DIFFERENCES IN THE FERRIHEMOGLOBIN-FORMING CAPABILITIES AND CARCINOGENICITIES BETWEEN MONOCYCLIC AND POLYCYCLIC N-ACYLARYLAMINES AND THEIR DERIVATIVES

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1. INTRODUCTION

Ferrihemoglobin-formation *in vivo* by an N-acylarylamine as an expression of acute toxicity is rarely an effect of the administered compound itself, but mostly produced by one or more metabolites. Thus, ferrihemoglobinemia observed in humans after ingestion of large doses of phenacetin (1) is thought to be effected by the molecular species arising from N-deacetylation and subsequent N-, ring- or(and) alkylhydroxylation of the intermediary arylamine (2,3), or, ferrihemoglobinemia observed in humans after large doses of prilocaine (4) is thought to be due to the molecular species produced by N-deacylation and subsequent ring-hydroxylation of the intermediary arylamine (5). In the activation of N-acylarylamines, therefore, at least two enzyme systems are involved, namely soluble and(or) bound N-arylamidases (esterases) and oxygen-activating enzymes, such as mixed-function oxygenases and(or) mixed-function amine oxidases (6). However, as will be discussed here, as an alternative possibility, the N-arylacetamide may be N-hydroxylated and then further oxidized by oxygen to yield the active molecule which effects the oxidation of hemoglobin. The active molecule has a limited life-time only, since it is consumed in the reaction of hemoglobin-Fe^{2+} with oxygen. But if it is regenerated, for example by accepting reducing equivalents, its activity can be observed for several cycles of activation and inactivation, until finally irreversible changes of its chemical structure terminate its activity. Thus, kinetics of ferrihemoglobin-formation are an appropriate tool for the recognition of differences in the rate of activation and inactivation among the various compounds tested for their ferrihemoglobin-forming capability.

What is true for ferrihemoglobin-formation is also accepted today for carcinogenicity. Carcinogenicity as an expression of subchronic or chronic toxicity of an N-acylarylamine is also rarely an effect of the administered compound itself, but of the ultimate carcinogen (7) produced by N-hydroxylation and subsequent esterification (8) and N-deacylation (9). N-Deacylation transforms N-hydroxy-N-acyl-arylamines into aryl hydroxylamines, which are also formed *in vivo* from arylamines by N-hydroxylation or from nitro- and nitrosoarenes by reduction. Since in the fluorene series all these derivatives lack an N-acyl group and the common intermediate, N-hydroxy-2-aminofluorene, was shown to be N-acetylated *in vivo*, on the other hand, N-deacetylation of hydroxamic acids was demonstrated *in vivo* (9) as well as *in vitro* (10, 11), either metabolic pathway can lead to the primary lesion. In fact, only the former has been pursued in the past and the latter been neglected, although experimental evidence for its significance has accumulated. Thus, ferrihemoglobin-formation and carcinogenicity have in common

the requirement of metabolic activation of the N-acylarylamine to yield the active molecule responsible for the toxic effects. Experiments with certain monocyclic and polycyclic N-acylarylamines and their derivatives, carried out by our group during the last ten years, have revealed differences in both, the ferrihemoglobin-forming and carcinogenic capabilities, of which we want to give a report.

2. COMPOUNDS AND ABBREVIATIONS IN THE TEXT, see *Chart 1*

Arylamines. Aniline, benzenamine (= A), Ia; 4-phenetidine, 4-ethoxybenzenamine (= 4-EA), IIa; 4-chloroaniline, 4-chlorobenzenamine (= 4-ClA), IIIa; 3.4-dichloroaniline, 3.4-dichlorobenzenamine (= 3.4-Cl_2A), IVa, 4-aminobiphenyl, (l,l'biphenyl)-4-amine (= 4-AB), Va; 2-aminofluorene, 9H-fluoren-2-amine (= 2-AF), VIa.

Arylhydroxylamines. Phenylhydroxylamine, N-hydroxy-benzenamine (= NOH-A), Ib; 4-ethoxyphenylhydroxylamine, N-hydroxy-4-ethoxybenzenamine (= NOH-4EA), IIb; 4-chlorophenylhydroxylamine, N-hydroxy-4-chlorobenzenamine (= NOH-4ClA), IIIb; 3.4-dichlorophenylhydroxylamine, N-hydroxy-3.4-dichlorobenzenamine (= NOH-3.4Cl_2A), IVb; N-hydroxy-4-aminobiphenyl, N-hydroxy-(l,l'biphenyl)-4-amine (= NOH-4AB), Vb; N-hydroxy-2-aminofluorene, N-hydroxy-9H-fluoren-2-amine (= NOH-2AF).

N-Acylarylamines. Acetanilide, N-phenyl-acetamide (= AA), Ic; 4-acetophenetidine, phenacetin, N-(4-ethoxyphenyl)-acetamide (=PH), IIc; 4-chloroacetanilide, N-(4-chlorophenyl)-acetamide (=4-ClAA), IIIc; 3.4-dichloroacetanilide, N-(3.4-dichlorophenyl)-acetamide (= 3.4-Cl_2AA), IVc; 4-acetylaminobiphenyl, N-(1,1'-biphenyl)-4yl acetamide (= 4-AAB), Vc; 2-acetylaminofluorene, N-(9H-fluoren-2yl)acetamide (= 2-AAF), VIc; 4-chloropropionanilide, N-(4-chlorophenyl-propanamide) (= 4-ClPA), 3.4-dichloropropionanilide, N-(3.4-dichlorophenyl)-propanamide (= 3.4-Cl_2PA).

N-Arylacetohydroxamic acids (= N-hydroxy-N-arylacetamides). N-Acetylphenylhydroxylamine, N-phenyl-N-hydroxy-acetamide (NOH-AA), Id; N-hydroxyphenacetin, N-(4-ethoxyphenyl)-N-hydroxy-acetamide (= NHP), IId; N-hydroxy-4-chloroacetanilide, N-(4-chlorophenyl)-N-hydroxy-acetamide (NOH-4ClAA), IIId; N-hydroxy-3.4-dichloroacetanilide, N-(3.4-dichlorophenyl)-N-hydroxy-acetamide (= NOH-3.4Cl_2AA), IVd; N-hydroxy-4-acetylaminobiphenyl, N-(1,1'-biphenyl)-4yl-N-hydroxy-acetamide (= NOH-4AAB), Vd; N-hydroxy-2-acetylaminofluorene, N-(9H-fluoren-2yl)-N-hydroxy-acetamide (= NOH-2AAF), VId.

3. FERRIHEMOGLOBIN-FORMATION *IN VIVO* AND *IN VITRO*

3.1 Arylamines.

The ferrihemoglobin-forming capabilities of A, 4-ClA, 4-EA, 3.4-Cl_2A, 4-AB, and 2-AF in different species are well known and have been reviewed by Kiese (12). However, only A (13), 4-EA (14), and 4-ClA (15) have been tested in the rat. Since the purpose of this paper is to compare the ferrihemoglobin-forming capabilities of six biologically relevant arylamines and their derivatives (see Chart 1, Ia-VIa), they had to be re-examined or examined, respectively, under identical conditions. Female Sprague Dawley rats served as the animal species for the experiments, whose results are shown in Figure 1

Measurement of the ferrihemoglobin concentration according to Evelyn and Malloy (16) for 4 h following i.p.-injection of various arylamines suspended in 0.25% agar revealed distinct differences, not only in the ferrihemoglobin-forming capabilities, but also in the initial hemoglobin oxidation rates as well as in the duration of action. The most active compound was 4-AB, followed by 4-ClA and 4-EA. The additional Cl-atom in 3-position of the benzene ring decreased the ferrihemoglobin-forming activity of 4-ClA by more than 50%. The least active arylamines were aniline and 2-AF. Whereas the initial velocities of hemoglobin oxidation by 4-AB, 4-ClA, and 4-EA were quite similar, that of a similar dose of 3.4-Cl_2A was slower. 4-Fold higher doses of 2-AF produced an initial velocity, which was even much slower than that of 3.4-Cl_2A and similar to that produced by a 15-fold higher dose of A. Also large differences in the duration of activity were noted, since A and 4-AB were active for 3h and their activity declined only slowly. In order to explain the differences in the kinetics of ferrihemoglobin-formation, it is necessary to search the blood for those metabolites, which are known to produce ferrihemoglobin also *in vitro*, i.e., arylhydroxylamines or nitrosoarenes and aminophenols. An overview of the pertinent literature reveals that apparently no reports are available on N-hydroxy and ring-hydroxy derivatives found in the blood of rats injected with an arylamine. The favoured animal species, on which blood analyses have been performed, were cats and dogs, because these species are much more sensitive to ferrihemoglobin-formation than the rat, probably because ferrihemoglobin is less rapidly reduced in these species (12). But data are available on the N-hydroxylation by rat liver microsomes *in vitro* of most of the relevant arylamines, whereas information on ring-hydroxylation is scanty. With this in mind, it is attempted to correlate differences in the ferrihemoglobin-forming capabilities *in vivo* with differences in the N-hydroxylating activities of rat liver microsomes *in vitro* of various

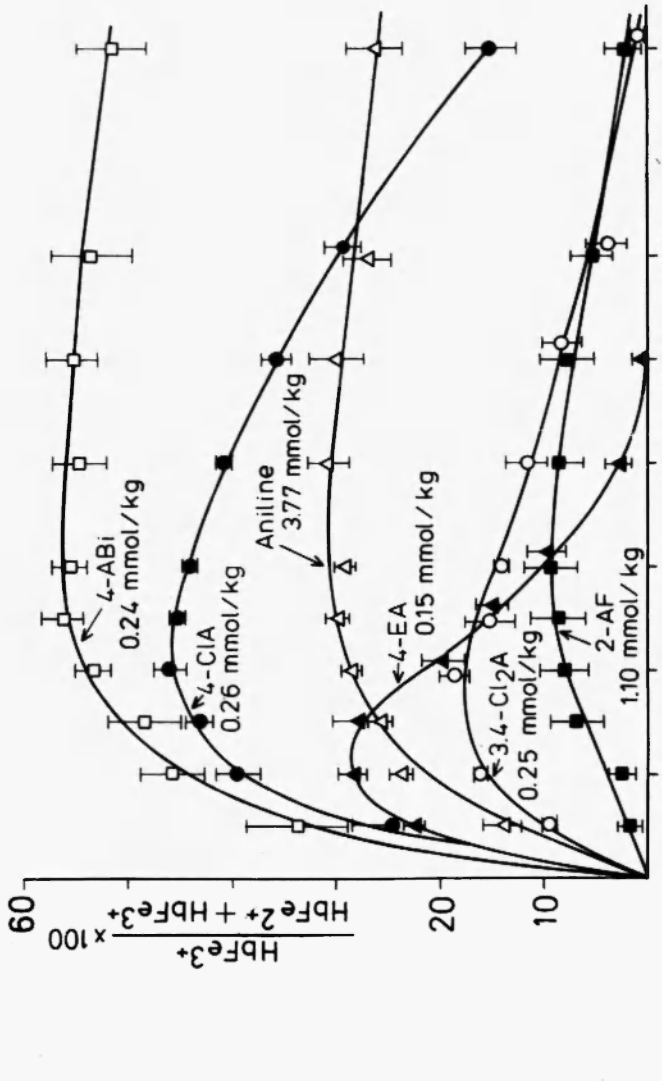

Fig. 1. Kinetics of ferrihemoglobin-formation by various arylamines. Ferrihemoglobin-formation in female Sprague Dawley rats after i.p.-injection of the doses of various arylamines indicated in the graph. Symbols indicate means from experiments on 3 animals used for each compound, bars indicate S.E. For i.p.-injection, the pure compounds were suspended in 0.25% agar prepared from 0.9% NaCl. At times indicated in the graph, 3 drops of blood were taken from the tail vein and ferrihemoglobin determined according to Evelyn and Malloy (16).

arylamines. If there is no information available on the rat, results of experiments on rabbit liver microsomes are given instead, although information from one species may not be relevant for another (17-20).

Kiese and Uehleke (20), who compared the rate of N-hydroxylation of A, 4-ClA, and 4-EA by rat liver microsomes, found 0.5 µg nitrosobenzene, 2.0 µg 4-nitroso-ethoxybenzene, and 2.3 µg 4-chloronitrosobenzene per ml, respectively, after 20 min incubation at 37° of the corresponding arylamines, indication, that the N-hydroxylation of the unsubstituted arylamine was very slow, but increased with the nature of the substituent in 4-position. Similar results were reported by Smith and Gorrod (18), who determined the N-hydroxylation of A and 4-ClA by hepatic microsomes from 5 different species and found V_{max} values of 0.17 and 0.59 nmoles per min per mg protein for A and 4-ClA, respectively, produced by the rat.

Since a similar order was found in the ferrihemoglobin-forming capabilities of the corresponding arylamines, it is most likely due to N-hydroxylation, that these arylamines oxidized hemoglobin *in vivo*. Baader et al. (2), who studied the ferrihemoglobin-forming capabilities of nitrosobenzene, 4-nitroso-ethoxybenzene, and 4-chloro-nitrosobenzene in dogs, could explain the kinetics of ferrihemoglobin-formation in this species by A and 4-ClA with the concentration of the corresponding nitrosarene determined in blood, but cast some doubt on the proposition, that 4-nitroso-ethoxybenzene and 4-aminophenol alone can explain the kinetics of ferrihemoglobin-formation in dogs after administration of 4-EA. Although N-hydroxylation of A *in vitro* by rat liver microsomes is so slow, Eyer et al. succeeded in demonstrating the N-hydroxylation of A in rat liver during recirculating perfusion in the presence of red cells (21).

An additional Cl-atom in 3-position caused a 2.5-fold decrease of the rate of N-hydroxylation of 4-ClA, for liver microsomes from phenobarbital-induced rabbits produced 0.42 µmoles NOH-4ClA and 0.17 µmoles NOH-3.4Cl_2A per min per mg protein per nmol cytochrome P-450 at 37° (initial velocities) (22). The lower ferrihemoglobin-forming capability and slower initial rate of hemoglobin-oxidation in the rat of 3.4-Cl_2A as compared to that of 4-ClA suggest, that it is probably also the N-hydroxy derivative which effects hemoglobin oxidation in the rat. The ferrihemoglobin-forming capabilities in rats of some ring-hydroxylated derivatives, such as 4-chloro-2-hydroxybenzenamine or 3.4-dichloro-6-hydroxy-benzenamine, which were also produced in low yield by rabbit liver microsomes (22), were so low that their contribution to the observed rate of hemoglobin-oxidation by 4-ClA and 3.4-Cl_2A can be neglected.

The N-hydroxy derivatives of 4-AB and 2-AF (determined as

nitrosoarenes) were found in the blood of rabbits, dogs, cats or guinea pigs after injection of 4-AB and 2-AF, respectively, but no such reports on the rat are available (12). Several reports on the N-hydroxylation *in vitro* of 4-AB and 2-AF by rat liver microsomes are available (18, 19, 23-25). Uehleke, who studied the N-hydroxylation in vitro of carcinogenic arylamines by rat liver microsomes, found that 4-AB was oxygenated more rapidly than 2-AF or A (23). Uehleke and Nestel found that rat liver microsomes produced less NOH-4AB (0.39 nmoles per min per mg protein) than rabbit liver microsomes (0.80), ring-hydroxylated metabolites were not searched for (24).

McMahon et al. (19), who studied the time course of the metabolism of 4-AB *in vitro* with hepatic preparations from various species, found that the 9000 x g supernatant fraction of 250 mg rat liver wet weight equivalent produced 2.5 nmoles NOH-4AB per min, whereas the hepatic enzymes from the Syrian hamster, rabbit, guinea pig, and C3H mouse were much more active, and that treatment of male Fischer rats with phenobarbital scarcely affected N-hydroxylation activity, but that a single i.p.-injection of Aroclor 1254 caused an 11-fold increase of N-hydroxylation activity. McMahon et al. also detected 3 ring-hydroxylated metabolites, namely, 3-hydroxy-4AB, 2'-hydroxy-4AB, and 4'-hydroxy-4AB in the 9000 x g incubates of enzyme preparations from Aroclor-treated rats. Ring-hydroxylated derivatives of 4-AB, which had been detected before in incubates of rabbit liver microsomes (26), have not been tested for their ferrihemoglobin forming activity in the rat. 4-Nitrosobiphenyl oxidized hemoglobin in beef and cat erythrocytes *in vitro* more rapidly than did nitrosobenzene (24). These data suggested that the N-hydroxy derivative of 4-AB mainly contributed to the ferrihemoglobin-formation in the rat by 4-AB, but do not suffice to explain the observed kinetics, since the other candidates have not been tested.

No other report on the ferrihemoglobin-forming capability of 2-AF in the rat is available. The ferrihemoglobin-forming capabilities of 2-AF and 4-AB, however, were evaluated by von Jagow et al. (27) after i.v. injection into dogs and rabbits and by Kiese and Wiedemann (28) after i.p.-injection into guinea pigs. In dogs and rabbits 2-AF had a lower ferrihemoglobin-forming capability than 4-AB. When the blood was searched for the N-hydroxy derivatives, lower concentrations of 2-nitrosofluorene than of 4-nitrosobiphenyl were found, indication for an arylhydroxylamine-induced ferrihemoglobin-formation by these arylamines. After i.p.-injection of doses of 25 to 500 mg 2-AF into guinea pigs, very small concentrations of 2-nitrosofluorene (< 0.1 µg/ml) were determined only in those animals injected with the highest dose. These results suggested that the low ferrihemoglobin-forming capability of 2-AF in the rat may also be due to very low concentrations of NOH-2AF

or 2-nitrosofluorene in the blood. Such low concentrations of the N-hydroxy derivative can be explained with either very slow rates of N-hydroxylation *in vivo* or rapid disposal of the N-hydroxy derivative. When 2-nitrosofluorene and nitrosobenzene were injected i.v. into guinea pigs to evaluate their ferrihemoglobin-forming capabilities, 2-nitrosofluorene was found to be less active than the monocyclic nitrosoarene (28). But whereas nitrosobenzene rapidly disappeared from the blood, nitrosofluorene displayed a biphasic elimination kinetics, the first rapid phase with an app. $t_{1/2}$ = 2 min and the second slower phase with a $t_{1/2}$ = 36 min. N-Hydroxylation *in vitro* of 2-AF by rat liver microsomes was slower than of 4-AB, but faster than of A (23). An interesting observation was reported by Hultin (25): when slices of rat liver were incubated with ^{14}C-labeled A and 2-AF in the presence of NADPH, 2-AF-linked ^{14}C was incorporated to a much higher extent into the tissue than A-linked ^{14}C. Ring-hydroxylation of 2-AF *in vitro* by rat liver microsomes was reported by Booth and Boyland (29), by which 2-aminofluoren-7ol was formed, whose ferrihemoglobin-forming activity has not been tested since. These results indicate, that, although N-hydroxylation *in vivo* of 2-AF was slower than that of 4-AB, a slow N-hydroxylation of 2-AF in the rat alone cannot explain its low ferrihemoglobin--forming capability, but rather a low availability of its N-hydroxy derivative for oxyhemoglobin, see section 3.6.

3.2 Arylhydroxylamines.

The effect of synthetic N-hydroxylation of the selected six arylamines on hemiglobin-formation *in vivo* was tested on female Sprague Dawley rats. Although the doses of the arylhydroxylamines (see Chart 1, Ib--VIb), indicated in the graph (Figure 2), were injected as rapidly as possible to avoid autoxidation (11), it is expected that a certain fraction of the sensitive NOH-4EA and NOH-2AF entered the peritoneum as primary nitroxides, nitrosoarenes or other molecular species. However, primary nitroxides and nitrosoarenes, unless highly electrophilic, are converted back to the corresponding arylhydroxylamines *in vivo* by reduction. Since the electrophilic NOH-2AF or 2-nitrosofluorene was shown to combine with the guanine moieties of RNA and DNA *in vitro* at pH 5-6 (30), with unsaturated fatty acyl residues of microsomal lipids (31), and with plasma proteins and erythrocytes (32), no such reports are available with NOH-4AB or 4-nitrosobiphenyl.

