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Blood protein conjugates and acetylation of aromatic amines

New findings on biological monitoring

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Summary. Internal stress of aromatic amines has so far been evaluated by their determination in blood or urine and by the degree of methemoglobin formation. Animal experiments have shown that these materials can form adducts and conjugates with proteins and nucleic acids. Our investigations show that these processes can also occur in human metabolism. For this the degree of such a formation of protein conjugates depends on an individually different potential for acetylation. In a positive sense it influences the magnitude and the rate of renal excretion of aminoaromates and their conjugates and metabolites formed by this metabolism. In contrast, only free non-acetylated aminoaromates can lead to the formation of conjugates with hemoglobin. These aminoaromates or their metabolites can then be detected quantitatively in intact erythrocytes during their lifespan. The degree of this protein conjugate formation correlates inversely with the magnitude of the acetylation potential depending on the availability of free non-acetylated aminoaromates. According to these results a clearer assessment of past stress or the presence of strain can be obtained with Biological Monitoring by a single determination of such hemoglobin adducts rather than by the traditional quantitative determination of aminoaromates or their metabolites in blood and/or urine or the methemoglobin concentration.

Key words: Aromatic amines – Acetylation – Fast and slow acetylators – Conjugation of aniline with proteins in erythrocytes – Separation of erythrocytes – Aniline – Biological Monitoring

Introduction

Measuring concentrations of industrial material in accessible body fluids, biological monitoring (BM), can provide "stress profiles"[1] which indicate whether or not and to what extent strain[1] levels exist. Qualitative and quantitative detec-

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1 The terms "stress" or "strain" of industrial material refer to the concept devised by the DFG Study Group "Classification of BAT values" [25]. (= "Aufstellung von BAT-Werten")

tion of industrial material and metabolites in urine or blood has until now been the main form of analysis, but advances in methodology and application of the results of basic biochemical research now enable more detailed observations to be made. They are an integral part of occupational medical examinations (OME) for specific substances.

Several monographs and reviews have reported connections between chemical structure of aminoaromates (AA), biotransformation and conjugation on the one hand and the resulting mechanism of excretion on the other hand [2, 3, 23, 43]. Accordingly in general more lipophilic substances are biotransformed into more hydrophilic substances and then conjugated, thus lowering their toxicity and increasing urinary excretion. The standard method in industrial medicine for monitoring internal stress of AA, in addition to less specific clinical chemistry and hematological tests, is the measurement of methemoglobin (Met-Hb), plus analysis of AA-metabolites in the urine.

In the case of accidentally developing cyanosis a correlation is often lacking between the severity of Met-Hb formation and the level of AA in blood or urine. The detection of the binding of AA and their metabolites to hemoglobin (Hb) in animal experiments was first reported in 1970 [4, 13, 19, 31–33, 35, 46].

The ratio of binding between Hb and proteins or nucleic acids in different tissues is constant at a given time point throughout the entire dose range. This means that metabolites, which would bind to hemoglobin and those which could react with nucleic acids, are formed in a constant proportion.

The results of these animal experiments have done a great deal in satisfying the requirements for using the measurement of Hb conjugates as an additional means of occupational medicinal evaluation following AA exposure.

Objectives

As much as 60% of aniline absorbed by humans is oxidized dose-dependently to give o- and p-aminophenol [22, 38] and following conjugation with glucuronic acid or sulfuric acid is eliminated via the urine [40]. The oxidative attack on the amino-function of aniline is quite definitely limited. The resulting metabolites, phenylhydroxylamine and nitrosobenzene, become responsible for the toxic effects of aniline [21].

Their characteristic effect is Met-Hb formation. Approximately 1% of the aniline absorbed is excreted unchanged in the urine. This percentage may increase markedly as the levels of the substance increase, e.g. in cases of acute intoxication [34, 37].

It is not aniline itself, but phenylhydroxylamine which, following oxidation to form nitrosobenzene, effects the formation of Met-Hb [8]. In the circular process caused by continuous regeneration of phenylhydroxylamine from nitrosobenzene and subsequent re-oxidation, even minor exposure to aniline can cause relatively large quantities of Met-Hb. A hydroxylamine-reductase can act as a catalyst in reducing phenylhydroxylamine back to aniline which is then excreted renally in a free or conjugated form. Detoxication of phenylhydroxylamine clearly involves rearrangement into o- and p-aminophenol followed by renal elimination [15]. More detailed information is shown in Fig. 1.

