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METABOLITES OF 4-CHLOROANILINE AND CHLOROACETANILIDES PRODUCED BY RABBITS AND PIGS*

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Abstract—From the urine of rabbits collected 24 hr after the injection of 4-chloroacetanilide, 18 per cent of the material was recovered as 4-chloroglycolanilide and 21 per cent as 4-chlorooxanilic acid. Weekly repetition of the dose increased the proportion of 4-chlorooxanilic acid to about 27 per cent. After the injection of 4-chloroaniline, 3 per cent was found in the urine as 4-chloroglycolanilide and 3 per cent as 4-chlorooxanilic acid. Weekly repetition substantially increased the yield of the metabolites, 4-chloroglycolanilide amounting to about 17 per cent and 4-chlorooxanilic acid to about 8 per cent of the dose of 4-chloroaniline.

Pigs were found to excrete only 11 per cent of the 4-chloroacetanilide injected as 4-chloroglycolanilide. No 4-chlorooxanilic acid was detected in the pig's urine after the injection of 4-chloroacetanilide or 4-chloroglycolanilide. These results and the recovery of 80 per cent of intraperitoneally injected 4-chlorooxanilic acid in the urine demonstrate that the pig, in contrast to the rabbit, is unable to oxidize 4-chloroglycolanilide to 4-chlorooxanilic acid. The low yield of 4-chloroglycolanilide in the pig's urine is, at least partly, explained by the rapid decomposition of 4-chloroglycolanilide observed with pig liver and kidney homogenates *in vitro*.

N-Hydroxy-4-chloroacetanilide was not discovered in the urine of rabbits injected with 4-chloroacetanilide.

Results of experiments with *m*-chloroacetanilide and *o*-chloroacetanilide on rabbits demonstrate the importance of the chlorine being substituted in the 4-position for the hydroxylation of the acetic acid, less than 0·1 per cent of a dose of *m*-chloroacetanilide and no detectable trace of *o*-chloroacetanilide being found as glycol analog in the urine.

3-Amino-7-chlorophenoxazone-2 was isolated from the urine of rabbits injected with 4-chloroaniline after the urine had been incubated with glucuronidase-sulfatase.

THE METABOLISM of chloroanilines and chloroacetanilides has become of interest for several reasons. 4-Chloroacetanilide was found in preparations of phenacetin.[1] It causes ferrihemoglobinemia[2] and, possibly, some other effects observed in phenacetin habitues.[3] After the absorption of 4-chloroacetanilide, ferrihemoglobin is formed in the same measure as 4-chloroaniline is produced from 4-chloroacetanilide and transformed into active metabolites, i.e. the *N*-hydroxy derivative and its nitroso analog.[4] These active metabolites were found to accumulate in the blood of dogs to concentrations that allowed the isolation of an *N*-hydroxy arylamine from the blood of dogs after being oxidized to the nitroso analog.[5,6]

Although the hydrolysis of 4-chloroacetanilide opens the way to the production of active metabolites of 4-chloroaniline it is not a major route of biochemical transformation of this compound. Only recently it was discovered that a large proportion

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of a dose of 4-chloroacetanilide is transformed in rabbits to 4-chloroglycolanilide and 4-chlorooxanilic acid.[7]

Experiments reported in this paper show that this major metabolic route of 4-chloroacetanilide is affected in the rabbit by the repeated administration of 4-chloroaniline or 4-chloroacetanilide and that it may be different in other species. Results with other chloroacetanilides indicate the structures needed for a rich yield of hydroxylated acetic acid in acetanilides.

In earlier studies of the metabolism of 4-chloroaniline and 4-chloroacetanilide in rabbits only minor metabolites were detected. Rabbits have been found to excrete small proportions of 4-chloroaniline as *N*-glucuronide,[8] 2-hydroxy derivative,[9] and about 0·4 per cent as *N*-hydroxy derivative.[10] A larger proportion of *N*-hydroxy derivative, about 3 per cent is found in pig urine.[11]

Studies of the metabolism of ring-substituted monochloroacetanilides in rats[12] showed that 62 per cent of 4-chloroacetanilide is excreted as the *o*-hydroxy derivative, a total of 70 per cent of the dose of 4-chloroacetanilide being recovered from the urine.

