Studies in Detoxication

39. NITRO COMPOUNDS. (a) THE METABOLISM OF *o*-, *m*- AND *p*-NITROPHENOLS IN THE RABBIT. (b) THE GLUCURONIDES OF THE MONONITROPHENOLS AND OBSERVATIONS ON THE ANOMALOUS OPTICAL ROTATIONS OF TRIACETYL β -*o*-NITROPHENYLGLUCURONIDE AND ITS METHYL ESTER

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As a result of the discovery that nitro compounds occur in nature there is likely to be a renewed interest in their fate in the body. The antibiotic chloromycetin (chloramphenicol) has been shown to be a *p*-substituted nitrobenzene, and the plant glycosides, hiptagin and karakin, have been shown by Carter & McChesney (1949) to contain β -nitropropionic acid (hiptagenic acid).

It is possible that the toxicity and perhaps the physiological effects of nitro compounds are related to the modifications which the nitro group may undergo *in vivo*. The work of Channon, Mills & Williams (1944) on the metabolism of 2:4:6-trinitrotoluene (TNT) has shown that the aromatic nitro group may be reduced *in vivo* to a hydroxylamino and then to an amino group, and it appears that the biological reduction of the nitro group follows the path, $\text{NO}_2 \rightarrow \text{NO} \rightarrow \text{NHOH} \rightarrow \text{NH}_2$. Evidence in support of this path has been obtained by Bueding & Jolliffe (1946) who showed that reduced coenzyme I plus purified Straub flavoprotein probably converts TNT to a nitroso compound, that xanthine oxidase can convert TNT to the hydroxylamine and that whole tissue (rat liver and diaphragm) is necessary to convert TNT to the amine. In some compounds, however, the nitro group may be relatively resistant to reduction *in vivo*, for Glazko, Wolf, Dill & Bratton (1949) have reported that little if any chloromycetin is reduced in man (see also Glazko, Dill & Rebstock, 1950).

Our intention in the present work was to study the extent of reduction of the three mononitrophenols and attempt to relate it to their orientation and to the fact that *p*-nitrophenol is much more toxic than the *ortho*-compound. Beutner & Block (1943) and Dietrich & Beutner (1946) have shown that *o*-nitrophenol is relatively non-toxic and is largely excreted by rabbits with its nitro group unaltered. We also wished to isolate and characterize the glucuronides of the nitrophenols as a preliminary to a study on the metabolism of nitrobenzene. Furthermore, tetra-acetyl β -*o*-nitrophenyl-D-glucoside shows an anomalous positive optical rotation (Glaser & Wulwek, 1924; Montgomery, Richtmyer

& Hudson, 1942), and we were desirous of finding out whether or not a similar rotation would be shown by the corresponding acetylated β -glucuronide.

METHODS

Materials. The *o*-, *m*- and *p*-nitrophenols used in this work were commercial products which were purified by recrystallization from ligroin or aqueous ethanol and had m.p. 45°, 96° and 140° respectively.

Determination of *o*-nitrophenol. This phenol can readily be determined colorimetrically using the yellow colour produced in alkaline solution. A straight-line calibration curve was obtained when the colour from 0.1 to 1 mg. of *o*-nitrophenol in 10 ml. 0.05 N-NaOH (0.1 or 5 N-NaOH can be used without affecting the colour) was measured in the Spekker absorptiometer using Ilford Spectrum blue filter no. 602. By this method, 0.1 mg. in 10 ml. solution could be estimated with an error of $\pm 1\%$. For the estimation of free *o*-nitrophenol in urine, the phenol was steam-distilled out of the urine at about pH 6.5. Quantities of the order of 0.5–1.0 mg. of the phenol could be recovered quantitatively from 10 ml. of urine, if the volume of distillate were not less than 10 ml.; usually 90–100% were recovered in the first 10 ml.

Rabbits were given *o*-nitrophenol suspended in water, by stomach tube. The 24 hr. urine (10 ml., pH 6.5) was placed in a micro-Kjeldahl distillation apparatus. Steam was then passed through the apparatus until 10 ml. of distillate were collected. The free *o*-nitrophenol usually distills in the first few ml. and the operation is complete in 4–5 min. The distillate was then treated with 1 ml. 2 N-NaOH and the yellow colour measured in the Spekker absorptiometer. The results are given in Table 1, from which it can be concluded that nearly all the *o*-nitrophenol excreted is in a combined form.