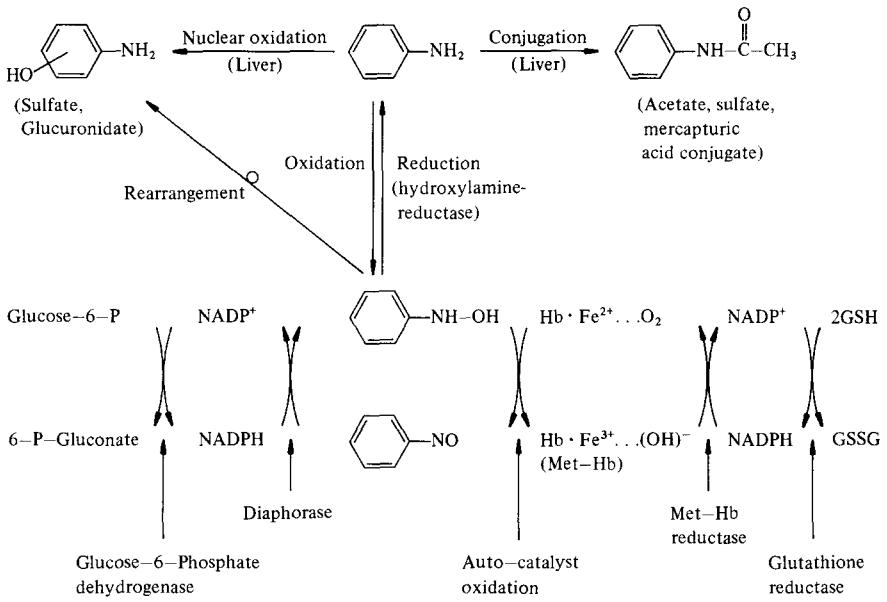


Fig. 1. Metabolism of aniline [3, 5, 9, 15]

In addition to the investigations of the metabolism of AA, specifically of aniline, under the aspect of Met-Hb formation [8, 23, 43] further metabolic pathways have been investigated since around 1970, at first by animal experiments [6, 24, 17, 32, 35] and since about 1981 also under occupational medical aspects with a view to the formation of conjugates with the sulfhydrylgroups of Hb and glutathione (G-SH).

Details of the AA metabolism in human erythrocytes (Eryc) were first reported for 4-aminodiphenyl [42] and in analogue have been outlined for aniline in Fig. 2.

Following intermediary disproportionation, the conjugate is present as an anilide of sulfinic acid bound to the SH group of a cysteine residue of Hb and glutathione. The glutathione-sulfinic anilides are dialysable [17]. When conversion by hydrolysis takes place, free aniline again emerges from the sulfinic acid anilide of Hb and this can be detected by analysis [32, 42]. Eryc are hence an important target organ for internal stress of aniline.

Internal stress of aniline can thus be directly assessed by the demonstration of conjugation of aniline metabolites onto erythrocyte proteins [17, 42]. These indicators outlast the acute phase of intoxication in the order of Eryc life time.

The suitability of Eryc as a target organ and DNA correlative for the detection and evaluation of internal tolerance levels for industrial materials has already been discussed by us in a study of internal chromium (VI) stress [27]. This report also goes into further detail about the life expectancy of Eryc with conjugates.

The extent of AA-Hb conjugate formation is thought to depend on the structure of the aryl residue [11]. From a toxicological point of view, the possi-

