- Daccomo, G. (1891). *Jber. Fortschr. Chem.* p. 1377.
Dakin, H. D. (1909). *J. biol. Chem.* **42**, 489.
Duff, J. C. (1941). *J. chem. Soc.* p. 547.
Feigl, F. (1954). *Spot Tests*, 2 (*Organic Applications*), 168. Amsterdam: Elsevier.
Fewster, M. E. & Hall, D. A. (1951). *Nature, Lond.*, **168**, 78.
Goldacre, P. L. & Galston, A. W. (1953). *Arch. Biochem. Biophys.* **43**, 169.
Groves, L. G., Turner, E. E. & Sharp, G. I. (1929). *J. chem. Soc.* p. 516.
Hodgson, H. H. & Wignall, J. S. (1926). *J. chem. Soc.* p. 2078.
Hodgson, H. H. & Wignall, J. S. (1927). *J. chem. Soc.* p. 2217.
Holleman, A. F. (1917). *Rec. Trav. chim. Pays-Bas*, **37**, 96.
Levy, S. (1883). *Ber. deutsch. chem. Ges.* **16**, 1445.
Mosso, U. (1887). *Jber. Fortschr. Chem.* p. 1301.
Otto, R. (1868). *Liebig's Ann.* **145**, 323.
Parke, D. V. (1955). *Biochem. J.* **59**, 422.
Smith, J. N., Spencer, B. & Williams, R. T. (1950). *Biochem. J.* **47**, 284.
Spencer, B. & Williams, R. T. (1950). *Biochem. J.* **47**, 279.
Willstätter, R. & Müller, H. E. (1911). *Ber. deutsch. chem. Ges.* **44**, 2184.

Studies in Detoxication

64. THE SYNTHESIS OF THE ISOMERIC DICHLOROPHENYLMERCAPTURIC ACIDS

By D. V. PARKE

Department of Biochemistry, St Mary's Hospital Medical School, London, W. 2

(Received 16 July 1954)

Recent work in this laboratory on the metabolism of the *o*-, *m*- and *p*-dichlorobenzenes in rabbits (Azouz, Parke & Williams, 1953, 1955; Parke & Williams, 1955) has shown that mercapturic acids are minor metabolites (*ca.* 5% of the dose) of the *ortho* and *meta* isomers. In order to identify these acids, the synthesis of the six isomeric L-dichlorophenylmercapturic acids became necessary. The present paper describes the synthesis and properties of these compounds and the corresponding dichlorophenyl-L-cysteines. At the same time, the mercapturic acid fraction obtained after feeding chlorobenzene to rabbits was examined chromatographically to ascertain whether the *o*- and *m*-chlorophenylmercapturic acids were present in addition to the principal metabolite, *p*-chlorophenylmercapturic acid (Spencer & Williams, 1950*a, b*). The *o*- and *m*-chlorophenylmercapturic acids and the corresponding chlorophenyl-L-cysteines were therefore synthesized. Since the completion of this work, a paper by West & Mathura (1954) on the synthesis of these latter compounds has appeared.

EXPERIMENTAL

Melting points are corrected.

Materials. L-Cystine was purified by dissolving 10 g. in 200 ml. $N-H_2SO_4$, filtering, and reprecipitating with a saturated solution of ammonium acetate. After 1 hr. at 0°, the cystine was filtered off, washed with water, and dried in air. 2-Chloroaniline (b.p. 209°) and 3-chloroaniline (b.p. 230°) were purchased and purified by distillation. 2,3-Dichloroaniline (m.p. 24°), 2,4-dichloroaniline (m.p. 64°), 2,5-dichloroaniline (m.p. 52°), and 3,4-dichloroaniline (m.p. 72°) were purchased and were purified by distillation

in steam. 2,6-Dichloroaniline (m.p. 39°) was prepared from 3:5-dichlorosulphanilamide as described by Seikel (1944). 3:5-Dichloroaniline (m.p. 50°) was prepared from 3:5-dichloronitrobenzene (Roberts & Turner, 1927) according to Dyson, George & Hunter (1926), and was purified by steam distillation.

