phthalic anhydride, suspended in urine pH 2–3 for 24 hr. and then continuously extracted with ether, yielded

phthalic acid in amounts indicating considerable conversion. No appreciable amount of phthalic acid was formed if phthalic anhydride was left to stand in urine at pH 7–8. It seems clear, therefore, that any phthalic anhydride isolated can be regarded as a metabolite whereas phthalic acid may be, wholly or in part, an artifact. Phthalamic acid forms no phthalic anhydride or phthalic acid on standing in urine at pH 7–8, but forms phthalic acid at pH 2–3.

o-*Hydroxymethylbenzoic acid and phthalide.* Examination of the urine of rabbits which had received either of these compounds failed to reveal the presence of either phthalic anhydride or phthalic acid. Only phthalide or *o*-hydroxymethylbenzoic acid could be isolated.

m-*Tolunitrile.* The urine of rabbits dosed with this compound was examined by methods similar to those described below for *p*-tolunitrile urine. From the ether-soluble acid fraction colourless needles, m.p. 217°, were obtained and identified as *m*-cyanobenzoic acid. The mixed m.p. with an authentic sample (m.p. 217°) was 217°. (Found: N, 9·9. Calc. for $C_8H_5O_2N$: N, 9·5 %.) Average yield, 61 % of the dose. No *m*-cyanohippuric, *iso*phthalamic or *iso*phthalic acid could be detected.

m-*Cyanobenzoic acid.* 1 g. of this acid was given to each of ten rabbits. The pooled 24 hr. urine was acidified (pH 3) and exhaustively extracted with ether. Only *m*-cyanobenzoic acid could be isolated from the extract. Yield, 6·6 g. (66 % of dose).

p-*Tolunitrile.* Several large-scale experiments were carried out with urine from rabbits dosed with *p*-tolunitrile. While the results obtained for the major metabolite were consistent, the amounts of the minor metabolites isolated varied considerably. (1) The ether-soluble acid obtained by continuous extraction with ether of *p*-tolunitrile urine at pH 2–3 was fractionated by refluxing with several successive portions of toluene (50 ml. each). The soluble material was crystallized from water giving colourless leaflets m.p. 216°, mixed m.p. with authentic *p*-cyanobenzoic acid (m.p. 219°) 219°. (Found: N, 9·7; equiv. (by titration) 147. Calc. for $C_8H_5O_2N$: N, 9·5 %; equiv. 147.) Yield 48–60 % of the dose. The material insoluble in toluene was a brown tar. This was dissolved in hot water and purified with charcoal. In one experiment, the cooled solution deposited 50 mg. (from 4 g. *p*-tolunitrile) of crystals which did not melt, but sublimed at 250–300°. These were shown to be terephthalamic acid by comparison with an authentic specimen and by conversion to the methyl ester (m.p. 201–203°). (Found: N, 8·5. Calc. for $C_8H_7O_3N$: N, 8·5 %.) It is unlikely that terephthalamic acid was an artifact since none was formed when added *p*-cyanobenzoic acid was extracted from acidified normal urine and then treated by the toluene fractionation procedure as above. (2) In another experiment the mother liquors from the recrystallization of *p*-cyanobenzoic acid deposited shining yellow plates, m.p. 190°, shown by comparison (mixed m.p.) with an authentic sample to be *p*-cyanohippuric acid. (Found: N, 13·3. Calc. for $C_{10}H_8O_3N_2$: N, 13·7 %.) A further small amount was obtained from the fraction of the ether-soluble acid insoluble in toluene. Total yield 700 mg. from 4 g. *p*-tolunitrile (10 % of the dose). (3) In another experiment all three metabolites were isolated. The yields were 6·6 g. *p*-cyanobenzoic acid, 20 mg. *p*-cyanohippuric acid, and 50 mg. terephthalamic acid from 8·8 g. *p*-tolunitrile, i.e. 60, 0·13 and 0·4 %, respectively, of the dose. The urine after acid extraction was hydrolysed by boiling for 1 hr. with conc. HCl (0·2 vol.) and again extracted with ether. Treatment of the crystalline extract with boiling water left an insoluble residue (100 mg.) which did not melt, but sublimed at 250–300°. It contained no N and was shown to be terephthalic acid by comparison with an authentic specimen and conversion to its dimethyl ester, m.p. 140°, mixed m.p. with authentic sample (m.p. 141°) 140°. It is probable that the terephthalic acid is an artifact formed from unextracted terephthalamic acid so that the total yield of the latter could be assessed as 1·2 % of the dose. The amount (10 % of the dose) of *p*-cyanohippuric acid isolated in the second experiment compared to that (0·0, 0·13 %) obtained in the other experiments requires comment. It may be relevant that in the second experiment the animals were dosed under fasting conditions before receiving their daily food ration, whereas in the others they were dosed after having consumed some food.