MATERIALS AND METHODS

Materials

The preparation and properties of 4-chloroacetanilide, 4-chloroglycolanilide, and 4-chlorooxanilic acid were described in an earlier paper.[7]

N-Hydroxy-4-chloroacetanilide was prepared by hydrogenation of 4-chloronitrobenzene in the presence of acetic anhydride following the procedure of Cramer *et al.*[13] for the synthesis of *N*-hydroxy-2-fluorenylacetamid. To 4·7 g of 4-chloronitrobenzene in 60 ml of ethyl acetate 3 drops of triethylamine, 10 ml of acetic anhydride, and 100 mg of charcoal containing 5 per cent of palladium was added. The mixture was shaken with hydrogen until half the theoretical amount had been taken up. The catalyst was removed by filtration. The solution was heated with 100 ml of about 15% aqueous ammonia solution for 20 min. After being separated from the aqueous phase the ethyl acetate was evaporated under reduced pressure. The residue was dissolved in ether. The *N*-hydroxy compound was extracted by shaking the ether three times with 10 ml of 2 N sodium hydroxide and returned into ether after the aqueous solution had been adjusted to pH 6·0. *N*-Hydroxy-4-chloroacetanilide crystallized on evaporation of the ether under reduced pressure. After recrystallization from ethyl acetate [m.p. 111–112° (corr.)] Baumgarten *et al.*,[14] who prepared the substance by acetylation of 4-chlorophenylhydroxylamine, report m.p. 113° (corr.). The absorption maximum in methanol was found at 257 nm; $\log \epsilon = 4{\cdot}15$. On addition of alkali the maximum shifted to 298 nm. The i.r. absorption in KI shows a broad band at 3150^{-1} cm (—OH) and bands at 1620, 1638 cm^{-1} (—CO—N).

o-Chloroacetanilide was prepared from commercial 2-chloroaniline and acetic anhydride. After recrystallization from 25% acetic acid [m.p. 86–87·5° (corr.)] Roberts *et al.*[15] found m.p. 87–88°.

m-Chloroacetanilide was prepared from commercial 3-chloroaniline and acetic anhydride. After recrystallization from 50% acetic acid [m.p. 76° (corr.)] Roberts *et al.*[15] report m.p 77–78°.

o-Chloroglycolanilide was synthetized from 2-chloroaniline and glycolic acid monohydrate according to method B in Shapiro *et al.*[16] After recrystallization from

10% acetic acid [m.p. 86° (corr.)] Shapiro *et al.*[16] found m.p. 86–87°. The n.m.r. spectrum (in deuterochloroform) determined with the Varian HA 100 showed these signals:

—OH	3·2 ppm (1) broad
—CO—CH_2—O—	4·31 ppm (2) s
3–H, 4–H, 5–H	between 7·0 and 7·5 ppm (3)
6–H	8·4 ppm (1) J = 8 Hz and 2 Hz
—NH—	9·0 ppm (1) broad

m-Chloroglycolanilide, a new compound, was synthesized from 3-chloroaniline and glycolic acid monohydrate also according to Shapiro's method B. After being recrystallized from 10% acetic acid and dried over P_2O_5 the product was found to melt at 108° (corr.). The solution in methanol showed absorption maxima at 285, 277 and 243 nm; $\log \epsilon_{243} = 4{\cdot}16$. The i.r. absorption in KBr showed the following bands: 3390 (—OH), 3290 (—NH), 1665 (—CONH—), 1078 cm^{-1} (—CH_2OH). The following n.m.r. signals in deutero-chloroform were observed with the Varian HA–100:

—OH	ca. 2·5 ppm (broad)
—CO—CH_2—O—	4·28 ppm (2) s
4–H, 5–H, 6–H	between 7·0 and 7·5 ppm (3)
2–H	7·7 ppm (1) indistruct t J = 2 Hz
—NH—	8·3 ppm (1) broad

METHODS

Male rabbits of mixed strains were housed individually in Acme metabolic cages and were fed Altromin standard diet and water *ad lib.*

Male miniature pigs supplied by Versuchsgut Friedland der Universität Göttingen were also housed individually in stainless steel metabolic cages and fed Feldmochinger Ferkelstarter, a diet rich in protein; tetracyclin and stabilizers were omitted.

For intraperitoneal injection the acetanilides were suspended in a solution of 0·25% agar agar in 0·9% sodium chloride solution.

An investigation into the nature of the glycolanilide conjugates excreted in the urine showed that a variety of "glucuronidase preparations" under comparable conditions produces different amounts of glycolanilide from the same urine. This is illustrated by the data presented in Table 1. It was, furthermore, observed that it takes several incubations of many hours to completely split the glycolanilide conjugates. The incubation with as much as 120 units of glucuronidase per ml did not completely split the conjugates in 6 hr. After extraction of the free glycolanilide and incubation with another dose of enzyme another 15 per cent of the first yield was recovered. Therefore, in the experiments described below the urine samples to be analysed for a glycolanilide, after being centrifuged, were incubated at pH 6·8 and 37° with 10 units of glucuronidase Sigma bacterial type I per ml and a small amount of chloroform for 16–18 hr. Then the