Preparation of dichlorophenylmercapturic acids. The isomeric *S*-dichlorophenyl-L-cysteines were synthesized from L-cystine (5 g.) and the corresponding dichloroanilines (7.5 g.) according to Parke & Williams (1951). Improved yields were obtained if the solution of cysteine cuprous mercaptide was filtered hot, to remove traces of copper oxides, before cooling to 0° and coupling with the diazotized dichloroanilines. The *S*-dichlorophenyl-L-cysteines (yield, 0.5–1.2 g.) were isolated by repeated extraction with 50% aqueous ethanol, in which cystine is sparingly soluble. These compounds were acetylated in pyridine with acetic anhydride to the corresponding mercapturic acids (Zbarsky & Young, 1943), which were then recrystallized from water or aqueous ethanol. A more efficient procedure for the isolation of pure dichlorophenyl-L-cysteines in quantity was to acetylate the crude dichlorophenylcysteine–cystine mixture to give the L-dichlorophenylmercapturic acid (yield, 1.5–4.5 g.) which was isolated, purified and subsequently hydrolysed with acid to the corresponding dichlorophenyl-L-cysteine.

Acid hydrolysis of dichlorophenylmercapturic acids. The pure L-dichlorophenylmercapturic acids (1 g.) were hydrolysed to the corresponding *S*-dichlorophenyl-L-cysteines by heating under reflux with 50 ml. $2N-H_2SO_4$ for 1–2 hr. 2:5-Dichlorophenylmercapturic acid was more resistant to acid hydrolysis than the other isomers, and 50 ml. of $5N-H_2SO_4$ was used in this case. The hydrolysed solutions were neutralized with $2N$ ammonia, and then adjusted to pH 5 with $2N$ acetic acid. The precipitated dichlorophenylcysteines were filtered off and recrystallized from aqueous ethanol. In no instance was there any evidence of racemization of the dichlorophenyl-L-cysteines, since the products obtained by acid hydrolysis of the mercapturic acids were

Table 1. Properties of the L-chlorophenyl- and L-dichlorophenyl-mercapturic acids

Isomer	Yield from 5 g. cystine (g.)	M.p. (°)	Solubility in water (%)	[α] _D ²⁰ * in ethanol (c, 1.0) (°)	pK	Found (%)					SH Equiv.	
						C	H	N	Cl			
<i>Chlorophenylmercapturic acids</i>												
<i>ortho</i> (rhombs) [†]	2.5	151	0.10	-14	3.4	48.3	4.2	5.2	13.4	11.9	265	
<i>meta</i> (rhombs) [†]	4.0	165	0.14 } at 10°	-19	3.5	48.2	4.8	4.8	13.2	12.5	271	
<i>para</i>	—	153 [‡]	0.06 } (C ₁₁ H ₁₃ O ₃ NCIS requires	-20 [‡]	3.8§	—	—	—	—	—	—	
<i>Dichlorophenylmercapturic acids</i>												
2.3- (needles)	1.8	146	—	0	3.7	43.0	3.8	4.5	23.4	10.1	314	
2.4- (needles)	3.2	160	—	-9	3.5	42.9	3.7	4.5	23.1	10.0	317	
2.5- (rosettes of needles)	3.8	156	—	-9	3.8	43.4	3.4	4.3	22.6	10.5	311	
2.6- (rhombs)	4.5	191	0.15 } at 20°	+10	4.0	43.0	3.5	4.6	23.2	10.7	296	
3.4- (needles)	1.5	149	0.04	-8	3.4	42.8	3.6	4.3	22.6	10.0	320	
3.5- (needles)	2.0	150	—	-11	3.5	42.7	3.4	4.4	22.4	10.3	317	
			(C ₁₁ H ₁₁ O ₃ NCIS requires			42.9	3.6	4.5	23.0	10.4	308)	
* Error $\pm 2^\circ$.												
[†] West & Mathura (1954) give for <i>ortho</i> compound, m.p. 146.5-147.5° and [α] _D ²⁰ -6.1° and for <i>meta</i> compound m.p. 160.5-161° and [α] _D ²⁰ -11°.												