Table 1. *Free o-nitrophenol in rabbit urine*

Rabbit no.	Dose of <i>o</i> -nitrophenol		Free <i>o</i> -nitrophenol	
	(g.)	(mg./kg.)	(mg.)	(% of dose)
29	0.6	200	1.74	0.29
32	0.6	200	3.12	0.52
33	0.6	200	8.22	1.37
9	1.0	450	13.0	1.3

Determination of total nitro groups with titanous chloride. A convenient amount of urine (1–5 ml.) containing the nitro

compound was boiled with 10 ml. of conc. HCl for 10 min. in a stream of CO₂ and then a measured excess amount of acid titanous chloride added. (The titanous chloride solution was prepared by boiling 50 ml. commercial (20%) TiCl₃ with 100 ml. conc. HCl for 1 min. and making up to 2 l. with water. The solution was kept in an inert atmosphere.) The mixture was kept boiling for a further 10 min. in the atmosphere of CO₂ and then the excess titanous chloride was estimated with standard ferric alum (0.3 M) using potassium thiocyanate as indicator near the end point. At the same time, a blank titration was performed on the titanous chloride solution omitting the urine. The difference between the two titrations gives the amount of reducible material in the urine calculated as nitro compound.

Samples of the three mononitrophenols (2–3 mg. in 10–20 ml. solution) could be recovered from pure solutions within 2% of the calculated values. The error in the recoveries of these phenols from urine was slightly greater than this, and normal urines contained reducible material equivalent to 0–10 mg. of nitrophenol per 100 ml. However, at the dose levels used in the present experiments, the error introduced by this material can be neglected. The titanous chloride reagent estimates all compounds of higher oxidation level than aminophenol, i.e. nitrophenol, nitrosophenol and hydroxylaminophenol. If appreciable amounts of the nitroso and hydroxylamino compounds are present, there will be a tendency to low values for nitro compound not reduced to amino, since the intermediates require only a fraction of the titanous chloride needed for an equivalent amount of nitro compound. As will be clear from the results (Table 2) the amounts of these intermediates appearing in the urine cannot be of much significance.

Determination of *p*-aminophenol. *p*-Aminophenol can readily be determined in quantities of 10–100 µg. in volumes up to 10 ml. by means of the indophenol reaction if K₃Fe(CN)₆ is used as the oxidizing agent. Hypochlorite, hypobromite, H₂O₂ and KSO₄ were tried as oxidizing agents, but were all found unsatisfactory. The procedure adopted was to take a suitable volume (up to 10 ml.) of *p*-aminophenol solution and make it alkaline with 5 ml. N-Na₂CO₃. Then 1 ml. of 5% (w/v) aqueous phenol was added followed by 5 ml. of 0.1% (w/v) aqueous K₃Fe(CN)₆. The volume was made up to 25 ml. with water, and after 30 min. the blue colour was measured in the Spekker absorptiometer using Ilford Spectrum orange filter no. 607. (In the presence of *p*-nitrophenol the solution is green in colour, but with the use of the filter the aminophenol can be estimated in the presence of nitrophenol.) The calibration curve was a straight line.

For the determination of *p*-aminophenol in *p*-nitrophenol urine rather more ferricyanide is necessary than in pure solution. A mixture of 1 ml. urine and 1 ml. conc. HCl was heated on a boiling-water bath for 2 hr. to hydrolyse the conjugated forms of the aminophenol. The solution was then diluted to 100 ml. with water. To 10 ml. of this diluted urine, was added 1 ml. 5% phenol, 5 ml. N-Na₂CO₃ and 8 ml. of 0.1% K₃Fe(CN)₆. After making up to 25 ml. and keeping 30 min. the colour was read in the Spekker absorptiometer as above. The recoveries of *p*-aminophenol from pure solutions of *p*-aminophenylglucuronide were 86–96% (average 93%). (See Table 2 for results.)

Determination of *m*-aminophenol. The determination of this phenol was carried out by diazotization and coupling with naphthylethylenediamine as follows (cf. Bratton & Marshall (1939) procedure for sulphanilamide) as follows:

a solution of *m*-aminophenol (10 ml.) containing up to 250 µg. of the phenol was treated with 0.5 ml. 4N-HCl and 1 ml. 0.1% (w/v) NaNO₂ solution. Diazotization was allowed to proceed for 10 min., after which time 1 ml. of 0.5% (w/v) ammonium sulphamate was added and the mixture allowed to stand 2 min. Then 1 ml. of a 1% aqueous solution of *N*-(1-naphthyl) ethylenediamine hydrochloride was added as coupling reagent and the red colour allowed to develop for 4 hr. After diluting with water to 25 ml. the colour intensity was read in the Spekker absorptiometer using an Ilford Spectrum yellow-green filter no. 605. The standard curve obtained was a straight line.

