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OXIDATION OF ACETANILIDES TO GLYCOLANILIDES AND OXANILIC ACIDS IN RABBITS

MANFRED KIESE and WERNER LENK

Pharmakologisches Institut der Universität München,
Munich, Germany

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Abstract—In the urine of rabbits injected with various acetanilides, the corresponding glycolanilides were found. Approximately 5 per cent of the given acetanilides appeared in the urine as 4-chloro-glycolanilide, 4-bromo-glycolanilide and 4-propionyl-glycolanilide. They were isolated and their structures were established by u.v., i.r. and NMR spectra. Less than 1 per cent of acetanilide and phenacetin were excreted as glycolanilides. No 4-propyl-glycolanilide was found in the urine after the administration of 4-propyl-acetanilide. The effect of various substituents in 4-position on the biochemical hydroxylation of acetic acid in acetanilides is discussed. Rabbits dosed with 4-chloro-acetanilide or 4-propionyl-acetanilide were found to excrete several per cent of the doses as the corresponding oxanilic acids.

INTRODUCTION

IN experiments with *p*-acetylaminopropiophenone (4-propionyl-acetanilide) it was discovered that rabbits ω-hydroxylate the acetic acid residue of this compound, nearly 5 per cent of the dose injected intraperitoneally being excreted in the urine as 4-propionyl-glycolanilide.[1] Schauer *et al.*[2] observed acetic acid also to be hydroxylated when bound in another acetamide, namely *N*-acetylneuramic acid.

This paper reports the results of experiments with several acetanilides which were carried out in order to determine the influence of substituents on the ω-hydroxylation of the acetic acid residue. The behavior of phenacetin appeared of particular interest. The metabolism of this compound, which has been used as a drug for 80 yr, has been studied repeatedly, but its fate in the body is not yet completely elucidated. The *O*-dealkylation to *p*-acetaminophenol and hydrolysis of a small fraction have been known for some time.[3,4] The hydroxylation of small fractions of a dose to 2- and 3-hydroxy-4-ethoxyacetanilide was only recently discovered.[5,6]

Acetanilide is known to be mainly hydroxylated to *p*-acetaminophenol and to be partly hydrolyzed. Rat liver microsomes hydroxylate acetanilide to a small extent in *o*- and *m*-position.[7,8] The influence of a halogen in 4-position on the hydroxylation in this position is known from experiments *in vitro*. Liver microsomes from various species hydroxylate 4-halogeno anilines and acetanilides much more slowly in 4-position than the halogen-free compounds.[9–11] The experiments reported in this paper also show that glycolanilides may be further oxidized in rabbits.

MATERIALS AND METHODS

Materials

Commercial acetanilide and 4-ethoxy-acetanilide were used in the experiments. The arylamines used for preparing the *N*-acetyl and *N*-glycoloyl derivatives were also commercial products, except 4-propylaniline. 4-Propyl-acetanilide was synthezised from 4-propylaniline[12] and acetic anhydride, m.p. 95·5–96·5°; Itaya *et al.*[12] give m.p. 94–95°. 4-Propionyl-acetanilide was prepared from 4-amino-propiophenone and acetic anhydride, m.p. 172°; Hartung and Foster[13] give m.p. 172–173°. 4-Chloro-acetanilide was prepared from 4-chloro-aniline and acetic anhydride, m.p. 181°; Ingold *et al.*[14] give m.p. 175°.

4-Bromo-acetanilide was prepared from 4-bromo-aniline and acetic anhydride, m.p. 166–168°; Sidgwick[15] gives m.p. 168°.

Glycolanilides were prepared from the anilines and glycolic acid following Shapiro *et al.*[16] procedure B. The melting points and absorbance maxima of our preparations are shown in Table 1. The structures of the compounds were proved by the NMR data presented in Table 2.