* Error $\pm 2^\circ$.[†] West & Mathura (1954) give for *ortho* compound, m.p. 146.5–147.5° and [α]_D²⁰ -6.1° and for *meta* compound m.p. 160.5–161° and [α]_D²⁰ -11°.[‡] Figures quoted from Parke & Williams (1951).[§] Figure quoted from Robinson *et al.* (1953).|| [α]_D²⁰ +13°.

identical with those obtained directly from L-cystine and the diazotized dichloroanilines. The properties of the *dichlorophenylmercapturic acids* and *dichlorophenylcysteines*, and the *o*- and *m*-chlorophenylmercapturic acids and chlorophenylcysteines are given in Tables 1 and 2.

Alkaline hydrolysis of the mercapturic acids. The SH content of the mercapturic acids after alkaline hydrolysis was determined by titration with 0.01 N-I₂ after heating at 100° for 40 min. in *n*-NaOH (Parke & Williams, 1951). All compounds were quantitatively decomposed into the corresponding thiophenols by this treatment, and may therefore be estimated by the iodometric procedure (cf. Stekol, 1936).

Determination of pK_a. The apparent dissociation constants of the mercapturic acids in aqueous solution were determined in the usual manner from the potentiometric titration curves (cf. Robinson, Smith & Williams, 1953) (see Table 1).

Fig. 1. The ultraviolet absorption spectra of the L-chlorophenylmercapturic acids in ethanol: A, *ortho* ($\lambda_{\max}$ 255 m μ , ϵ 8600); B, *meta* ($\lambda_{\max}$ 256 m μ , ϵ 8000); C, *para* ($\lambda_{\max}$ 260–261 m μ , ϵ 11200) (Parke & Williams, 1951).

Fig. 2. The ultraviolet absorption spectra of the chlorophenyl-L-cysteines in 0.1N-NaOH (—): A, *ortho* ($\lambda_{\max}$ 252–253 m μ , ϵ 8400); B, *meta* ($\lambda_{\max}$ 257 m μ , ϵ 9500); C, *para* ($\lambda_{\max}$ 260 m μ , ϵ 12600); and in 0.1N-HCl (---): A, *ortho* ($\lambda_{\max}$ 250 m μ , ϵ 5900); B, *meta* ($\lambda_{\max}$ 256 m μ , ϵ 7000); C, *para* ($\lambda_{\max}$ 257 m μ , ϵ 10600).

Table 2. *Properties of the S-chlorophenyl- and S-dichlorophenyl-L-cysteines*

Isomer	Yield* (g.)	M.p. (decomp.) (°)	[α] _D ²⁰ † in 0.1N-NaOH (c, 1.0) (°)	Found (%)			
				C	H	N	Cl
<i>Chlorophenyl-L-cysteines</i>							
<i>ortho</i> (plates)	1.1	184	+4	—	—	6.2	14.9
<i>meta</i> (needles)	1.5	183	+14	—	—	6.2	14.7
<i>para</i>	—	190†	+18†	—	—	—	—
		(C ₆ H ₁₀ O ₂ NCIS requires				6.1	15.3)
<i>Dichlorophenyl-L-cysteines</i>							
2:3- (plates)	0.8	184	+12	40.3	3.3	5.2	26.2
2:4- (plates)	1.0	180	+15	—	—	4.9	26.0
2:5- (needles)	1.2	178	+10	—	—	5.2	26.0
2:6- (needles)	1.5	175	-15	—	—	5.4	26.6
3:4- (plates)	0.5	177	+17	40.7	3.2	5.3	26.3
3:5- (plates)	0.8	181	+14	—	—	5.2	27.1
		(C ₆ H ₈ O ₂ NCIS ₂ requires				5.3	26.6)
				40.6	3.4		

* Obtained directly from L-cystine (5 g.).

† Quoted from Parke & Williams (1951).

† Error $\pm 2^\circ$.