TABLE 1. AMOUNT OF 4-CHLOROGLYCOLANILIDE FOUND IN 300 ml OF POOLED URINE FROM RABBITS, WHICH WERE INTRAPERITONEALLY INJECTED WITH 50 mg 4-CHLOROACETANILIDE/kg, AFTER 5 hr INCUBATION WITH 22,500 UNITS OF VARIOUS GLUCURONIDASE PREPARATIONS AT 37°

Enzyme preparation	pH	4-Chloroglycolanilide isolated (mg)
Sigma bacterial type I with chloroform	6·8	8·7
Boehringer–Mannheim	4·5	2·0
Glusulase Endo Laboratories	5·0	2·2

urine sample was extracted three times with an equal volume of ether. The incubation with freshly added glucuronidase and the extraction were repeated until no more glycolanilide was found in the extract.

The combined ether extracts were reduced *in vacuo* to about 300 ml. After being extracted with 15 ml of 1 N sodium hydroxide and being dried over sodium sulfate the ether was evaporated *in vacuo*. The residue was dissolved in a mixture of chloroform and methanol and applied to a thin-layer of silica gel GF_{254}. The chromatograms were developed several times with a mixture of 95 vol. of chloroform and 5 vol. of methanol, until the glycolanilide had separated from other substances.

The silica gel was extracted four times with methanol. The glycolanilide content of the solution was determined by its u.v. absorption. When urines containing known amounts of synthetic 4-chloroglycolanilide were worked up according to the complete analytical procedure an average 88 per cent of the glycolanilide was recovered. The data reported below is corrected on the basis of this recovery.

In some experiments the unchanged 4-chloroacetanilide excreted in the urine was determined. It was eluted from the thin-layers used for isolating 4-chloroglycolanilide and measured by means of its u.v. absorption.

For determining oxanilic acid, a urine sample from which the glycolanilide had been extracted or another aliquote of the urine not treated with glucuronidase was acidified with hydrochloric acid to pH below 3 and extracted three times with an equal volume of ether. After being dried with sodium sulfate the ether volume was reduced to 300 ml and shaken with 10 ml of saturated sodium bicarbonate solution. On standing for 3–4 days at 4° coarse crystals had formed. They were sucked off, dried on the air and weighed.

With the ratio of 300 ml of ether to 10 ml of saturated sodium bicarbonate, which was kept constant in all experiments, an average loss of 8·2 mg of 4-chlorooxanilic acid was observed when a known amount of oxanilic acid was added to the urine and recovered by the above method. The data reported below is corrected for this loss.

Microsomes were prepared from rabbit's liver and kidney according to the method of Von Jagow *et al.*[17] They were suspended in 0·1 M tris buffer pH 7·4 and fortified with 0·12 mM NADP, 10 mM glucose-6-phosphate, 350 I.U. glucose-6-phosphate dehydrogenase per liter, 6 mM magnesium chloride, and 12 mM nicotinamide.

For determining the rate of enzymic hydrolysis of the acylanilides liver and kidney homogenates were prepared according to Von Jagow *et al.*[17] The tissue pulp was mixed with 2 vol. of 0·1 M phosphate pH 7·4 and incubated with 10^{-3} M acylanilides. The

concentration of the anilines liberated after 10, 20 and 40 min was determined according to Brodie and Axelrod's[18] method.

RESULTS

1. *Urinary excretion of 4-chloroglycolanilide and 4-chlorooxanilic acid by rabbits after repeated intraperitoneal injections of 4-chloroacetanilide*

In two series of experiments, three and six rabbits were intraperitoneally injected weekly with 50 mg 4-chloroacetanilide/kg for 5 weeks. One of the animals of the first series died 2 days after the 3rd injection.

The results of the 2 series of experiments are summarized in Fig. 1. It presents the average portions of the dose of 4-chloroacetanilide found as 4-chloroglycolanilide and