TABLE 1. MELTING POINTS (CORRECTED), ABSORPTION MAXIMA, AND MOLAR ABSORPTION COEFFICIENTS (ϵ) IN METHANOL OF SOME GLYCOLANILIDES

	Melting point	Melting point according Shapiro *et al.*[16]	Absorbance λ max mμ	$\epsilon \times 10^{-3}$ for λ max
Glycolanilide	92–93°	91–92°	241	13·5
4-Ethoxy-glycolanilide	152°	149–150°	249	14·8
4-Propyl-glycolanilide	91–92°	—	245	15·8
4-Propionyl-glycolanilide	129°	—	280	20·2
4-Chloro-glycolanilide	169–170°	169–170°	248	18·2
4-Bromo-glycolanilide	178–180°	177–178°	249	19·1

4-Chloro-oxanilic acid (Formula I) was obtained by heating 1·3 g of oxalic acid dihydrate and 1·3 g of melted 4-chloro-aniline to 150° for 1·5 hr. The solid cake was finely ground, suspended in 100 ml of 2 N HCl, and extracted with ether, until the solid substance had disappeared. On shaking the combined ether extracts with a saturated sodium bicarbonate solution 1·91 g of the sparingly soluble sodium salt of 4-chloro-oxanilic acid separated. Part of the crop was recrystallized from boiling water. The colorless needles showed no change in shape and color on heating up to 300°. In aqueous solution the sodium salt had an absorption maximum at 261 mμ.

The free acid, recrystallized from ethylacetate, melted at 194°. G. Piccinini and A. Delpiano[17] give the m.p. 190–191°. The methanolic solution showed an absorption maximum at 268 mμ, which on addition of 2 drops of 2 N NaOH shifted to 261 mμ.

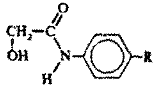
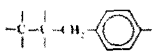
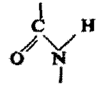
Analysis. $C_8H_6Cl\ NO_3$ (199·6)

calc. C 48·14% H 3·03% Cl 17·76 N 7·02%
found C 48·27% H 3·25% Cl 17·46 N 6·75%

4-Propionyl-oxanilic acid (Formula II) was prepared by heating a finely ground mixture of 1·5 g 4-aminopropiophenone and 1·3 g oxalic acid dihydrate for 2 hr to 140°. The solid cake was worked up as described above. After recrystallization of the sodium salt from boiling water pale yellow crystals were obtained, which on heating began to decompose at 290° without melting. The aqueous solution showed an absorption maximum at 290 mμ.

The free acid crystallized from ethylacetate in pale yellow crystals. They melted

TABLE 2.

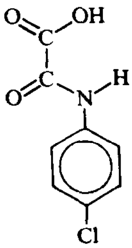
	$CH_3-\overset{\vert}{\underset{\vert}{C}}-\left[\begin{array}{c}-O-\\-CH_2-\\-CO-\end{array}\right]$	$-\overset{\vert}{\underset{\vert}{C}}-\overset{\vert}{\underset{\vert}{C}}H_2-\left[\begin{array}{c}-O-\\-CH_2-\\-CO\end{array}\right]$		$-O-CH_2-\overset{O}{\overset{\Vert}{C}}-$	$HO-\overset{\vert}{\underset{\vert}{C}}-\overset{O}{\overset{\Vert}{C}}-$	arom. H	
I $R = -O-CH_2-CH_3$	1·34 ppm (3) t J ~ 7Hz	4·03 ppm (2) q J ~ 7Hz	—	3·99 ppm (2) s	5·66 ppm (1) broad exchangeable against D	ca. 6·9 ppm (4) ca. 7·6 ppm $\}A_2B_2$	9·52 ppm (1) broad
II $R = -CH_2-CH_2-CH_3$	0·89 ppm (3) t J ~ 7Hz	1·58 ppm (2) multiplett	2·51 ppm the signal was superimposed by that of the nondeuterated solvent	4·00 ppm (2) s	5·6 ppm (1) broad exchangeable against D	ca. 7·1 ppm (4) ca. 7·6 ppm $\}A_2B_2$	9·51 ppm (1) broad
III $R = -\overset{O}{\overset{\Vert}{C}}-CH_2-CH_3$	1·08 ppm (3) t J ~ 7Hz	2·99 ppm (2) q J ~ 7Hz	—	4·05 ppm (2) d; J ~ 6Hz after exchange against D: (2) s	5·69 ppm (1) t; J ~ 6Hz exchangeable against D	ca. 7·9 ppm (4)	10·0 ppm (1) broad slow exchange against D
IV $R = -Cl$	—	—	—	4·03 ppm (2) d; J ~ 6Hz after exchange against D: (2) s	5·68 ppm (1) t; J ~ 6Hz exchangeable against D	ca. 7·4 ppm (4) ca. 7·8 ppm $\}A_2B_2$	9·80 ppm (1) broad exchangeable against D
V $R = -Br$	—	—	—	4·02 ppm (2) d; J ~ 6Hz after exchange against D: (2) s	5·66 ppm (1) t; J ~ 6Hz exchangeable against D	ca. 7·5 ppm (4) ca. 7·7 ppm $\}A_2B_2$	9·76 ppm (1) broad exchangeable against D

