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ROUTES OF METABOLISM OF [^{36}Cl] RING-SUBSTITUTED MONOCHLOROACETANILIDES*†

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Abstract—The excreted monohydroxy and deacetylated metabolites of 2-, 3- and 4-[^{36}Cl]chloroacetanilide in the urine of rats were determined. The extent of *ortho*-hydroxylation (relative to the amino group) decreased in the same order as the relative toxicities and antipyretic activities of the administered drugs: 4- 3- 2-. Neither total hydroxylation nor *para*-hydroxylation followed this order. Deacetylation of the parent compound increased as the toxicity and antipyretic activity decreased. Conjugation of the hydroxylated metabolites as etheral sulfates and glucuronides was observed.

POSSIBLE relationships between metabolism and carcinogenicity of aromatic amines have been suggested by several investigators[1, 2] and the importance of the *o*-hydroxylated metabolites has been discussed in some detail.[3] These studies on the metabolism of carcinogenic amines have been extended also to simpler amines,[4] and it is possible that a systematic study with compounds of less complicated structure than the carcinogens may offer a clue to the problem of the metabolism of aromatic amines as related to physiological response.

It has been established that the analgesic and antipyretic drug, acetanilide, is metabolized by deacetylation, hydroxylation and conjugation as glucuronides or ethereal sulfates. Studies *in vitro* by Bray *et al.*[5] indicate that substitution in the various positions in acetanilide affects the extent of deacetylation markedly. Ring substitutions would also be expected to affect the position and extent of hydroxylation. In turn, such differences in hydroxylation and deacetylation might influence certain pharmacological properties of the compound. In fact, studies by Argus *et al.*[6] showed that the position of chloro-substitution in the ring altered the toxicity and antipyretic activity of acetanilide. This information suggested that the isomeric chloroacetanilides might be of value in studying the relationship between metabolism and these biological activities. The use of chlorine as the substituent in the aromatic ring of acetanilide gives the added advantage of permitting the use of the radioactive isotope, ^{36}Cl, as a tracer in inverse isotope dilution experiments for identifying metabolites.

The present study describes the quantitative determination or identification of metabolites of 2-, 3- and 4-[^{36}Cl]chloroacetanilide in the urine of rats receiving these compounds. The possible relationship between routes of metabolism and observed differences in the biological effects of the administered compounds is discussed.

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EXPERIMENTAL

Materials

Melting points of all compounds are uncorrected.

Radioactive compounds. 4-[^{36}Cl]Chloroacetanilide melting at 175–177 °C and having a specific activity of $3{\cdot}4 \times 10^4$ counts/min per mg was prepared according to Gryder *et al.*[7] The synthesis of 3-[^{36}Cl]chloroacetanilide (m.p. 73 °C; specific activity $1{\cdot}5 \times 10^4$ counts/min per mg) and 2-[^{36}Cl]chloroacetanilide (m.p. 88 °C; specific activity $2{\cdot}1 \times 10^4$ counts/min per mg) were described previously.[8]

Metabolites of 4-chloroacetanilide. 4-Chloroaniline and 4-chloroacetanilide (Eastman) were recrystallized from 50% aqueous ethanol to sharp melting points of 72 °C and 178 °C, respectively, in agreement with the literature.[9, 10] 4-Chloro-2-hydroxyaniline was prepared by the method of Hodgson and Kershaw.[11] Acetylation of this compound in benzene with equimolar portions of acetic anhydride yielded 4-chloro-2-hydroxyacetanilide, m.p. 190°.