p-*Cyanobenzoic acid.* 1 g. of this compound was administered to each of five rabbits. The pooled 24 hr. urine was acidified (pH 3) and continuously extracted with ether. The extract on purification yielded only *p*-cyanobenzoic and *p*-cyanohippuric acids in amounts corresponding to 50 and 2 %, respectively, of the dose.

p-*Cyanohippuric acid.* This was administered to rabbits in doses of 1 g. and the acidified urines treated as in the previous section. Only *p*-cyanobenzoic acid (yield 50 % of dose) was isolated, suggesting that the glycine conjugate is hydrolysed in the gut. No glycine conjugate was detected in the ether-soluble acid.

Terephthalamic acid. The ether-soluble acid isolated from acidified terephthalamic acid urine by continuous extraction for a week was triturated with hot ethanol to remove tarry material. The white residue contained N and after further purification was shown to be terephthalamic acid. Yield 2·9 g. (58 % of the dose). No terephthalic acid could be detected.

The yields of the compounds isolated from urine after administration of the tolunitriles and related compounds are summarized in Table 2. The values given are those for the highest yields obtained.

Table 2. *Metabolites isolated from urine after administration of tolunitriles and related compounds, expressed as percentage of dose*

Compound administered	Cyanobenzoic acid	Cyanohippuric acid	Carbamyl benzoic acid	Benzene dicarboxylic acid	Phthalic anhydride	Phthalide
o-Tolunitrile	0	0	0	3·5	Trace	21
Phthalamic acid	—	—	—	68	7	—
m-Tolunitrile	61	0	0	0	—	—
m-Cyanobenzoic acid	66	0	0	0	—	—
p-Tolunitrile	60	10	0·9	0·8	—	—
p-Cyanobenzoic acid	50	2	0	0	—	—
p-Cyanohippuric acid	50	0	0	0	—	—
Terephthalamic acid	—	—	58	0	—	—

Metabolism of benzonitrile

Analyses were carried out on benzonitrile urines as described for the tolunitriles. Benzonitrile is, however, an extremely toxic compound, and animals receiving it did not consume the whole of their food ration for several days after dosage. The baseline excretion values of the metabolites estimated are, therefore, likely to be unreliable. The excretion of ethereal sulphate was definitely increased and the percentages of the dose apparently excreted in this form were found to be 20–21 %. It is likely that these results are low. The metabolism of benzonitrile appears to be a slow process, since the increase in ethereal sulphate excretion persisted for 2 days after dosage. The ether-soluble acid results were too variable to justify any conclusion drawn from them. As already mentioned, benzonitrile caused in some animals an enormous increase in the excretion of reducing material which was usually not apparent until 3 or 4 days after dosage and which lasted up to 3 or 4 further days. During this period as much as 7–8 g. extra reducing material (calculated as glucuronic acid) was excreted, a considerable part of which was shown to be due to glucuronic acid by the quantitative naphthoresorcinol method of Hanson, Mills & Williams (1944). Thus the reducing values of benzonitrile urine cannot be used as a reliable indication of the metabolic fate of the compound.