The kinetics of ferrihemoglobin-formation by six arylhydroxylamines, shown in Figure 2, indicate that, not unexpected, much lower doses of the arylhydroxylamines produced similar ferrihemoglobin concentrations (as compared to arylamines), and that already 5 min after

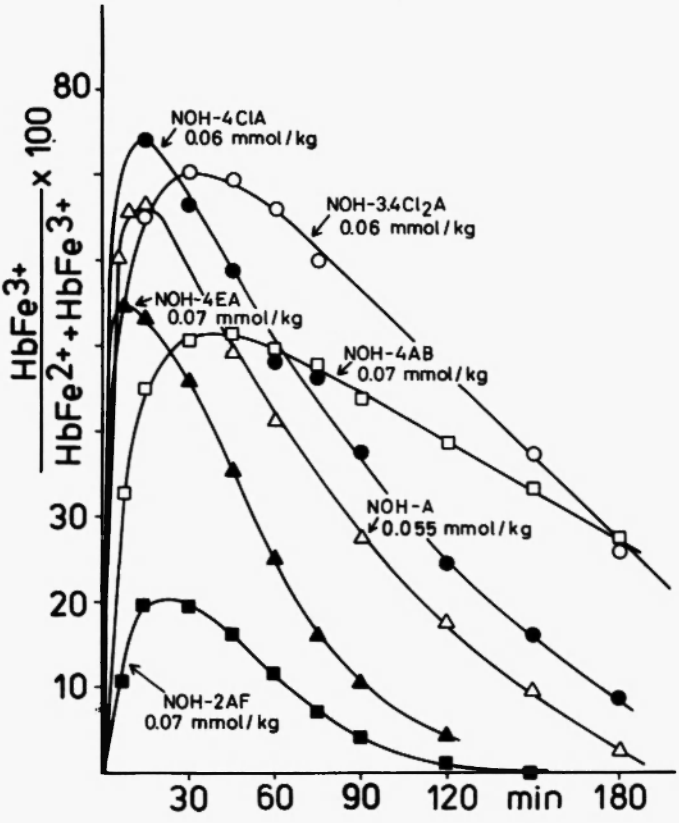

Fig. 2. Kinetics of ferrihemoglobin-formation by various arylhydroxylamines. Ferrihemoglobin-formation in female Sprague Dawley rats after i.p.-injection of the doses of various arylhydroxylamines indicated in the graph. Symbols indicate means from experiments on 6(NOH-4EA, NOH-4ClA, and NOH-2AF) or 3(NOH-A, NOH-3.4Cl_2A, NOH-4AB) animals. For i.p.-injection, the pure compounds were weighed and suspended in 0.25% agar as quickly as possible to avoid autoxidation. For details see caption of *Fig.1.*

i.p. injection high concentrations of hemoglobin-Fe^{3+}, at least with the four monocyclic arylhydroxylamines, were determined. This is in agreement with the assumption, that arylhydroxylamines are the ultimate ferrihemoglobin-forming agents and need no further metabolic activation (33), but this is also indication for a rapid invasion of the blood and instantaneous reaction of the active molecule with oxyhemoglobin. However, the molar dose ratio of arylhydroxylamine to arylamine to

produce the same maximal ferrihemoglobin concentration *in vivo* varied over a wide range: NOH-A: A = 1 :136, NOH-4EA: 4-EA = 1 :4, NOH-4ClA: 4-ClA = 1 : 7, NOH-3.4Cl_2A: 3.4-Cl_2A 1 :10, NOH-4AB: 4-AB = 1 :3, and NOH-2AF: 2-AF = 1 :41. Factors which determine the size of the dose ratio are, among others: the rate of N-hydroxylation of the arylamine *in vivo*, availability and the relative metabolic stability (reactivity) of the active molecule in the blood either in the reduced or oxidized state.

High dose ratios found for NOH-4AB/4-AB, NOH-4EA/4-EA, and NOH-4ClA/4-ClA are an indication for high metabolic activation rate and (or) high availability-of the active molecule for hemoglobin-Fe^{2+}, low dose ratios consequently an indication for either low activation rate and (or) reduced availability due to limited solubility or competing reactions. A very slow N-hydroxylation of aniline in rats as compared to 4-EA or 4-ClA is probably one of the explanations for the very low dose ratio found for NOH-A/A, if one accepts, that the results of *in vitro* experiments with rat liver microsomes (18, 20, 23) resemble formation rates *in vivo*. The results with NOH-2AF however suggest, that it is not due to a very slow N-hydroxylation of 2-AF *in vivo* alone, but rather a low availability of the active molecule, that NOH-2AF was the least active compound tested. Factors, which explain impaired availability are discussed in sections 3.1 and 3.6.

3.3 N-Acylarylamines.

It is generally accepted today, that N-acylarylamines do not themselves produce ferrihemoglobin, but after metabolic activation *in vivo*. In the first step of activation, an N-acylarylamine is N-deacylated to yield the corresponding arylamine, which is then either N- or ring-hydroxylated or both to yield the active molecule(s). Evidence for N-deacylation *in vivo* as a prerequisite for ferrihemoglobin-forming activity was presented by Heymann et al. (34) and Singleton and Murphy (35). Heymann et al. found that simultaneous i.v.-injection of bis(4-nitrophenyl)-phosphate, an effective inhibitor of N-arylamidases(esterases) *in vivo* (36), and i.p.-injection of either AA or PH into Wistar rats significantly reduced the extent of ferrihemoglobin-formation by 80-85% or 50%, respectively. Singleton and Murphy found that i.p.-injection of 125 mg tri-o-tolylphosphate per kg 18 h prior to i.p.-injection of various doses of 3.4-Cl_2PA (propanil) into mice reduced ferrihemoglobin concentrations by 68 or 85% respectively. Our results of experiments on the effect of bis(4-nitrophenyl)-phosphate on the ferrihemoglobin-formation *in vivo* by NOH-4ClAA are described in section 3.5.

Since two successive steps are required for the activation of N-acylarylamines, the onset of ferrihemoglobin-formation is expected to be even more delayed than with arylamines. And indeed, as can be seen from the kinetics of ferrihemoglobin-formation by six N-acylarylamines tested (see Chart 1, I-Vc), shown in Figure 3, obviously the onset of ferrihemoglobin-formation was more delayed than with arylamines, and the kinetic curves showed a sigmoid characteristic indication of a slowly increasing concentration with time of the active molecule in the blood. The six N-acylarylamines tested were: AA, 4-ClAA, PH, 4-ClPA, 3.4-Cl_2PA(propanil), and 4-AAB. 2-AAF was not tested because of its expected low ferrihemoglobin-forming capability.

When the dose of 2.58 mmol AA per kg was injected i.p. into rats, severe depression of the CNS (with loss of the righting reflex and ataxia), but only very little (up to 10%) ferrihemoglobin-formation was observed. The ratio of molar doses of AA and A which produced the same maximal ferrihemoglobin concentration was found to be 2 :1, indication not only for a very slow N-hydroxylation (see 3.1), but also a slow N-deacetylation in this species. PH-induced ferrihemoglobin-formation in the rat has been shown to be directly proportional to the concentration of 4-EA in the blood (37). Rates of N-deacetylation *in vitro* of AA and PH by rat liver homogenate determined by Heymann et al. (34) have shown that AA and PH were N-deacetylated with the same rate (1.12 versus 1.10 nmoles per min per mg protein). The substituted derivatives exceeded the unsubstituted acetanilide in their ferrihemoglobinogenic activity in the order AA < PH < 4-ClAA. The same order was found in the rate of N-hydroxylation *in vitro* of A, 4-EA, and 4-ClA by rat liver microsomes (20), which suggests, that the rate of N-deacetylation *in vivo* of these acetanilides may be quite similar. A hint that this may be true for AA and PH was given by Heymann et al. (34). However, the initial oxidation rate was higher with PH than with 4-ClAA, indication that PH was N-deacetylated *in vivo* much faster than 4-ClAA, since 4-EA and 4-ClA showed the same initial oxidation rate *in vivo* (Fig.1). Reports on the N-deacetylation of 4-ClAA *in vitro* by rat liver enzymes which could support this assumption, are not available. Of special interest is the finding, that the N-propionyl derivative of 4-ClA produced 10-fold higher ferrihemoglobin concentrations *in vivo* than the analogous derivative of 3.4-Cl_2A. As was reported earlier (38), 3.4-Cl_2PA (propanil) was N-deacylated much faster by liver microsomes from phenobarbital-induced rabbits than 4-ClPA (9.7 nmoles versus 2.8 nmoles per min per mg protein), but 4-ClA was N-hydroxylated 3-fold faster than 3.4-Cl_2A. Apparently differences in the rate of N-deacylation are not as critical as are the differences in the rate of N-hydroxylation.

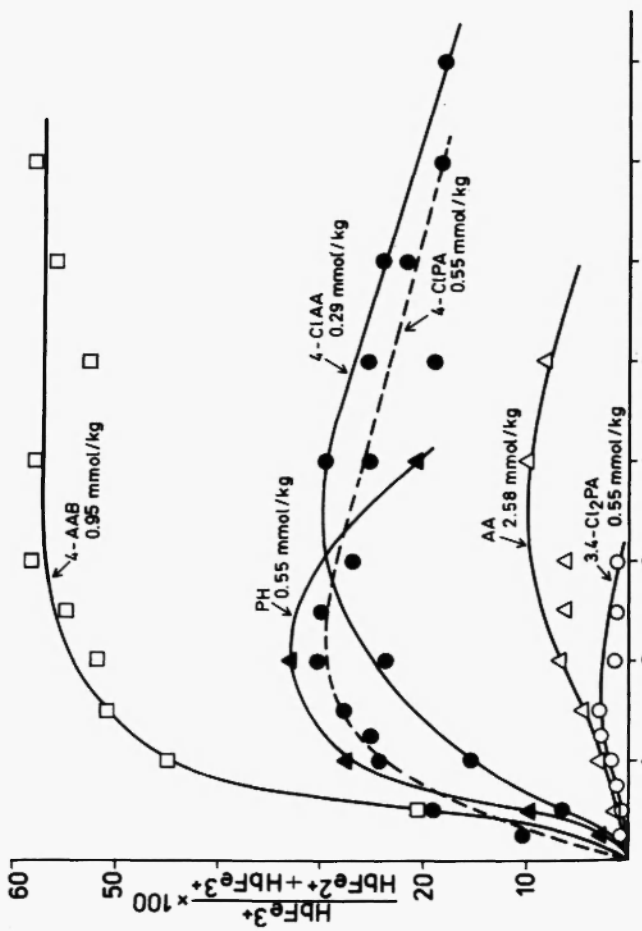

Fig. 3. Kinetics of ferrihemoglobin-formation by various N-acylarylamines. Ferrihemoglobin-formation in female Sprague Dawley rats after i.p.-injection of the doses of various N-acylarylamines indicated in the graph. Symbols indicate means from experiments on 3 animals for each compound. For i.p.-injection, the pure compounds were finely ground and suspended in 0.25% agar. For other details see caption of *Fig. 1*.

Chart 1. Formula scheme of the compounds dealt with in this article.

The ferrihemoglobin-forming capability of 4-AAB was tested before in the rat. Miller et al. (39), who noted ferrihemoglobin-formation in adult and weanling female rats after i.p.-injection of 0.27 mmol NOH-4AAB per kg, did not observe any effect after i.p.-injection of the same dose of 4-AAB. However, a dose of 0.88 mmol 4-AAB per kg produced a maximal ferrihemoglobin concentration of 57%, which was constant for 5 h, see Fig. 3.

It is not known, which metabolites are present in the blood of rats injected with 4-AAB, but it is known that NOH-4AAB (as a conjugate) is a urinary metabolite of both 4-AB and 4-AAB in the rat (Miller et al. 1961). Ring-hydroxylated metabolites were detected, but not identified. Studies on the metabolism *in vitro* of 4-AAB by rabbit liver microsomes have shown that 4-AAB was N-hydroxylated to yield NOH-4AAB, that NOH-4AAB was N-deacetylated to yield NOH-4AB, which was either reduced to 4-AB or isomerized to 4-amino-3-hydroxybiphenyl (26). Thus, a mechanism of ferrihemoglobin-formation different from that established for monocyclic N-acylarylamines may function for the polycyclic N-arylacetamides, in that the N-arylacetamide is N-hydroxylated *in vivo* to yield the hydroxamic acid, which can be N-deacetylated either enzymatically or by oxidation to yield the active arylhydroxylamine or nitrosoarene, see also section 3.5.

3.4 N-Arylacetohydroxamic Acids (N-Hydroxy-N-Arylacetamides)

Whereas N-hydroxy derivatives of certain monocyclic N-arylacetamides, such as 4-ClAA and PH were found in very low amounts only in incubates of liver microsomes from rabbits (41, 43), hamsters (40, 42), and mice (44), and in the urine of hamsters (46), and rats (47),larger amounts of the N-hydroxy derivatives of the polycyclic N-arylacetamides 4-AAB and 2-AAF were produced *in vitro* by liver microsomes from rabbits (26, 49, 50) and rats (48) and consequently, higher amounts of NOH-4AAB (up to 3.9% (39) or 7% (52) of dose) or NOH-2AAF (up to 15% (51) or 30% (53)) were found in the urine of rats or rabbits, respectively. The high ferrihemoglobin-forming capabilities of the monocyclic hydroxamic acids NOH-4ClAA and NHP were discovered, when we determined their acute and chronic toxicities in the rat (54). This discovery has stimulated a study on the ferrihemoglobin-forming capabilities of certain monocyclic and polycyclic N-arylacetohydroxamic acids (see Chart 1, I-VId), whose results are shown in *Figure 4.*

15 Min after i.p.-injection of the doses of NOH-AA, NOH-4-ClAA, and NHP indicated in the graph, similar ferrihemoglobin concentrations were determined as 5 min after i.p.-injection of much smaller doses of the corresponding arylhydroxylamines, suggesting that N-arylaceto-

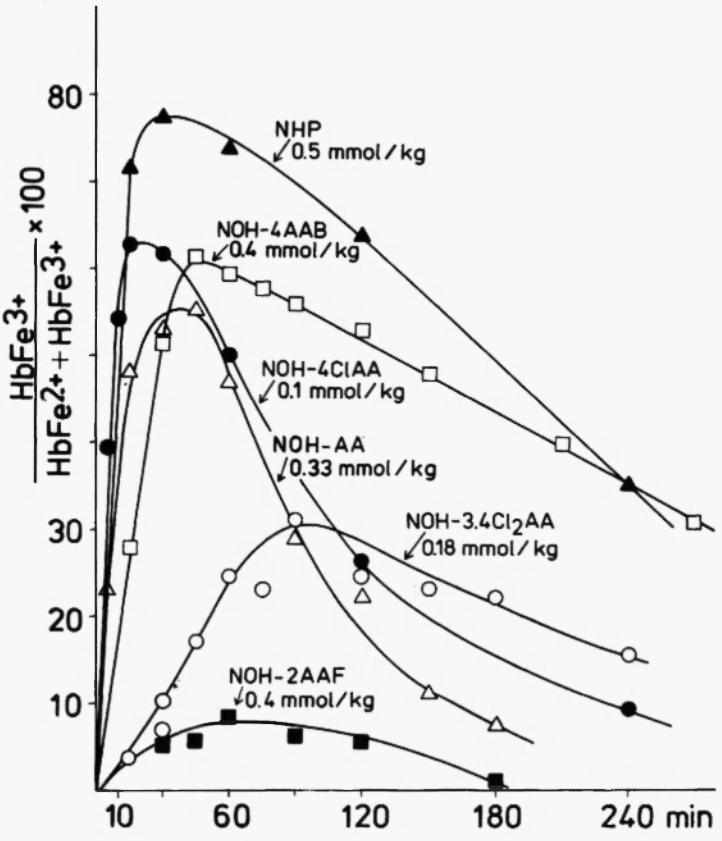

Fig. 4. Kinetics of ferrihemoglobin-formation by various N-arylacetohydroxamic acids (N-hydroxy-N-arylacetamides).

Ferrihemoglobin-formation in female Sprague Dawley rats after i.p.-injection of the doses of various N-arylacetohydroxamic acids indicated in the graph. Symbols indicate means from experiments on 9(NOH-2AAF), 6(NHP, NOH-4AAB), and 3(NOH-AA, NOH-4ClAA, NOH-3.4-Cl_2AA) animals. For i.p.-injection, the pure compounds were finely ground and suspended in 0.25% agar. For other details see caption of *Fig.1.*

hydroxamic acids do not themselves produce ferrihemoglobin, but after N-deacetylation *in vivo*, a mechanism first proposed by Kiese and coworkers (55, 56). The ferrihemoglobin-forming capability of an N-arylacetohydroxamic acid would then depend on the rate of absorption from the peritoneum into blood, on the rate of N-deacetylation *in vivo*, and on the metabolic stability of the liberated arylhydroxylamine or its oxidation product nitrosoarene.

If one compares the doses of N-arylacetohydroxamic acids which produced maximal ferrihemoglobin concentrations *in vivo* with the doses of the corresponding arylhydroxylamines (Fig. 2) which produced the same ferrihemoglobin concentrations, one finds that synthetic N-acetylation reduced the ferrihemoglobin-forming activity of the arylhydroxylamines tested, but that the molar dose ratio of N-arylacetohydroxamic acid to arylhydroxylamine for the same maximal ferrihemoglobin concentration varied from 2 :1 for NOH-4ClAA: NOH-4ClA to 14 :1 for NOH-2AAF :NOH-2AF:

NOH-4ClAA:	NOH-4ClA	= 2	: 1
NHP:	NOH-4EA	= 5	: 1
NOH-4AAB:	NOH-4AB	= 5	: 1
NOH-3.4Cl_2 AA:	NOH-Cl_2A	= 6.8	: 1
NOH-AA:	NOH-A	= 7.3	: 1
NOH-2AAF:	NOH-2AF	= 14	: 1

The highest availability of the liberated arylhydroxylamine for oxyhemoglobin *in vivo* was observed with NOH-4ClAA and the lowest with NOH-2AAF. Factors, which determine the availability of the active arylhydroxylamine in the blood for oxyhemoglobin are: i) the solubility of the N-arylacetohydroxamic acid in aqueous solutions, ii) the yield, with which an arylhydroxylamine is produced from the parent hydroxamic acid, and iii) the disposition to react with other molecules. Since i) and iii) are discussed in section 3.6, only ii) is discussed here. If the enzymatic N-deacetylation of N-arylacetohydroxamic acids in the liver alone were involved in the formation of the active arylhydroxylamine, then another dose ratio would have been observed, since a certain order was observed for the rates of N-deacetylation of various N-arylacetohydroxamic acids *in vitro* by microsomal and cytosolic enzymes from rat liver (11). Furthermore, it is presently not known, to which extent arylhydroxylamines, liberated in the liver, are released into the blood and become available for oxyhemoglobin. Eyer et al. have demonstrated recently, that, although N-hydroxylation of aniline by rat liver is so slow, minute amounts of NOH-A were released from rat liver during recirculating perfusion by measuring their effect on red cells (21). However, on their way from the peritoneum to red blood cells the liver is bypassed, and hydroxamic acids are converted into nitrosoarenes as soon as they invade the blood. Since we have found a rapid concentration-dependent ferrihemoglobin-formation and nitrosoarene-formation, we favour the hypothesis, that nitrosoarenes are formed from hydroxamic acids by oxidation in the blood.

3.5 Differences in the mechanism of hemoglobin-oxidation between arylhydroxylamines and N-arylacetohydroxamic acids.

3.5.1 Earlier Studies

Results of experiments *in vivo* and *in vitro* on the coupled reaction of arylhydroxylamines with oxyhemoglobin were reported by Kiese and coworkers (12) in the fifties. In the absence of reducing equivalents, for example with crystallized horse hemoglobin or hemolysate, phenylhydroxylamine caused the oxidation of 0.8 equivalents of hemoglobin-Fe^{2+} and was oxidized itself to nitrosoarene. However, in suspensions of erythrocytes, supplemented with glucose, or *in vivo*, phenylhydroxylamine caused the oxidation of several hundred equivalents of hemoglobin-Fe^{2+}. This apparent catalytic effect of the arylhydroxylamine in the presence of reducing equivalents was explained with the metabolic regeneration of the arylhydroxylamine from the nitrosoarene, which continued until either all hemoglobin-Fe^{2+} was consumed or the arylhydroxylamine/nitrosoarene had been inactivated, for example, by further reduction to the arylamine or condensation with glutathione (57).

Reports on the mechanism by which N-arylacetohydroxamic acids cause oxidation of hemoglobin *in vivo* and *in vitro* by Heubner et al. (58) and by Kiese and coworkers (55, 56) also appeared in the fifties. It was found, that NOH-AA rapidly oxidized hemoglobin in cats, and that the blood contained high concentrations of nitrosobenzene, but that the onset of action was delayed as compared with the arylhydroxylamine. In dogs, ferrihemoglobin-formation by NOH-AA was much slower and lower nitrosobenzene concentrations were found in the blood, indication that the hydroxamic acid did not itself produce ferrihemoglobin, but after N-deacetylation *in vivo*.

In contrast, *in vitro* experiments with N-acetyl-, N-butyryl, and N-benzoyl-phenylhydroxylamine have shown that they oxidized hemoglobin-Fe^{2+} much slower than phenylhydroxylamine, that the rate of oxidation was proportional to the hemoglobin-Fe^{2+} and hydroxamic acid concentration, and that more than stoichiometric amounts of hemoglobin-Fe^{2+} were oxidized. These results suggested that hydroxamic acids themselves, at least *in vitro*, could also catalyze the oxidation of hemoglobin-Fe^{2+}, but at a much lower rate.