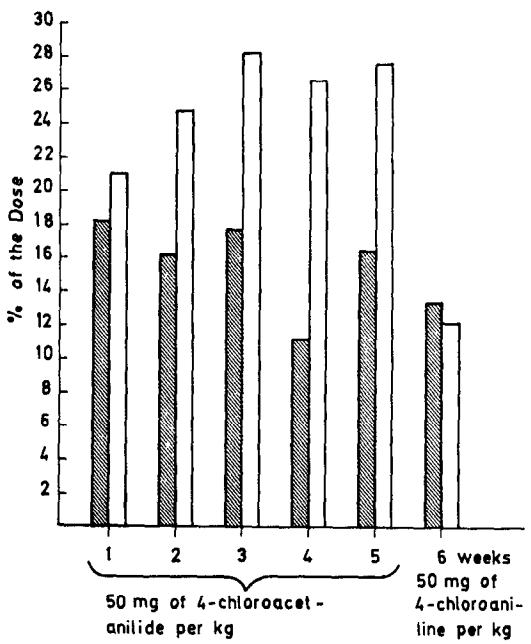


FIG. 1. 4-Chloroglycolanilide and 4-chlorooxanilic acid found in the urine of nine rabbits after weekly intraperitoneal injections of 50 mg of 4-chloroacetanilide/kg.
The first five pairs of columns indicate the average portions of the metabolites found in the urine collected during 24 hr following the injection.
The sixth pair of columns indicates the portions of the same metabolites found in the urine after six of the rabbits treated five times with 4-chloroacetanilide had been injected with 50 mg of 4-chloroaniline/kg.
The hatched column shows 4-chloroglycolanilide and the other one 4-chlorooxanilic acid.

4-chlorooxanilic acid in the urine collected during 24 hr after the injection. Not included in Fig. 1 is the amount of unchanged 4-chloroacetanilide recovered from the urine. Its proportion of the dose dropped from 2·6 per cent after the first injection to 0·1 per cent after the 5th injection of 4-chloroacetanilide.

The amount of 4-chloroglycolanilide found in the first day's urine, i.e. 18 per cent of the dose of 4-chloroacetanilide, did not increase after several injections. The amount

of 4-chlorooxanilic acid, however, was found to increase from about 20 per cent to nearly 30 per cent; the portion remaining the same after two more injections.

A week after the 5th injection of 4-chloroacetanilide six rabbits were injected with 50 mg 4-chloroaniline/kg. As may be seen in Fig. 1, about 13 per cent of the dose was recovered from the urine as 4-chloroglycolanilide and nearly the same portion as 4-chlorooxanilic acid. This may be compared with the excretion of the same metabolites by rabbits not previously treated with 4-chloroacetanilide as shown in Fig. 2.

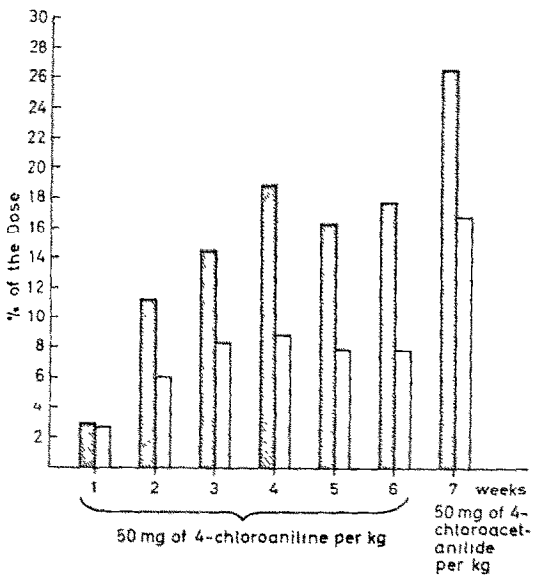


FIG. 2. 4-Chloroglycolic acid and 4-chlorooxanilic acid found in the urine of nine rabbits after weekly intraperitoneal injections of 50 mg of 4-chloroaniline/kg. The first six pairs of columns indicate the average portions of metabolites found in the urine collected during 24 hr following the injection. The seventh pair of columns shows the portions of the same metabolites found in the urine after a dose of 50 mg of 4-chloroacetanilide/kg given a week after the last dose of 4-chloroaniline. The hatched column shows 4-chloroglycolanilide and the other one 4-chlorooxanilic acid.

2. *Urinary excretion of 4-chloroglycolanilide and 4-chlorooxanilic acid by rabbits after repeated intraperitoneal injections of 4-chloroaniline*

4-Chloroglycolanilide and 4-chlorooxanilic acid were also found in the urine of nine rabbits injected intraperitoneally with 50 mg 4-chloroaniline/kg weekly. As may be seen in Fig. 2, the amounts of these metabolites were small after the first injection, amounting to only 3 per cent each of the 4-chloroaniline dose. After two more doses, however, the amount of 4-chloroglycolanilide in the urine has increased to nearly 20 per cent of the 4-chloroaniline injected, i.e. the same portion which was observed with rabbits injected with 4-chloroacetanilide; see Fig. 1.

Unlike rabbits treated with 4-chloroacetanilide, the rabbits dosed with 4-chloroaniline were found to excrete much less 4-chlorooxanilic acid than 4-chloroglycolanilide in the urine.