Metabolites of 3-chloroacetanilide. 3-Chloroaniline (Eastman) was purified by distillation; the fraction distilling at 226–227 °C at atmospheric pressure was used.[9] 3-Chloroacetanilide (Eastman) was purified (m.p. 73 °C)[12] by recrystallization from 50% aqueous ethanol. 3-Chloro-4-hydroxyaniline was prepared by the reduction of 3-chloro-4-hydroxynitrobenzene (Eastman) with sodium hydrosulfite in 10% aqueous sodium hydroxide, according to the method of Christiansen.[13] The crude compound was recrystallized from water in the presence of sodium bisulfite to m.p. 153 °C. Acetylation with equimolar quantities of acetic anhydride in benzene yielded 3-chloro-4-hydroxyacetanilide (m.p. 143 °C).[13] 3-Chloro-6-hydroxyaniline was prepared similarly from 3-chloro-6-hydroxynitrobenzene (Eastman). It was rapidly recrystallized from water in the presence of a small amount of sodium hydrosulfite to prevent oxidation. The gleaming white amine, stable in air, melted at 140 °C; the m.p. recorded by Mottier[14] for this compound was 138 °C. 3-Chloro-6-hydroxyacetanilide (m.p. 185 °C)[14] was obtained in good yield by acetylation of the amine with acetic anhydride in benzene.

Metabolites of 2-chloroacetanilide. 2-Chloroaniline (Eastman) was purified by distillation. The fraction distilling at 206–207 °C at atmospheric pressure was taken.[12] 2-Chloroacctanilide (Eastman) was recrystallized (m.p. 88 °C)[12] from 50% aqueous ethanol. 2-Chloro-4-hydroxyaniline was prepared by the method of Hodgson and Kershaw,[11] m.p. 160°. 2-Chloro-4-hydroxyacetanilide was prepared by acetylation of the amine with equimolar quantities of acetic anhydride and recrystallized from absolute ethanol to m.p. 124–125 °C; Hurst and Thorpe[15] report m.p. 121 °C. The infrared spectrum, however, had one strong carbonyl band at 5·9 μ, a finding indicating the presence of only one acetyl group, and a broad medium band centered at 3 μ, an observation which demonstrates the presence of a free hydroxy group. The compound was soluble in dilute aqueous sodium hydroxide, a circumstance which indicates that the hydroxy group was unsubstituted (Found: N, 7·5; Cl, 19·7; Calc. for $C_8H_8O_2NCl$: N, 7·5; Cl, 19·1%). 2-Chloro-6-hydroxyaniline was synthesized by reduction with sodium hydrosulfite of 2-chloro-6-hydroxynitrobenzene, in a manner similar to that used by Christiansen[13] for 3-chloro-4-hydroxyaniline. The intermediate nitro compound was prepared by the method of Hodgson and Moore.[16] 2-Chloro-6-hydroxyacetanilide was obtained by acetylation of the corresponding amine hydrochloride in water. The amine was suspended in water and 10 N Cl was added dropwise until the compound

dissolved. The solution was warmed to 50 °C and 1·15 Equiv. of acetic anhydride were added while the solution was stirred vigorously. Saturated aqueous sodium acetate solution, in amount sufficient to neutralize the acidity of the solution and to precipitate the product, was added immediately. The compound was washed with water and recrystallized from aqueous ethanol, m.p. 190–191 °C (Found: Cl, 19·2; Calc. for $C_8H_8O_2NCl$: Cl, 19·1%). The infrared spectrum revealed one strong carbonyl band at 6 μ and a broad medium (hydroxy) band centered at 3·2 μ; hence, acetylation did not affect the hydroxyl group.

Enzymes. β-Glucuronidase (bacterial powder, 25,000 units/g) was purchased from the Sigma Company, St. Louis, Mo. Takadiastase, a plant sulfatase, was purchased from Parke, Davis and Company, Detroit, Mich.