For the estimation of total *m*-aminophenol in *m*-nitrophenol urine, 0.5 ml. urine and 0.5 ml. of 4N-HCl were heated in a boiling-water bath for 4 hr. (The *m*-aminophenol may be present in the urine as the glucuronide and ethereal sulphate and as *N*-acetyl conjugates. The ethereal sulphates are readily hydrolysed in about 15 min., but the glucuronides require prolonged heating for complete hydrolysis. Under the above conditions of hydrolysis, recoveries of *m*-aminophenol from its glucuronide at levels of 100–200 µg. were 96–106% (average 100%); high results are obtained if the hydrolysis is not quite complete, for the glucuronide gives more colour than the free phenol.) The hydrolysed urine was then diluted with water to 10 ml. and the procedure described in the preceding paragraph followed. (See Table 2 for results.)

Determination of *o*-aminophenylglucuronide. The *o*-aminophenol in *o*-nitrophenol urine is present as the glucuronide and the ethereal sulphate. The total amount of *o*-aminophenol in the urine is difficult to assess because *o*-aminophenylglucuronide is relatively resistant to acid hydrolysis (Hanson, Mills & Williams, 1944). The amount of *o*-aminophenylglucuronide in the urine, however, can readily be assessed by diazotization and coupling by the Bratton & Marshall procedure. In this procedure the *o*-aminophenol occurring as ethereal sulphate is not estimated since *o*-aminophenylsulphuric acid readily loses its sulphate group on diazotization (Burkhardt & Wood, 1929) to yield *o*-aminophenol which gives no colour with the Bratton & Marshall reagents. The extent of reduction of *o*-nitrophenol could therefore only be partly assessed.

The method used was as follows (Smith & Williams, 1949). The urine (0.5 ml.), 0.5 ml. 4N-HCl and 3 ml. of water were mixed and heated in a test tube in a boiling-water bath for 1 hr. This procedure deacetylates any *o*-acetamidophenylglucuronide and hydrolyses ethereal sulphates. The solution was cooled diluted to 10 ml. and diazotized with 1 ml. of 0.1% NaNO₂ solution. The remainder of the method was as described above, except that the colour was allowed to develop for 24 hr. The amount of *o*-aminophenylglucuronide present was then read from a standard curve produced by subjecting known amounts of *o*-aminophenylglucuronide to the same treatment. In this way 10–60 µg. of *o*-aminophenol as its glucuronide could be readily estimated, and the recoveries were 100 ± 5%.

Glucuronic acid was determined by the method of Hanson *et al.* (1944).

Ethereal sulphates were estimated turbidimetrically according to Sperber (1948). Inorganic sulphate was removed from the urine with acid BaCl₂ solution, the excess of the latter being removed by Na₂CO₃ solution and centrifuging. Samples of the supernatant were hydrolysed, and the liberated sulphate estimated turbidimetrically after the addition of solid BaCl₂·2H₂O using the Spekker absorptio-

Table 2. *Excretion of nitro compounds and amino derivatives after the feeding of o-, m- and p-nitrophenols to rabbits*

Nitrophenol	Rabbit no.	Dose (mg./kg.)	Amounts excreted (% of dose)		
			Unchanged nitro group*	Reduced to amino compound†	Total
<i>Ortho</i>	5	330	77.9	3.5	81.4
	7	330	83.1	1.8	84.9
	12	330	85.7	2.4	88.1
<i>Meta</i>	5	200	70.3	6.9	77.2
	7	200	68.0	8.0	76.0
	16	200	78.5	11.0	89.5
	17	200	86.2	9.2	95.4
	20	200	69.8	12.9	82.7
<i>Para</i>	15	200	88.5	10.8	99.3
	16	200	91.7	13.2	104.9
	17	200	82.0	18.5	100.5

* The excretion of nitro compounds was virtually complete in 1 day.

† Estimated for 2 days after dosing.

meter with Ilford Spectrum orange filter no. 607. The Spekker readings were referred to a standard curve prepared with potassium *p*-nitrophenylsulphate. The results of glucuronic acid and ethereal sulphate determinations are given in Table 3.