In view of Smith & Williams's (1950) more detailed study our qualitative investigation was confined to examination of extracts of benzonitrile urine by paper chromatography. Using a mixture of *n*-butanol (4 parts), pyridine (8 parts), saturated aqueous NaCl (5 parts) and NH_3 (sp.gr. 0·880, 1 part) as developing solvent and diazotized *p*-nitraniline as detecting reagent, *o*- and *m*-cyanophenols (R_F 0·8 and 0·9, respectively, after 15 hr.) were detected in extracts of hydrolysed benzonitrile urine. *m*-Cyanophenol was also detected using a mixture of light petroleum (b.p. 60–80°) (5 parts), water (1 part) and glacial acetic acid (5 parts) as solvent. (R_F 0·1 after 5 hr.) It is likely that the *p*-isomer was also present, but it was not detected since it gives no colour with any of the spraying reagents used. The results of Smith & Williams (1950) suggest that only *m*- and *p*-cyanophenols are excreted as ethereal sulphates, so that the *o*-isomer probably forms only a glucuronide. The chromatograms suggested that the amount of *o*-cyanophenol present was of the same order as that of *m*-cyanophenol.

DISCUSSION

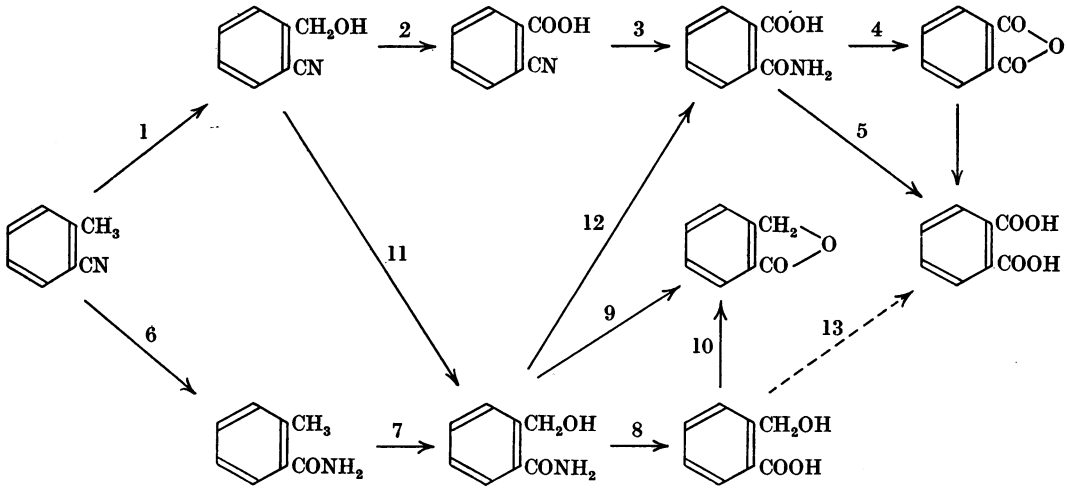
From the foregoing results it is clear that the nitrile group may be hydrolysed in the rabbit although with difficulty in some compounds. Smith & Williams (1950), on the basis of the isolation of 500 mg. benzoic acid from urine, claimed that about 10 % of benzonitrile is hydrolysed. It is doubtful, however, whether the isolation of benzoic acid under these circumstances proves that it is a metabolite of benzonitrile. It is unlikely that 500 mg. of benzoic acid would have been isolated from 10 days' normal rabbit urine, although such an amount could be present, since isolation procedures from urine are usually far from efficient. We have, however, isolated comparably large amounts of benzoic acid from the urine of rabbits which had received 3:5-xylen-1-ol, *o*-toluamide, *o*-tolunitrile and 2-methylbenzoxazole. Benzoic acid would not be expected as a metabolite of any of these. Smith & Williams (1950) also reported the isolation of benzoylglucuronide from benzonitrile urine. If benzoic acid had been formed to such an extent that benzoylglucuronide was excreted in sufficient quantity to be isolated, it would have been expected that an even greater amount of hippuric acid would also have been formed, since it is well known that in the rabbit the conjugation of benzoic acid with glycine takes place to a much greater extent than conjugation with glucuronic acid. Smith & Williams (1950) did not comment upon the amount of hippuric acid isolated. It seems, therefore, reasonable to attribute their findings to the inhibition of glycine conjugation by the administration of benzonitrile. The formation of cyanophenols from benzonitrile in the amounts which they claimed indicates that hydroxylation takes place more readily than does hydrolysis of the nitrile group.