Methods

Care of animals and collection of urine. Male Sprague–Dawley rats (390–490 g) were kept in metabolism cages that permit the collection of urine free from feces. All animals had free access to Purina Laboratory Chow and water. The animals were kept in an air-conditioned room at a constant temperature of 22 °C. The rats were injected intraperitoneally with either 4-, or 3- or 2-[^{36}Cl]chloroacetanilide in a single dose: 100 mg per kg of body weight. The compounds were suspended in a 2 per cent methyl cellulose solution. Urine samples from animals receiving the same compound were pooled; two rats were used for 2-[^{36}Cl]chloroacetanilide and for the 4-isomer, while three rats were administered the 3-isomer. The urine was collected for a period of 72 hr after injection, in vials which were packed in dry ice; thus, the urine was frozen as soon as it was delivered into the vial. The vials were changed every 4 hr, in order that the urine was given no opportunity to thaw. Thus, bacterial or enzymatic action was prevented. The radioactivity in each 4 hr urine sample was determined by counting duplicate planchets, each plated with 0·1 ml of urine. The most active urine samples were pooled for use in quantitative determinations and qualitative identifications of metabolites, in inverse isotope dilution experiments.

General counting procedure. A carefully weighed amount (approximately 200 mg) of the postulated metabolite was added to a sample (3 or 5 ml) of urine (giving 33,800 to 78,000 counts/min) and the inactive compound was brought into equilibrium with any chemically identical radioactive material in the urine sample by refluxing for 5 hr (equilibrium was not established in some of the qualitative studies in which a milder procedure was used). The metabolite was precipitated by cooling and brought to constant specific radioactivity by successive crystallizations from several solvents. In some cases this was done following conversion to the acetyl derivative. For determination of specific activity, 0·2 ml of a solution (5 mg/ml) in 95% aqueous ethanol was plated on duplicate planchets in an almost infinitely thin layer.

To determine when the constancy of radioactivity was reached in some cases, in which several recrystallizations were made from the same solvent, activity of the recrystallizing medium was taken. The metabolite was dissolved, allowed to crystallize slowly in a centrifuge tube, left for 12 hr at 5 °C ,and then centrifuged for 5 min. The supernatant fraction was removed immediately with a sintered-glass filter stick and 0·1 ml was plated on duplicate planchets. Each sample was plated at the same temperature, thus insuring equal saturation of the solvent with the solute. When the activity of the recrystallizing medium was constant for at least two recrystallations,

the solid phase was also assumed to have constant activity; the specific activity of the crystallized metabolite was then determined.

All planchets were counted for at least 4840 counts or, if the activity was low, for a period of at least 2 hr. Background was counted for periods of 2 hr on three or more occasions during each day. All measurements were made in a Q-gas counting chamber with a thin window (Nuclear Corporation of Chicago, model D-47) and an automatic sample changer (model C-110) and a scaler (model 183) with an efficiency of 20 per cent, as calculated against a ^{60}Co standard. The amount of metabolite (expressed as percentage of radioactivity in the urine) was calculated with the aid of the formula:

$$\% \text{ metabolite} = \frac{(\text{mg non-radioactive compound added}) \times (\text{specific activity of compound recovered in counts/min per mg})}{\text{total activity of the urine sample in counts/min}} \times 100$$

Determination of the metabolites. Quantitative determinations were made of: (a) the unchanged parent compounds; (b) the N-deacetylated parent compounds; (c) the total hydroxy metabolites of each hydroxyaniline derivative, in order to determine the total amount of hydroxylation in a given position; and (d) inorganic chloride ion; in addition, qualitative studies were made to determine the presence in the urine of: (e) the unconjugated monohydroxy derivatives of the parent compounds and of the N-deacetylated compounds; and (f) the glucuronides and etheral sulfates of the monohydroxy derivatives of the parent compounds and N-deacetylated compounds. These estimations of the individual, conjugated and unconjugated, monohydroxy derivatives are considered as only qualitative since the sum of the recoveries of the individual metabolites hydroxylated in a given position did not equal the total hydroxy derivative obtained following complete acid hydrolysis. Although the same general methods were used as for the quantitative studies, it is possible that enzyme hydrolysis was incomplete and equilibrium between non-radioactive and radioactive metabolites could not always be established.