Table 3. *Glucuronic acid and ethereal sulphate conjugations of the mononitrophenols*

Nitrophenol	Rabbit no.	Dose (mg./kg.)	Glucuronide (% of dose)	Ethereal sulphate (% of dose)
<i>Ortho</i>	29	200	65.5	12.2
	35	200	76.0	7.3
	36	200	70.5	13.0
<i>Meta</i>	29	200	71.5	28.0
	36	150	82.5	16.0
	40	200	81.5	14.0
<i>Para</i>	29	200	59.0	12.5
	36	200	61.5	20.5
	40	150	74.0	15.0

Detection of the minor metabolites of the nitrophenols by paper chromatography and spectroscopy

Reference compounds. 3-Nitrocatechol (m.p. 86°) and 4-nitrocatechol (m.p. 168–170°) were prepared according to Weselsky & Benedikt (1882) and 2-nitroresorcinol (m.p. 85°) according to Kauffman & Pay (1904). Nitroquinol (m.p.

132°) was obtained by demethylation with concentrated HBr of nitroquinol dimethyl ether prepared according to Mühlhäuser (1881).

Paper chromatography. The above compounds and the mononitrophenols were readily separated by paper partition chromatography using benzene, acetic acid and water (1:1:2 by volume) and Barcham Green no. 401 paper. For the determination of R_F values (see Table 4) the compounds were applied to the paper (which had been equilibrated to the solvent vapour for 24 hr.) in ethereal solution, and the chromatogram run for 15 hr. at 20°. Under these conditions, *o*-nitrophenol and 2-nitroresorcinol volatilized and gave no spot on spraying the paper. If the chromatogram was run at 0°, however, *o*-nitrophenol could be kept on the paper and formed a spot near the solvent front, but since this phenol is readily identified in small amounts by steam distillation and spectroscopy, its chromatographic identification was not pursued at this point. For development of the spots on the paper, it was sprayed with *N*-NaOH, with which each phenol gives a distinctive colour (see Table 4). *p*-Nitrophenol and 4-nitroquinol, however, have the same R_F value, but in the nitrophenol urines examined, they could not be expected to appear together.

For more precise identification and approximate estimation of the dihydroxy compounds the spots on the unsprayed paper were cut out under illumination by ultraviolet light. In this light, the phenols quenched the weak background fluorescence of the paper and showed up as

Table 4. *Chromatography of the nitrophenols*

(Solvent: benzene, acetic acid, water (1:1:2, v/v). Chromatograms run for 15 hr.)

Compound	R_F value	Colour of spot with <i>N</i> -NaOH	Absorption spectrum in 0.05 <i>N</i> -NaOH of the phenol in the visible region	
			$\lambda_{\max.}$ (m μ .)	$\epsilon_{\max.}$
4-Nitrocatechol	0.21	Red	510	10,500
4-Nitrophenol	0.62	Canary yellow	—	—
Nitroquinol	0.62	Purple	540	3,900
3-Nitrophenol	0.65	Yellow	—	—
3-Nitrocatechol	0.68	Mauve	—	—

dark spots which could be cut out with minimum contamination from other materials from urine.

For spectroscopic identification, a chromatogram was prepared with a spot of urine extract running alongside the authentic phenols. Suitable small areas of the paper were cut out as small pointed strips (2.5×10 cm.) and the phenols eluted chromatographically into graduated flasks. The monohydric phenols were rapidly eluted with ethanol, whereas the dihydric phenols required 0.1 N-NaOH for rapid elution. The solutions of the dihydric phenols were then diluted with water so that the NaOH concentration was 0.05 N and the absorption spectra of the solutions in the $500\text{ m}\mu$ region determined in a Unicam spectrophotometer. The relevant data for the two dihydric phenols found are given in Table 4.

Feeding experiments. *p*-Nitrophenol (250 mg.) was fed in aqueous solution to a rabbit. The 24 hr. urine was treated with 0.1 vol. of conc. HCl and heated at 100° for 20 min. to hydrolyse ethereal sulphates. After cooling, the urine was continuously extracted for 6 hr. with ether. Aminophenols were removed from the extract with 2 N-HCl and the residual nitrophenols transferred to 2 N-NaOH . The latter was acidified and the nitrophenols transferred to 10 ml. ether which was then used for paper chromatography.

In *p*-nitrophenol urine only one dihydric phenol was found in the ethereal sulphate fraction, namely 4-nitrocatechol. The 4-nitrocatechol spot was cut out and eluted as above. In this way, it was found that roughly 0.1 – 0.2% of the *p*-nitrophenol fed appeared in the ethereal sulphate fraction as 4-nitrocatechol.