From the behaviour of the tolunitriles it is clear that modification of the nitrile group does occur, although only to a small extent with the *p*-isomer and possibly not at all with the *m*-isomer. In these two compounds the methyl group is more readily oxidized. In view of the isolation of phthalide and phthalic acid from *o*-tolunitrile urine and of terephthalamic and terephthalic acid from *p*-tolunitrile urine, we must modify our earlier opinion (Bray, Humphris & Thorpe, 1949; Thorpe, 1950) that in compounds containing two potential centres for conjugation only one is modified in a single molecule. Both potential centres must have been changed, in order to form the benzenedicarboxylic acid derivatives which were isolated as metabolites.

The only metabolites of *o*-tolunitrile isolated were phthalide and phthalic acid. No evidence was obtained of the excretion of *o*-cyanobenzoic or *o*-toluic acids. The available information relevant to the fate *o*-tolunitrile in the rabbit can be summarized as follows: (*a*) *o*-Hydroxymethylbenzoic acid (or phthalide) is known to be a metabolite of *o*-toluamide. It is probable that *o*-hydroxymethylbenzamide is an intermediate in this reaction (Bray, Thorpe & Wood, 1949). (*b*) Ether extracts of *o*-toluamide urines only give a very feeble fluorescein test so that at the most only minute amounts of phthalic anhydride (or acid) are formed in the rabbit from *o*-toluamide. (*c*) When phthalide or *o*-hydroxymethylbenzoic acid is given to a rabbit no phthalic anhydride or phthalic acid can be detected. (*d*) *o*-Toluic acid gives rise in the rabbit to neither phthalide (Bray, Thorpe & Wood, 1949), nor phthalic anhydride (or acid). (*e*) Only phthalic acid and its anhydride were isolated from the urine when phthalamic acid was given to the rabbit. (*f*) *o*-Hydroxymethylbenzoic acid is readily converted to

phthalide in acid solution. The fact that phthalide is often isolated in better yield after the urine has been treated with acid (standing at pH 2·3 or under hydrolysis conditions) favours the view that *o*-hydroxymethylbenzoic acid may be the primary metabolite, but there is as yet no conclusive evidence. (*g*) Phthalic acid may be an artifact formed from phthalamic acid or from phthalic anhydride owing to acid treatment of the urine.

The scheme below has been drawn up, taking these facts into consideration and provides an explanation for the observed metabolites and artifacts. It cannot at present be decided whether phthalide is formed via reactions 1 and 11 or 6 and 7, since we have so far



failed to synthesize *o*-hydroxymethylbenzonitrile and *o*-hydroxymethylbenzamide. It seems probable that the formation of phthalic acid proceeds via *o*-cyanobenzoic acid (reactions 1, 2 and 3) although none of the intermediates has actually been isolated. In view of its lability, isolation of *o*-cyanobenzoic acid could hardly be expected. Phthalic acid may be regarded as an artifact derived from either phthalamic acid or phthalic anhydride. Phthalamic acid is readily converted to phthalic anhydride (or acid) by the rabbit. The alternative route via reactions 1, 11 (or 6, 7) and 12 is improbable since only traces of phthalic acid are formed from *o*-toluamide. An argument against the route 1, 11 (or 6, 7), 8 and 13 is that *o*-hydroxymethylbenzoic acid does not give rise to phthalic acid. It must, however, be admitted that there is no direct evidence for the carbamyl intermediates in the scheme.

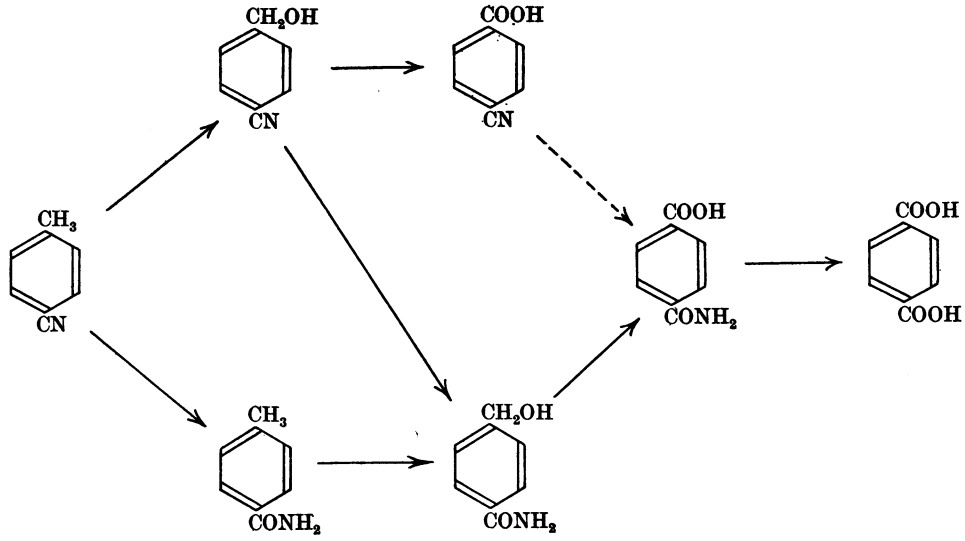
The only metabolite of *m*-tolunitrile identified was *m*-cyanobenzoic acid. This was isolated in such large amount that it is reasonable to assume that ether-soluble acid excreted (92 % of dose) was mainly composed of this compound, since no glycine conjugation could be detected.