(a) *Unchanged parent compounds.* For addition to the urine samples, 2-chloroacetanilide and 4-chloroacetanilide were dissolved in acetone, and 3-chloroacetanilide was dissolved in 95% aqueous ethanol. Following refluxing, all three compounds precipitated on cooling to 5 °C. 2-Chloroacetanilide was purified by successive crystallization from 50% aqueous ethanol to radioactive constancy of the recrystallizing medium. 3-Chloroacetanilide was recrystallized successively from 50% aqueous ethanol, 50% aqueous methanol, 30% aqueous acetic acid and four times from 25% aqueous ethanol. 4-Chloroacetanilide was recrystallized from 50% aqueous ethanol and further purified by deacetylation and reacetylation as follows: the compound was was refluxed 30 min in 10% sodium hydroxide to remove the acetyl group, the reaction mixture was then cooled, made neutral with 2 N H_2SO_4 and extracted with benzene; the 4-chloroaniline was reacetylated with acetic anhydride in the extraction solvent. The solvent was distilled off and the 4-chloroacetanilide recrystallized to constant radioactivity from 50% aqueous methanol and twice from 50% aqueous ethanol.

(b) N-*Deacetylated parent compounds.* 2-Chloroaniline and 3-chloroaniline were dissolved in 95% aqueous ethanol. After refluxing with the urine samples, the solutions were cooled to 50 °C and the compounds precipitated as the acetyl derivatives in the following manner: HCl was added to form the amine hydrochloride, and acetic acid and a saturated aqueous solution of sodium acetate were added in immediate succession.

The 2-chloroacetanilide was recrystallized six times from 50% aqueous ethanol and the 3-chloroacetanilide was successively crystallized from 50%aqueous ethanol, 25% aqueous methanol, 25% aqueous acetic acid, and six times from 25% aqueous ethanol to constant activity of the recrystallizing solvent. 4-Chloroaniline was added to the urine in solution in acetone. After refluxing, the compound, which precipitated on cooling, was recrystallized from 50% aqueous ethanol and acetylated in benzene with acetic anhydride. The 4-chloroacetanilide was successively crystallized from 30% aqueous methanol and three times from 50% aqueous ethanol. For the calculations, the weights of the acetyl derivatives equivalent to the non-radioactive chloroanilines added, were used.

(c) *Total hydroxy metabolites.* The radioactive urine samples were each refluxed with 5 ml of 10 N H_2SO_4 for 1 hr to assure removal of the acetyl, sulfate and glucuronide moieties from the hydroxy derivatives. The solutions were then cooled and made neutral with 10 N NaOH. A carefully weighed amount (about 200 mg) of the monohydroxyaniline metabolite to be tested for was added in acetone and the mixtures were refluxed for 5 hr. At the end of the refluxing period the reaction mixtures were cooled and sodium sulfate was removed by filtration and twice washed with acetone to remove any occluded metabolite. In each case, acetone washings and filtrate were combined and the volume of solvent was reduced by distillation; 2-chloro-6-hydroxyaniline and 4-chloro-2-hydroxyaniline precipitated when the solutions were cooled to 5 °C. The former compound was recrystallized from 50% aqueous ethanol and then seven times from water (containing sodium hydrosulfite). The latter compound was recrystallized six times from water (in the presence of sodium hydrosulfite). To precipitate the 2-chloro-4-hydroxyaniline it was necessary to adjust the pH of the solution to 8. The product was recrystallized from water (containing sodium hydrosulfite) to constant activity of the recrystallizing medium. 3-Chloro-4-hydroxyaniline and 3-chloro-6-hydroxyaniline precipitated with the sodium sulfate. Repeated washings with water dissolved the inorganic sulfate. 3-Chloro-4-hydroxyaniline was recrystallized from 50% aqueous ethanol and then seven times from water (containing sodium hydrosulfite); 3-chloro-6-hydroxyaniline was successively crystallized from water, 50% aqueous ethanol, 50% aqueous methanol, and five times from water to constant activity of the solvent.