Similar experiments were carried out with *o*- and *m*-nitrophenols. The former yielded small amounts of nitroquinol, (approx. 0.03% of the dose), and the latter 4-nitrocatechol (approx. 0.03%) and traces of nitroquinol ($<0.002\%$) as ethereal sulphates.

Elbs persulphate oxidation of the mononitrophenols

Baker & Brown (1948) have suggested that a free radical, the sulphate ion radical, is involved in the Elbs persulphate oxidation of phenols. The possibility that biological hydroxylation is a free radical process has been discussed by us (Smith & Williams, 1950; Smith, 1950), and it was interesting, therefore, to find out whether the Elbs persulphate oxidation of the mononitrophenols produced the ethereal sulphates of the same dihydric phenols as those found biologically. In the persulphate oxidation an ethereal sulphate group is introduced into the phenol by alkaline persulphate, and hydrolysis of the hydroxyphenylsulphate produced, yields a diphenol. In the present work the diphenols were not all isolated, but were detected chromatographically by the technique described above.

A mixture of the *o*-, *m*- or *p*-nitrophenol (2 g.), KOH (4 g.), KSO_4 (4 g.) and water (100 ml.) was allowed to stand at room temperature for 48 hr. A further 2 g. of the persulphate was added and the mixture left a further 24 hr. The mixture was then acidified at 0° and extracted with ether to remove unchanged nitrophenol. The aqueous residue was then heated under reflux at 100° for 40 min. to hydrolyse ethereal sulphates and after cooling the liberated phenols were extracted with ether. The ether extract was

evaporated and the dihydric phenols present identified by paper chromatography using benzene, acetic acid and water ($1:1:2$) as described above. *o*-Nitrophenol yielded nitroquinol and traces of 3-nitrocatechol (both have been isolated by Forrest & Petrow, 1950); *m*-nitrophenol yielded mainly nitroquinol with appreciable amounts of 3- and 4-nitrocatechol; and *p*-nitrophenol yielded 4-nitrocatechol only (see Table 6). In large-scale experiments potassium 4-hydroxy-3-nitrophenylsulphate was easily isolated from the Elbs persulphate oxidation of *o*-nitrophenol, and potassium 4-hydroxy-2-nitrophenylsulphate from *m*-nitrophenol (unpublished observations).

The glucuronides of the mononitrophenols

(Melting points are uncorrected; $[\alpha]_D$ was measured in 2 dm. tubes and where $c=1$ the error is $\pm 1^\circ$, and $c=0.1$, $\pm 5^\circ$.)

***o*-Nitrophenol.** A total of 12 g. of the phenol was fed in water by stomach tube to six rabbits. The 24 hr. urine was acidic ($\text{pH } 6$), gave a strong naphthoresorcinol test and a positive diazo test for aromatic amino compounds and was slightly reducing to Fehling's and Benedict's solutions and ammoniacal AgNO_3 . The glucuronide fraction was isolated in the usual way with basic lead acetate. After removal of the lead with H_2S and then concentrating, a crude glucuronide gum was obtained which weighed 8.9 g. and contained much inorganic material. Extraction of this gum with 95% ethanol yielded 2 g. of purified gum which would not crystallize. The gum (1.2 g.) was dissolved in 95% methanol and added dropwise to an ethereal solution of diazomethane. After keeping an hour, the ether was evaporated leaving a neutral gummy methyl ester. The latter was acetylated with pyridine (5 ml.) and acetic anhydride (8 ml.) and the mixture kept overnight. The product was then poured into water whereupon the triacetyl β -*o*-nitrophenylglucuronide methyl ester crystallized (0.7 g.). A further yield of 1 g. of this ester was obtained from the ethanol-insoluble part of the glucuronide gum (total yield, 4.3% of the dose). The ester was recrystallized from aqueous acetone and formed blunt needles, m.p. 172° and $[\alpha]_D^{25} + 18.5^\circ$ ($c=2$ in CHCl_3). (Found: C, 50.7 ; H, 4.3 ; N, 3.3 ; OCH_3 , 7.1 ; COCH_3 , 28.1 . $\text{C}_{15}\text{H}_{21}\text{O}_{12}\text{N}$ requires C, 50.1 ; H, 4.7 ; N, 3.1 ; OCH_3 , 6.8 ; COCH_3 , 28.4% .)