Fig. 3. The ultraviolet absorption spectra of the L-dichlorophenylmercapturic acids in ethanol: A, 2:3- ($\lambda_{\max}$. 258 m μ ., ϵ 11 200); B, 2:4- ($\lambda_{\max}$. 261–262 m μ ., ϵ 13 800); C, 2:5- ($\lambda_{\max}$. 256 and 259 m μ ., ϵ 8600 and 9600); D, 2:6- ($\lambda_{\max}$. 278 m μ ., ϵ 2800); E, 3:4- ($\lambda_{\max}$. 265 m μ ., ϵ 14 500); F, 3:5- ($\lambda_{\max}$. 265 m μ ., ϵ 9800).

Light absorption. The ultraviolet absorption spectra of the mercapturic acids in ethanol, and of the phenylcysteines in 0.1N-NaOH and in 0.1N-HCl, were determined with a Unicam spectrophotometer, Model SP. 500 (see Figs. 1–5).

Solubility. The chlorophenylmercapturic acids and the dichlorophenylmercapturic acids are colourless compounds, soluble in ethanol, less soluble in ether and CHCl₃, and only sparingly soluble in cold water. The solubilities of the dichlorophenylmercapturic acids in ether are equal to or greater than those in CHCl₃, so that ether could be used for the extraction of these compounds from urine. Chloroform is usually the solvent used for extracting mercapturic acids from urine.

Fig. 4. The ultraviolet absorption spectra of the dichlorophenyl-L-cysteines in 0.1N-NaOH: A, 2:3- ($\lambda_{\max}$. 255 m μ ., ϵ 9700); B, 2:4- ($\lambda_{\max}$. 260 m μ ., ϵ 10 800); C, 2:5- ($\lambda_{\max}$. 252 and 256–257 m μ ., ϵ 8000 and 8400); D, 2:6- ($\lambda_{\max}$. 225 and 272–273 m μ ., ϵ 19 300 and 5200); E, 3:4- ($\lambda_{\max}$. 262 m μ ., ϵ 15 000); F, 3:5- ($\lambda_{\max}$. 258 and 262 m μ ., ϵ 8000 and 8700).

DISCUSSION

The six isomers of *S*-dichlorophenyl-L-cysteine have been prepared from cystine in yields of about 10 % and have been acetylated to the corresponding L-dichlorophenylmercapturic acids in almost theoretical yields. The pK_a values of these mercapturic acids all lie between 3.4 and 4.0, which is in agreement with the work of Robinson *et al.* (1953). The

Fig. 5. The ultraviolet absorption spectra of the dichlorophenyl-L-cysteines in 0.1N-HCl: A, 2:3- ($\lambda_{\max}$, 256 m μ , ϵ 5700); B, 2:4- ($\lambda_{\max}$, 257 m μ , ϵ 7900); C, 2:5- ($\lambda_{\max}$, 250 and 255-256 m μ , ϵ 5400 and 5700); D, 2:6- ($\lambda_{\max}$, 225 and 268-270 m μ , ϵ 22000 and 5000); E, 3:4- ($\lambda_{\max}$, 258 m μ , ϵ 10750); F, 3:5- ($\lambda_{\max}$, 258 m μ , ϵ 6250).

effect of the chlorine atoms in the aromatic ring on the pK_a value is small, as might be expected from a comparison with the β -(chlorophenyl)-propionic acids (Dippy, 1939), where the introduction of chlorine increases the dissociation of β -phenylpropionic acid (pK_a , 4.66) by only a slight degree (*ortho*-chlorophenylpropionic acid, pK_a 4.58; *meta*-, pK_a 4.59; *para*-, pK_a 4.61). The pK_a values of the dichlorophenylmercapturic acids are, moreover, affected by the relative positions of the two chlorine atoms to the acetylcysteine moiety, the pK_a increasing as the direction of the resultant dipole of the chlorine atoms changes from the *para* to the *ortho* position.