3.5.2 *Experiments in vitro to elucidate the mechanism of ferrihemoglobin-formation by arylhydroxylamines and N-arylacetohydrox amid acids.*

On addition of NOH-4ClA to solutions of purified human hemoglobin, instantaneous ferrihemoglobin-formation occurred, see Figure 5B. Second order rate constants of the rapid oxidation measured in an Aminco-Morrow stopped-flow apparatus were determined to 7844 ± 300 l x mol^{-1} sec^{-1}. The second phase of the oxidation was much slower and is also seen from Figure 5B, but has not been analyzed so far. On variation of the arylhydroxylamine concentration molar ratios of arylhydroxylamine to hemoglobin-Fe^{2+} of 1 :0.7 to 1 :3 were measured. From an Arrhenius plot of the second order rate constants determined at various temperatures an E_a of 3.5 kcal/Grad/mol was obtained. Analysis of the reaction mixture 2 min after addition of NOH-4ClA to 5.8 mM hemoglobin Fe^{2+} to give a final concentration of 6.0 mM has shown that 3.2mM ferrihemoglobin and 2.35 mM 4-chloronitrosobenzene were formed. In addition, amounts of app. 0.1 mM of 4-ClA and 4.4' bisazoxychlorobenzene were found. The formation of 4-ClA during the rapid oxidation of NOH-4ClA gives a hint of the mechanism of the complex reaction. When NOH-4ClAA was added to solutions of purified human hemoglobin, oxidation of hemoglobin-Fe^{2+} proceeded much slower, see Figure 5A. Second order rate constants measured in a Zeiss PMQ 3 spectrophotometer were determined to 3.45 ± 0.23 l x mol^{-1} x sec.^{1}. When the hydroxamic acid concentration was succesively decreased, molar ratios of hydroxamic acid to hemoglobin-Fe^{2+} oxidized increased from 1 :16 to 1 :45, indication for an increase of the stationary concentration of the active molecule with decreasing concentration of NOH--4ClAA. This is indication for a second order decay of the active molecule. Acetyl 4-chlorophenyl nitroxide was shown to display a second order decay kinetics (59). From an Arrhenius plot an activation energy E_a = 12.7 kcal/Grad/mol was determined. When we searched a reaction mixture of hemoglobin-Fe^{2+} and NOH-4ClAA (3 :1 molar ratio) incubated at 37° for metabolites of NOH-4ClAA by HPLC, we detected the presence of 4-ClAA, 4-chloronitrosobenzene (=4-ClNOB), and 4-chloronitrobenzene (=4-$ClNO_2$B) in the ether extracts taken at 2 and 60 min incubation. Since recovery experiments have shown that during a 60 min incubation at 37°, 80% of the added 4-$ClNO_2$B disappeared, and that the loss of the more volatile 4-ClNOB was nearly complete, we have carried out the subsequent experiments in a closed system, thereby reducing the loss of 4-$ClNO_2$B to 14%. The results of an analysis of the reaction mixture incubated in a closed system at 2 and 60 min were:

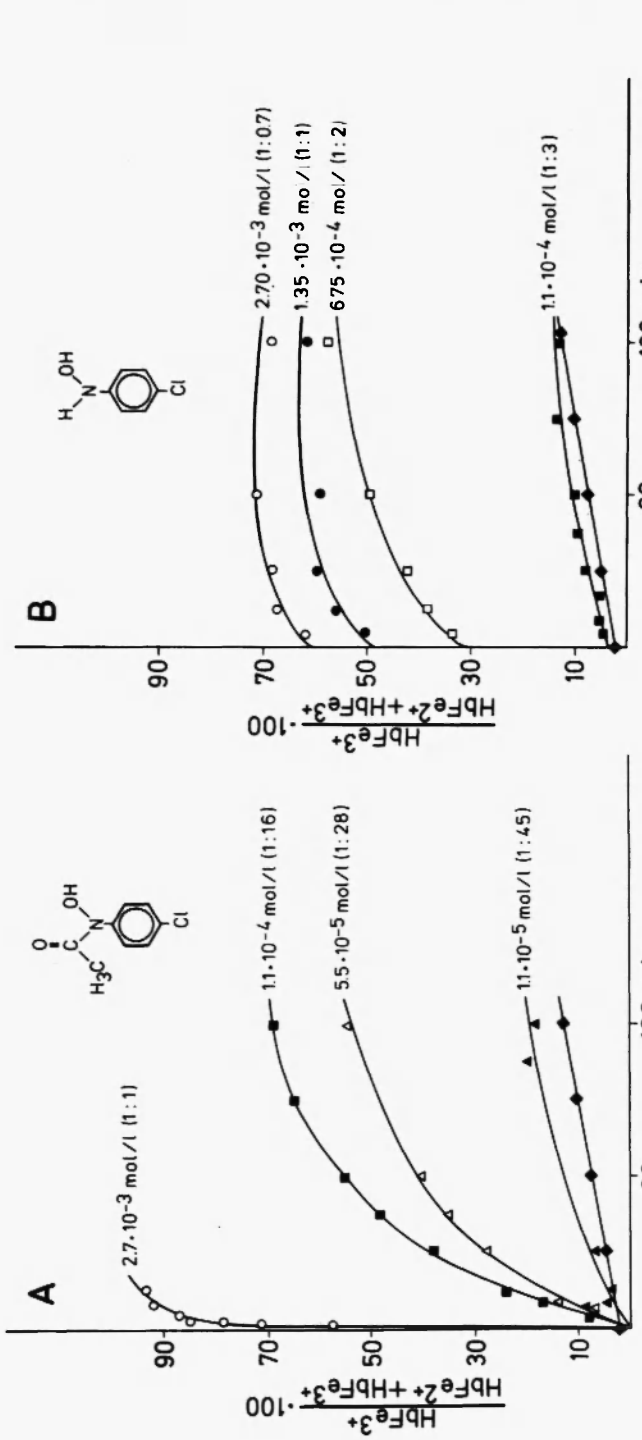

Fig. 5. Kinetics of ferrihemoglobin-formation *in vitro* by NOH-4ClAA(A) and NOH-4ClA(B). A. A dilution of purified human hemoglobin (2.24×10^{-3} M hemoglobin Fe^{2+} in 0.2 M phosphate buffer pH 7.4 was incubated at 37° with NOH-4ClAA (concentrations given in the graph); symbols indicate results from single experiments and are corrected on the basis of hemoglobin autoxidation under identical conditions. B. Under the same conditions the effect of different concentrations of NOH-4ClA on the kinetics of ferrihemoglobin-formation was studied; symbols indicate the results of single experiments and are corrected on the basis of hemoglobin autoxidation under identical conditions; autoxidation: △ ▲

$HbFe^{3+}$ (45.3, 100%), NOH-4ClAA (85, 75%), 4-ClAA (2.2, 4.0%), 4-ClNOB (1.3, 0.6%), and 4-$ClNO_2$B (1.0, 0.2%).

The discovery that erythrocytes converted hydroxamic acids into N-aryl-acetamides was recently made by Weisburger et al. (32) who investigated the metabolic fate of NOH-2AAF in the blood of rats. They found 2-AAF in the plasma as well as on the erythrocyte membrane and thought it was a product of reduction. Our experiments have shown that various N-arylacetohydroxamic acids were converted into N-aryl-acetamides in suspensions of erythrocytes from various species. At first we also suggested that it was due to a reduction, that N-aryl-acetohydroxamic acids were converted into N-arylacetamides, although we could not explain why neither NADPH nor an NADPH-regenerating system could reduce hydroxamic acids to N-arylacetamides (59).

However, papers on the chemical oxidation of various hydroxamic acids and secondary alkylhydroxylamines by Forrester et al. (60), Nagakawa et al. (61), and Banks et al. (62) have shown that nitroso and nitroarenes as well as N-arylacetamides and alkylamines arise from the decay of secondary nitroxides. N-Arylacetamides, although formally classified as reduction products of N-arylacetohydroxamic acids, are in fact oxidation products of hydroxamic acids which arise from the decay of the secondary nitroxides, which are formed by the withdrawel of one electron. Our experiments on the formation and decay of aromatic secondary nitroxides (59) have shown a close analogy between the chemical oxidation of hydroxamic acids by PbO_2, Ag_2O, $KMnO_4$ or nickelperoxide and the biological oxidation by oxyhemoglobin, in which a dioxygen species is the oxidans. Therefore we can summarize the catalytic function and the metabolic fate of N-arylacetohydroxamic acids as follows, see also Chart 2: the hydroxamic acid (I) in a solution of oxyhemoglobin is oxidized by the electrophilic dioxygen species ($O_2^{\bullet 1-}$) via one-electron transfer to yield the secondary nitroxide (II), which, by accepting one electron from hemoglobin-Fe^{2+}, is converted back into the hydroxamic acid and can enter a new catalytic cycle. However, since secondary nitroxides (II) have a limited life-span only (59), they decay with a second order rate, and from the disproportionation emerge nitrosoarene (III) and starting material. Nitrosoarene reacts with the secondary nitroxide to yield the complex (IV), whose decay yields nitroarene (V) and N-arylacetamide (VI).

The postulated one-electron transfer is in agreement with the view of L.Michaelis (63), that all oxidations of organic molecules proceed in successive univalent steps, the intermediate being a radical, which is the prerequisite for the reversibility of an oxidation reduction system. The postulated mechanism is also in agreement with the laws of thermodynamics, since electrons can flow from N-arylacetohydroxamic acids

($E_{1/2}$ = + 600 ± 30 mV (depending on the aryl residue) (59) to the dioxygen species to yield either H_2O_2 ($E_{1/2}$ = + 900 mV (64)) or H_2O ($E_{1/2}$ = + 800 to + 1350 mV (64) and from hemoglobin-Fe^{2+} ($E_{1/2}$ = + 125 mV (65)) to the secondary nitroxide.

When we extended our experiments on the mechanism of hydroxamic acid induced ferrihemoglobin-formation to suspensions of rat erythrocytes, we found the same metabolites of NOH-4ClAA in the ether extracts taken after 2 and 60 min of incubation at 37°, namely 4-ClAA, 4-chloronitrosobenzene, and 4-chloronitrobenzene, indication that the oxidative mechanism is also functioning in erythrocytes.

3.5.3 *Experiments in vivo to elucidate the mechanism of ferrihemoglobin-formation by arylhydroxylamines and N-arylracetohydrox amic acids.*

Experiments designed to elucidate whether the oxidative mechanism, by which N-arylacetohydroxamic acids effect oxidation of hemoglobin, is also functioning *in vivo*, have to consider that the nitro- and nitrosoarenes formed by that mechanism, are not as inert as they are in the *in vitro* experiments, but become active after being reduced to arylhydroxylamines. Ferrihemoglobin-formation *in vivo* by N-arylacetohydroxamid acids, therefore, is expected to be effected partly by the hydroxamic acid itself and partly by the nitro- and nitrosoarene after reduction to the active arylhydroxylamine. The questions to be answered are: how much ferrihemoglobin is produced by the hydroxamic acid itself and how much by the oxidation products, and secondly, how are nitrosoarenes formed, if there is any detected in the blood, from enzymatic or oxidative degradation of the hydroxamic acid? To the first question: the concentration of ferrihemoglobin and of the hydroxamic acid and its metabolites in the blood was determined 3 min after i.p.-injection of 50 mg NOH-4ClAA per kg into rats and in another series of experiments the concentration of ferrihemoglobin and of NOH-4ClA and its metabolites 3 min after i.p.-injection of 8mg NOH-4ClA per kg. Two methods were used to determine the concentration of NOH-4ClAA and of NOH-4ClA and their metabolites, namely i) the method of Herr and Kiese (66), applicable to arylamines and their diazotizable derivatives, and ii) analysis of the ether extracts of blood by HPLC. These ether extracts contained only the neutral and basic metabolites, since NOH-4ClAA had been removed before by shaking with 2 M NaOH. NOH-4ClAA was determined either by Herr and Kiese or directly by reading the u.v.-absorbance at 255 nm of a methanol solution of NOH-4ClAA. As the following results of a typical experiment show, similar ferrihemoglobin and 4-chloronitrosobenzene concentrations were deter-

mined in the blood after injection of either NOH-4ClAA or NOH-4ClA:

Rat 1 (50 mg NOH-4ClAA per kg i.p.) analysis of blood after 3 min: $HbFe^{3+}$: 34.5%; $C_{NOH\ 4ClAA}$ = 17.8 µg/ml; concentration of metabolites in the ether extracts of blood: 4-ClA = 1.3 µg/ml;4-ClAA = 1.3µg/ml, 4-chloronitrobenzene = 0.4µg/ml, and 4-chloronitrosobenzene =0.5 µg/ml; 4-chloronitrosobenzene bound to hemoglobin-Fe^{2+}, determined by Herr and Kiese (66): 2.9 µg/ml; total 4-chloronitrosobenzene: 3.2 µg/ml.

Rat 2 (8mg NOH-4ClA per kg) analysis of blood after 3 min: $HbFe^{3+}$: 32.4%; concentration of metabolites in the ether extracts of blood: 4-ClA = 0.4 µg/ml; 2 unknown metabolites, 4-chloronitrosobenzene = 0.3 µg/ml;4-chloronitrosobenzene bound to hemoglobin-Fe^{2+} = 2.7 µg/ml; total 4-chloronitrosobenzene = 3.0 µg/ml.

These results indicated that the nitrosoarene formed from the hydroxamic acid was solely responsible for the oxidation of hemoglobin *in vivo* and that the hydroxamic acid itself scarcely contributed to the ferrihemoglobin-formation, because its action is so much slower than that of an arylhydroxylamine. As to the second question, how 4-chloronitrosobenzene was formed from NOH-4ClAA, either enzymatically or by oxidative degradation, we have carried out experiments *in vivo* with the esterase inhibitor bis(4-nitrophenyl)phosphoric acid ester = BNPP *in vivo*, see also 3.3. In four experiments we have i.p. injected 50mg BNPP per kg into rats 30 min prior to i.p.-injection of 50mg NOH-4ClAA per kg and the same number of animals with 50mg NOH-4ClAA per kg only. The blood was analyzed 10 and 15 min after injection of NOH-4ClAA, but no statistically significant differences in the concentrations of ferrihemoglobin and 4-chloronitrosobenzene determined by Herr and Kiese were found.

When we measured the kinetics of ferrihemoglobin-formation in rats after i.p.-injection of either 10mg NOH-4ClAA per kg or 10 mg NOH-4ClAA together with 50mg BNPP per kg (3 days apart with the same animals), we observed differences in the means, but without statistical significance on the 5% level. Although enzymatic N-deacetylation of some mono- and polycyclic N-arylacetohydroxamic acids by microsomal and cytosolic enzymes from rat and rabbit liver has been measured recently (11), these results indicated, that BNPP was without effect on the ferrihemoglobin-formation by NOH-4ClAA in the rat. Most likely 4-chloronitrosobenzene was formed by oxidation of NOH-4ClAA. The presence of 4-ClAA in the blood of rats injected with NOH-4ClAA supports such an hypothesis.

Chart 2. Formula scheme to illustrate the proposed mechanism of hemoglobin oxidation by N-arylacetohydroxamic acids. In the first section those reactions are outlined, by which the catalytic cycle of hemoglobin oxidation is sustained. In the second section those reactions are shown, by which the active molecule is inactivated *in vivo*, since the nitrosoarene as well as the nitroarene can be reduced to yield the highly active arylhydroxylamine.

3.6 *Differences in the ferrihemoglobin-forming capabilities between monocyclic and polycyclic arylamines and their derivatives.*

It is evident from Fig. 1, 2, and 4, that large differences in the ferrihemoglobin-forming capabilities only exist between the monocyclic compounds and 2-AF and its derivatives tested. As was discussed in section 3.1, the low ferrihemoglobin-forming capability of 2-AF in the rat may have two reasons, either very slow N-hydroxylation or very low availability of the N-hydroxy derivative for oxyhemoglobin or both.

In a recent study on the N- and ring-hydroxylation of (9-^{14}C)-2-AF and (U-^{14}C)-4-ClA *in vitro* by liver microsomes from untreated and phenobarbital-induced rabbits, the extent of ^{14}C-binding to microsomal lipids and proteins was determined (67). The N-hydroxy derivative of 4-ClA was stable enough to survive a 60 min incubation at 37° and was determined as NOH-4ClAA. In contrast, only part of the N-hydroxy derivative of 2-AF was determined as NOH-2AAF, another part being identified as 2.2'-bisazoxyfluorene. Little 4-ClA-derived ^{14}C-binding, but considerable 2-AF-derived (9-^{14}C)-binding to microsomes was measured, evidence, that a third part of NOH-2AF had reacted with microsomal macromolecules. Rapid reaction of NOH-2AAF or its metabolites with macromolecules in plasma and high affinity for erythrocytes was reported by Weisburger et al. (32). These results indicated that the high reactivity of NOH-2AF or its oxidation products may be an explanation of the impaired availability for oxyhemoglobin.

Differences in the ferrihemoglobin-forming capabilities between NOH-4ClAA and NOH-2AAF also became evident by a direct comparison in the rat.

1.Two rats, 100 mg NOH-4ClAA per kg; analysis of blood after 4 min:
Rat 1: $HbFe^{3+}$ = 58.5%; $C_{NOH\ 4ClAA}$ = 64.3 µg/ml;HPLC-analysis of the ether extract containing neutral and basic metabolites: 4-ClA = 4.8 µg/ml; 4-ClAA = 1.9 µg/ml; 4-chloronitrosobenzene =2.5 µg/ml; 4-chloronitrobenzene =0.5 µg/ml; 4-chloronitrosobenzene bound to hemoglobin-Fe^{2+} = 1.5 µg/ml.
Rat 2: $HbFe^{3+}$ = 60.7%; $C_{NOH\ 4ClAA}$ =77.9 µg/ml;HPLC-analysis of the ether extract containing neutral and basic metabolites: 4-ClA =6.2 µg/ml; 4-ClAA =2.1 µg/ml; 4-chloronitrosobenzene =2.3 µg/ml; 4-chloronitrobenzene = 0.5 µg/ml; 4-chloronitrosobenzene bound to hemoglobinFe^{2+} = 2.7 µg/ml.

2.Two rats 100 mg NOH-2AAF per kg; analysis of blood after 4 min and after 30 min:
After 4 min
Rat 1: $HbFe^{3+}$ = 0; traces of 2-AAF were detected by HPLC, but not quantified.

Rat 2: $HbFe^{3+}$ = 0
After 30 min
Rat 3: $HbFe^{3+}$ = 0; $c_{NOH\ 2AAF}$ = 2.1 μg/ml; HPLC-analysis of the ether extract containing neutral and basic metabolites: 2-AAF = 0.1 μg/ml.
Rat 4: $HbFe^{3+}$ = 3.9%; $c_{NOH\ 2AAF}$=5.3 μg/ml; HPLC-analysis of the ether containing neutral and basic metabolites: 2-AAF = 0.6 μg/ml; traces of two unidentified metabolites were observed.
These results indicated that NOH-4ClAA rapidly invaded the blood from the peritoneum and was readily oxidized there to 4-chloronitrosobenzene and 4-chloronitrobenzene, whose reduction to the arylhydroxylamine caused the rapid onset and large extent of ferrihemoglobin-formation; in contrast, the concentration of free NOH-2AAF in the blood increased only very slowly, after 30min being still 23-fold lower than that of NOH-4ClAA after 4 min. Because extractable 2-nitro and 2-ni trosofluorene were present in the blood only in trace amounts, they could not be unambiguously identified by HPLC in the ether extracts, while 2-AAF was present in substantial amounts. Since one of the factors which determine the rate of invasion of blood is the solubility in aqueous solutions, we have determined the solubility of NOH-4ClAA, NHP, NOH-4AAB, and NOH-2AAF in Krebs-Ringer phosphate buffer pH 7.4 and found a molar ratio of 91 :52 :1 :1, respectively, at room temperature. Therefore it is due to their excellent solubility in aqueous solutions *and* the unimpaired availability of their oxidation products for oxyhemoglobin, that monocyclic hydroxamic acids rapidly invaded the blood from the peritoneum and massively produced ferrihemoglobin after oxidation. In contrast, invasion of the blood by the sparingly hydrosoluble, but perhaps protein-bound polycyclic hydroxamic acids NOH 4AAB and NOH-2AAF was sluggish and only small concentrations of the oxidation products 2-AAF, 2-nitroso-, and 2-nitrofluorene were formed. But since 2-AAF is only slowly metabolized, it accumulated, while 2-nitroso- and 2-nitrofluorene, being consumed in competing reactions, escaped their function as ferrihemoglobinogenic agents. The excellent ferrihemoglobinogenic activity of NOH-4AAB *in vivo* is indication, that 4-nitroso- and 4-nitrobiphenyl, although probably present in the same small concentrations, are less reactive than the corresponding fluorene derivatives and can sustain the catalytic cycle of hemoglobin oxidation for a long time. Experiments are underway to elaborate these differences.

3.7. *Correlation between the ferrihemoglobinogenic activity of certain mono- and polycyclic arylamines and related compounds and their carcinogenicity.*

In a more detailed study, Neish has shown that there was no correlation between the ferrihemoglobinogenic capability of ten azobenzene derivatives and their carcinogenic activity in the rat (68). Later on Radomski and Brill compared the ferrihemoglobinogenic capability of 4-AB, of 1- and of 2-naphtylamine with the rate of N-hydroxylation *in vivo* and with the carcinogenic activity in the dog (69), implicating the N-hydroxy derivative in either effect. They found high ferrihemoglobinogenic and carcinogenic activity of 4-AB and large amounts of NOH-4AB in the urine, but decreasing amounts of the N-hydroxy derivatives of 2- and 1-Naphthylamine in the urine (in this order) and consequently lower and negligible ferrihemoglobinogenic and carcinogenic activity, respectively, in the dog. Thus, with this arbitrary selection of arylamines, the authors found a correlation between the two effects, whereas in this paper, where we describe the same effects of another arbitrary selection of arylamines, we do not find such a correlation. This is deduced from the finding, that 2-AF and its derivatives, known as potent carcinogens, were found to be the least active ferrihemoglobinogenic compounds, whereas the monocyclic compounds tested were very active ferrihemoglobin-forming agents, but very weak carcinogens. The properties of 4-AB, representing a third group of arylamines, prevents us from discussing a modified theory of correlation.

4. CARCINOGENICITY

Results of tests for carcinogenic activity of arylamines and related compounds discussed in this paper may be found in (70-78). These books contain either compilations of data from reports published up to 1967 (70-73), up to 1972 (77) and 1974 (78) or monographs on chemical carcinogenesis (74-76), in which some of the relevant contributions may be found. Pertinent contributions which have appeared after 1967 and those which have escaped compilation, are enlisted in the subsequent references.