The above triacetyl methyl ester (0.5 g.) was dissolved in 25 ml. ethanol and the solution saturated with NH_3 at 0° . The mixture was kept for 2 days at room temperature and then again saturated with gaseous NH_3 . After keeping a further 24 hr., the solution was allowed to evaporate spontaneously and residue recrystallized from ethanol. The β -*o*-nitrophenylglucuronidamide separated very slowly from the ethanol as little nodules of small needles, m.p. 173 – 175° and $[\alpha]_D^{25} - 63.8^\circ \pm 2^\circ$ ($c=0.14$ in ethanol; 4 dm. tube). (Found: C, 45.9 ; H, 4.4 ; N, 8.7 . $\text{C}_{12}\text{H}_{14}\text{O}_9\text{N}_2$ requires C, 45.9 ; H, 4.5 ; N, 8.9% .)

Triacetyl β -*o*-nitrophenylglucuronic acid was prepared by dissolving 1 g. of the glucuronide gum in 5 ml. acetic anhydride and treating the mixture with 5 drops of perchloric acid (60%). The mixture was kept for 0.5 hr. at room temperature and then carefully diluted with water. A buff precipitate separated which was filtered and washed with benzene. On recrystallization from aqueous ethanol, the triacetyl acid (0.54 g.) was obtained as buff needles, m.p. 195 – 196° and $[\alpha]_D^{20} + 31.2^\circ$ ($c=1$ in CHCl_3). (Found: C, 48.9 ; H, 4.3 ; N, 3.3 . $\text{C}_{15}\text{H}_{19}\text{O}_{12}\text{N}$ requires C, 49.0 ; H, 4.3 ; N, 3.2% .)

m-Nitrophenol. From the glucuronide fraction of the urine of three rabbits which had each received 0.6 g. of *m*-nitrophenol there was isolated after acetylation and methylation of the glucuronide gum 0.5 g. (8.5% of dose) of triacetyl β -*m*-nitrophenyl glucuronide methyl ester. After recrystallization from absolute ethanol the compound formed needles, m.p. 159–160° and $[\alpha]_D^{20} - 42.6^\circ$ ($c=0.8$ in CHCl_3). (Found: C, 50.7; H, 4.7; N, 3.4; OCH_3 , 6.8. $\text{C}_{19}\text{H}_{21}\text{O}_{12}\text{N}$ requires C, 50.1; H, 4.7; N, 3.1; OCH_3 , 6.8%.) The above ester (0.2 g.) was dissolved in 10 ml. methanol and the solution saturated with dry NH_3 at 0°. After keeping overnight in a stoppered flask and then concentrating, β -*m*-nitrophenylglucuronidamide (0.1 g. or 72%) was obtained as blunt needles. It was recrystallized from methanol containing a few drops of water and elementary analysis showed it to be a hemihydrate. It had m.p. 206° and showed $[\alpha]_D^{20} - 91^\circ$ ($c=0.2$ in methanol). (Found: C, 44.7; H, 4.3; N, 8.6; $\text{C}_{12}\text{H}_{14}\text{O}_8\text{N}_2 \cdot 0.5 \text{H}_2\text{O}$ requires C, 44.6; H, 4.4; N, 8.7%.)

p-Nitrophenol. Owing to its toxicity this phenol could only be fed at dose levels not exceeding 0.2 g./kg. As before, the glucuronide was isolated as triacetyl β -*p*-nitrophenylglucuronide methyl ester (0.5 g. from 1.2 g. of *p*-nitrophenol or 11.4% of dose). On recrystallizing from ethanol, the compound formed cigar-shaped crystals, m.p. 146° and $[\alpha]_D^{20} - 51^\circ$ ($c=0.9$ in CHCl_3). (Found: C, 50.2; H, 4.6; N, 3.5; OCH_3 , 6.7. $\text{C}_{19}\text{H}_{21}\text{O}_{12}\text{N}$ requires C, 50.1; H, 4.7; N, 3.1; OCH_3 , 6.8%.) β -*p*-Nitrophenylglucuronidamide was obtained as above and formed needles from methanol (yield 75%); m.p. 210° and $[\alpha]_D^{20} - 96^\circ$ ($c=0.17$ in methanol). (Found: C, 44.0; H, 5.0; N, 8.4. $\text{C}_{12}\text{H}_{14}\text{O}_8\text{N}_2 \cdot \text{H}_2\text{O}$ requires C, 43.4; H, 4.9; N, 8.4%.)