A test dose of 50 mg 4-chloroacetanilide/kg given a week after the 6th injection of 4-chloroaniline produced larger portions of 4-chloroglycolanilide and 4-chloro-

oxanilic acid than the injection of 4-chloroaniline. In contrast to the rabbits repeatedly injected with 4-chloroacetanilide, 4-chloroglycolanilide was found to exceed 4-chlorooxanilic acid by 60 per cent.

3. *Urinary excretion of 4-chloroglycolanilide by pigs after the intraperitonel injection of 4-chloroacetanilide or 4-chloroaniline*

One of two pigs injected with 50 mg 4-chloroaniline/kg died 9 hr later, the other one survived. The urine collected during 9 and 24 hr, respectively, was incubated twice with glucuronidase Sigma and once with glucuronidase Boehringer. From the ether extracts 54 and 63 mg of 4-chloroglycolanilide was isolated, corresponding to 1·8 and 2·9 per cent of the 4-chloroaniline dose. Melting points and i.r. spectra (in KI) of the metabolite and synthetic 4-chloroglycolanilide were found to be identical.

Since a dose of 50 mg 4-chloroaniline/kg proved to be highly toxic, another pig was injected with 20 mg 4-chloroaniline/kg. From 1720 ml of urine collected in 24 hr 99·8 mg of 4-chloroglycolanilide was isolated corresponding to 8·6 per cent of the 4-chloraniline dose.

The urines collected in the three experiments were acidified to pH below 3 and extracted with ether. The reduced volumes of the ether extracts were shaken with saturated sodium bicarbonate solution. No precipitation of 4-chlorooxanilic acid as sodium salt was observed after several days standing at 4°. If any, less than 1 per cent of the 4-chloroaniline injected was excreted as 4-chlorooxanilic acid.

After the injection of 20 mg 4-chloroacetanilide/kg 11 per cent of the dose was recovered as 4-chloroglycolanilide from the pig's urine collected in 24 hr. Again 4-chlorooxanilic acid was not found in the urine. As has been observed in experiments with rabbits[7] the excretion of 4-chloroglycolanilide was found to go on for several days after a single dose of 4-chloroacetanilide, 1·5 mg of 4-chloroglycolanilide being isolated from the urine collected on the 7th days after a dose of 440 mg 4-chloroacetanilide.

4. *4-Chloroglycolanilide and 4-chlorooxanilic acid in the urine of rabbits and pigs injected with 4-chloroglycolanilide or 4-chlorooxanilic acid*

4-Chlorooxanilic acid was found to be a metabolite of 4-chloroacetanilide in the rabbit, but not in the pig. In order to further elucidate the conversion of 4-chloroglycolanilide to 4-chlorooxanilic acid either compound was injected into rabbits and pigs.

A male rabbit weighing 3·9 kg was intraperitoneally injected with 50 mg 4-chlorooxanilic acid/kg. From the urine collected in 24 hr 183 mg of the sodium salt of 4-chlorooxanilic acid was isolated, i.e. 94·5 per cent of the 4-chlorooxanilic acid injected.

Pigs were found also to excrete 4-chlorooxanilic acid into the urine, though not as rapidly as rabbits. The results of an experiment with a pig presented in Table 2 show that 65 per cent of a dose of 20 mg 4-chlorooxanilic acid/kg is excreted during the first day, a total of about 80 per cent being found in the urine produced in 3 days.

After the injection of 4-chloroglycolanilide a pig was found to excrete a total of 6·6 per cent of the dose in the urine. The data in Table 3 shows that the excretion is going on for 3 days, only 3 per cent of the dose being excreted on the first day. 800 ml of the first day's urine and 1000 ml of the third day's urine was analysed for 4-chlorooxanilic acid, but none was found.

TABLE 2. URINARY EXCRETION OF 4-CHLOROOXANILIC ACID BY A PIG, WEIGHING 74·5 kg, AFTER THE INTRAPERITONEAL INJECTION OF 1·5 g 4-CHLOROOXANILIC ACID

Hours after injection	Urine excreted (ml)	Urine analysed (ml)	Na-salt of 4-chlorooxanilic acid isolated (mg)*	4-Chlorooxanilic acid in total urine (mg)†	Dose administered (%)
0–24	680	340	545	998	66·5
24–48	2600	800	39	138	9·2
48–72	2100	700	23	84	5·6
				Total 1220	81·3

* Not corrected.
† Corrected on the basis of the recoveries obtained by the addition of known amounts.