4.1 *Structure-Activity Relationships*

From the observation that, unlike polycyclic hydrocarbons, certain arylamines did not induce tumors at the site of application, but at a distant site, such as liver, mammary gland, ear duct, intestines, urinary bladder or smooth muscle, the concept was born, that these compounds

do not themselves produce tumors, after metabolic activation which followed systemic absorption from the site of application. Ortho-hydroxylation was first thought to be the required step of metabolic activation, because the dog, being the most sensitive species towards the carcinogenic effect of 2-naphtylamine, excreted the largest amounts of 2-amino-1-naphthol in the urine. When certain o-aminophenols were tested for their ability to produce bladder cancer after being implanted in a paraffin or cholesterol pellet in the bladder, they were found active (74, 79). However, when it was recognized, that the vehicle itself was active, the validity of these results was questioned. The discovery of NOH-2AAF as a urinary metabolite of 2-AAF in the rat and its higher activity as compared to 2-AAF opened a new approach to the problem (80).

Lack of carcinogenicity of an arylamine or its N-acyl derivative was shown to be due to either very slow N-hydroxylation *in vivo* or inactivity of the endogenously formed N-hydroxy derivative. Synthetic N-hydroxy derivatives, such as N-2-fluorenyl-benzohydroxamic acid or N-hydroxy-N-2-fluorenylbenzenesulfonamide (81) were found to be very active, whereas the parent N-acylarylamines were not, obviously, because they are not N-hydroxylated *in vivo*. However, NOH-AA and N-hydroxy-phenylbenzene-sulfonamide were inactive. N-Hydroxylation, therefore, was recognized as a necessary, but not sufficient prerequisite for carcinogenicity. Whereas the N-hydroxy derivative of 2-AAF was more reactive than 2-AAF in male rat liver *in vivo* as measured by the amount of 2-AAF or 2-AF bound to hepatic proteins, DNA or RNA, it showed only limited reactivity *in vitro* at pH 7.4, indication for the requirement of a second activation step, by which the proximate carcinogen NOH-2AAF is transformed into the ultimate carcinogen. Positive results obtained with the benzoic ester of the hypothetic N-hydroxy-N-methyl-4-aminoazobenzene suggested that the acetyl- or sulphate ester or the O-glucuronide of NOH-2AAF might be the ultimate carcinogen (82). Whereas the enhanced toxicity, carcinogenicity, and reactivity *in vitro* at pH 7.4 of the synthetic O-acetates of NOH-2AAF and 4-AAB apparently supported this hypothesis (82, 83), lack of carcinogenic activity of the sulphate esters weakened it (82, 8). Lack of carcinogenic activity, therefore, would have been due to low affinities of certain N-hydroxy-N-arylacetamides to those enzymes, which converted them into reactive esters *in vivo*. However, when the ability of the sulphate esters of NOH-2AAF, NHP, NOH-4ClAA, and NOH-AA to bind covalently to microsomal protein *in vitro* was studied, NHP exceeded even NOH-2AAF in activity and NOH-4ClAA and NOH-AA were inactive (84). This is indication that not the functional groups attached to the N- and 0-atom of N-hydroxy N-arylacetamides govern the reactivity and carcinogenicity, but the physico-chemical properties of the aryl moiety.

Differences in the electron donor and acceptor properties among various aryl residues appear to be responsible for the differences in the reactivity of the N-atom. The fluorene nucleus was found to exceed even the biphenyl and phenanthrene nuclei in its reactivity with nucleophiles *in vitro* (83), indication for its unexpectedly high electron donor properties, for which the pKa value of 4.64 for 2-AF (as compared to 4.35, 3.91, and 4.62 for 4-AB, 2-aminophenanthrene, and A, respectively (85) and the second order rate constant of 0.88 1 xmol $^{-1}$xsec $^{-1}$ for the decay of acetyl 2-fluorenyl nitroxide in toluene solution versus 0.11 and 0.34 for acetyl 4-aminobiphenyl- and -2-aminophenanthryl nitroxide,respectively,give additional support(59). The "para-principle", postulated by Druckrey in 1952 (86), that only such arylamines are carcinogenic in which the amino group is attached to the terminal C-atom of the longest conjugated carbon chain, appears to mean an enhanced reactivity of the N-atom, which is caused by the (+)I-effect of the polycyclic nuclei.

4.2 Carcinogenicity of monocyclic arylamines and related compounds.

Reports on the nephrotoxic effects of large doses of phenacetin-containing analgesics in humans, which have appeared since 1953 (87) and the reports on the enhanced carcinogenicity of the N-hydroxy derivatives of 4-AAB or 2-AAF (88, 39), caused Nery in 1971 to speculate that NHP, the N-hydroxy derivative of phenacetin, may be responsible for the toxic effects (89). Potter et al. (90) assumed the N-hydroxy derivative of paracetamol to be the active molecule which caused hepatic necroses in humans after administration of large doses of paracetamol. These hypotheses, which reflected a change of the view on the carcinogenicity of monocyclic arylamine derivatives, have stimulated several groups to synthesize and test the suspected compounds for their acute and chronic toxicities.

4.2.1 Aniline and some derivatives.

The carcinogenic activity of A was tested on mice, rats, rabbits, and dogs by various routes, such as s.c., i.p., p.o., local, inhalation, and bladder implantation (70-76, 78). Because most of the results were negative and a careful analysis of some positive results has shown that they are not convincing, A was not thought to be carcinogenic, until recently, it was shown that in the Fischer 344 rat, aniline hydrochloride produced several types of mesenchymal tumors, primarily in the spleen. In the low dose group fed 0.3% aniline hydrochloride in the feed, the incidence was 2%, and in the high dose group fed 0.6%, 14% (91).

Two groups have independently shown that NOH-A i.p. injected into rats did not induce tumors (78).

Rats, injected s.c. with a total amount of 0.2 mmol nitrosobenzene per rat in 16 doses over 8 weeks, did not develop tumors by 8 months (Miller et al. 1965) (73, 78).

Of the ring-hydroxylated derivatives of A tested, o-(71, 78)as well as p-aminophenol (70, 71, 78) was not active when fed to rats or painted on the skin of rabbits.

The carcinogenicity of AA was tested on dogs and rats (70, 75, 76). In the experiments on dogs, 9-144 mg per kg were ingested with food, but after at least 110 days, no tumors were observed. Whereas Holtzman rats fed massive doses of AA (8.1 mmol per kg) for 8 months did not develop tumors, the much lower doses of 2-AAF (1.62 mmol per kg) produced various tumors in most of the animals.

Female rats, injected i.p. 3 times weekly for 4 weeks with 45 mg benzanilide per kg (total dose: 0.37 mmol per rat), did not develop tumors by 34 weeks (92).

Of the female rats, injected i.p. 3 times weekly with 45 mg benzenesulfanilide per kg for 4 weeks (total dose: 0.254 mmol per rat), 2/12 animals developed tumors of the mammary gland by 12 months (81).

No NOH-AA was detected by chromatographic analysis of the urine of dogs fed 500 mg AA per animal (93).

When 12 doses of 45 mg of either NOH-AA or N-hydroxy-phenylbenzenesulfonamide per kg were injected i.p. to female Holtzman rats (total dose of either compound: 0.25 mmol per rat), 1/12 and 2/12 animals, respectively, had developed tumors of the mammary gland by 12 months Gutmann et al. (81).

Female Holtzman rats injected i.p. with a total amount of 0.33 mmol N-hydroxy-benzamide per rat in 12 doses for 4 weeks, did not develop tumors, by 34 weeks (92).

These results indicated that synthetic N-hydroxylation of N-acyl derivatives of a monocyclic arylamine (A) did not enhance their carcinogenic activity; N-hydroxylation therefore was recognized as a necessary, but not sufficient prerequisite for carcinogenicity.

Obviously, the size of the phenyl residue did not exceed a limiting size required for oncogenicity.

Of the ring-hydroxylated derivatives of AA, o- and p-hydroxyacetanilide were tested for carcinogenic activity (76, 94). The finding that o-hydroxyacetanilide produced pituitary adenomas in Buffalo rats was considered unreliable, because untreated rats yielded variable tumors, too. No evidence was obtained for the carcinogenicity of p-hydroxyacetanilide (paracetamol) in the rat, but large doses produced severe liver necrosis in male Sprague Dawley rats and male mice (76). Recently

Johansson reported the results of a test on the carcinogenicity of p-hydroxyacetanilide in Wistar rats. After feeding the animals for 117 days with a diet containing 0.535% of this drug, no tumors were found (94). Peters et al. (95) studied the effect of large doses (1500-3000 mg per kg) p-hydroxyacetanilide administered by stomach tube to male Wistar rats for 60 days. They detected degenerative histological alterations in cortical proximal and distal tubules, but no inflammatory changes or medullary or papillary lesions due to the compound, indication that p-hydroxyacetanilide lacks nephrotoxicity in the rat.

4.2.2 4-Phenetidine and some derivatives.

We are not aware of any reports on the carcinogenic activity of 4-EA or its N-hydroxy, its ring-hydroxylated derivatives or of 4-nitrosophenetole. However, the effects of a 13 weeks' ingestion of 0.25 ml 4-EA per 100 ml drinking water by male Osborne-Mendel rats was studied (96). Besides Heinz bodies in red cells and many reticulocytes, enlargement of the spleen with distended sinuses engorged with erythrocytes, dark brown deposits of hemosiderin in the spleen, in the renal tubular epithelium, and in the Kupffer cells, and deposits of dark brown hemosiderin-negative pigment in the distal tubular epithelium were observed.

Since it is beyond the scope of this survey to review the clinical reports on the nephrotoxic effects in man of large doses of phenacetin containing analgesics, the reader is recommended the article of J.H. Shelley (97). Reports on the carcinogenic activity of phenacetin in laboratory animals are conflicting (70, 72, 75, 76, 94-100). Experiments on dogs (1944) and on Wistar rats (1954) have shown that PH is not tumorigenic. In the study on BDI and BDIII rats, 30 animals received 40-50 mg PH daily with food, so that after a 20 months' administration, each animal had received a total dose of 22 g. After 31 months, when the experiment was terminated, no tumors were observed.

In the medium-term toxicity tests reported by Peters et al. (95), male white Wistar rats received either æsmallæ (600 mg PH per kg daily) or ælargeæ doses (1500, 2250, and 3000 mg PH per kg daily) per stomach tube for 60 days. (In comparison: a human male of 70 kg weight would have to ingest doses of PH between 42 and 210 g daily!; prescribed doses of PH: 1 -2 g; doses of PH ingested in cases of phenacetin abuse reported by Shahidi (101): 2 -5 g!). In spite of such high doses of PH, only degenerative histological alterations in cortical proximal and distal tubules, but no major nephrotoxic effects were observed. Two long-term toxicity tests were reported by Johansson and Angervall (98) and Johansson (94). In the former study, Sprague Dawley rats were fed for

110 weeks with a diet containing 0.535% PH. High incidence of urothelial hyperplasia of the renal papillae, but no renal pelvic tumors were observed. In the latter study the effect of 0.535% PH in the diet was compared with that of a combination of PH with phenazone and (or) caffeine. After 117 weeks of administration the experiment was terminated and the animals examined histologically.

PH produced 31 malignant tumors, including renal pelvic tumors, tumors of the renal parenchyma, squamous-cell carcinomas in the head and neck region, and forestomach tumors. In combination with phenazone and caffeine, hepatomas were found in addition, indication of alterations of the metabolism of PH towards more toxic metabolites of PH. Nakanishi et al. (99) studied the toxic effects of PH in rats treated with a sub-carcinogenic dose of N-butyl-N-(4-hydroxy-butyl) nitrosamine and found an increased incidence of bladder tumors. Isaka et al. (100) also reported epithelial tumors of the urinary tract, including renal pelvic tumors and urothelial hyperplasia in 30% of the animals induced by long-term feeding of Sprague Dawley rats with a diet containing 2.5% PH.

Metabolic studies aimed at detecting NHP as a urinary metabolite of PH have shown, that in the urine of dogs fed 500 mg PH per animal, no NHP was detected by chromatographic analysis (93).

McLean et al. (47) succeeded to determine the amount of NHP excreted in the urine of male Wistar rats given 500 mg PH per kg by gavage to be 0.023% of the dose.

N-hydroxyphenacetin, which had been suspected by Nery in 1971 (89) to be the metabolite of PH responsible for the nephrotoxicity of large doses of phenacetin-containing analgesics in man, was tested by Calder et al. (102) for acute nephrotoxicity in 13 female Wistar rats after a single i.v.-injection of 1.0 mmol NHP per kg. The substance was injected in aqueous solution, if necessary, as the sodium salt or as alcohol-water-tween 80 mixture. Already 48 h later 3 out of 13 animals showed necroses of proximal convoluted tubules of grade 2, grade 4 denoting the most severe lesion. However, these authors did not present data on control experiments. Furthermore, the dose of 1 mmol per kg (= 195 mg per kg) is expected to have caused severe cyanosis and even the death of animals, see also Fig.4 and the subsequent studies.

When the nephrotoxicity of 2-hydroxyphenacetin was tested under the same conditions (dose i.v.-injected: 1.1 mmol per kg), no signs of renal damage were detected.

NHP was also tested by Calder et al. (103) for carcinogenic activity in a long-term experiment on male albino rats. Groups of 15 to 24 animals were fed either normal diet or diet containing 0.05, 0.1 or 0.5% NHP for 18 months. Tumors of the liver were found in all animals fed NHP, the

earliest tumors found at the highest dose after 38 weeks of ingestion. One animal with a carcinoma in the kidney and metastases in the liver, lung, and in the bone marrow was found, no tumors were found in control animals. This study suffers from the lack of information on the systemic availability of orally administered and on the urinary excretion of NHP. Attempts to identify and quantify NHP in the urine have failed, because it decomposed during analysis. The subchronic toxicity of NHP in Sprague Dawley rats was also studied by our group (54). 4 Groups of 15 rats (7 males, 8 females) were used for the test. One group received i.p.-injections of 50 mg NHP per kg twice weekly, another group the same dose injected s.c.. NHP was suspended in 0.25% agar prepared from 0.9% NaCl. The rats of the two other groups received i.p.- or s.c.-injections of 0.25% agar solution only. After a 16 weeks' treatment, 10 animals and 5 controls were sacrificed and their organs examined. Then the dose was increased to 150 mg NHP per kg, but severe cyanosis developed in all animals, and one died. Therefore the dose was reduced to 100mg per kg for the remaining 12 weeks of administration, during which period the symptoms of cyanosis were still present. After a total of 29 weeks' treatment, the remaining animals were sacrificed and the organs examined. Inspection of the kidneys, liver, lung or skin did not give evidence for pathological changes, which were not also observed in control animals. In a second experiment one group of 10 male and another of 10 female Sprague Dawley rats were injected s.c. with 100 mg NHP per kg in suspension of olive oil twice daily for five days a week. The same number of animals received s.c.injections of olive oil only. After a 12 weeks' treatment, the animals were sacrificed and the organs examined. The epithelial cells of the tubules of all experimental, but of only one control animal contained a finely dispersed brown pigment varying in shade, which did not stain with Prussian Blue, indication of a lipochrome pigment. When the livers were searched for the same pigment, only the sinus cells of 6 experimental animals contained this pigment. No evidence was found for symptoms of chronic interstitial nephritis or tumor growth in the kidney. In these experiments it was also found, that NHP rapidly disappeared from the blood of rats after i.p.-injection of 100 mg NHP per kg, a plasma half-life of 14 min being determined. NHP was found in the 24 h urine of the experimental rats of the first experiment, 1.1% of the dose being recovered unchanged and further 0.6% after enzymatic cleavage of conjugates in the urine.

4.2.3 4-Chloroaniline and some derivatives.

Since the 4-chloroaniline moiety is part of the antimalarial drug paludrine and can be liberated chemically by acids, Elson et al. (104)

have determined the acute toxicity of 4-ClA in mice and rats and its chronic toxicity in rats. Doses of 4 mg 4-ClA per rat per day were administered over a period of 6 months, but no pathological change attributable to the compound was detected.

1-Chloro-4-nitrobenzene was tested for long-term toxicity on male Charles River rats and Ham/ICR mice of both sex on Purina diet containing two levels, the higher being the maximum tolerated dose and the lower, one-half that level (105). Rats were fed a diet containing 2-4 g per kg diet for 3 months and 1-2 g for 15 months, mice 4-8 g per kg diet for 3 months and 2-4 g for 15 months. 1-Chloro-4-nitrobenzene was ineffective in male rats, but male and female mice showed vascular tumors at the high dose level and liver tumors at the low dose level. Since 1-chloro-4-nitrobenzene is reduced *in vivo* to the corresponding arylhydroxylamine, the carcinogenic effect in mice may be related in fact to the latter compound.

Because 4-chloroacetanilide was a contaminant of phenacetin, its subchronic toxicity was tested in male Osborne-Mendel rats (96). The animals were fed a standard diet containing 0.25 g 4-ClAA per 100 g Purina chow for 13 weeks. Then the animals were sacrificed and the organs examined. The results pointed to a compensated hemolytic process with Heinz bodies in the erythrocytes, enlargement of the spleen with distended sinuses engorged with erythrocytes and increase of hemosiderin in the spleen, Kupffer cells, and tubular epithelia.

The chronic toxicity of NOH-4ClAA in Sprague Dawley rats was tested by Fischbach et al. (54). A group of ten rats (5 males, 5 females) was injected i.p. twice weekly for 16 weeks with a suspension of NOH-4ClAA in 0.25% agar, a second group received the same dose of 20 mg per kg s.c., and a third and a fourth group received i.p. or s.c. injections, respectively, of the corresponding amounts of agar-NaCl-solution only. After a 16 weeks' treatment, each rat had received a total dose of 640 mg, i.e., 3.5 mmol NOH-4ClAA per kg. Two years after the first injection the surviving 17 animals were sacrificed and the sites of application as well as the organs of these and of the animals which had died before examined microscopically, but no pathological changes due to the compound were detected.

4.2.4 3.4-Dichloroaniline and some derivatives.

We are not aware of published reports on the chronic toxicity of 3.4-Cl_2A and related compounds in animals.

Reports on incidents which have occurred during the production of 3.4-Cl_2A are available (106). In both cases the intoxicated workers showed severe and persistent chloracne, an effect attributed to 3.4.3'.4'-tetrachloroazoxybenzene (= TCAOB) and (or) -azobenzene. TCAOB was found to share with TCDD the ability to induce arylhy-

drocarbon hydroxylase and to cause chloracne, but it is not known, whether it has also hepatotoxic and mutagenic properties.

4.3 Carcinogenicity of polycyclic arylamines and their derivatives.

In the preceding section it was shown, that it is difficult to assess the carcinogenicity of marginally active compounds or even to significantly prove the inactivity of compounds. Such difficulties do not arise from active compounds. Thus, the first experiments to test the chronic toxicity of 2-AAF or 4-AAB and related compounds have unambiguously proved the carcinogenicity of these compounds, but in spite of 40 years of continued scientific interest the mechanism, by which they induce cancer, has not been elucidated yet.

It is accepted today that 2-AAF, 4-AAB, and certain other polycyclic N-arylacetamides, such as 2-acetylaminophenanthrene and trans-4-acetylaminostilbene, induce cancer after metabolic activation by N-hydroxylation and subsequent further activation as discussed in 4.1. However, not only the N-acetyl derivatives of 4-AB and 2-AF are carcinogenic, but also the arylamines themselves, their N-hydroxy derivatives, and the corresponding nitroso- and nitroarenes -as far as they have been tested. Since these compounds can be either N-hydroxylated or reduced *in vivo*, arylhydroxylamines constitute the common intermediate.

The ability of liver cytosol from the hamster, guinea pig, mouse and rat, but not from the dog, to N-acetylate 2-AF, 4-AB, and 2-naphtylamine *in vitro*, was demonstrated by Lower and Bryan (107). Rats fed 4-AB with the diet excreted the same metabolites in the urine as after ingestion of 2-AAF, NOH-4AAB being identified as a urinary metabo lite of 4-AB (39), and rats injected i.v. with NOH-2AF excreted mainly N-acetylated derivatives in the urine, indication for the N-acetylation in vivo of 4-AB and 2-AF and their hydroxy derivatives (108). Since the dog obviously cannot N-acetylate aromatic amines and derivatives, N-hydroxy metabolites of aromatic amines or their glucuronides are thought to produce bladder cancer in this species. In contrast, rats dosed with 4-AB did not develop bladder tumors, probably because it was N-acetylated in this species. Thus it is reasonable to explain the observed differences in tissue susceptibilites towards tumorigenesis by aromatic amines in various species with different active molecules, those which lack and those which have an N-acetyl group.

Gutmann et al.(9, 92) have demonstrated that N-deacylation of N-acyl-2-fluorenylhydroxylamines *in vivo* is a prerequisite for carcinogenicity, and that N-phenyl-2-fluorenylhydroxylamine, which cannot be metabolized to NOH-2AF *in vivo*, is inactive in the male and only

marginally active in the female rat. Since evidence has accumulated that polycyclic arylhydroxylamines are obligatory intermediates for the carcinogenicity of arylamines and N-acylarylhydroxylamines, it appears desirable to pursue the metabolic fate of these reactive compounds.

4.3.1 4-Aminobiphenyl and Some Derivatives.

4.3.1.1 4-Aminobiphenyl

The carcinogenic activity of 4-AB in rats, dogs, hamsters, rabbits, and mice after s.c.-injection or oral administration is well documented (71-77, 109), however, after skin-painting of mice, no tumors were observed (110). In 1955 4-AB was recognized as a strong occupational bladder carcinogen in humans and withdrawn from commercial use.

In 1954 4-AB was first demonstrated to produce bladder tumors in dogs after ingestion on 6 days a week for 33 months. This result was confirmed by Deichmann et al. in 1958. Search of the urine of dogs for metabolites of 4-AB gave 70-80% 4-amino-3-biphenylsulfate (and glucuronide), 5-10% N-glucuronide of NOH-4AB, appr. 10-15% of the free amine, and trace amounts of NOH-4AB and 4-nitrosobiphenyl. The N-glucuronide of NOH-4AB was suspected to be the active metabolite in the urinary tract and later shown to be mutagenic in the Ames test.