(d) *Inorganic chloride ion.* A sample of urine was placed in a 15 ml conical centrifuge tube and acidified with 6 N nitric acid. The urine was heated to 50 °C in a water bath and 0·1 M silver nitrate was added dropwise. Coagulation of the formed silver chloride was induced by stirring and heating. When no further precipitation occurred, the solid was spun down. The supernatant fraction was tested for unprecipitated chloride ion and was discarded when none was detected. The precipitated silver chloride was washed three times with 6 ml of water and once with 95% aqueous ethanol, dissolved in 12 N ammonium hydroxide, and filtered by means of a filter stick into a clean tube. The filter stick was thoroughly washed with 6 N ammonium hydroxide. The silver chloride was reprecipitated with nitric acid, and the procedure of washing, re-solution, and transfer to a clean tube was repeated twice; finally, transfer was made to a tared tube. The final precipitate of silver chloride was dried at 120 °C for 4 hr. The carefully weighed material was then dissolved in such an amount of 12 N ammonium hydroxide that each 0·1 ml contained 1 mg of silver chloride; 0·1 ml was plated on each of two planchets and the radioactivity was recorded. The process was repeated until constant

radioactivity of the silver chloride was reached. The percentage of radioactivity in the urine attributable to [^{36}Cl]chloride ion was determined from the formula:

$$\% \text{ chloride ion} = \frac{\text{specific activity of AgCl in counts/min per mg} \times \text{mg AgCl}}{\text{total activity of urine sample in counts/min}} \times 100$$

(e) *Unconjugated monohydroxy derivatives.* The hydroxychloroacetanilides and hydroxychloroanilines were added to the urine samples in solution in 95% aqueous ethanol. These mixtures were then refluxed for 5 hr, except for 4-chloro-2-hydroxyacetanilide and 4-chloro-2-hydroxyaniline which were stirred at room temperature for 1½ hr. Cooling of the solutions to 5 °C was sufficient to precipitate each compound. 2-Chloro-4-hydroxyacetanilide, 2-chloro-4-hydroxyaniline and 4-chloro-2-hydroxyaniline were repeatedly crystallized from water; with the aniline compounds sodium hydrosulfite was present. The other unconjugated monohydroxy derivatives were brought to constant specific activity by successive crystallizations as follows: 2-chloro-6-hydroxyacetanilide—50% aqueous acetone, 50% aqueous ethanol, 25% aqueous methanol, and twice more from 50% aqueous ethanol; 2-chloro-6-hydroxyaniline—twice from 50% aqueous ethanol and five times from water containing sodium hydrosulfite; 3-chloro-4-hydroxyacetanilide—25% aqueous ethanol, 25% aqueous acetic acid, 25% aqueous methanol, and six times from 25% aqueous ethanol; 3-chloro-4-hydroxyaniline—25% aqueous ethanol, 25% aqueous methanol, 25% aqueous acetic acid, and five times from water containing sodium hydrosulfite; 3-chloro-6-hydroxyacetanilide—50% aqueous ethanol, 50% aqueous methanol, 50% aqueous acetic acid, and again five times from 50% aqueous ethanol; 3-chloro-6-hydroxyaniline—25% aqueous ethanol and six times from water containing sodium hydrosulfite; 4-chloro-2-hydroxyacetanilide—30% aqueous methanol, 25% acetic acid, and four times from 50% aqueous ethanol.