The specific optical rotations of the dichlorophenyl-L-cysteines also decrease in this same order. With the mercapturic acids, however, the specific rotation is made more positive by the presence of *ortho*-substituted chlorine, viz., L-phenylmercapturic acid, $[\alpha]_D -21^\circ$; 2-chlorophenylmercapturic acid, $[\alpha]_D -14^\circ$; 2:3-dichlorophenylmercapturic acid, $[\alpha]_D 0^\circ$; 2:6-dichlorophenylmercapturic acid, $[\alpha]_D +10^\circ$; and 2:3:5:6-tetrachlorophenylmercapturic acid, $[\alpha]_D +33^\circ$ (Bray, Hybs, James & Thorpe, 1953). The ultraviolet absorption maxima of the dichlorophenylcysteines and the dichlorophenyl-

mercapturic acids show a slight decrease in wavelength as the resultant chlorine dipoles change from the *para* to the *ortho* position, but an anomaly is provided by the 2:6- isomers, which have an absorption spectrum similar to that of non-ionized 2:6-dichlorophenol (absorption maximum in 0.1N-HCl, 2760 Å; ϵ , 2250; cf. Parke & Williams, 1955). This suggests that the two chlorine atoms vicinal to the cysteine or acetylcysteine moiety cause steric inhibition of uniplanarity of the molecule, which results in a change both in wavelength and intensity of the absorption band (cf. Braude, Sondheimer & Forbes, 1954). The solubilities of the dichlorophenylmercapturic acids in water also increase steadily from the 3:4- isomer to the 2:6- isomer and the 2:5- and 2:6-dichlorophenylcysteines are noticeably more soluble than the other isomers in 50 % aqueous ethanol. A similar relationship between optical activity, dissociation constants, and solubilities has been observed by Pauly (1921) in a series of (-)-menthol esters.

SUMMARY

1. The six isomeric *S*-dichlorophenyl-L-cysteines and the corresponding L-dichlorophenylmercapturic acids have been prepared in about 10 % yield from L-cystine.

2. The dichlorophenylmercapturic acids are completely hydrolysed by heating in *N*-NaOH at 100° for 40 min., and may be estimated iodometrically.

3. The apparent pK_a values of *o*- and *m*-chlorophenylmercapturic acids and the dichlorophenylmercapturic acids have been measured, and range from 3.4 to 4.0.

4. The ultraviolet absorption spectra of the *o*- and *m*-chlorophenylmercapturic acids, the isomeric dichlorophenylmercapturic acids and the corresponding *S*-phenylcysteines have been determined. The absorption maxima are related to the relative positions of the aromatic halogen atoms. The wavelength and intensity of the absorption maxima of L-2:6-dichlorophenylmercapturic acid and the corresponding *S*-phenylcysteine are anomalous, and indicate steric inhibition of molecular uniplanarity.

I am grateful to Professor R. T. Williams for his interest in the work. The expenses of this work were in part defrayed by a grant to Professor Williams from the Medical Research Council.

REFERENCES

- Azouz, W. M., Parke, D. V. & Williams, R. T. (1953). *Biochem. J.* **55**, 146.
- Azouz, W. M., Parke, D. V. & Williams, R. T. (1955). *Biochem. J.* **59**, 410.
- Braude, E. A., Sondheimer, F. & Forbes, W. F. (1954). *Nature, Lond.*, **173**, 117.

- Bray, H. G., Hybs, Z., James, S. P. & Thorpe, W. V. (1953). *Biochem. J.* **53**, 266.
- Dippy, J. F. J. (1939). *Chem. Rev.* **25**, 151.
- Dyson, G. H., George, H. J. & Hunter, R. F. (1926). *J. chem. Soc.* p. 3043.
- Parke, D. V. & Williams, R. T. (1951). *Biochem. J.* **48**, 624.
- Parke, D. V. & Williams, R. T. (1955). *Biochem. J.* **59**, 415.
- Pauly, H. (1921). *Z. anorg. Chem.* **119**, 271.
- Roberts, E. & Turner, E. E. (1927). *J. chem. Soc.* p. 1843.
- Robinson, D., Smith, J. N. & Williams, R. T. (1953). *Biochem. J.* **55**, 151.
- Seikel, M. K. (1944). *Org. Synth.* **24**, 47.
- Spencer, B. & Williams, R. T. (1950*a*). *Biochem. J.* **47**, 279.
- Spencer, B. & Williams, R. T. (1950*b*). *Biochem. J.* **47**, 284.
- Stekol, J. A. (1936). *J. biol. Chem.* **113**, 279.
- West, H. D. & Mathura, G. R. (1954). *J. biol. Chem.* **208**, 315.
- Zbarsky, S. H. & Young, L. (1943). *J. biol. Chem.* **151**, 211.