Rabbits given 4-AB orally developed bladder papillomas and carcinomas, the earliest carcinoma being observed four years after the first application. Rabbits injected i.p. with 100 mg 4-AB per kg excreted larger amounts of NOH-4AB into the urine (20%) than dogs after i.v.-injection of 80 mg 4-AB per kg (1.1%) (27).

Rats s.c. injected daily with 4-AB (total dose: 3.6.-5.8 g per kg) developed tumors of the mammary gland and of the intestines. The finding that rats fed 4-AB excreted the same metabolites into the urine as after application of 4-AAB is indication for the N-acetylation of this arylamine *in vivo* (Miller et al. 1961). 3 s.c.-Injections of 200 µg 4-AB into newborn mice produced hepatomas in 19/20 male, but only 4/23 female animals. In addition, 1 pulmonary adenoma, 1 thymic lymphoma, and 1 lymphosarcoma of the spleen were detected. Oral administration of 4-AB to mice (1 mg per mouse per week) for 38 weeks produced bladder carcinoma in 2/12 animals 90 weeks after the first dose. In an experiment with a different strain of mice, 1.5 mg 4-AB was orally administered per mouse per day for 52 weeks. Besides bladder carcinoma in 1/21 male animals, a higher frequency of hepatomas in both male and female mice was observed.

These results suggested that a correlation exists between the meta-

bolism of 4-AB *in vivo* and the tissue susceptibility towards tumorigenesis in so far, as a low extent of N-acetylation *in vivo* of 4-AB and its hydroxy derivatives favours the formation of bladder tumors (dogs, rabbits) and a large extent of N-acetylation favours the formation of mammary and intestinal carcinomas (rats).

4.3.1.2 N-Hydroxy-4-aminobiphenyl

NOH-4AB and certain ring-hydroxylated derivatives of 4-AB were tested in relation to 4-AB by Gorrod et al. (111) in newborn mice to implicate any of them as the proximate carcinogen of 4-AB. Three 200 µg doses of NOH-4AB per rat were injected s.c. on each of the first 3 days of life and the surviving animals sacrificed between 38 and 52 weeks after the first injection. 14/19 male and 3/33 female animals injected with NOH-4AB showed hepatomas and only 1 a generalized malignant lymphoma. In comparison: 19/28 male and 4/23 female mice injected with 4-AB were found with hepatomas, 1 with a pulmonary adenoma, with a thymic lymphoma, and 1 with a lymphosarcoma of the spleen. These results indicated, that 4-AB was the most active of the compounds tested, but that NOH-4AB was as active as the ring-hydroxylated compounds, thus, the attempt to implicate any of these compounds as the proximate carcinogen of 4-AB had failed.

Since rat skin was shown to be sensitive to tumorigenesis by topical application of certain carcinogens, the skin of Osborne-Mendel and Sprague Dawley rats was painted with 1.5 mg NOH-4AB per rat in acetone solution twice weekly for 52 weeks in relation to the effects of 4-AB and 4-nitrosobiphenyl. From the negative result obtained after an additional year of observation it was concluded, that the skin of these strains must be considered refractory to tumor induction by the dose applied. Boyland et al. (73) reported on results of 2 studies on the carcinogenic activity of NOH-4AB in stearic acid pellets implanted into the bladder of mice for 40 weeks. While the results of the first study were inconclusive, 6 bladder carcinomas and 1 adenoma or papilloma of the bladder were detected in the animals of the second study, yielding a tumor incidence of 13%.

4.3.1.3 4-Nitrosobiphenyl

Information on the carcinogenic activity of 4-nitrosobiphenyl (=4-NOB) is scanty. 4-NOB was tested for carcinogenic activity by Brill et al. (110) by topical application on the skin of Osborne-Mendel and Sprague Dawley rats in relation to the effects of 4-AB and NOH-4AB. The animals were painted twice weekly with 1.5 mg 4-NOB in

acetone solution for 52 weeks. After an additional year of observation, no tumors were detected. 4-NOB was shown to have mutagenic activity in the Ames test (112).

4.3.1.4 4-Nitrobiphenyl

4-Nitrobiphenyl was considered a moderately active carcinogen (72, 73, 75, 76, 78). Deichmann et al. (1958, 1965) reported on the effects of oral administration of 300 mg doses of 4-nitrobiphenyl to female mongrel dogs by capsule 3 times per week for life (total dose: 10 g per kg), i.e., 33 months. The effects consisted in carcinomas of the bladder epithelium in 2/4 animals. In the later study it was shown that a total dose of 0.77 g per kg did not suffice to induce tumors in 6 dogs within 3 years. Small amounts of non-acetylated N-hydroxy derivatives of 4-AB were detected by Radomski et al. (1973) in the urine of dogs and monkeys given a single dose of 5 mg 4-nitrobiphenyl per kg.

4.3.1.5 Ring-hydroxylated derivatives of 4-aminobiphenyl

Reports on the carcinogenic activity of ring-hydroxylated derivatives of 4-AB are conflicting (72, 73, 75, 77). Gorrod et al. (111) tested the carcinogenic activity of 3-hydroxy- and of 4'-hydroxy-4-aminobiphenyl by injecting newborn mice s.c. on each of the first 3 days of life with 200 μg doses of these two compounds. The incidence of hepatomas determined after 48 to 52 weeks was 12/19 male and 0/29 female mice injected with 3-hydroxy- and 12/18 male and 2/26 female animals injected with 4'-hydroxy-4-aminobiphenyl; in addition, 2 animals injected with 3-hydroxy-4-aminobiphenyl had pulmonary adenomas, and of the mice injected with 4'-hydroxy-4-aminobiphenyl, 1 had a pulmonary adenoma and 1 a generalized malignant lymphoma, indication for carcinogenic activity of these compounds which was lower than that of 4-AB.

Rats of both sexes fed a diet containing 1.62 mmol 3-hydroxy-4-aminobiphenyl per kg grain diet for 8 months, did not develop tumors, but 3/7 female animals were found with fibroadenomas of the mammary gland by 13 months (Miller et al 1956). Since such fibroadenomas were occasionally observed in control rats as well, this compound was classified as not active.

3-Hydroxy-4-aminobiphenyl and 4-amino-3-biphenylsulphate in paraffin wax pellets implanted into the bladders of mice were found to be active by 40 weeks, 9/38 and 10/32 animals, respectively, had bladder carcinomas (Bonser et al. 1956, 1963).

4'-Hydroxy-4-aminobiphenyl in paraffin wax pellets implanted into

the bladder of mice did not produce tumors by 40 weeks, but 4 squamous cell metaplasias of the bladder were found (Bonser et al. 1963).

4.3.1.6 4-Acetylaminobiphenyl and other N-acyl derivatives of 4-aminobiphenyl

The carcinogenic activity of 4-AAB was tested on rats of both sexes and on dogs by i.m.-injection, ingestion with the feed or by skin painting (71-73, 75, 76).

When 4-acetylaminobiphenyl was fed to rats of both sexes in a grain diet containing 1.62 mmol 4-AAB per kg diet for 8 months, the induction of adenocarcinomas of the mammary gland was observed in 19/21 or 10/10 female animals, of ear duct tumors only in 2/21 or 1/10 female animals, and of adenocarcinomas of the epithelium of the small intestines in none of the male, but in 2/21 or 0/10 female animals, and of no hepatomas (Miller et al. 1956). In an earlier report, Miller et al. (1954) observed 1 papillary intraductal cystadenoma besides adenomas of the mammary gland, which were described as quite malignant, 1 fibroadenoma of the mammary gland, 1 adenoma of the lung, and 1 leiomyosarcoma of the uterus. Other authors have detected bladder tumors in Wistar rats fed a diet containing 0.05% 4-AAB (Tuba et al. 1953) and sarcomas of the skin (Druckrey et al., unpublished). Miller et al. (1956) have compared the carcinogenic activity of 4-AAB and 2-AAF in the rat: 4-AAB was found as active as 2-AAF in the induction of adenocarcinomas of the mammary gland, but in the ear duct, small intestine, and liver, 4-AAB was much less active than 2-AAF, so that any generalization would have to be made separately for each tissue. Gutmann et al. (81) have tested the carcinogenic activity of N-4-biphenylylbenzamide and N-4-biphenylylbenzenesulfonamide injected i.p. into immature female Holtzman rats and found moderate (33% tumor incidence) or weak (17%) carcinogenic activity, respectively. Synthetic N-hydroxylation enhanced the toxicity and carcinogenicity of these compounds.

The metabolism of 4-AAB *in vitro* was studied with rabbit liver microsomes by Booth and Boyland (26). In the presence of KF, NOH-4AAB was the only metabolite, but in its absence, in addition NOH-4AB, 4-AB, 4-amino-3-hydroxy-, and 4-amino-4'-hydroxybiphenyl were formed. The metabolism of 4-AAB in dogs and rats was studied by Miller et al. (39). Dogs given 4-AAB by capsules excreted 7-20% of the dose as NOH-4AAB.

Continuous feeding of 0.04% 4-AAB in the diet to adult rats of both sexes for 12 weeks caused a decrease of the urinary excretion of NOH-4AAB from 3.9% of the 2nd week to 1.1% of the 8th week, just the opposite of what was observed with 2-AAF. Besides NOH-4AAB,

4-AAB and several other zones were noted on the paper chromatograms, which stained with phenol and diazo reagent but were not further investigated. Rabbits given a dose of 50 mg 4-AAB per kg p.o. excreted 7% as NOH-4AAB and 0.4% as glycolamide (52).

4.3.1.7 N-hydroxy-4-acetylaminobiphenyl and other N-acyl-4-biphenylhydroxylamines

The carcinogenic activity of NOH-4AAB was tested in relation to 4-AAB on rats by either the oral route or i.p.-injection or on mice by implantation of stearic acid or cholesterol pellets in the bladder (73, 75-77).

N-OH-4AAB was shown to be as active a mammary carcinogen as the parent 4-AAB in young female rats fed 0.043% NOH-4AAB in the diet for 16 weeks, frequencies being 14/19 versus 15/16, but it was more active in inducing ear duct carcinomas (6/19 versus 1/16) and forestomach papillomas than the parent 4-AAB. One of the rats with a forestomach papilloma also had a squamous-cell carcinoma of the forestomach. In contrast to the effect of 2-AAF or NOH-2AAF, neither 4-AAB nor NOH-4AAB showed hepatocarcinogenic activity.

Doses of 0.1 mmol NOH-4AAB or 4-AAB per kg in 0.9% NaCl-solution containing 1.75% gum acacia injected i.p. into adult male rats 3 times weekly for 5 months produced 1/4 ear duct tumor by 10 months or no tumors (0/4) by 11 months, respectively (Miller et al. 1961). These results indicated that NOH-4AAB exceeded its parent N-arylacetamide in its local effect at the site of application.

Gutmann et al. (81) tested the carcinogenic activity of N-hydroxy-4-biphenylylbenzamide and N-hydroxy-4-biphenylylbenzenesulfonamide in relation to the parent N-acylarylamine on immature female Holtzman rats. 12 Doses of 2.3 mg of either hydroxamic acid per kg body weight injected i.p. 3 times weekly for 4 weeks (total dose:.0.194 and 0.190 mmol per rat, respectively) produced 5/12 versus 1/11 intraperitoneal pleomorphic sarcomas and 8/12 versus 5/11 tumors of the mammary gland, respectively, by 12 months. This is indication for the enhanced carcinogenicity (tumor incidences given for comparisons; 33 versus 100% and 17 versus 55%) of the synthetic hydroxamic acids. The same authors compared the relative carcinogenicities of various fluorenyl- and 4-biphenylyl-acylhydroxylamines for the mammary gland of the immature female rat and found that those compounds in which the N-atom occupied a p- or extended p-postition relative to the system of conjugated double bonds induced predominantly malignant lesions.

The metabolism of NOH-4AAB *in vitro* was studied with the microsomal and supernatant fraction of rabbit liver homogenate(11, 25) and of

rat liver homogenate (11). Booth and Boyland (1964) detected 4-acetyl-lamino-3-hydroxybiphenyl in cytosolic incubates of NOH-4AAB, indication for an isomerizing enzyme activity, whose activity depended on NAD^+, NADH or NADPH. No metabolites were found in microsomal incubates of NOH-4AAB in the presence of KF, but in its absence, NOH-4AB, 4-AB, and 4-amino-3-hydroxybiphenyl were detected, indication for N-deacetylation and subsequent reduction or isomerization of the intermediary arylhydroxylamine. Rat liver microsomes were found to be less active than rabbit liver microsomes in N-deacetylating NOH-4AAB. Microsomal N-deacetylation of various N-arylacetohydroxamic acids was shown to be an enzyme-catalyzed reaction, since the formation of nitrosoarenes or bisazoxyarenes via arylhydroxylamines could be blocked by either paraoxon or omission of oxygen. Whereas the cytosolic fraction of rat liver homogenate was not very active in the reduction of mono- and polycyclic N-arylacetohydroxamic acids to N-arylacetamides, the corresponding rabbit liver preparation produced larger amounts of the polycyclic (4-AAB, 2AAF) than of the monocyclic (4-ClAA, PH) N-arylacetamides, indication for species and substrate differences in the reduction of N-arylacetohydroxamic acids (11).

Studies on the metabolism of NOH-4AAB *in vivo* have shown that continuous feeding of a diet containing 0.043% NOH-4AAB to adult male rats for several weeks caused a decrease in the urinary excretion of NOH-4AAB (as a conjugate) from 5% after 2 weeks to 2.1% after 8 weeks (Miller et al. 1961).

4.3.1.9 O-Acyl and -glucuronyl derivatives of N-hydroxy-4-acetyl-aminobiphenyl

From the observation that the benzoic ester of N-hydroxy-N-methyl-4-aminoazobenzene reacted at neutral pH with nucleophiles, such as methionine moieties, proteins, and nucleic acids *in vitro*, the concept was tested, that the esters of N-arylacetohydroxamic acids may also react with these macromolecules *in vitro*. Thus, N-acyloxy-N-arylacetamides were considered the ultimate carcinogens so long searched for.

Miller et al. (82) compared the carcinogenic activity of 4 polycyclic N-acetoxy-N-arylacetamides in male rats with that of the corresponding N-arylacetohydroxamic acids. They found that rats s.c.-injected with 15 weekly doses of 5 μmoles N-acetoxy-4-acetylaminobiphenyl in 0.2 ml trioctanoin developed 5/16 sarcomas at the injection site versus 0/16 with NOH-4AAB by 16 months. The local activity was found to decrease in the order 4-stilbenyl, 2-phenanthryl, 2-fluorenyl, 4-biphenylyl.

The sulphate ester of NOH-4AAB isolated in pure form, repeatedly

injected s.c. into rats (total dose; 350 μmoles per rat) had only low activity, 12% of the animals had sarcomas as compared to 63% with NOH-4AAB, Miller and Miller (113).

0-Glucuronides of N-arylacetohydroxamic acids as well as of arylhydroxylamines were considered transport forms of the carcinogens, from which the ultimate carcinogen is formed after deconjugation. No reports on the carcinogenic activity of the N-GlO-4AAB are available.

4.3.2 2-Aminofluorene and Some Derivatives

4.3.2.1 2-Aminofluorene

The carcinogenic activity of 2-AF in mice and rats is well documented (70-73). Mice or rats painted with benzene or acetone solutions of 2-AF, fed 2-AF in the diet for at least 10 months or injected s.c. or i.p., developed hepatomas, cholangiomas,mammary carcinomas, ovarian carcinomas, tumors of the ear duct, small intestines, and of colon, kidney and bladder carcinomas, fibrosarcomas of the kidney and bone, papillomas of the forestomach, epithelioma of the skin, pituitary adenomas, and lung adenomas. 2-AF in paraffin pellets implanted into the bladders of mice produced hyperplasia of bladder epithelioma (20/41) and inflammatory and(or) squamous metaplasia of bladder (21/41), however, no difference was observed from paraffin alone. In contrast, guinea pigs fed 2-AF in the diet, were refractory to the carcinogenic activity of 2-AF (72).

Miller et al. (1954) compared the carcinogenic activity of 2-AF, 2-AAF, and 2-nitrofluorene in Holtzman rats by feeding 1.62 mmol of each compound per kg diet for 8 months to animals of both sexes. By 10 months the tumor incidences of 2-AF and 2-AAF were the same, 2-nitrofluorene being much less active, but 2-AF showed a higher latent period. The hepatic tumors induced by 2-AF, 2-AAF, 2-nitrofluorene, and 7-fluoro-2-acetylaminofluorene had in common histological characteristics, a slow development, and a less malignancy as compared to those induced by azo dyes. The finding of Morris et al. (1950) that most of the skin tumors induced by 2-AF were not in the painted area, and that the other tumors appeared at sites distant from the site of application, was taken as evidence that 2-AF did not produce tumors itself, but after metabolic activation after absorption from the site of application. Differences in the tumor incidences between 2-AF, 2-AAF or 2-nitrofluorene, painted on the back or ingested by rats may be explained with differences in the efficacy of absorption and(or) differences in the availability of the ultimate carcinogen at the various sites of tumor development.

Enzymatic N-acetylation 2-AF *in vitro* by liver cytosol from various species was studied by Lower and Bryan (107). Of the 3 substrates (2-AF, 4-AB, and 2-aminonaphthaline) tested, 2-AF was the better substrate and the enzymatic activity increased in the order dog < rat < mouse < guinea pig < hamster. Uehleke reported the N-hydroxylation *in vitro* of 2-AF and of 4 other arylamines by rat liver microsomes (23). Booth and Boyland detected 7-hydroxy-2-aminofluorene as a metabolite of 2-AF in incubates with rat liver microsomes (29). The metabolism of (9-^{14}C)-2AF and (U-^{14}C)-4-ClA *in vitro* by rabbit liver microsomes was studied by McLean and Lenk (67). While hepatic microsomes from untreated animals produced more NOH-2AF (and its oxidation products) than NOH-4ClA, phenobarbital-treatment caused a larger increase of the N-hydroxylation of 4-ClA than of 2-AF, indication for different substrate affinities in the native and induced forms of cytochrome P-450 which catalyzed the N-hydroxylation of these arylamines.

The refractoriness of the guinea pig to orally administered 2-AF has stimulated Kiese et al. (114) to study the N-hydroxylation of 2-AF in guinea pigs *in vitro* and *in vivo*. Guinea pig liver microsomes N-hydroxylated 2-AF *in vitro*, 2-10 µg NOH-2AF per ml incubate being pro duced in 40 min. Following i.p.-injection of 100 mg 2-AF per kg, guinea pigs excreted 0.5% of the dose as NOH-2AF, whereas dogs and rabbits excreted 0.2 and 2.2%, respectively (27). Following i.p.-injection of doses of 25-500 mg 2-AF per kg into guinea pigs, NOH-2AF was detected only in the blood samples from the animals injected with the highest dose in trace amounts (0.1 µg per ml blood). These results indicated that, at least in the guinea pig, there is a relationship between the lack of carcinogenic and low ferrihemoglobinogenic activity, because the N-hydroxy derivative was so rapidly disposed, that only traces were found in the blood and urine (28).

No N-acetylated metabolites were detected in the urine of dogs following ingestion of 150 mg 2-AF or NOH-2AF in capsules (93). No data are available on the carcinogenic activity of 2-AF in the dog.

4.3.2.2 N-Hydroxy-2-aminofluorene

Reports on the carcinogenic activity of N-hydroxy-2-aminofluorene are available (73, 82, 115). Miller et al. (1964) detected peritoneal sarcomas in guinea pigs injected i.p. with NOH-2AF and Boyland et al. (1964) determined the tumor incidence of NOH-2AF implanted into the bladder of mice after a 40 weeks' exposition to be 28% for one and 6% for another group. Miller et al. (1969) compared the carcinogenic activity of NOH-2AF and its oxidation product, 2-nitrosofluorene (= 2-NOF) with that of NOH-2AAF, 2-AAF, and 2-AF in rats. The incidence of

sarcomas at the site of s.c.-injection was higher with NOH-2AF (5/17) and 2-NOF (10/17) than with 2-AAF or 2-AF (0/17,0/17) but lower or of the same size as that of NOH-2AAF (12/17). The local effect of NOH-2AF and 2-NOF is indication that they are at least proximate carcinogens as is NOH-2AAF, but possibly they are closer to the ultimate carcinogen than NOH-2AAF. Irving and Wiseman (1971) assessed the carcinogenic activity and systemic toxicity of NOH-2AF in relation to NOH-2AAF and the glucuronides of either compound in young female rats. Rats injected s.c. with 28 doses of 12.7 mmoles NOH-2AF per rat in tricaprylin for 9 weeks caused the death of 15/16 animals by 13 months as compared to 16/16 and 0/16 animals for NOH-2AAF and the O-glucuronide of NOH-2AAF, respectively. Local activity was evidenced by the appearance of sarcomas at the site of injection in 5/16 animals after 2 months (for comparison: NOH-2AAF: 10/16). No mammary tumors, but one ear duct carcinoma was found. These results indicated that NOH-2AF exceeded NOH-2AAF in its systemic toxicity and that its local activity was similar as that of NOH-2AAF, but that its activity at distant target organs was much weaker than that of NOH-2AAF, indication for an enhanced reactivity.

The metabolic fate of i.v.-injected (9-^{14}C)-NOH-2AF in male and female Fischer rats was investigated by Weisburger et al. (108). The identified urinary metabolites were mostly in the N-acetylated form and comprised NOH-2AAF as well as ring-hydroxylated derivatives of 2-AAF (7-, 5-, and 3-hydroxy). Following injection of NOH-2AF into dogs, no N-acetyl derivatives were detected in the urine (93).