The optical rotation of triacetyl β -*o*-nitrophenylglucuronide methyl ester

Tetra-acetyl β -*o*-nitrophenylglucoside has an abnormal positive rotation (Glaser & Wulwek, 1924; Montgomery *et al.* 1942) and Pigman (1944) showed that when the temperature was raised the rotation of the tetra-acetate in nitrobenzene became less positive and eventually became negative. The trend of the curve relating rotation to temperature suggested that at sufficiently high temperatures, the rotation would approach the value for the *m*- and *p*-isomers. It was suggested that the abnormal rotation was due to weak bonds between groups in the molecule and that at the higher temperature the vibrational energy of the molecules is greater and tends to disrupt these weak bonds. The following observations show that triacetyl β -*o*-nitrophenylglucuronide methyl ester behaves in a similar manner to the corresponding tetra-acetyl glucoside.

The specific rotation of a 0.63% solution of triacetyl β -*o*-nitrophenylglucuronide methyl ester in cyclohexanone was measured in a 2 dm. jacketed polarimeter tube in a Bellingham and Stanley Model A polarimeter. The temperature of the water circulating through the tube was raised and readings of the rotation were taken at intervals of 10° from 20 to 90°. Six readings were taken at each temperature which varied by no more than 0.5° during each set of readings. The temperature was kept steady for several minutes before readings were taken to ensure uniform heating.

After reaching 90°, the solution was allowed to cool and readings again taken at intervals of 10°. No corrections were made for the expansion of the solution or polarimeter tube, since the error introduced by neglecting them is relatively small. The coefficient of cubical expansion of cyclohexanone is 0.00091, and in our highest reading this would cause an error of 0.7%. Our results (Fig. 1) show that the rotation of triacetyl β -*o*-nitrophenylglucuronide methyl ester becomes

Fig. 1. The specific optical rotations at different temperatures of triacetyl β -nitrophenyl-D-glucuronide methyl esters in cyclohexanone. O, ortho compound; M, meta compound.

less positive on raising the temperature and passes through zero at about 65°. In a similar way the effect of temperature on the rotation of a 0.432% solution of triacetyl β -*m*-nitrophenylglucuronide methyl ester in cyclohexanone was studied (see Fig. 1). In this case there was little change in rotation between 23 and 92°.

DISCUSSION

The early work of Meyer (1905) and Baumann & Herter (1877–8) had shown qualitatively that the *o*-, *m*- and *p*-nitrophenols conjugated *in vivo* with glucuronic and sulphuric acids. The present work shows that in doses of 0.2–0.3 g./kg. the conjugation is almost complete (Table 5), the major product in all three cases being the nitrophenyl glucuronide. Meyer (1905) was doubtful whether *o*-nitrophenol was reduced *in vivo*, although he was able to detect reduction of the *m*- and *p*-isomers. Table 5 shows that all three nitrophenols undergo reduction which is not extensive and which appears to be slightly greater for the *p*- than for the *m*- and *o*-isomers. *p*-Nitrophenol is much more toxic than its isomers (see Oettingen, 1949); *o*-nitrophenol, on the other hand, is a relatively innocuous substance (Beutner & Block, 1943). It seems unlikely, however, that

Table 5. *Metabolism of mononitrophenols—Summary*

Nitrophenol	Percentage of dose excreted as				
	Nitro compounds <i>N</i>	Amino compounds <i>A</i>	(<i>N</i> + <i>A</i>)*	Glucuronides <i>G</i>	Ethereal sulphates <i>E</i>
<i>ortho</i>	82	3†	85	71	11
<i>meta</i>	74	10	84	78	19
<i>para</i>	87	14	101	65	16
					(<i>G</i> + <i>E</i>)*
					82
					98
					81

* (*N* + *A*) should be roughly equal to (*G* + *E*), since the amounts of free phenols excreted were very small.

† This figure is low since only *o*-aminophenol combined with glucuronic acid was estimated. To allow for *o*-aminophenol combined with sulphuric acid, this figure could probably be doubled.

Table 6. *Orientation of biological hydroxylation and of Elbs persulphate oxidation of the mononitrophenols*

Nitrophenol	Dihydric phenols* produced by			
	Biological hydroxylation		Elbs persulphate oxidation	

* The dihydric phenols are given in the quantitative order found experimentally.

the toxicity of *p*-nitrophenol can be explained in terms of the extent of its metabolic reduction, since this is not very much more than for its isomers. In any case, the product of the reduction, *p*-aminophenol, is not very toxic. However, the intermediates in the reduction, *p*-nitrosophenol and *p*-hydroxylaminophenol, may be more toxic, for Bueding & Jolliffe (1946) have shown, with TNT, that the intermediate products between the nitro compound and the amine inhibit respiration. We have observed that toxic doses of *p*-nitrophenol are very rapidly fatal, and this suggests that it is toxic *per se* and not as a result of metabolic changes. The latter very probably detoxicate *p*-nitrophenol.