(f) *Glucuronides and ethereal sulfates of the monohydroxy derivatives.* Each radioactive urine sample, added to a 5 ml portion of potassium phosphate buffer (pH 6·8), was incubated 24 hr at 37·5 °C with 5 ml of either 1% aqueous β-glucuronidase or 1% aqueous Takadiastase to remove the glucuronide or ethereal sulfate moieties. After incubation each mixture was filtered to remove any excess enzyme. The inverse isotope dilution technique was then applied in the same manner as for unconjugated hydroxy derivatives described in (e) above. The amount of hydroxy metabolite present in the samples after enzymatic hydrolysis was calculated with the aid of the isotope dilution formula. In each case this value was then subtracted from the amount of corresponding free hydroxy metabolite previously determined, in order to establish the presence of the metabolite freed by the enzyme.

RESULTS AND DISCUSSION

In our qualitative studies attempt was made to identify those monohydroxy metabolites which could be expected on purely chemical grounds from the fact that amino, acetylamino and chloro substituents are *ortho–para* directing, the amino group being the most powerful in this respect and the chloro group the least.

4-Chloro-2-hydroxyacetanilide and the deacetylated derivative, 4-chloro-2-hydroxyaniline were found unconjugated and also as the glucuronides and ethereal sulfates. The pattern of conjugation was different for the hydroxy metabolites of 2-chloroacetanilide and the hydroxy metabolites of 3-chloroacetanilide. Both 3-chloro-4-hydroxyacetanilide and its deacetylated derivative were found unconjugated. and also

as ethereal sulfates. 3-Chloro-4-hydroxyacetanilide, but not 3-chloro-4-hydroxyaniline, was conjugated as the glucuronide. A variation exists with 3-chloro-6-hydroxyacetanilide and its deacetylated derivative. Here both metabolites were found unconjugated and conjugated as the glucuronides, but only the acetylated compound existed as the ethereal sulfate. Neither 2-chloro-4-hydroxyaniline nor 2-chloro-6-hydroxyaniline was found unconjugated; both gave glucuronides and ethereal sulfates. On the other hand, of the corresponding acetylated metabolites, 2-chloro-4-hydroxyacetanilide was identified in all three forms, while 2-chloro-6-hydroxyacetanilide was found only as the ethereal sulfate. This last compound was the only N-acetylated hydroxy metabolite of the five isomers investigated which was not found in an unconjugated form. Each of the administered chloroacetanilides was also excreted in the urine partly as the deacetylated chloroaniline. Thus, N-deacetylation of the chloroacetanilides can occur independently from hydroxylation.

The results of the quantitative determinations of excreted metabolites are given in Table 1. In previous investigations[6] the administered compounds were found to have

TABLE 1. QUANTITATIVE RECOVERY OF RADIOACTIVITY FROM THE URINE OF RATS INJECTED WITH 4-, 3- OR 2-[^{36}Cl]CHLOROACETANILIDE

(Expressed as percentage of radioactivity in urine)

Compound	4-Chloro-acetanilide	3-Chloro-acetanilide	2-Chloro-acetanilide
Unchanged chloroacetanilides	4·63	1·34	3·18
Chloroanilines	2·53	8·26	28·28
4-Hydroxy derivatives (*para* to amino group)	—	57·32	45·10
2-Hydroxy derivatives (*ortho* to amino group)	61·96	—	—
6-Hydroxy derivatives (*ortho* to amino group)	—	26·49	8·53
Total hydroxylation (sum of lines 3, 4 and 5)	61·96	83·81	53·63
Inorganic chloride ion	1·18	1·98	0·62
Total percentage	70·30	95·39	85·71

the following decreasing order of both toxicity and antipyretic activity: 4-chloroacetanilide, 3-chloroacetanilide, 2-chloroacetanilide. For convenience in discussion, the parent compounds have been listed in Table 1 in this decreasing order. The amount of unchanged parent compound in each case was less than 5 per cent and did not follow the pattern of toxicity. The amounts of deacetylated parent compounds (i.e. 4-, 3- and 2-chloroaniline; line (2) followed a pattern: the most toxic compound, 4-chloroacetanilide, was the least deacetylated, while the least toxic compound, 2-chloroacetanilide, was deacetylated to the greatest extent. This order is contrary to the expectation that the more deacetylated chloroaniline formed, the more toxic the parent compound should be, since it is known that aniline is much more toxic than acetanilide.[17] This apparent inverse relation between the extent of deacetylation and toxicity of the substituted acetanilides may be attributable to increased solubility in body fluids and, thus, to faster elimination of the compound as a result of deacetylation. In fact, more rapid elimination occurs with 2-chloroacetanilide than with