The terephthalic acid isolated from hydrolysed *p*-tolunitrile urine was almost certainly an artifact. Terephthalamic acid appears to be a true metabolite of *p*-tolunitrile. This acid was also identified as a metabolite of *p*-toluamide (Bray, Thorpe & Wood, 1949). Terephthalamic acid might be formed from *p*-tolunitrile either via *p*-cyanobenzoic acid or via *p*-hydroxymethylbenzamide as shown in the scheme (p. 198). Since we failed to detect any terephthalamic acid as a metabolite of *p*-cyanobenzoic acid, it seems probable that the small amount of terephthalamic acid produced from *p*-tolunitrile has been formed either via *p*-toluamide or via *p*-hydroxymethylbenzonitrile. The main metabolic reaction is the formation of *p*-cyanobenzoic acid which appears to be readily eliminated either as such or conjugated with glycine.

The study of the tolunitriles has shown that the stability of the nitrile group is considerably affected by the position of the methyl group. In the ortho compound at least 23 % is hydrolysed to carboxyl, in the para more than 1 %, but in the meta either none, or so little as to prevent isolation of the metabolites. There is no doubt that the methyl group is oxidized readily in the meta and para isomers, but whether the apparent absence of any hydrolysis of the nitrile group in the meta compound is due to the formation or excretion of *m*-cyanobenzoic acid with exceptional ease, leaving little opportunity for the action of the enzyme system concerned with hydrolysis of the nitrile group, must be left for further study. The position with regard to the *o*-tolunitrile is as unsatisfactory as that which we found with *o*-toluamide (Bray, Thorpe & Wood, 1949) in that not more than one half of the dose has been accounted for. The substance (or substances) responsible for the increased excretion of ethereal sulphate and ether glucuronide has not been identified. There is no

support for the idea that hydrogen cyanide is liberated leaving *o*-cresol (cf. Adeline *et al.* 1926). Had the increased elimination of ethereal sulphate been due mainly to *o*-cresol, a larger excretion of ether glucuronide would have been expected (cf. Bray *et al.* 1950*b*).

certain, however, whether the role of the enzyme *in vivo* is hydrolytic or synthetic, or whether the synthesis of glucuronic acid is brought about by the same process as that responsible for the formation of glucuronides. Deichmann, Kitzmiller & Witherup (1945) have observed that the ability of rabbits to



Benzonitrile and *o*-tolunitrile may now be added to the list of compounds which cause in some rabbits the excretion of large amounts of non-fermentable reducing material which is probably mainly glucuronic acid. The other compounds are: *o*- and *m*-acetotoluidides (Bray & Thorpe, 1948), *o*-xylene (Bray, Humphris & Thorpe, 1949), *o*-toluamide (Bray, Thorpe & Wood, 1949), *o*-aminophenol, benzoxazolone (unpublished results). Smith & Williams (1949*a*, *b*) have made similar observations with aniline, *p*-phenetidine and the anisidines. They suggest that the reducing material, which they showed to be glucuronic acid, resulted from the breakdown of labile glucuronides, the nature of which could not be elucidated. In some of our experiments, however, the amount of reducing material was much too great to be accounted for by the dose administered and its excretion, which was not an invariable response, often persisted for periods of several days after the dose, whereas glucuronides formed as a result of conjugation of administered foreign compounds are usually excreted rapidly. It is of interest that Levvy, Kerr & Campbell (1948) have observed an increase in the glucuronidase activity *in vitro* of the livers and kidneys of mice which had been treated with menthol, carbon tetrachloride, chloroform, mercuric nitrate, chloroform, uranyl acetate, yellow phosphorus, or phenylarsenoxide, and suggest that it is a consequence of cell proliferation following tissue damage. It is not yet