The hydroxylation of the nitrophenols

Paper chromatography has shown that the nitrophenols are very slightly oxidized *in vivo* to dihydric nitrophenols. The extent of this oxidation, however, is less than 0.5% of the dose, and is unimportant from a practical point of view. The dihydric phenols produced are, however, interesting from the theoretical aspect, for the second hydroxyl group is introduced *o*- and/or *p*- to the existing hydroxyl group (cf. Smith, 1950), and its orientation is similar to that produced during the Elbs persulphate oxida-

tion of the mononitrophenols (Table 6). Baker & Brown (1948) have suggested that the Elbs persulphate oxidation involves the sulphate ion radical, $\cdot\text{OSO}_3^-$. We have earlier suggested that the orientation of biological hydroxylation is similar to that found in free radical substitution (Smith & Williams, 1950; see also discussion by Stein & Weiss, 1950).

The anomalous optical rotation of β -o-nitrophenylglucuronide derivatives

The optical rotations of the derivatives of the β -nitrophenylglucuronides are given in Table 7. Both the triacetyl acid and triacetyl methyl ester of the *ortho*-derivative show anomalous positive rotations, whereas the corresponding *meta*- and *para*-isomers show the expected negative rotation characteristic of β -glycosides. The anomalous effect is obviously due to the presence of the three acetyl groups in the sugar ring combined with *ortho*-substitution of the aromatic ring by a positive group, for the effect disappears in the unacetylated glucuronide as shown by the normal negative rotation of β -o-nitrophenylglucuronidamide. The effect also tends to disappear on heating the solution, a point which has already been discussed. Abnormal positive rotations in the β -D-glucoside

series have also been observed with the tetracetates of *o*-chloromethyl-, *o*-bromomethyl-, *o*-iodomethyl-, 2:6-dinitro- and 2:4-dinitro- substituted β -D-phenylglucosides where the *ortho*-substituent is positive (Kunz, 1926; Pigman, 1944).

Table 7. Optical rotations of β -nitrophenyl-D-glucuronide derivatives

(For details of temperature and concentration, see text)

	Glucuronid- amide ([α] _D in methanol)	Triacetyl glucuronide methyl ester ([α] _D in CHCl ₃)	Triacetyl glucuronic acid ([α] _D in CHCl ₃)
β - <i>o</i> -Nitrophenyl	-63.8°	+18.5°	+31.2°
β - <i>m</i> -Nitrophenyl	-91°	-42.6°	—
β - <i>p</i> -Nitrophenyl	-96°	-51.0°	—

SUMMARY

1. A study has been made of the metabolism of the *o*-, *m*- and *p*-nitrophenols in the rabbit.

2. All three phenols are excreted in conjugated forms, there being only small amounts (< 1 %) of the unchanged free phenols excreted. The main metabolites are the nitrophenylglucuronides which account for about 70 % of the dose. The corresponding ethereal sulphates are also excreted.

3. The nitro group of the nitrophenols undergoes reduction *in vivo* only to a minor extent, and some

80 % of it is excreted unchanged. The extent of reduction is about 6 % for *o*-, 10 % for *m*- and 14 % for *p*-nitrophenol.

4. The nitrophenols also undergo oxidation to a very small extent (< 1 %); *o*- yields traces of nitroquinol, *m*-, nitroquinol and 4-nitrocatechol and *p*-, 4-nitrocatechol. The dihydric phenols produced are similar to those formed during the Elbs persulphate oxidation of the mononitrophenols. The significance of the similarity in relation to free radicals is discussed.

5. The paper chromatography of the mono- and di-hydric nitrophenols has been studied.

6. The β -glucuronides of the nitrophenols have been isolated and characterized as amides and triacetyl methyl esters.

7. The abnormal positive rotations of triacetyl β -*o*-nitrophenyl-D-glucuronide and its methyl ester have been investigated, and it has been shown that in the case of the ester this rotation becomes negative on raising the temperature, a behaviour reminiscent of tetra-acetyl β -*o*-nitrophenylglucoside.

8. The metabolic results have been discussed in relation to the toxicity of *p*-nitrophenol and the non-toxicity of *o*-nitrophenol.

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