NMR signals observed with various glycolanilides in d_6-dimethylsulfoxide. Compounds I, II, and III were synthetic compounds. Compound IV and V have been isolated from urine of rabbits dosed with the corresponding acetanilides; the synthetic compounds produced the same signals. NMR spectrometer Varian HA-100 was used for studying compound I, II, IV, and V, and model HA-60 for compound III.

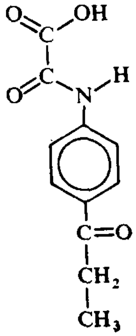
at 184° (decomp.). The absorption maximum in methanol was found to be at 291 mμ. There was no shift of the absorption maximum on addition of 2 drops 2 N NaOH.

Analysis. $C_{11}H_{11}NO_4$ (221·21)

calc. C 59·72% H 5·01% N 6·33%
found* C 59·21% H 4·89% N 6·81%



I



II

METHODS

Male rabbits of mixed strains were supplied by two breeders. The animals showed differences in their metabolic capacities and are designated as group A and B. They were housed individually in Acme metabolic cages and were fed Altromin standard diet and water.

Doses of 50 mg of the acetanilides per kg suspended with an ultra-turrax in a solution of 0·25% agar agar and 0·9% sodium chloride were injected i.p. In each experiment the urine of several rabbits excreted during the 24 hr following the injection of an acetanilide was collected. After being centrifuged the clear urine was separated from the precipitate, adjusted to pH 6·8, and incubated for 6 hr at 37° with 100 units β-glucuronidase Sigma from bacteria type I per ml urine.

At room temperature the urine was three times extracted with equal volumes of ether and twice with equal volumes of ethyl acetate. Then the urine was acidified with conc. hydrochloric acid to pH below 3 and twice extracted with equal volumes of ethyl acetate.

Each extract was dried over sodium sulfate. Its volume was reduced *in vacuo* to about 200 ml. In order to remove phenols the extracts were washed twice with $\frac{1}{16}$ volume of 1 N sodium hydroxide. After being dried again the organic solvents were evaporated *in vacuo*. The residues were dissolved in a mixture of chloroform and methanol. A small portion was applied to a thin layer of silica gel GF_{254} (STAHL) and tested for glycolanilides, synthetic glycolanilide being applied alongside the extract. The chromatograms were developed several times with a mixture of 95 volumes chloroform and 5 volumes methanol, until the substances had separated. Substances with the same R_f value as the synthetic glycolanilides were eluted with methanol. If

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* The sample was dried at 80–100° i.h. vac.

the absorption spectrum of the substance and the R_f value on another TLC were identical to those of the synthetic substance, this was taken as proof of the presence of a glycolanilide in the urine.

When more than 10 mg of the glycolanilide was found in the urine, the substance was isolated by preparative TLC analog to the analytical procedure. The constitution of the crystalline substance was proved by the identity of its u.v.-, i.r.- and NMR-spectra with those of the synthetic substance.