TABLE 3. URINARY EXCRETION OF 4-CHLOROGLYCOLANILIDE BY A PIG, WEIGHING 66 kg. AFTER THE INTRAPERITONEAL INJECTION OF 1·7 g 4-CHLOROGLYCOLANILIDE

Hours after injection	Urine excreted (ml)	Urine analysed (ml)	4-Chloroglycolanilide isolated (mg)*	4-Chloroglycolanilide in total urine (mg)†	Dose administered (%)
0–24	1590	200	5·7	51·4	3·0
24–48	870	200	1·8	8·8	0·5
48–72	3200	600	8·6	52·0	3·1
72–96	2020	300	0	0	—
				Total 112·2	6·6

* Not corrected.
† Corrected on the basis of the recoveries obtained by the addition of known amounts.

As may be seen in Table 4, rabbits excrete nearly 30 per cent of a dose of 4-chloroglycolanilide as such and nearly 20 per cent as 4-chlorooxanilic acid. This data differs only a little from the proportions of the metabolites found when 4-chloroacetanilide is administered.

5. *Search for* N-*hydroxy*-4-*chloroacetanilide in the urine of rabbits injected with* 4-*chloroacetanilide*

In view of the large proportion of *N*-hydroxy-2-fluorenylacetamide found in the urine of rabbits after the administration of 2-fluorenylacetamide[19] *N*-hydroxy-4-chloroacetanilide was expected to be an urinary metabolite of 4-chloroacetanilide.

From six rabbits injected with 920 mg of 4-chloroacetanilide (50 mg/kg) 806 ml of urine was collected in 24 hr; 275 ml of the urine was adjusted to pH 6·8 and incubated for 18 hr at 37° with 25 units of glucuronidase Sigma per ml and a few drops of chloroform. The urine was adjusted to pH 6·0 and extracted three times with ether. After being reduced to 300 ml under reduced pressure the ether was extracted three times with 10 ml of 2 N sodium hydroxide. The aqueous solution was acidified to pH 6 and extracted three times with ether. After evaporation of the ether the residue was

TABLE 4. URINARY EXCRETION OF 4-CHLOROGLYCOLANILIDE AND 4-CHLOROOXANILIC ACID BY THREE RABBITS INTRAPERITONEALLY INJECTED WITH 550 mg 4-CHLOROGLYCOLANILIDE (50 mg/kg)

	Urine		4-Chloroglycolanilide			4-Chlorooxanilic acid			
Hours after injection	excreted (ml)	analysed (ml)	Isolated (mg)*	In total urine (mg)†	Dose administered (%)	Urine analysed (ml)	Na-salt isolated (mg)*	In total urine (mg)†	Dose administered (%)
0–24	350	100	38·0	150·0	27·3	150	42·2	118·3	18·0
24–48	405	100	1·9	8·7	1·4	200	—	—	—
			Total	158·7	28·7			118·3	18·0

* Not corrected.
† Corrected on the basis of the recoveries obtained by the addition of known amounts.

repeatedly chromatographed on silica gel with a mixture of 95 vol. chloroform and 5 vol. methanol. No spot was observed with the R_f of synthetic *N*-hydroxy-4-chloroacetanilide. The urine was adjusted to pH 6·2 and incubated with 1 ml of the Boehringer glucuronidase-arylsulfatase preparation. Again TLC did not show any trace of *N*-hydroxy-4-chloroacetanilide in the urine. One mg of the hydroxamic acid was easily detected on the thin-layer. When 2 mg of *N*-hydroxy-4-chloroacetanilide was added to 275 ml of urine and carried through the whole procedure of incubation, extraction and chromatography, 83 per cent was recovered from the thin-layer. Therefore, the excretion of *N*-hydroxy-4-chloroacetanilide, if any, amounted to much less than 0·1 per cent of the dose of 4-chloroacetanilide injected.

6. *Microsomal hydroxylation of 4-chloroacetanilide to 4-chloroglycolanilide*

Microsomes were prepared from the livers and kidneys of rabbits which had been six times injected with 50 mg of 4-chloroaniline/kg and once with 50 mg of 4-chloroacetanilide. A suspension of liver microsomes with 1·6 mg protein/ml and a suspension of kidney microsomes with 17 mg protein/ml were incubated for 1 hr at 37° with 0·01 M 4-chloroacetanilide. The suspensions were extracted three times with ether and three times with ethyl acetate. 4-Chloroglycolanilide was isolated from the extracts by TLC. From the u.v. absorption of the eluates, its concentration was calculated as being 2·3 μg/ml in the liver microsome suspension and 5·1 μg/ml in the kidney microsome suspension.

7. *Decomposition of acylanilides by liver and kidney homogenates*

It has been known for 60 years that acetanilides are decomposed by liver and kidney tissue *in vitro*.[20] Since the catalyst of the reaction does not seem to be highly specific[21] the hydrolysis of acetanilide, 4-chloroacetanilide, 4-chloroglycolanilide and 4-chlorooxanilic acid was studied. The results are shown in Table 5. The reaction rates are calculated from the concentrations of aniline found after 10 min incubation. For comparison the rate of hydrolysis of acetanilide has been determined. 4-Chloroacetanilide was decomposed much more slowly than acetanilide.