4.3.2.3 2-Nitrosofluorene

Reports on the carcinogenic activity of 2-nitrosofluorene (= 2-NOF) are available (73, 75, 76). 2-NOF was tested for carcinogenic activity on rats by s.c.-injection (82, 116), i.p.-injection (81) or by oral administration (9, 117).

Of the female rats injected s.c. twice weekly for 8 weeks with 2.5 mg 2-NOF per rat in 0.2 ml tricaprylin (total dose: 0.2 mmol per rat), 10/20 animals developed sarcomas at the site of injection and 9/20 animals mammary carcinomas by 8 months (116). Of the male rats injected s.c. once weekly for 6 weeks with 2-NOF in 0.2 ml trioctanoin (total dose: 0.075 mmol per rat), 10/17 animals developed sarcomas at the injection site by 14 months (82).

A total amount of 0.082 mmol 2-NOF in 12 doses injected i.p. into each female Holtzman rat produced tumors of the mammary gland in 9/12 animals by 12 months (81). Whereas no tumors were observed after oral application by stomach tube of a total dose of 1.95 mmol

2-NOF per rat for 7.1 months (9), oral application of a total dose of 12.6 mmol 2-NOF per animal to Wistar rats of both sexes caused the development of tumors in 4/10 animals. Among them were 2 ear duct tumors, 1 hepatoma, 2 carcinomas of the forestomach, 1 adenocarcinoma in the intestines in males,and 1 skin carcinoma of a female animal (117). These results indicated that 2-NOF is an active carcinogen not only at the site of application, but also at distant sites, therefore 2-NOF was considered an "ultimate" carcinogen.

Kiese and Wiedemann (28) compared the rate of elimination of 2-NOF from the blood of guinea pigs with that of nitrosobenzene and 4-nitrosopropiophenone. When nitrosobenzene was infused at a rate of 0.5 mg per kg and min, the concentration of nitrosobenzene rapidly increased to 4-5 µg per ml blood and remained there for 1 h. In contrast, 2-NOF infused at rates of 0.25, 0.5, 1.0, and 1.5 mg per kg and min, as well as N-hydroxy-4-aminopropiophenone infused at rates of 0.1, 0.15, and 0.2 mg per kg and min, accumulated in the blood as long as the infusion lasted, but the rate of accumulation of 2-NOF increased 4-fold on doubling the rate of infusion, whereas the rate of accumulation of 4-nitrosopropiophenone increased even 10-fold on doubling the rate of infusion. These results indicated that, due to a rapid elimination of nitrosobenzene from the blood, the steady state concentration was maintained at a rather low level, whereas with 2-NOF and 4-nitrosopropiophenone, the rate of disappearance from the blood was so slow, that steady state concentrations could not be reached with the 4 infusion rates, indication for either a very low capacity of metabolism or a very high binding capacity of the blood for these two compounds. The same authors found that, although 2-NOF rapidly disappeared from suspensions of guinea pig red cells, only small amounts of 2-AF were recovered. This is indication for either an as yet unknown metabolic pathway or high binding capacity of 2-NOF to erythrocytes. Evidence for both high binding capacity of plasma and of erythrocytes for NOH-2AAF and its metabolites was presented by Weisburger et al. (32) who studied the transport of chemical carcinogens by blood.

4.3.2.4 2-Nitrofluorene

The carcinogenic activity of 2-nitrofluorene in the rat was tested either by the oral route or topically by painting the scapular region of the skin (70, 71). The report of Morris et al. (1950) indicated that of the rats fed 0.05% 2-nitrofluorene in the diet for 23 weeks (total dose: 756 mg per rat), 1/9 had a mammary and 1/9 an ear duct carcinoma by 44 weeks. 6/10 Rats painted with 2% 2-nitrofluorene in acetone (total dose: 69 mg) were found with the following pathological findings by 80

weeks: mammary and pituitary adenomas, lung lymphosarcomas, subcutaneous fibroma, and anaplastic carcinoma of the salivary gland. Miller et al. (1955) reported on a higher tumor incidence with Holtzman rats of both sexes fed higher doses of 2-nitrofluorene (1.62 mmol per kg diet) for 8 months. 4/9 Mammary gland tumors and in male rats 1/9 liver tumor, 1 tumor of the small intestines, and 1 adenocarcinoma of the lung were detected, 2 of 2 females and 5 of 7 males examined were found with papillomas of the forestomach, indication for local activity at the site of application. 2-Nitrofluorene was found less active in this experiment than 2-AAF or 2-AF in the production of tumors at various sites. To confirm the results of the first experiment, 20 male rats were fed 1.62 mmol 2-nitrofluorene per kg of diet for 12 months: 17/18 of the surviving rats had squamous-cell carcinomas in the forestomach, characterized as malignant. In addition, 13/18, 4/18, 2/18, and 1/18 animals had tumors of the liver, ear duct, small intestine, and mammary gland, respectively, by 12 months.

4.3.2.5 Ring-hydroxylated derivatives of 2-aminofluorene

Among the 8 possible ring-hydroxylated derivatives of 2-AF, only 2-amino-1-, -3-, -5-, and -7-hydroxyfluorene have been tested for carcinogenic activity on mice by the bladder implantation test (73). 2-Amino-1- and -7-hydroxyfluorene were tested in cholesterol pellets in an experiment of 40 weeks' duration. Bladder papillomas and carcinomas are found in animals treated with either compound (Bonser et al. 1963). 2-Amino-1-, -3-, -5-, and -7-hydroxyfluorene hydrochloride were tested in paraffin pellets in an experiment of 40 weeks' duration. All four aminofluorenols were found to produce hyperplasia of bladder epitheliomas, inflammatory and (or) squamous metaplasia of bladder, bladder papillomas, -carcinomas, and adenocarcinoma of thigh. Results obtained with the implantation method are in doubt, since no difference from paraffin alone was observed (Irving et al. 1963).

Information on ring-hydroxylated derivatives of 2-AF found in either microsomal incubates of or as urinary metabolites of 2-AF, 2-AAF or NOH-2AAF is scanty. 2-Amino-7-hydroxyfluorene was detected in microsomal incubates with 2-AF (29). In the absence of KF, 2-amino-7- and -1-hydroxyfluorene were detected as metabolites *in vitro* of NOH-2AAF and the latter compound as a metabolite *in vitro* of either 2-AF or NOH-2AF (26). Weisburger et al. (108) determined the amount of 2-amino-7-hydroxyfluorene in the urine of rats i.v.-injected with 10 mg (9-^{14}C)-NOH-2AF per kg to be 0.9 (males) or 2.3 (females)% of dose. Weisburger et al. (118) isolated substantial amounts of 2-amino-7-hydroxyfluorene either conjugated or free from the urine of rats inject-

ed with 10 mg (9-^{14}C)-NOH-2AAF per kg by means of paper chromatrography.

4.3.2.6 2-Acetylaminofluorene and other N-acyl derivatives of 2-aminofluorene

A vast number of reports on the carcinogenicity of N-acyl derivatives of 2-AF in various species is available (70-76), the majority dealing with 2-acetylaminofluorene. The following N-acyl derivatives of 2-AF were tested: formyl, glycyl, acetyl, fluoroacetyl, trifluoroacetyl, diacetyl, succinyl, benzoyl, o-phthalyl, benzenesulfonyl, and p-toluenesulfonyl. Of these, the benzamide, benzenesulfonamide, and p-toluenesulfonamide were inactive or weakly active in rats, because they are not N-hydroxylated *in vivo*. Since the other N-acyl derivatives of 2-AF produced similar effects in rats as did 2-AAF, it is sufficient to discuss only 2-AAF.

The carcinogenic properties of 2-AAF were tested in the following animal species, routes of administration given in brackets: rat (s.c., p.o., i.p., intratesticular, intracecal, intragastrical, intratracheal, intubation, implantation in both lobes of the thyroid gland or in the left interior bronchus), mouse (skin painting, p.o., s.c., intragastrical), rabbit (p.o., i.p.), dog (p.o.) cat (p.o.) guinea pig (skin painting, p.o., i.p., s.c.), hamster (p.o., i.p.), steppe lemming (intragastrical, implantation s.c., topically interscapular), fowl (white Leghorn) (injected into crop), frog (p.o.) tadpole (in water), salamander (s.c.), and monkey (p.o).

The guinea pig and steppe lemming proved to be refractory to the carcinogenic activity of 2-AAF, apparently because it is not N-hydroxylated *in vivo*.

Male rats fed 1.62 mmol 2-AAF per kg diet for 8 months, developed hepatic tumors (malignant hepatomas and cholangiomas) 24/26, ear duct tumors 11/26, and tumors of the small intestines 13/26 by 10 months. Female rats had no liver tumors, but adenomas of the mammary gland 22/27, ear duct tumors 19/27, adenocarcinomas of the small intestines 6/27, and epidermoic cysts with basal- and squamous cell elements of the roof of the mouth 1/27.

Rabbits fed 70 mg 2-AAF daily for 56 weeks showed hyperplasia and squamous metaplasia of the epithelium of the urinary tract 10/19, tumors of the urinary tract 3/19, and 1 adenocarcinoma of the uterus. Of the rabbits injected i.p. with 30 mg 2-AAF per kg for 40 weeks, 2/14 had tumors in the abdominal wall 15 cm from the site of injection, 1 had a carcinoma of the urinary bladder, but no peritoneal tumors were detected.

Dogs fed 8.3-10.9 mg 2-AAF in the diet (total dose: 90-200 g) deve-

loped hepatocarcinomas, bladder papillomas, and adenomas of the ovary or pancreas by 68-90 months. In another test of 91 months, 2-AAF was given orally to dogs. 4/5 Animals had hepatic and urinary cystic tumors.

These results indicated species-dependent organ susceptibilities of 2-AAF. According to the mechanism of chemical carcinogenesis accepted today, differences in organ susceptibilities should reflect differences in the metabolism of the causative agent, but a convincing model has not yet been developed. Since 2-AAF has only weak local activity, metabolic activation was recognized as a mandatory step. N-Hydroxylation was found to produce a molecular species which exceeded the parent N-arylacetamide by far in its local and systemic activity and which was considered therefore a proximate carcinogen.

Reports of several investigators on the metabolism of 2-AAF *in vitro* and *in vivo* have shown that 2-AAF is N-hydroxylated in those species, in which it is carcinogenic, thus differences in the formation, distribution, metabolization, and elimination may explain the differences in organ susceptibilities in various species. The urinary excretion of NOH-2AAF as a glucuronide by rats increased with continued administration of a diet containing 0.03% 2-AAF from traces in the first days to 10-15% after 6 weeks (Miller et al. 1960). By the 3rd day the urinary NOH-2AAF excreted by rabbits fed daily doses of 70 mg 2-AAF for 371 days had increased from 13% to 30% and remained there for at least 32 days and there after decreased to 10-20% for the remaining period (Irving 1962). These results indicated that the glucuronide of NOH-2AAF was the major urinary metabolite of 2-AAF in the rat as well as in the rabbit, but they did not give an explanation for the susceptibility of the epithelial lining of the urinary tract of the rabbit to 2-AAF carcinogenicity.

But 2-AAF is also ring-hydroxylated, the 1-, 3-, 5-, 7-, 8-, and 9-hydroxy derivatives being found in microsomal incubates or in the urine of various species. A recent report has shown, that these monoxy derivatives of 2-AAF undergo secondary oxygenation to form monohydroxy derivatives of 2-acetylaminofluoren-9-one (= 2-AAF-9-one) as well as dihydroxy derivatives of unknown structure (119). In addition, N-deacetylation and hydroxylation of the acetic moiety can occur, so that a complex mixture of metabolites is formed, which has resisted so far any complete qualitative and quantitative analysis.

Ring-hydroxylation of 2-AAF in 9-position produces 9-hydroxy-2-acetylaminofluorene (= 2-AAF-9-ol), and its oxidation 2-AAF-9-one, two compounds, which have been tested for carcinogenic activity on the rat and found to be active (71, 72, 75, 76). Rats of both sexes, fed 1.62 mmoles of 2-AAF, 2-AAF-9-one or 2-AAF-9-ol per kg of diet for 8 months, developed tumors at 4 different sites, incidences given in brack-

ets in the same order: liver (males: 24/26, 4/9, 2/9); mammary gland (22/27, 6/9, 1/9); ear duct (females: 19/27, 1/9, 0/9); small intestine (males: 13/26, 1/9, 0/9) by 10 months. These results indicated, that hydroxylation of C-9 decreased the carcinogenic activity of 2-AAF at the four target organs tested, and that 2-AAF-9-one was as active as 2-AAF only on the mammary gland, but less active on the liver, ear duct, and small intestines. An interesting correlation is established by comparison of the carcinogenicity and the hydrosolubility of these compounds: whereas the carcinogenicity decreased in the order 2-AAF, 2-AAF-9-one, 2-AAF-9-ol, hydrosolubility and molecular thickness increased in the same order (75).

There is evidence that 2-AAF-9-one was N-hydroxylated *in vitro* by hepatic microsomes from rabbits repeatedly injected i.p. with 3-methylcholanthrene (120).

4.3.2.7 N-Hydroxy-2-acetylaminofluorene and other N-acyl-2-fluorenylhydroxylamines

The carcinogenic activity of N-hydroxy-2-acetylaminofluorene and of its chelates with Co^{2+}, Cu^{2+}, Fe^{3+}, Mn^{2+}, Ni^{2+}, and Zn^{2+} even in those species which are refractory to the effect of the parent N-arylacetamide was established by a large number of reports (73, 75, 76). In contrast to the only marginally local activity of 2-AAF, NOH-2AAF produced tumors at the site of application, such as carcinoma of the forestomach in the mouse, rat, and hamster following oral application, peritoneal sarcomas in rats, rabbits, and guinea pigs following i.p.-injection, bladder tumors in mice following implantation into the urinary bladder, sarcomas at the site of s.c.-injection into rats, and malignant skin tumors at the site of painting. The metal chelates of NOH-2AAF were much more effective in inducing sarcomas at the injection site than was NOH-2AAF itself, probably due to a prolonged exposition time of the surrounding tissue.

Based on equimolar doses, NOH-2AAF was more toxic to the rat and the rabbit than 2-AAF when fed in the diet or injected i.p., but NOH-2AAF injected i.p. produced more extensive changes than given orally. The enhanced toxicity of NOH-2AAF was manifested by a greater weight loss, a lesser gain,and a higher mortality: following i.p.-injection of 223 μmoles NOH-2AAF or 2-AAF per kg as aqueous suspensions 3 times at 48 h intervals into male rats, 4/16 rats injected with NOH-2AAF died within 48 h after the 1 st injection and 5 more within the next week; in contrast, none of the 13 rats injected with 2-AAF died during this time.

As to the systemic carcinogenic activity of orally given NOH-2AAF in

the rat, malignant hepatomas, squamous-cell carcinomas of the ear duct, and adenocarcinomas of the small intestines were as frequent as with 2-AAF, but in contrast to the rats fed 2-AAF, the animals showed a latent period in the development of mammary tumors. However, NOH-2AAF injected i.p. produced more squamous-cell carcinomas of the ear duct (8/29) and adenocarcinomas of the small intestinal epithelium (6/29) than did i.p.injected 2-AAF (2/24, 1/24).

Liver damage was more extensive in male rats injected i.p. with NOH-2AAF, but the incidence of mammary tumors (at 4 months) was not higher than with 2-AAF.

Of the 42 rabbits given 2-AAF, 5 (12%), but only 1/31 (3%) given NOH-2AAF orally or i.p. developed carcinoma of the bladder; one of the animals injected i.p. with NOH-2AAF was found with a sarcoma in the wall of the cecum and one with an ear duct tumor. NOH-2AAF was classified as being more toxic and carcinogenic for the rabbit at the site of injection as was 2-AAF (Irving et al. 1966).

Similar conclusions were drawn by Miller et al. (1964) for the effects of NOH-2AAF in mice, hamsters, and guinea pigs. However, as was the case with rabbits, mice were more susceptible to the effect of 2-AAF on the epithelium of the urinary tract than NOH-2AAF, 28% of the animals fed 2-AAF, but only 18% fed NOH-2AAF had papillomas or carcinomas of the urinary bladder.

Guinea pigs fed NOH-2AAF (0.048 or 0.034% in the diet) did not develop tumors of the stomach, but died of carcinoma of the small intestines between the 17th and 28th month. NOH-2AAF injected i.p. produced severe chronic peritonitis with extensive fibrous adhesions, which caused the death of the animals.

Extensive peritonitis with and without ascites associated with peritoneal sarcomas observed in hamsters following i.p.-injection of NOH-2AAF, caused the death of the animals.

Whereas 3-methylcholanthrene protected rats against tumor development by 2-AAF (Miller et al. 1958), it did not protect rats against the effects of NOH-2AAF when fed in the diet (0.032%) for 12 weeks (Miller et al. 1961). 3-Methylcholanthrene, known to stimulate the N- and ring-hydroxylation *in vitro* of 2-AAF by rat, hamster, mouse, and rabbit liver microsomes (48), decreased the urinary excretion of NOH-2AAF and of 7-hydroxy-2-acetylaminofluorene, but increased the excretion of 3- and 5-hydroxy-2-acetylaminofluorene.

In order to test the hypothesis that 2-acetylaminofluorene-N-sulphate (= 2-AAF-N-sulphate) is an ultimate carcinogenic metabolite of 2-AAF in rat liver, male rats were injected i.p. with 50 mg NOH 2AAF per kg and 1 M Na_2SO_4, which caused an increase in toxicity (13/15 versus

9/15 animals dead) and an increase of liverbound 1- and 3-(methion-2-yl)-2AAF (121).

Studies on the metabolism of NOH-2AAF *in vitro* by the microsomal and cytosolic fraction of rat and rabbit liver have shown that NOH-2AAF is N-deacetylated enzymatically, reduced to 2-AAF or isomerized to yield 1- and 3-hydroxy-2-acetylaminofluorene (10, 11,122). The metabolism of NOH-2AAF in the rat has shown, that the same metabolites were found in the urine as after administration of 2-AAF,, namely NOH-2AAF, 2-AAF, 1-, 3-, 5-, and 7-hydroxy-2-acetylaminofluorene, except, that the amount of NOH-2AAF (as a glucuronide) was much higher (51, 118). However, due to improved chromatographic techniques, in addition small amounts of 8-hydroxy-2-acetylaminofluorene and of secondary oxygenation products were found in the urine of rats injected i.p. with a mixture of NOH-2AAF and (9-^{14}C)-2AAF (119, 120). 3-Methylthio-2-fluorenylacetamide was described by Weisburger et al. (32) as a metabolite of NOH-2AAF in the plasma of rats.

Other synthetic N-acyl- or N-aryl- derivatives of NOH-2AF which have been tested for carcinogenic activity were: N-hydroxy-2-fluorenylbenzamide (= NOH-BAF) (92, 81) N-hydroxy-2-fluorenylbenzenesulfonamide (= NOH-2FBS) (81, 9), and N-phenyl-2-fluorenylhydroxylamine (9).

Whereas 2-fluorenylbenzamide was found to be only slightly active in rats following i.p.-injection of 45 mg per kg 3 times weekly for 4 weeks (a total dose of 0.2 or 0.31 mmol per rat) by 20 or 24 weeks, its N-hydroxy derivative (= NOH-BAF) was very active when injected i.p. at a total dose of 0.15 or 0.21 mmol per rat. NOH-BAF was quite toxic to rats as was NOH-2AAF and produced fast growing peritoneal sarcomas in 8/11 female and 6/6 male rats. In addition, adenocarcinomas of the mammary gland were observed in 3/8 animals.

Whereas N-2-fluorenylbenzenesulfonamide (= 2-FBS) and -p-tosylsulfonamide (123) were found to be weakly active or inactive, the synthetic N-hydroxy derivative (= NOH-2FBS) gave the same tumor incidence of 100% in immature female and male Holtzman rats as did NOH-2AAF (81). Female rats injected i.p. with a total dose of 0.085-0.154 mmol NOH-2FBS per rat developed peritoneal sarcomas, tumors of the mammary gland, one subcutaneous squamous cell carcinoma, one metastatic adenocarcinoma of the lung,and one renal cell carcinoma by 12 months. Of the male rats injected i.p. with a total dose of 0.057 mmol NOH-2FBS per rat, one had a tumor of the mammary gland and 7 had tumors at other sites, such as skin, small intestines, abdominal cavity, and pancreas. But NOH-2FBS was also toxic to rats and caused a large number of premature deaths. A total dose of 0.49 or 2.41 mmol NOH-2FBS per male rat given orally for 2-7 months, gave tumor

incidences of 40 or 60%, respectively, suggesting, that NOH-2FBS was less active when given p.o. All male rats showed preneoplastic changes of the liver and one a tumor of the mammary gland by 12 months. These results indicated that neither the toxicity nor the carcinogenic activity were affected on substitution on the acetyl for the benzoyl or benzenesulfonyl group in NOH-2AAF.

Studies on the metabolic fate of NOH-BAF, ^{14}C-labeled at the α-C-atom of the benzoyl moiety, and of a mixture of NOH-2FB^{35}S and NOH 2-(9-^{14}C)-FBS in the rat have shown that 5% NOH-BAF was N-deacylated and that NOH-2FBS was rapidly and extensively N-deacylated by the mammary gland, liver, and kidney, the first two being target tissues for the compounds. Thus, as one prerequisite for carcinogenic activity of N-acylarylhydroxylamines was recognized the N-deacylation, which yielded the obligatory intermediate NOH-2AF and(or) 2-NOF. But a total dose of 1.95 mmol 2-NOF per rat given orally to male rats for 6-7 months, was inactive by 12 months. However, half that dose of 0.082 mmol 2-NOF injected i.p. into female rats, gave a tumor incidence of 82% by 12 months. N-Phenyl-2-fluorenylhydroxylamine, which cannot be N-deacylated, displayed only weak activity in the female and was inactive in the male rat after i.p.-injection.