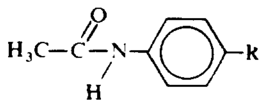
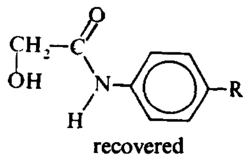
Oxanilic acids were isolated from the ethyl acetate extracts taken from the urines after they had been acidified to pH below 3 in those experiments in which the glycolanilides had been completely extracted from the neutral urine. These extracts were not treated with 0·1 N sodium hydroxide. They were dried with sodium sulfate. A brown oil remained after the ethyl acetate had been evaporated *in vacuo*. On shaking the oil with 200 ml of ether and 15 ml of a saturated solution of sodium bicarbonate a solid substance separated. It was filtered off and recrystallized twice from boiling water.

RESULTS

The results of the determinations of glycolanilides are summarized in Table 3. The highest yields of glycolanilides, as much as 10 and 6 per cent of the acetanilide injected, were observed with 4-chloro- and 4-bromo-acetanilide. In both cases the glycolanilide was found in the extracts taken from neutral urine and from the strongly acidified urine.

From 1850 ml urine of 8 rabbits of group B injected with 4-chloroacetanilide 65·6 mg crystalline 4-chloro-glycolanilide was obtained, m.p. 169–171° (corr.). The NMR spectrum is described in Table 2. U.v.- and i.r.-spectrum (in KI) were identical with those of the synthetic compound. The isolation of additional 17·5 mg 4-chloro-glycolanilide which was determined by the extinction at 248 mμ increased the total

TABLE 3. GLYCOLANILIDES FOUND IN THE URINE OF RABBITS WHICH WAS EXCRETED IN THE 24 hr FOLLOWING THE i.p. INJECTION OF 50 mg OF THE CORRESPONDING ACETANILIDES per kg

Compound applied 	g	Number of rabbits	Group	Urine in 24 hr ml	 recovered	
					mg	% of parent compound
$R = -H$	1·37	8	B	2075	1·7	0·1
$-O-CH_2-CH_3$	6·96	16	B	8115	5·0	0·07
$-CH_2-CH_2-CH_3$	1·25	6	A	1110	—	—
	0·59	3	B	350	—	—
$-CO-CH_2-CH_3$	3·26	12	A	2445	176·6	5·2*
	1·66	10	B	2620	3·6	0·2
$-Cl$	1·09	6	A	1100	126·8	10·6
	1·19	8	B	1850	83·1	6·4
$-Br$	0·81	5	B	1275	62·2	5·7

* In experiments by von Jagow *et al.* (1968) 4·7% of the compound applied was found in the urine as glycol analog.

The experiments marked A or B were carried out with rabbits supplied by different breeders.

yield of the first 24 hr to 83·1 mg. Rabbits of group A excreted nearly twice as much 4-chloro-glycolanilide after injection of the same dose of 4-chloro-acetanilide. These amounts of 4-chloro-glycolanilide are only a fraction of the total amount produced from a dose of 4-chloro-acetanilide. In an experiment with three rabbits of group B the daily excretion of 4-chloro-glycolanilide was determined for 15 days after the i.p. injection of 50 mg 4-chloro-acetanilide per kg. The results presented in Fig. 1 show that during the second day about half as much 4-chloro-glycolanilide is excreted as during the first day and that small amounts of 4-chloro-glycolanilide are found in the urine excreted 15 days after the injection of the 4-chloro-acetanilide. In the 15 days following the injection of 4-chloro-acetanilide a total of about 8 per cent of the dose was found in the urine as 4-chloro-glycolanilide.