Rabbit's tissues were found to split 4-chloroglycolanilide a little more rapidly than 4-chloroacetanilide. But pig's tissues decomposed it as fast as acetanilide. 4-Chlorooxanilic acid was virtually stable in kidney homogenate and was very slowly hydrolyzed by liver homogenate.

TABLE 5. DECOMPOSITION OF SOME ACYLANILIDES BY HOMOGENATES PREPARED FROM RABBIT'S AND PIG'S LIVER AND KIDNEY

	10^{-11} moles decomposed per mg protein per min by			
	Liver		Kidney	
Substrate	Rabbit	Pig	Rabbit	Pig
Acetanilide	10·6	24·8	7·3	35·1
4-Chloroacetanilide	3·0	12·8	2·1	11·6
4-Chloroglycolanilide	5·5	26·0	5·0	32·0
4-Chlorooxanilic acid	0·7	1·4	0·5	0·9
4-Chloropropionanilide	27·8	48·5	5·7	46·4
L(−)4-Chlorolactanilide	16·7	27·2	6·4	20·6

The figures are the means of three experiments.

It may be pointed out that pig liver and kidney homogenates decomposed 4-chloroacetanilide and 4-chloroglycolanilide four to six times more rapidly than the homogenates from rabbit's tissues. The rate of hydrolysis by pig liver homogenate was studied with 3×10^{-4} to 10^{-2} M acetanilide, 4-chloroacetanilide, and 4-chloroglycolanilide. Lineweaver–Burk plots of the results showed a similar affinity of all three substrates for the enzyme(s), the apparent K_m being nearly 10^{-2} M in each case. The following V_{max} values were calculated: acetanilide 85, 4-chloroacetanilide 32, and 4-chloroglycolanilide 90 $\times 10^{-11}$ moles/mg protein/min.

8. *3-Amino-7-chlorophenoxazone-2 in the urine of rabbits injected with 4-chloroaniline*

When the urine produced by rabbits after the intraperitoneal injection of 50 mg of 4-chloroaniline/kg was incubated for 12–24 hr at pH 6·2 with glucuronidase-sulfatase Boehringer and extracted with ether, a yellow-red dye was present in the ether. The dye was not removed by extracting the ether with 0·1 N sodium hydroxide. In TLC it readily separated from other components. The dye was eluted with methanol and the methanol evaporated *in vacuo*. The dye thus collected from the urine of nine rabbits after five administrations of 4-chloroaniline was dissolved in chloroform. On slow evaporation of the chloroform at room temperature violet-red crystals formed. After recrystallization by the same procedure 5 mg of the crystalline dye was collected.

The recrystallized dye was found to sublimate at 270° and to decompose between 295 and 300°. Dissolved in methanol it showed absorption maxima at 236, 420–422, 433–436 nm and an inflection at 272 nm. On addition of hydrochloric acid the absorption maxima shifted to 235, 444–446, 460–462 nm and inflections appeared at 265 and 505 nm.

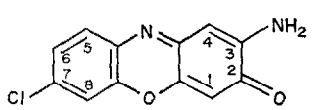
The i.r. absorption (in KI) showed bands at 3405, 3300 cm^{-1} ($—NH_2$), and a wide one at 1600–1550 cm^{-1} caused by quinone–CO. The n.m.r. spectrum in d_6-dimethylsulfoxide recorded with the Varian HA–100 showed the following signals:

1–H 6·36 ppm (1) s
4–H 6·36 ppm (1) s
3–NH_2 6·9 ppm (2) s exchangeable against D
5–H 7·44 ppm (1) q J = 8·5 Hz and 2 Hz
6–H 7·7 ppm (1) J = 8·5 Hz
8–H 7·65 ppm (1) J = 2 Hz

after the addition of d_6-benzene:

1–H, 4–H 6·36 and 6·73 ppm, (each 1), s

The data proves 3-amino-7-chlorophenoxazone-2 to be the structure of the dye, see Fig. 3.



3-Amino-7-chlorophenoxazone-2

FIG. 3.

9. *Metabolites of* m-*chloroacetanilide and* o-*chloroacetanilide*

Two groups of five and six rabbits were twice injected with 50 mg of *m*-chloroacetanilide/kg. In each of the four experiments about 1 l. of urine was collected during the first day. *m*-Chloroglycolanilide was isolated by TLC and identified by the R_f and u.v. absorbance only. A total of 2·8 mg of *m*-chloroglycolanilide was found in the four experiments, corresponding to 0·07 per cent of the total dose of 3·86 g of *m*-chloroacetanilide.