As another prerequisite for carcinogenic activity was recognized the position of the N-atom and the size of the aryl moiety of various N-acylarylhydroxylamines, since the carcinogenicity decreased in the order NOH-2AAF> NOH-3AAF > NOH-4AAF (124).

4.3.2.8 Ring-hydroxylated derivatives of 2-Acetylaminofluorene

Of the ring-hydroxylated derivatives of 2-AAF with phenolic character, 1- and 7-hydroxy-2-acetylaminofluorene were tested for carcinogenic activity on rats (70, 73). Rats were either fed 0.07% 7-hydroxy-2-acetylaminofluorene in the diet (Hoch-Ligeti 1947) or injected i.p. with 45 mg per kg in gum acacia (0.9% NaCl) 3 times a week for 4 weeks (92). No animals with tumors were found by 71 or 34 weeks, respectively, but in the animals of the first experiment retrobulbar hepatomas and 2/35 adenomas in lungs were found. However, synthetic N-hydroxylation of 7-hydroxy-2-acetylaminofluorene restored the carcinogenic activity in rats, another proof for the significance of N-hydroxylation for carcinogenic activity. Doses of 40 mg1-hydroxy-2-acetylaminofluorene per kg in 0.4 ml gum acacia (injected i.p.) 3 times a week into female rats did not induce mammary tumors by 23 weeks (Miller et al. 1961). No tumors likewise were found by 34 weeks in female rats injected i.p. 3 times a week for 4 weeks with 45 mg of 1- or 3-hydroxy-2-benzoylaminofluorene per kg(92).

These results indicated that the hydroxylation of aromatic C-atoms of the fluorene nucleus effected either by a direct insertion reaction or by isomerization of the N-hydroxy derivative completely or mostly abolished the carcinogenic activity of 2-AAF and N-hydroxy-2-benzoylaminofluorene, but that synthetic N-hydroxylation restored it.

4.3.2.9 O-Acyl and -glucuronyl derivatives of N-hydroxy-2-acetylaminofluorene and of other N-acyl-2-fluorenylhydroxylamines

Searching for the ultimate carcinogen of 2-AAF, the following O-acyl derivatives of NOH-2AAF have been tested for carcinogenic activity in the rat either by s.c.-injection or oral administration: N-acetoxy-2-acetylaminofluorene (= N-AcO-2AAF) (8, 82), N-benzoyloxy-2-acetylaminofluorene (82), N-myristoyloxy-2-acetylaminofluorene (= N-MyO-2AAF) (8), 2-Acetylaminofluorene-N-sulphate (2-AAF-N-sulphate) (8, 82), and the O-glucuronide of NOH-2AAF (= N-GlO-2AAF) (115), its Na or Cu salts (82, 125), and its triacetyl methyl ester (125). In addition, the following O-acyl derivatives of NOH-2AF with other N-acyl groups than acetyl were tested: N-acetoxy-2-fluorenylbenzamide (= N-AcO-2BAF) (81), N-acetoxy-N-myristoyl-2-aminofluorene (= N-AcO-2MyAF) (8), and N-myristoyloxy-N-myristoyl-2-aminofluorene (= 2-MyO-2MyAF) (8).

It was found that s.c.-injection of a total dose of 0.075 mmol of N-AcO-2AAF or N-benzoyloxy-2-acetylaminofluorene into male rats produced more sarcomas at the site of injection than after injection of equimolar doses of NOH-2AAF, and that the enhanced toxicity of the latter compound caused the death of 5/17 animals. These enhanced toxicities and carcinogenicities were found to correlate well with their high reactivities and mutagenicities for transforming DNA (82, 126). A total dose of 3.75 mmol N-AcO-2BAF per rat given orally for months, exceeded NOH-2BAF and 2-BAF in its toxicity in so far, as it caused the death of 6/10 animals by 7 months (versus 0/12 and 0/12, respectively), but gave a tumor incidence of 20% only compared with 75% and 0% for NOH-2BAF and 2-BAF, respectively (81).

Bartsch et al. (8) tested the effect of enhanced lipophilicity of long-chain N,O-bis acyl derivatives of NOH-2AAF on the carcinogenic activity *in vivo*, on the reactivity *in vitro* with methionine and the DNA of cultured human fibroblasts, and on the mutagenicity.

Of the six groups of male rats injected s.c. with a total amount of 64 μmoles per rat of NOH-2AAF, N-AcO-2AAF, N-MyO-2AAF, N-AcO-2MyAF, N-MyO-2MyAF or NOH-2MyAF in divided doses, the four groups injected with the long-chain N- and(or) O-acyl substituents

showed a high incidence of sarcomas at the site of injection by 5 months, incidences given in the same order: 1/18, 0/18, 18/18, 15/18, 12/18, and 14/18, respectively. An expression of the enhanced toxicity of these higher lipophilic compounds was the high mortality, which caused the death of all animals by 9 months. Of the five N-, O-acyl derivatives of NOH-2AF tested for reactivity with nucleophiles, N-AcO-2AAF was the most reactive and N-MyO-2MyAF the least reactive compound.

2-AAF-N-sulphate, which has been implicated as an ultimate carcinogen of 2-AAF because of its high reactivity *in vitro* with nucleophiles was tested for carcinogenic activity on male rats (8, 82). Since 2-AAF-N-sulphate is a very labile compound and rapidly decomposed in aqueous medium ($t_{1/2}$ of less than a minute) (126), only a 70% pure preparation was available for the s.c. injections. A total amount of 100 μmoles 2-AAF-N-sulphate per rat in divided doses was neither toxic nor did it produce any sarcomas at the injection site, but doses of 200 and 400 μmoles per rat caused the death of 6/16 and 6/16 animals, respectively, indication for moderate toxicity, but only weak carcinogenic activity.

Since the O-glucuronide of NOH-2AAF, being the main urinary metabolite of 2-AAF as well as NOH-2AAF in several species, was also considered an ultimate carcinogen of 2-AAF because of its "esterlike" structure and reactivity *in vitro* with nucleophiles, it was also tested on rats (82, 115, 125).

Female rats injected s.c. with 0.2 μmoles of the sodium or cupric salt or the triacetyl methyl ester of N-GlO-2AAF in trioctanoin per rat in divided doses for 8 weeks survived for a longer time and developed less tumors than the animals injected s.c. with an equimolar dose of NOH-2AAF. By 14 to 15 months 4/16 rats injected with the triacetyl methyl ester of N-GlO-2AAF and 1/16 rat in each group injected with either the Na or Cu salt of N-GlO-2AAF developed sarcomas at the injection site. For comparison: by 12 months 9/16 animals injected with NOH-2AAF had developed sarcomas at the injection site, indication for an unexpectedly low local activity. As for the systemic carcinogenicity, 1/16 animals injected with the Cu salt of N-GlO-2AAF had developed a mammary tumor by 12 months.

Similar results were reported on by Irving and Wiseman (115). Female rats injected s.c. with 28 doses of 12.5 to 12.7 μmoles of N-GlO-2AAF per rat within 9 weeks either in 0.9% NaCl solution or in tricaprylin responded differently by 13 months: whereas N-GlO-2AAF in 0.9% NaCl solution gave a tumor incidence of 8/16, N-GlO-2AAF in tricaprylin gave a value of 0/16 as compared with 15/16 and 16/16 for NOH-2AAF in either 0.9% NaCl solution or in tricaprylin, respectively. In addition, 1 animal had a sarcoma at the injection site, 4 a mamma-

ry, and 3 ear duct tumors. These results did not support the idea, that the O-glucuronide is an ultimate carcinogen of 2-AAF. The reason for its unexpectedly low toxicity and carcinogenic activity as compared to the esters of NOH-2AAF can partly be explained with its structure as an acetal, which lacks the -I-effect of the carbonyl group of the esters.

5. CONCLUSIONS

1.There is evidence that N-hydroxylation is the sufficient prerequisite for the ferrihemoglobinogenic activity of arylamines *in vivo*, but the reactivity of the endogenously formed arylhydroxylamine may limit its activity as is the case with NOH-2AF.

2.N-arylacetohydroxamic acids which catalyze the oxidation of $HbFe^{2+}$ by the dioxygen species of oxyhemoglobin *in vitro*, are oxidatively or (and) enzymatically N-deacetylated *in vivo* to yield nitrosoarenes and(or) arylhydroxylamines which are the actual active molecules *in vivo*, since nitrosoarenes are rapidly reduced to arylhydroxylamines.

3.There is abundant evidence that N-hydroxylation of carcinogenic aryl amines and N-arylacetamides is the initial metabolic activation for the initiation of carcinogenesis. Since carcinogenic arylhydroxylamines, such as NOH-2AF, are N-acetylated and NOH-2AAF in turn is N-deacetylated in the rat, the further metabolic activation probably proceeds via NOH-2AAF. However, since the dog cannot N-acetylate arylamines or arylhydroxylamines, there appears to exist a still unknown metabolic pathway to the ultimate carcinogen, which may be relevant also for other species, since there is a species variation of organ susceptibilities to the carcinogenic activity of 2-AF, 2-AAF,and NOH-2AAF. Because NOH-2AAF displayed enhanced carcinogenic activity even at the local site of application as compared to 2-AAF, it was named a proximate carcinogen, although its reactivity *in vitro* was only low.

4. On the search for a derivative of NOH-2AAF with enhanced carcinogenicity, especially at the site of application, and reactivity *in vitro* with nucleophilic cell components, the concept of N-hydroxyesterification was developed. It was assumed that the final reactive molecule (= the ultimate carcinogen) arises from esterification *in vivo* of the N-arylacetohydroxamic acid at those sites which are susceptible to the carcinogenicity of NOH-2AAF. Thus, 2-AAF-N-sulphate was thought to arise from NOH-2AAF and PAPS by sulphotransferase activity located in liver cytosol. The high NOH-2AAF sulphotransferase activity and the high susceptibility to hepatocarcinogenesis by NOH-2AAF of male rat liver supported this idea. The female rat and other species tested which have lower NOH-2AAF sulphotransferase activity in the liver, are therefore not susceptible to hepatocarcinogenesis(127).

The synthetic O-acetate, -benzoate, and -sulphate of NOH-2AAF rapidly reacted with various nucleophiles *in vitro* and *in vivo*, but only the first two compounds showed enhanced carcinogenicity, 2-AAF-N-sulphate being only weakly active at the site of application, although large amounts of protein-bound 1- and 3-methionyl derivatives of 2-AAF were found at or near the site of injection. The short half life of 2-AAF-N-sulphate of less than 1 min may explain the hepatocarcinogenicity of NOH-2AAF in the male rat and its reaction with tissue proteins, but cannot explain the lack of local activity, if the extent of protein-bound methionyl-2AAF is related to carcinogenic activity. Because of its short life-time and ionic character it was also thought unlikely that 2-AAF-N-sulphate can penetrate cell membranes and alkylate the RNA and DNA of the nucleus, adduct-formation with the guanine moieties (128) thought to be the critical step for the initiation of carcinogenesis. If it is true that esterification with sulfuric acid at those sites, which are susceptible to the carcinogenic effects of NOH-2AAF, then the mammary and Zymbal's gland as well as the subcutaneous tissue are expected to display NOH-2AAF sulphotransferase activity too, but none was detected (129).

5.This is indication that another mechanism of activation of NOH-2AAF may function in these tissues, such as enzymatic or oxidative N-deacetylation, by which a cascade of rapid reactions is initiated which results in the production of insoluble derivatives of 2-AF. Such an hypothesis is supported by the finding of Gutmann et al.(9), that the mammary gland of the female rat had a higher NOH-2FBS N-arylamidase activity than the liver. Druckrey has pointed out that implanted foils of various polymers or cross-linking of nucleoproteins by bifunctional agents like p-benzoquinone can also initiate carcinogenesis (86).

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References

1 SMITH, P.K., Acetophenetidin, a critical bibliographic review. Interscience Publishers, New York, London, 1958.
2 BAADER, H., GIRGIS, S., KIESE, M., MENZEL, H. and SKROBOT, L., Der Einfluß des Lebensalters auf Umsetzungen von Phenacetin, p-Phenetidin, N-Acetyl-p-aminophenol, p-Aminophenol und Anilin im Hunde. *Naunyn-Schmiedeberg's Arch. exp.Path.Pharmakol.*, **241**, 317 (1961).
3 BÜCH, H., HÄUSER, H., PFLEGER, K. and RÜDIGER, W., Über die Ausscheidung eines noch nicht beschriebenen Phenacetin Metaboliten beim Menschen und bei der Ratte. *Naunyn-ryn-Schmiedeberg's Arch.exp.Path.Pharmakol.* **253**, 25 (1966).
4 MARTINDALE, The Extra Pharmacopoeia, 27th edition, London, 1977.
5 HJELM, M., RAGNARSSON, B. and WISTRAND, P., Biochemical effects of aromatic compounds. III. Ferrihaemoglobinaemia and the presence of p-hydroxy-o-toluidine in human blood after the administration of prilocaine. *Biochem.Pharmacol.*, **21**, 2825 (1972).
6 HLAVICA, P., Biological oxidation of nitrogen in organic compounds and disposition of N-oxidized products. In: CRC Critical Reviews in Biochemistry, Vol. 12, CRC Press, Inc., Boca Raton, Fl., 1982, p.39-101.
7 MILLER, E.C., MILLER, J.A. and ENOMOTO, M., The comparative carcinogenicities of 2-acetylaminofluorene and its N-hydroxy metabolite in mice, hamsters, and guinea pigs. *Cancer Res.*, **24**, 2018 (1964).
8 BARTSCH, H., MALAVEILE, C., STICH, H.F., MILLER, E.C. and MILLER J.A. Comparative electrophilicity, mutagenicity, DNA repair induction activity,and carcinogenicity of some N- and O-Acyl derivatives of hydroxy-2-aminofluorene. *Cancer Res.*, **37**, 1461 (1977).
9 MALEJKA-GIGANTI, D., GUTMANN, H.R., RYDELL, R.E. and YOST, Y., Activation of the carcinogen, N-hydroxy-2-fluorenylbenzenesulfonamide by desulfonylation to N-2-fluorenylhydroxylamine. *Cancer Res.*, **31**, 778 (1971).
10 IRVING, C.C., Enzymatic deacetylation of N-hydroxy-2-acetylamino-fluorene by liver microsomes. *Cancer Res.*, **26**, 1390 (1966).
11 LENK W. and SCHARMER U. Reaction of microsomal and cytosolic enzymes with N-arylacetohydroxamic acids. *Xenobiotica*, **10**, 573 (1980).
12 KIESE, M., Methemoglobinemia, a comprehensive treatise. CRC Press Inc., Cleveland, Ohio, 1974.
13 SMITH, P.K. Changes in blood pigments associated with the

prolonged administration of large doses of acetanilide and related compounds. *J.Pharmacol.exp.Ther.*, **70**, 171 (1940).

14 UEHLEKE, H. N-Hydroxylierung von p-Phenetidin in vivo und durch isolierte Mikrosomen aus Lebern und Nieren: Stimulierung durch Phenobarbital-Vorbehandlung. *Naunyn-Schmiedeberg's Arch. Pharmacol.*, **264**, 434 (1969).

15 SCOTT, A.I. and ECCLESTON, E. Investigation of the general toxic and haematological effects of para-chloroaniline in several species. *Proc.Eur.Soc.Stud.Drug Toxicity*, **8**, 195 (1967).

16 EVELYN, K.A. and MALLOY, H.T. Microdetermination of oxyhemoglobin, methemoglobin, and sulfhemoglobin in a single sample of blood. *J.Biol.Chem*, **126**, 655 (1938).

17 DEBACKERE, M. and UEHLEKE, H. C- And N-hydroxylation of aromatic amines by isolated liver microsomes of different species. In: Some Factors Affecting Drug Toxicity, Vol. VI of Proc.Eur.Soc. Study of Drug Toxicity, Excerpta Medica, Intern.-Congr.Series 81, p.39-43, (1964).

18 SMITH, M.R. and GORROD, J.W. The microsomal N-oxidation of some primary aromatic amines. In: Biological Oxidation of Nitrogen, J.W.Gorrod ed., Elsevier Biomedical Press, Amsterdam, p.65-70, 1978.

19 MCMAHON, R.E., TURNER, J.C. and WHITAKER, G.W. The N-hydroxylation and ring-hydroxylation of 4-aminobiphenyl in vitro by hepatic mono-oxygenases from rat, mouse, hamster, rabbit and guinea pig. *Xenobiotica*, **10**, 469 (1980).

20 KIESE, M. and UEHLEKE, H. Der Ort der N-Oxidation des Anilins im höheren Tier. *Naunyn-Schmiedeberg's Arch.exp.-Path.Pharmakol.*, **242** 117 (1961).

21 EYER P., KAMPFFMEYER, H., MAISTER, H. and RÖSCH-OEHME, E. Biotransformation of nitrosobenzene, phenylhydroxylamine, and aniline in the isolated perfused rat liver. *Xenobiotica*, **10**, 499 (1980).

22 STERZL, H. Dissertation Universität München 1983.

23 UEHLEKE, H. N-Hydroxylation of carcinogenic amines in vivo and in vitro with liver microsomes. *Biochem.Pharmacol.*, **12**, 219 (1963).

24 UEHLEKE, H. and NESTEL, K. Hydroxylamino- and Nitrosobiphenyl: Biologische Oxidationsprodukte von 4-Aminobiphenyl und Zwischenprodukte der Reduktion von 4-Nitrobiphenyl. *Naunyn-Schmiedeberg's Arch.exp.Path. Pharmakol.*, **257**, 151 (1967).

25 HULTIN T. Reactions of 2-amino-9-^{14}C-fluorene with rat liver proteins *Acta Unio Intern.contra Cancrum (Louvain)*, **16**, 1115 (1960).

26 BOOTH J. and BOYLAND, E. The biochemistry of aromatic amines: l0. Enzymatic N-hydroxylation of arylamines and con-

version of arylhydroxylamines into o-aminophenols. *Biochem.J.*, **91**, 362 (1964).

27 VON JAGOW, R., KIESE, M. and RENNER, G. Urinary excretion of N-hydroxy derivatives of some aromatic amines by rabbits, guinea pigs, and dogs. *Biochem.Pharmacol.*, **15**, 1899 (1966).

28 KIESE, M. and WIEDEMANN, I. Elimination of N-hydroxy-arylamines from the blood of guinea pigs. *Biochem.Pharmacol.*, **17**, 1151 (1968).

29 BOOTH, J. and BOYLAND, E. The biochemistry of aromatic amines. 3. Enzymatic hydroxylation by rat liver microsomes. *Biochem. J.*, **66**, 73 (1957).

30 KRIEK, E. On the interaction of N-2-fluorenylhydroxylamine with nucleic-acids in vitro. *Biochem.Biophys.Res.Commun.*, **20**, 793 (1965).

31 FLOYD, R.A., SOONG, L.M., STUART, M.A. and REIGH, D.L. Free radicals and carcinogenesis. Some properties of the nitroxyl free radicals produced by covalent binding of 2-nitroso-fluorene to unsaturated lipids of membranes. *Archs.Biochem.Biophys.*, **185**, 450 (1978).

32 WEISBURGER, J.H. GRANTHAM, P.H. and WEISBURGER, E.K. The transport of chemical carcinogens by blood. Metabolites of N-2-fluorenylacetamide and N-hydroxy-N-2-fluorenyl acetamide as a function of time. In: Physico-Chemical Mechanisms of Carcinogenesis, E.D.Bergmann and B. Pullman eds., Proc. Int. Sympos., Israel Academy of Sciences and Humanities, p.262-283, Jerusalem 1969.

33 MILLER, J.A. Carcinogenesis by chemicals: An overview - G.H.A. Clowes Memorial Lecture. *Cancer Res.*, **30**, 559 (1970).

34 HEYMANN, E., KRISCH, K., BÜCH, H. and BUZELLO, W. Inhibition of phenacetin- and acetanilide-induced methemoglobinemia in the rat by the carboxylesterase inhibitor bis(p-nitrophenyl)phosphate. *Biochem.Pharmacol.*, **18**, 801 (1969).

35 SINGLETON, S.D. and MURPHY, S.D. Propanil(3.4-dichloropropionanilide)-induced methemoglobin-formation in mice in relation to acylamidase activity. *Toxicol.Appl.Pharmacol.*, **25**, 20 (1973).

36 HEYMANN, E. and KRISCH, K. Phosphorsäure-bis-(p-nitrophenylester), ein neuer Hemmstoff mikrosomaler Carboxylesterasen. *Hoppe-Seyler's Z.Physiol.Chem.*, **348**, 609 (1967).

37 BAETHKE, R. and MUELLER, B. Untersuchungen zur embryotoxischen Wirkung von Phenacetin im chronischen Versuch an Ratten. Kli.Wo., **43**, 364 (1965).

38 LENK, W. and STERZL, H. Biotransformation in vitro of the herbicide propanil by rabbit liver microsomes. In: Industrial and Environmental Xenobiotics. In vitro versus in vivo biotransforma-

tion and toxicity, J.R.Fouts and I.Gut eds.,Intern.Conf., Excerpta Medica, p.213-215, Amsterdam 1978.

39 MILLER, J.A., WYATT, C.S., MILLER, E.C. and HARTMANN, H.A. The N-hydroxylation of 4-acetylaminobiphenyl by the rat and dog and the strong carcinogenicity of N-hydroxy-4-acetylaminobiphenyl in the rat. *Cancer Res.*, **21**, 1465 (1961).