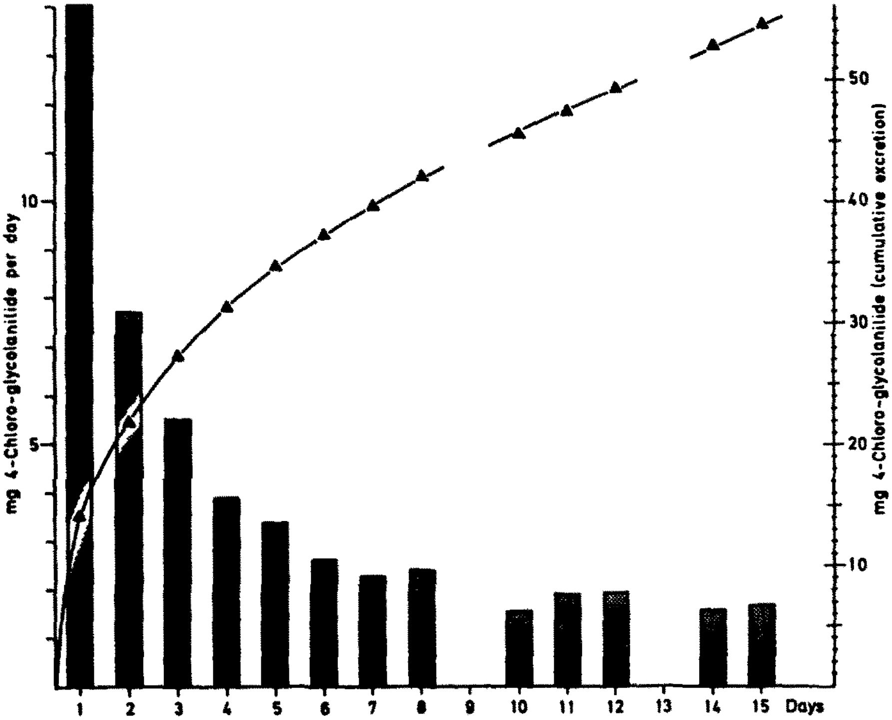


FIG. 1. Daily and cumulative excretion of 4-chloro-glycolanilide by three rabbits of group B injected i.p. with 0·6 g of 4-chloro-acetanilide, i.e. 50 mg per kg. The urines produced on days 9 and 13 were lost. For calculating the cumulative excretion the average of the excretion the day before and the day after was used.

The yield of crystalline 4-bromo-glycolanilide amounted to 55·2 mg; m.p. 177°. The u.v.- and i.r.-spectrum (KI) were identical with those of the synthetic compound; for NMR-spectrum see Table 2. In addition to the crystalline substance 7 mg 4-bromo-glycolanilide was isolated from the extracts.

The isolation of 4-propionyl-glycolanilide from the urine of rabbits injected with 4-propionyl-acetanilide has been earlier described.[1] The yield amounted to 4·7 per cent of the 4-propionyl-acetanilide injected. The experiments were repeated with rabbits of group B. They excreted only 0·2 per cent of the 4-propionyl-acetanilide as 4-propionyl-glycolanilide. The metabolite was found only in the ether extract taken from neutral urine and could be identified only by its u.v.-spectrum and R_f value. Then another experiment was carried out with rabbits of group A. It confirmed the earlier results of von Jagow *et al.*,[1] 5·2 per cent of the 4-propionylanilide being found in the urine as 4-propionyl-glycolanilide.

Rabbits of group A and B were injected with 4-propyl-acetanilide. 4-Propyl-glycolanilide was not detected in the urine.

The urine of the rabbits injected with phenacetin contained only 0·1 per cent of the dose as 4-ethoxy-glycolanilide. It was found only in the ether extracts taken from neutral urine.

Several TLC of the metabolite isolated from 8 l. of urine yielded 1 mg of pure crystalline substance. The i.r.- and u.v.-spectra of the substance were found to be identical with those of the synthetic compound.

Acetanilide was found to be excreted only to 0·1 per cent as glycolanilide. This was identified by its u.v.-spectrum and R_f value in TLC. Since these criteria proved reliable in the discovery of 4-substituted glycolanilides in urine, they also evince the appearance of glycolanilide in the urine.

Oxanilic acids have been isolated from the urine of rabbits dosed with 4-propionyl-acetanilide or 4-chloro-acetanilide. The extracts prepared from 2620 ml of urine produced by ten rabbits of group B in 24 hr following the i.p. injection of 1·66 g of 4-propionyl-acetanilide yielded 0·3 g of the sodium salt of 4-propionyl-oxanilic acid. This corresponds to 14·5 per cent of the 4-propionyl-acetanilide injected.