Tissue Fractionation Studies

3. FURTHER OBSERVATIONS ON THE BINDING OF ACID PHOSPHATASE* BY RAT-LIVER PARTICLES

By FRANÇOISE APPELMANS AND C. DE DUVE

Department of Physiological Chemistry, University of Louvain, Belgium

(Received 3 August 1954)

A considerable part of the unspecific acid phosphatase of rat liver is bound to cytoplasmic granules which are recovered mainly with the mitochondria, but also to some extent with the microsomes (Berthet & Duve, 1951). Recent experiments have shown that these granules form a separate group, entirely distinct from the cytochrome oxidase-containing mitochondria and from the glucose 6-phosphatase-bearing microsomes (Duve, Gianetto, Appelmans & Wattiaux, 1953; Appelmans, Wattiaux & Duve, 1955).

Fresh preparations show very little enzymic activity when assayed under conditions which preserve their morphological integrity; they are easily activated by a number of treatments, which all cause the simultaneous release of the enzyme in soluble form. Investigations bearing on this phenomenon led to the conclusion that acid phosphatase is retained within the granules by a membrane-like barrier, impermeable to sucrose and glycerophosphate, but not to a number of other substances, such as glycerol, glucose and various inorganic salts (Berthet, Berthet, Appelmans & Duve, 1951). The granules appeared to behave as typical osmotic systems.

The present paper deals with a more detailed study of the facts observed previously. In general, the new results afford additional support to the earlier conclusions. However, some of the concepts on which these were based have had to be re-examined.

METHODS

Washed mitochondria were isolated from 0.25M sucrose homogenates of rat liver by a slight modification of the method described previously (Berthet & Duve, 1951). The pressing of the liver through a wire mesh was omitted

and higher field-time values were used to ensure a better yield of phosphatase-containing granules. Using a Spinco Model L preparative ultracentrifuge, with rotor no. 40 ($r_{\max} = 8.1$ cm., $r_{\min} = 4.8$ cm.), the 1:10 cytoplasmic extract, free of nuclei, was usually centrifuged at 160000 g-min.; ($S_{\min} = 3500S$); the resuspended particles were washed twice at 130000 g-min. ($S_{\min} = 4400S$). (The use of the composite unit g-min. has been proposed by Duve & Berthet (1953). It is necessitated in the present case by the fact that the centrifugings are performed essentially by means of up-and-down runs, full advantage being taken of the force developed during acceleration and deceleration. The term $S_{\min}$ refers to the sedimentation constant, expressed in Svedberg units (10^{-13} sec.), of the lightest spherical particles which are completely sedimented under the conditions used.)

Acid phosphatase was measured as before, with β -glycerophosphate as substrate. Various commercial preparations were used, after it had been ascertained by means of periodate titration that they contained no more than 5% of the α isomer (which, unlike the β isomer, is significantly hydrolysed by the microsomal glucose 6-phosphatase).

Most of the assays were carried out in 10 min. at pH 5 and 37°, or for longer periods at pH 6.1 and 0°. As already shown (Berthet & Duve, 1951), little thermal activation occurs under these conditions, allowing for a selective estimation of the amount of free enzyme present in the incubation mixture. The total enzyme content of the preparations was measured in a similar manner on suspensions previously treated for 3 min. in a Waring Blendor (usually after tenfold dilution with distilled water), care being taken to prevent excessive heating of the preparation during this treatment. The reaction was stopped by the addition of 8% (w/v) trichloroacetic acid and inorganic P estimated on the filtrates by the method of Fiske & Subbarow (1925).

RESULTS

Osmotic activation

As reported previously (Berthet *et al.* 1951), treatment of granules with distilled water causes a rapid release of their acid phosphatase activity. The same

* Part 2 of this series: Berthet, Berthet, Appelmans & Duve (1951).