form and excrete glucuronides after administration of *cyclo*hexanone is not impaired by treatment with liver poisons, e.g. yellow phosphorus, and conclude that glucuronic acid excretion is not an indication of the functional integrity of the liver. The origin of the reducing material observed in our experiments, which certainly cannot be entirely derived from conjugates of metabolites of the compounds administered, is still obscure. Experimental study is rendered difficult by the uncertain response of the animals, but it is hoped to investigate this problem further.

SUMMARY

1. The metabolism of *o*-, *m*- and *p*-tolunitriles and benzonitrile in the rabbit has been studied.

2. *o*-Tolunitrile gives rise to phthalide and phthalic acid (21 and 3 % of the dose, respectively, by isolation) and also increases the excretion of ethereal sulphate by an amount equivalent to 12 % of the dose.

3. *m*- And *p*-tolunitriles are excreted almost entirely as ether-soluble acid (92 and 86 % of the dose, respectively). *p*-Cyanobenzoic and *p*-cyanohippuric acids (60 and 10 % of the dose, respectively) were isolated from *p*-tolunitrile urine. A small amount of terephthalamic acid (1·2 % of the dose) was also isolated. The only metabolite of *m*-tolunitrile detected was *m*-cyanobenzoic acid (61 % of the dose was isolated).

4. At least 20–21 % of a dose of benzonitrile is excreted as ethereal sulphate. *o*- And *m*-cyanophenols were detected by paper chromatography in ether extracts of hydrolysed benzonitrile urine. In some experiments the administration of benzonitrile gave rise to the excretion of very large amounts of reducing material.

5. Qualitative examination of the excretion products obtained after administration of some possible metabolites of the tolunitriles has been made. *m*-Cyanobenzoic acid was excreted unchanged (66 % of dose). No glycine conjugation could be detected. *p*-Cyanobenzoic acid was excreted mainly unchanged, but was conjugated to a small extent with glycine (50 and 2 % of the dose isolated, respectively). *p*-Cyanohippuric acid was excreted mainly as *p*-cyanobenzoic acid (50 % of the dose isolated); no glycine conjugate was detected. Phthalic anhydride and phthalic acid (7 and 86 % of the dose, respectively) were isolated after administration of phthalamic acid. Only unchanged terephthalamic acid (58 % of the dose) could be isolated after giving terephthalamic acid.

The micro-analyses were carried out by Drs Weiler and Strauss, Oxford, and by Miss B. G. Humphris.

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Biochemistry of Locusts

5. THE GREEN PIGMENT OF THE HAEMOLYMPH AND INTEGUMENT OF SOLITARY LOCUSTS (*LOCUSTA MIGRATORIA MIGRATORIOIDES*, R. & F., AND *SCHISTOCERCA GREGARIA*, FORSK.)

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In the solitary phase locusts are often green; this is not an essential characteristic of solitary insects because other colours, such as buff, are often encountered, but it is probably true to say that the green pigment does not occur in the integument of gregarious insects. Similarly, the haemolymph from solitary locusts is almost invariably bright green whilst that from gregarious locusts is golden-yellow; a light-green haemolymph is, however, very occasionally encountered in the latter. The present paper reports an investigation into the green pigment.

Green pigments are widespread in insects, but not a great deal is known about them. As early as 1882, Krukenberg considered that the green pigment of *Locusta* (*Tettigonia*) *viridissima* was different from chlorophyll although, later, Podiapolsky (1907) stated that the absorption spectrum of the pigment was somewhat similar to that of chlorophyll. Rearing experiments on a number of insect species showed, however, that the formation of the green pigment was independent of the amount of chlorophyll in the diet (Toumanoff, 1927; Giersberg, 1928); in particular, Faure (1932) noted that the produc-