4-chloroacetanilide. Thus, in the rat peak excretion occurred at 6 hr following intraperitoneal injection of 2-chloroacetanilide, but not until 24 hr with 4-chloroacetanilide.[18]

The total amount of hydroxylation in a given position, as determined after acid hydrolysis of the acetyl, glucuronide and ethereal sulfate derivatives, is given in lines (3), (4), (5) and (6) of Table 1. With 4-chloroacetanilide, hydroxylation cannot occur in the 4-position, since it is blocked by the chloro-sustitituent. The very small amount of inorganic chloride ion found in the urine (Table 1, line 7) shows that the block in the 4-position remains substantially intact. Initially, it was thought that the absence of *p*-hydroxylation may account for the high toxicity of 4-chloroacetanilide. For the two chloroacetanilides with the 4-position free, however, 2-chloroacetanilide exhibited less hydroxylation in the 4-position than did the more toxic 3-chloroacetanilide.

The extent of hydroxylation that occurred in the 2- and 6-positions (both of which are *ortho* to the amino group) followed the pattern of relative toxicity and antipyretic activity. 4-Chloroacetanilide exhibited the most extensive *ortho*-hydroxylation; this occurred to an extent almost two and a half times that found for 3-chloroacetanilide and more than seven times that found for 2-chloroacetanilide. Unfortunately, these experiments had to be terminated before the LD_{50} values for the 2- and 6-hydroxylated metabolites could be determined, since the results might have supported the suggestion that the toxicity of the parent compounds is related to the amount of *o*-hydroxy compound formed. The apparent relation of *o*-hydroxylation to the physiological activities of the chloroacetanilides is analogous to that found for the carcinogenic activity of certain aromatic amines. Bonser *et al.*[1] have shown a relationship between the amount of 2-aminonaphthalene that is excreted by an animal as an *o*-hydroxy metabolite, and the carcinogenic response to the parent compound. Weisburger *et al.*[2] have found that in a susceptible species, the rat, a much greater total *o*-hydroxylation of 2-acetylaminofluorene occurs than in a resistant species, the guinea pig.

The extent of total recovery of radioactivity (Table 1, line 8) indicates that metabolites other than those investigated must be present in the case of 4-chloroacetanilide and possibly in the case of the 2-isomer. Mercapturic acid formation may account in part for this low recovery. Although aromatic amines are not known to form mercapturic acids, halogen derivatives may be converted by certain mammalian species to this types of metabolite. Bray *et al.*[19] found that mercapturic acid formation is a minor metabolic pathway for 2- and 4-chloronitrobenzene, but not for 3-chloronitrobenzene. In the two former compounds, mercapturic acid apparently is formed by acetylcysteyldechlorination. On the other hand, mercapturic acids from monochlorobenzene and polychlorobenzene are products of acetylcysteyldeprotonation.[20] The small amount of free chloride ion excreted in the chloroacetanilide series (Table 1, line 7) suggests that if mercapturic acid formation takes place in these compounds it does so largely through acetylcysteyldeprotonation, rather than through acetylcysteyldechlorination. Other possible metabolites which were not tested for are polyhydroxy compounds, N-glucuronic acids, sulfamic acids and products of aromatic ring breakage.

In conclusion, no simple unifying rule can be formulated in the chloroacetanilide series which completely correlates toxicity, antipyretic activity and relative participation in the metabolic routes investigated. The only consistent pattern is that of

o-hydroxylation, which follows the same order as the relative toxicities and antipyretic activities.

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