After a dose of 50 mg of *o*-chloroacetanilide/kg 1100 ml of urine was collected from five rabbits. TLC of the ether extract did not show a spot with the same R_f as synthetic *o*-chloroglycolanilide.

DISCUSSION

Rabbits were found to produce 4-chloroglycolanilide and 4-chlorooxanilic acid, recently discovered metabolites,[7] in substantial amounts from 4-chloroacetanilide and from 4-chloraniline, 40 per cent of the former and 6 per cent of the latter being found in the urine as the glycolic acid and oxalic acid analog. 4-Chloroacetanilide as well as 4-chloroaniline stimulate their transformation into the two metabolites, the latter to a larger extent than the former. Although the administration of one compound also stimulates the transformation of the other one, there are differences in the stimulation process. These are the more remarkable as the stimulating compounds are closely related: 4-chloroacetanilide does not increase the portion excreted as 4-chloroglycolanilide, whereas three injections of 4-chloroaniline increase the portion excreted as 4-chloroglycolanilide 6-fold.

The experiments with pigs reveal an interesting species difference in the metabolism of 4-chloroacetanilide and 4-chloroaniline. Pigs do not transform 4-chloroglycolanilide into 4-chlorooxanilic acid. After the injection of a large dose of 4-chloroglycolanilide no 4-chlorooxanilic acid is found in the urine. However, if 4-chlorooxanilic acid is intraperitoneally injected it appears in the urine.

After the administration of 4-chloroacetanilide or 4-chloroglycolanilide the yield of 4-chloroglycolanilide in pig urine is much lower than in rabbit urine. This is, at least partly, explained by the rapid decomposition of 4-chloroglycolanilide observed in liver and kidney homogenates. Other pathways of 4-chloroglycolanilide metabolism exist in addition to conjugation.

This is true also with the rabbit, although the transformation to 4-chlorooxanilic acid is a major route. A large part of 4-chloroglycolanilide injected into rabbits is not recovered from the urine as such or 4-chlorooxanilic acid. Transformations other than the formation of conjugates or the oxidation to 4-chlorooxanilic acid seem to be more important when the concentration of 4-chloroglycolanilide in the rabbit is high, as is the case after the injection of a large dose (50 mg/kg) of chloroglycolanilide. Otherwise it is hard to understand, why hardly more 4-chloroglycolanilide and 4-chlorooxanilic acid are found in the urine after the injection of 4-chloroglycolanilide than after the injection of 4-chloroacetanilide.

4-Chloroacetanilide yields the highest portion of glycol analog of all *N*-arylacetamides studied so far. In addition to the acetanilides earlier studied,[7] meanwhile 4-phenylacetanilide, 4-cyclohexylacetanilide, *N*-2-naphthylacetamide, and *N*-2-fluorenylacetamide have been tested and found to yield only small portions of glycol

analog in the urine. But up to 10 per cent of 4-sulfamidoaniline or 4-sulfamidoacetanilide appears in the urine as 4-sulfamidoglycolanilide.[22] The results of experiments with the isomers of 4-chloroacetanilide demonstrate the importance of the 4-position being blocked by chlorine for the hydroxylation of the acetic acid. *m*-Chloroacetanilide was found to yield as little glycol analog as acetanilide itself.[7] In *o*-position the chlorine obviously blocks the hydroxylation of the acetic acid.

N-Hydroxy-4-chloroacetanilide was not detected in the urine of rabbits injected with 4-chloroacetanilide. Regarding the sensitivity of the method used it had to be concluded that less than 0·1 per cent of a dose of 4-chloroacetanilide, if any, is excreted as *N*-hydroxy derivative. This is of interest in view of the high yield of 4-chloroglycolanilide. As will be demonstrated and discussed in a forthcoming paper,[22] a reciprocal relationship seems to exist between the *N*-hydroxylation and the acetic acid hydroxylation in *N*-acyl arylamines.

3-Amino-7-chlorophenoxazone-2 is a new compound. Its formation in the urine after the incubation with glucuronidase and sulfatase is more likely than its being a metabolite. The incubation with the enzyme splits the conjugates of 2-hydroxy-4-chloroaniline, a metabolite of 4-chloroaniline.[9] The free compound then condenses to the phenoxazone.

The formation of 3-amino-7-ethoxyphenoxazone-2 from 3-hydroxy-4-aminophenetol, a metabolite of phenacetin in human urine, has been described by Büch *et al.*[23] Data upon which the elucidation of the structure is based may be found in Häuser.[24]

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