40 HINSON, J.A. and MITCHELL, J.R. N-Hydroxylation of phenacetin by hamster liver microsomes. *Drug Metab.Disp.*, **4**, 430 (1976).

41 FISCHBACH, T., LENK, W. and SACKERER, D. Additional routes in the metabolism of phenacetin. In: Biological Reactive Intermediates. Formation, Toxicity, and Inactivation, D.J.Jollow, J.J.Kocsis, R.Snyder, and H.Vainio eds., Proc.Intern.Conf., Plenum Press, p.380-386, New York 1977.

42 HINSON, J.A., MITCHELL, J.R. and JOLLOW, D.J. Microsomal N-hydroxylation of p-chloroacetanilide. *Mol.Pharmacol.*, **11**, 462 (1976).

43 LENK, W. unpublished results.

44 KAPETANOVIC, I.M., STRONG, J.M. and MIEYAL, J.J. Metabolic structure- activity relationship for a homologous series of phenacetin analogs. *J.Pharmacol.Exp.Ther.*, **209**, 20 (1979).

45 KIESE, M. and LENK, W. Metabolites of 4-chloroaniline and chloroacetanilides produced by rabbits and pigs. *Biochem.Pharmacol.*, **20**, 379 (1971).

46 HINSON J.A., MITCHELL, J.R. and JOLLOW, D.J. N-Hydroxylation of p-chloroacetanilide in hamsters. Biochem.Pharmacol., **25**, 599 (1976).

47 MCLEAN, S., DAVIES, N.W., WATSON, H., FAVRETTO, W.A. and BIGNALL, J.C. N-Hydroxyphenacetin, a new urinary metabolite of phenacetin in the rat. *Drug Metab.Disp.*, **9**, 255 (1981).

48 LOTLIKAR, P.D., ENOMOTO, M., MILLER, J.A. and MILLER, E.C. Species variations in the N- and ring-hydroxylation of 2-acetylaminofluorene and effects of 3-methylcholanthrene pretreatment. *Proc.Soc.Exp.Biol. Med.*, **125**, 341 (1967)

49 IRVING, C.C. Enzymatic N-hydroxylation of the carcinogen 2-acetylaminofluorene and the metabolism of N-hydroxy-2-acetylaminofluorene-9-^{14}C in vitro. *J.Biol.Chem.*, **239**, 1589 (1964).

50 BENKERT, K., FRIES. W., KIESE, M. and LENK, W. N-(9-hydroxy-9H-fluoren-2yl)-acetamide and N-(9-oxo--9H-fluoren-2yl)-acetamide: metabolites of N-(9H-fluoren-2yl)-acetamide. *Biochem.Pharmacol.*, **24**, 1375 (1975).

51 MILLER, J.A., CRAMER, J.W. and MILLER, E.C. The N- and ring-hydroxylation of 2-acetylaminofluorene during carcinogenesis in the rat. *Cancer Res.*, **20**, 950 (1960).

52 FRIES W., KIESE, M. and LENK, W. Oxidation of polycyclic

N-arylacetamides to glycolamides and hydroxamic acids in rabbits. *Xenobiotica*, **3**, 525 (1973).

53 IRVING C.C. N-Hydroxylation of 2-acetylaminofluorene in the rabbit. *Cancer Res.*, **22**, 867 (1962).

54 FISCHBACH, T., HERTLE, H., KIESE, M., LENK, W., STERZL, H. and MEISTER P. Toxicity of certain polycyclic and monocyclic N-arylacetohydroxamic acids in rats. In: Chemistry and Biology of Hydroxamic Acids, H.Kehl ed.,Proc.First Intern.Symp., S.Karger Publishers, p.140-148, New York 1982.

55 KIESE, M. and PLATTIG, K.H. Hämiglobinbildung durch Benzoylphenylhy-droxylamin. *Naunyn-Schmiedeberg's Arch.-exp.Path.Pharmakol.*, **235**, 373 (1959).

56 HUSTEDT, G. and KIESE, M. Umsetzungen von Acetanilid und Acetylphenyl-hydroxylamin im Organismus. Naunyn-Schmideberg's *Arch.exp.Path.Pharmakol.*, **236**, 435 (1959).

57 EYER P. and LIERHEIMER, E. Biotransformation of nitrosobenzene in the red cell and the role of glutathione. *Xenobiotica*, **10**, 517 (1980).

58 HEUBNER, W., WALLER, B. and ZIEGLER, C. Über die Bildung von Hämiglobin durch acetylierte Phenylhydroxylamine. *Hoppe-Seyler's Z.Physiol. Chem.*, **295**, 397 (1953).

59 RIEDL, M. The electrochemical properties of N-arylacetohydroxamic acids. Diplomarbeit Universität München 1983.

60 FORRESTER, A.R., OGILVY, M.M. and THOMSON, R.H. Mode of action of carcinogenic amines. Part I. Oxidation of N-arylhydroxamic acids. *J.Chem.Soc.(C)*, **1081**, 1970.

61 NAKAGAWA, K., ONOUE, H. and MINAMI, K. Oxidation with nickel peroxide. VI. Oxidation of N-substituted hydroxylamine derivatives with nickel peroxide. *Chem.Pharm.Bull.*, **17**, 835 (1969).

62 BANKS, R.E., HASZELDINE, R.N. and STEVENSON, M.J. Perfluoralkyl derivatives of bis trifluoromethylnitroxide. *J.Chem.-Soc.(C)*, **901**, 1966.

63 MICHAELIS, L. Fundamentals of Oxidation and Reduction. In: Currents in Biochemical Research. D.E.Green, ed., Interscience Publishers, Inc., New York, p.207-227, 1946.

64 BONNETT, R. Oxygen Activation and Tetrapyrroles. In: Essays in Biochemistry, P.N.Campbell and B.D.Marshall eds., publ. for the Biochem. Soc. by Academic Press, Vol.17, p.1-51, 1981.

65 ANTONINI, E., WYMAN, J., BRUNORI, M., TAYLOR, J.F., ROSSI-FANELLI, A. and CAPUTO, A. Studies on the oxidation-reduction potentials of heme proteins. *J.Biol.Chem.*, **239**, 907 (1964).

66 HERR, F. and KIESE, M. Bestimmung von Nitrosobenzol im Blute. *Naunyn Schmiedeberg's Arch.exp.Path.Pharmakol.*, **235**, 351 (1959).

67 MCLEAN, S. and LENK, W. Microsomal metabolism and binding of a carcinogenic arylamine, 2-aminofluorene(2AF), and a noncarcinogenic arylamine, 4-chloroaniline(4CA). *Clin.Exp. Pharmacol.Physiol.*, **9**, 472(1982).

68 NEISH, W.J.P. Lack of correlation between the methaemoglobinogenic activity of azo dyes and their carcinogenicity. *Naturwiss.*, **46**, 535(1959)

69 RADOMSKI, J.L. and BRILL, E. Bladder cancer induction by aromatic amines: role of N-hydroxy metabolites. *Science*, **167**, 992 (1970).

7O HARTWELL, J.L. Survey of compounds which have been tested for carcinogenic activity. Public Health Service Publication No.149, U.S.Government Printing Office, second edition, Washington: 1951.

71 SHUBIK, P. and HARTWELL, J.L. Survey of compounds which have been tested for carcinogenic activity. Public Health Service Publication No.149, Supplement 1, U.S.Government Printing Office, Washington: 1957.

72 SHUBIK, P. and HARTWELL, J.L. Survey of compounds which have been tested for carcinogenic activity. Public Health Service Publication No.149, Supplement 2, U.S.Government Printing Office, Washington:1969.

73 TRACOR/JITCO (John I.Thompson and Company, Rockville, Md. 20850). Survey of compounds which have been tested for carcinogenic activity. 1961-1967 Volume Section I, DHEW Publication No.73-35, Public Health Service Publication No.149.

74 CLAYSON, D.B. Chemical Carcinogenesis. J. and A.Churchill, Ltd., London 1962.

75 ARCOS, J.C. and ARGUS, M.F. Chemical Induction of Cancer. Structural bases and biological mechanisms. Vol.IIB, Academic Press, New York 1974.

76 CLAYSON, D.B. and GARNER, R.C. Carcinogenic aromatic amines and related compounds, p.366-461. In: Chemical Carcinogens, C.E.Searle ed., ACS Monograph 173, Washington, D.C. 1976.

77 WHO Int.Agency Res.Cancer. IARC Monogr. Evaluation of carcinogenic risk of chemicals to man, **1**, 69-91 (1972).

78 WHO Int.Agency Res.Cancer. IARC Monogr. Evaluation of carcinogenic risk of chemicals to man, **4**, 27-124 (1974).

79 CLAYSON D.B. JULL J.W. and BONSER, G.M. The testing of ortho hydroxy-amines and related compounds by bladder implantation and a discussion of their structural requirements for carcinogenic activity. *Brit.J.Cancer*, **12**, 222 (1958).

80 CRAMER, J.W., MILLER, J.A. and MILLER, E.C. N-Hydroxylation: a new metabolic reaction observed in the rat with the carcinogen 2-acetylaminofluorene. *J.Biol.Chem.*, **235**, 885 (1960).

81 GUTMANN, H.R., LEAF, D.S., YOST, Y., RYDELL, R.E. and CHEN, C.C. Structure-activity relationships of N-acylaryl-hydroxylamines in the rat. *Cancer Res.*, 30, 1485 (1970).

82 MILLER, J.A. and MILLER, E.C. The metabolic activation of carcinogenic aromatic amines and amides. *Progr.exp.Tumor Res.*, **11**, 273 (1969).

83 SCRIBNER, J.D. and NAIMY, N.K. Reactions of esters of N-hydroxy-2-acetylamidophenanthrene with cellular nucleophiles and the formation of free radicals upon decomposition of N-acetoxy-N-arylacetamides. *Cancer Res.*, **33**, 1159 (1973).

84 MULDER, G.J., HINSON, J.A. and GILLETTE, J.R. Generation of reactive metabolites of N-hydroxy-phenacetin by glucuronidation and sulfation. *Biochem. Pharmacol.*, **26**, 189 (1977).

85 ZUFALL, A. Die pK_a -Werte einiger biologisch relevanter Aryllamine und Arylhydroxylamine. Diplomarbeit Universität München 1981.

86 DRUCKREY, H. Konstitution und Wirkung cancerogener Substanzen. *Arzneimittelforsch.*, **2**, 503 (1952).

87 CARRO-CIAMPI, G. Phenacetin abuse: a review. *Toxicology*, **10**, 311 (1978)

88 MILLER, E.C., MILLER, J.A. and HARTMANN, H.A. N-Hydroxy-2-acetylaminofluorene: a metabolite of 2-acetylaminofluorene with increased carcinogenic activity in the rat. *Cancer Res.*, **21**, 815 (1961).

89 NERY, R. The possible role of N-hydroxylation in the biological effects of phenacetin. *Xenobiotica*, **1**, 27 (1971).

90 POTTER, W.Z., DAVIS, D.C., MITCHELL, J.R., JOLLOW, D.J., GILLETTE, J. R. and BRODIE, B.B. Acetaminophen-induced hepatic necrosis. III. Cytochrome-450-mediated covalent binding in vitro. *J.Pharmacol.Exp.Ther.*, **187**, 203 (1973).

91 National Cancer Institute, Carcinogenesis Techn.Rep.Series No.130,1978. Bioassay of aniline hydrochloride for possible carcinogenicity. U.S. Department of health, Education, and Welfare, DHEW publication No.78-1385.

92 GUTMANN, H.R., GALITSKI, S.B. and FOLEY, W.A. The conversion of non-carcinogenic aromatic amides to carcinogenic arylhydroxamic acids by synthetic N-hydroxylation. *Cancer Res.*, **27**, 1443 (1967).

93 POIRIER, L.A., MILLER, J.A. and MILLER, E.C. The N- and ring-hydroxylation of 2-acetylaminofluorene and the failure to detect N-acetylation of 2-aminofluorene in the dog. *Cancer Res.*, **23**, 790 (1963).

94 JOHANSSON, S.L. Carcinogenicity of analgesics: long-term treatment of Sprague-Dawley rats with phenacetin, phenazone, caffeine and paracetamol (acetamidophen). *Int.J.Cancer*, **27**, 521 (1981).

95 PETERS. G., BAECHTOLD-FOWLER, N., BONJOUR, J.P. et al. Review Article: General and renal toxicity of phenacetin, paracetamol and some antimitotic agents in the rat. *Arch.Toxikol.*, **28**, 225 (1972).

96 SCHNITZER, B and SMITH, E.B. Effects of the metabolites of phenacetin on the rat. *Arch.Path.*, **81**, 264 (1966).

97 SHELLEY, J.H. Phenacetin, through the looking glass. *Clin. Pharmacology Ther.*, **8**, 427 (1967).

98 JOHANSSON, S. and ANGERVALL, L. Urothelial changes of the renal papillae in Sprague-Dawley rats after longterm feeding of phenacetin *Acta path.microbiol.Scand.*, Sect.A, **84**, 375 (1976).

99 NAKANISHI, L., FUKUSHIMA, S., SHIBATA, M., SHIRAI, T., OGISO, T. and ITO, N. Effects of phenacetin and caffeine on the urinary bladder of rats treated with N-butyl-N-(4-hydroxybutyl)nitrosamin. *Gann*, **69**, 395 (1978).

100 ISAKA, H., YOSHII, H., OTSUYI, A., KOIKE, M., NAGAI, Y., KOURA, M., SUGIYASHU, K. and KANABAYASHI, T. Tumors of Sprague-Dawley rats induced by long-term feeding of phenacetin. *Gann*, **70**, 29 (1979).

101 SHAHIDI, N.T. Acetophenetidin-induced methemoglobinemia. *Ann.N.Y.Acad. Sci.*, **151**, 822 (1968).

102 CALDER, I.C., CREEK, M.J., WILLIAMS, P.J., FUNDER, C.C., GREEN, C.R., HAM, K.N. and TANGE, J.D. N-Hydroxylation of p-acetophenetidide as a factor in nephrotoxicity. *J.Med.Chem.*, **16**, 499 (1973).

103 CALDER, I.C., GOSS, D.E., WILLIAMS, P.J., FUNDER, C.C., GREEN, C.R., HAM, K.N. and TANGE, J.D. Neoplasia in the rat induced by N-hydroxyphenacetin, a metabolite of phenacetin. *Pathology*, **8**, 1 (1976).

104 ELSON, L.A., SPINKS, A. and TOTTEY, M.M. Some pharmacological properties of p-chloroaniline. *Biochem.J.*, **42**, XLIII (1948).

105 WEISBURGER, E.K., RUSSFIELD, A.B., HOMBURGER, F., WEISBURGER, J.H., BOGER, E., VANDONGEN, C.G. and CHU, K.C. Testing of twenty-one environmental aromatic amines or derivatives for long-term toxicity or carcinogenicity. *J.Environ.Pathol.Toxicol.*, **2**, 325 (1978).

106 POLAND A., GLOVER, E., KENDE, A.S., DEKAMP, M. and GIANDOMENICO, C.M. 3.4.3'.4'-Tetrachloroazoxybenzene and azobenzene: potent inducers of aryl hydrocarbon hydroxylase. *Science*, **194**, 627 (1976).

107 LOWER, G. and BRYAN, G.T. Enzymatic N-acetylation of carcinogenic aromatic amines by liver cytosol of species displaying different organ susceptibilities. *Biochem.Pharmacol.*, **22**, 1581 (1973).

108 WEISBURGER J.H., GRANTHAM, P.H. and WEIS-

BURGER, E.C. The metabolism of N-2-fluorenylhydroxylamine in male and female rats. *Biochem.Pharmacol.*, **15**, 833 (1966).

109 RADOMSKI, J.L. The primary aromatic amines: their biological properties and structure-activity relationships. *Ann.Rev.Pharmacol.Toxicol.*, **19**, 129 (1979).

110 BRILL, E., RADOMSKI, J.L. and MACDONALD, W.E. Failure of the N-oxidized metabolites of some carcinogenic amines to induce tumors in normal and wounded rat skin. *Res.Commun. Chem.Path.Pharmacol.*, **18**, 353 (1977).

111 GORROD, J.W., CARTER, R.L. and ROE, F.J.C. Induction of hepatomas by 4-aminobiphenyl and three of its hydroxylated derivatives administered to newborn mice. *J.Natl.Cancer Inst.*, **41**, 403 (1968).

112 AMES, B.N., LEE, F.D. and DURSTON, W.E. An improved bacterial test system for the detection and classification of mutagens and carcinogens. *Proc.Natl.Acad.Sci.USA*, **70**, 782 (1973).

113 MILLER, J.A. and MILLER, E.C. Metabolic activation of carcinogenic aromatic amines and amides via N-hydroxylation and N-hydroxyesterification and its relationship to ultimate carcinogens as electrophilic reactants. In: Physico-Chemical Mechanisms of Carcinogenesis, E.D.Bergmann and B. Pullman eds., Proc.Int. Sympos., Israel Academy of Sciences and Humanities, p.237-260, Jerusalem 1969.

114 KIESE, M., RENNER, G. and WIEDEMANN, I. N-Hydroxylation of 2-aminofluorene in the guinea pig and by guinea pig liver microsomes in vitro. *Naunyn-Schmiedeberg's Arch.Path. Pharmakol.*, **252**, 418 (1966).

115 IRVING, C.C. and WISEMAN, JR., R. Studies on the carcinogenicity of the glucuronides of N-hydroxy-2-acetylaminofluorene and N-2-fluorenylhydroxylamine in the rat. *Cancer Res.*, **31**, 1645 (1971).

116 MILLER, E.C., MCKECHNIE, D., POIRIER, M.M. and MILLER, J.A. Inhibition of amino acid incorporation in vitro by metabolites of 2-acetylaminofluorene and by certain nitroso com pounds. *Proc.Soc.Exp.Biol.Med.*, **120**, 538 (1965).

117 HECKER, E., TRAUT, M. and HOPP, M. Über die carcinogene Wirkung von 3-Amino-$\Delta^{1.3\,5.(10)}$-östratrienen und von 2-Nitrosofluoren. *Z.Krebsforsch.*, **71**, 81 (1968).

118 WEISBURGER E.K., GRANTHAM, P.H. and WEISBURGER, J.H. Differences in the metabolism of N-hydroxy-N-2-fluorenylacetamide in male and female rats. *Biochemistry*, **3**, 808 (1964).

119 LENK, W. and ROSENBAUER-THILMANN, R. Secondary oxygenation of the carcinogen 2-acetylaminofluorene (= 2-AAF) in vitro and in vivo. In: Microsomes, Drug Oxidations, and Chemical Carcinogenesis, M.J.Coon, A.H. Conney, R.W.Esta-

brook, H.V.Gelboin, J.R.Gillette, and P.J.O'Brien eds. Volume II, p.1227-1230, Academic Press, New York 1980.

120 ROSENBAUER-THILMANN, R. Sekundäre Oxygenierung des Carcinogens 2-Acetylaminofluoren in vitro und in vivo. Biotransformation von 2-Acetylaminofluorene und 2-Acetylaminofluoren-9on in vitro und in vivo. Dissertation Universität München 1978.

121 DEBAUN, J.R., SMITH, J.Y.R., MILLER, E.C. and MILLER, J.A. Reactivity in vivo of the carcinogen N-hydroxy-2-acetylaminofluorene: increase by sulfate ion. *Science*, **167**, 184 (1970).

122 LOTLIKAR, P.D., MILLER, E.C., MILLER, J.A. and MARGRETH, A. The enzymatic reduction of the N-hydroxy derivatives of 2-acetylaminofluorene and related carcinogens by tissue preparations. *Cancer Res.*, **25**, 1743 (1965).

123 RAY,F.E. and ARGUS,M.F. Studies on the metabolism, distribution, and excretion of 2-p-toluene sulfonamidofluorene-^{35}S in the rat. *Cancer Res.*, **11**, 783 (1951).

124 YOST, Y., GUTMANN, H.R. and RYDELL, R.E. The carcinogenicity of fluorenylhydroxamic acids and N-acetoxy-N-fluorenylacetamides for the rat as related to the reactivity of the esters toward nucleophiles. *Cancer Res.*, **35**, 447 (1975).

125 MILLER, E.C., LOTLIKAR, P.D., MILLER, J.A., BUTLER, B.W., IRVING, C.C. and HILL, J.T. Reactions in vitro of some tissue nucleophiles with the glucuronide of the carcinogen N-hydroxy-2-acetylaminofluorene. *Mol.Pharmacol.*, **4**, 147 (1968).

126 MAHER, V.M., MILLER, E.C., MILLER, J.A. and SZYBALSKI, W. Mutations and decreases in density of transforming DNA produced by derivatives of the carcinogens 2-acetylaminofluorene and N-methyl-4-aminoazobenzene. *Mol.Pharmacol.*, **4**, 411 (1968).

127 DEBAUN, J.R., MILLER, E.C. and MILLER, J.A. N-Hydroxy-2-acetylaminofluorene sulfotransferase: its probable role in carcinogenesis and in protein-(methion-S-yl) binding in rat liver. *Cancer Res.*, **30**, 577 (1970).

128 KRIEK, E. and WESTRA, J.G. Metabolic activation of aromatic amines and amides and interaction with nucleic acids. In: Chemical Carcinogenesis and DNA., P.L.Grover ed., Vol.11 p.1-28, CRC Press, Inc., Boca Raton, Fl., 1979.

129 IRVING, C.C., JANSS, D.H. and RUSSELL, L.T. Lack of N-hydroxy-2-acetylaminofluorene sulfotransferase activity in the mammary gland and Zymbal's gland of the rat. *Cancer Res.*, **31**, 387 (1971).