Application of NMR-spectroscopy (solvent: D_2O) to the purified metabolite revealed the structure of an acylated 4-aminopropiophenone the acyl radical lacking any protons non-exchangeable with D. Hence the acyl radical could consist of inorganic anions as well as of oxalic acid. The identification of the metabolite was achieved by comparison with an authentic sample of the synthesized sodium salt of 4-propionyl-oxanilic acid: both substances showed identical i.r.- (in KI) and u.v.-spectra, and in paper chromatography[18] (paper: S.a.S.2043 b, descending; system: EtOH:conc. $NH_4OH:H_2O = 80:4:6$) the spots on the paper chromatograms, which could be detected by spraying with a 0·03 per cent solution of methyl red in 0·05 N borate buffer pH 8·0[19] were found to have identical R_f values. 4-Chloro-oxanilic acid was isolated from the urine of three rabbits collected during the 24 hr following the i.p. injection of 600 mg 4-chloro-acetanilide. The excretion of 4-chloro-glycolanilide by these animals is shown in Table 4. From 400 ml of urine 27·3 mg of the sodium salt of 4-chloro-oxanilic acid was obtained. This corresponds to 3·4 per cent of the 4-chloro-acetanilide injected. After recrystallisation from boiling water the substance showed i.r.- (in KBr) and u.v.-absorption spectra identical with those of the synthetic substance.

DISCUSSION

The results of our experiments show that certain substituents in the *p*-position of acetanilide strongly influence the extent to which the acetic acid in acetanilide is hydroxylated to glycolic acid. A similar effect of the *p*-substitution has been observed with the *N*-hydroxylation of aniline. *p*-Chloro-aniline, *p*-ethoxy-aniline, and *p*-propionyl-aniline have been found to be *N*-hydroxylated more rapidly than aniline *in vivo* and by liver microsomes *in vitro*.[20–25] There is, however, a difference in the effect of the propyl residue in *p*-position. Whereas the hydroxylation of the acetic acid in acetanilide is diminished by the 4-propyl residue, 4-propylaniline is *N*-hydroxylated in rabbits and by liver microsomes from rabbits and pigs *in vitro* more rapidly than aniline.[25]

In discussing their experiments on the hydroxylation of alkyl and halogen substituted anilines and acetanilides by rabbit liver microsomes Daly *et al.*[10] emphasize the primary directing influence of the amino or acetamido substituent for aryl hydroxylation in this class of aromatic compounds. However, the substituent in 4-position, in particular halogen, not only blocks hydroxylation in this position but has a directing influence of its own. This is illustrated by experiments of Ullrich *et al.*[9] with rat liver microsomes. Substitution of a halogen in 4-position increased the yield of 2-hydroxy derivative by a factor of 3 with 4-bromo-acetanilide and by a factor of 6 with 4-fluoro-acetanilide.

Neither this paper nor the ones quoted can yet elucidate the mechanism by which some substituents in 4-position favor the hydroxylation of the acetic acid in acetanilide. When position 4 is blocked by a substituent which is hard to remove this may increase the rate of hydroxylation of other atoms because substrate and enzymes are not engaged in the rapid hydroxylation of the 4-position. Then the hydroxylation of the other atoms would be inhibited competitively by the rapid hydroxylation in 4-position of unsubstituted acetanilide. This view is supported by the results with 4-alkyl-acetanilides. The alkyl group is hydroxylated and, therefore, it does not direct the oxygenation to other positions.

Another explanation may be based on the vast experience which demonstrates that there are either several microsomal oxygenating systems or one unspecific oxygenating system with specific substrate guides.[26] By their affinity for certain conformations they determine which substances or which sites of a substance are offered to the enzyme for oxygenation. Substituents may change the affinity of a substance for substrate guides and thereby cause a change in the hydroxylation pattern.

The presence of a metabolite in the urine 15 days after the injection of 4-chloro-acetanilide demonstrates that rabbits eliminate 4-chloro-acetanilide very slowly. The transformation into compounds that are excreted in the urine seems to proceed slowly. From the amounts of 4-chloro-glycolanilide and 4-chloro-oxanilic found in the urine it has to be concluded that hydroxylation of the acetic acid and further oxidation to oxalic acid is a major path in 4-chloro-acetanilide metabolism. The same appears to be true of 4-propionyl-acetanilide of which an even larger portion is excreted as oxanilic acid.

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