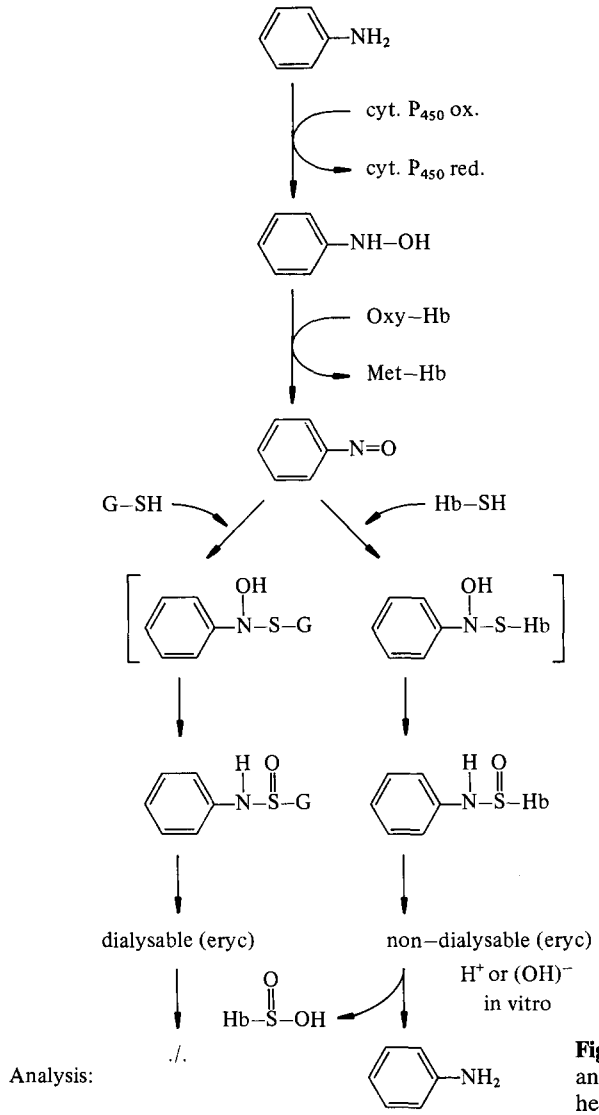


Fig. 2. Diagram of the formation of aniline-glutathione and aniline-hemoglobin conjugates

bility of an indicator of carcinogenic potential based on parallels between DNA and Hb binding has been discussed for some substances [4, 30, 35].

Experimental investigations in animals on AA showed isolated protein (Hb)-conjugates without nucleic acid conjugates, but no isolated nucleic acid conjugates without protein conjugates also being present [35].

Individual acetylation potential

Hb binding is only one aspect, however, of the metabolisation of foreign substances, specific of AA. In the problem of detoxification and elimination studies

over recent years into acetylation of Xenobiotics have provided substantial new information for pharmacology as well as industrial medicine [7, 45].

N-acetylation is a physiological metabolic process for aliphatic and aromatic amines, sulfonamides, amino acids, hydrazine and hydrazides. For this purpose the acetyl group is supplied by acetyl-CoA. The catalyst is the N-acetyltransferase present in the cytosol of the various cells [7]. The equation for the acetylation reaction is as follows:

$$R-NH_2 + CH_3-CO\sim S\text{-}CoA \rightarrow R-NH-\overset{\overset{O}{\|}}{C}-CH_3 + CoA-SH.$$

There are clearly differences in humans' ability to acetylize isoniazide and hence to eliminate by metabolism [14, 44]. Therefore, a distinction has been made between "fast acetylators" and "slow acetylators". A high acetylation rate leads to rapid renal elimination of isoniazide and hence low plasma levels, and vice versa. Among the Japanese and Eskimos fast acetylators are in the majority (85 and 95% of the population respectively) and in Europe, the two groups more or less hold the balance. The disposition to a specific level of N-acetyltransferase activity is hereditary [18, 28, 29].

For this reason the target of our investigations into the metabolic products of subjects exposed to AA was to differentiate between acetylized and free AA and its metabolites in plasma and Hb-conjugates. In this context, the acetylation is only one, albeit significant, step.

The starting point for our investigations was to differentiate the entire aromatic amine stress from the part of it binding to Eryc. A precondition for this is that AA or their metabolites can only bind to Eryc proteins if they have managed to permeate the Eryc membrane in a free, non-acetylized condition, i.e. they must be capable of reacting or ready to react.

To answer these questions we started by examining four points:

(1) What is the relation between the concentrations of AA in whole blood or urine and the binding of their metabolites to proteins in erythrocytes?
(2) Does the acetylation of AA affect its permeation of the Eryc membrane and its binding to Eryc and, if so, to what extent?
(3) Do repeated tests throw any light on the genetic determination of acetylation potential and what is the correlation between this potential and the degree of Hb binding by AA?
(4) Can the AA-binding observed in vivo be reproduced in vitro?

In response to these questions, whole blood and urine samples were taken from workers handling AA: in connection with OME and also following acute exposure. The individual AA content was determined in each of the compartments by various procedures.

Materials and methods

Processing of blood samples and spiking

To differentiate between the free and protein-conjugated AA, whole blood samples without and after spiking with AA were tested. The principle is that working rapidly in isotonic solutions should ensure that the Eryc remain intact.

Table 1. Spiking schedule for whole blood, plasma, erythrocytes

Volume of stock solution (µl)	Final volume of comparison standard (ml)	Concentration of comparison standard (µg/l)
0	5	0
2.5	5	5
5.0	5	10
25.0	5	50
50.0	5	100
250.0	5	500
500.0	5	1000

For the steps of dialysis, spiking and dilution isotonic saline with a content of 154.0 mmol Na^+ and Cl^- per one liter of water have to be used exclusively. For the purpose of simplification, in the following text the isotonic solutions have been designated 0.9% saline (NaCl), and they possess an osmolarity of 308 mosm/l. The blood samples are treated with heparin or EDTA to inhibit the coagulation.

The packed cell volume (PCV) is determined for the whole blood sample by a standard method [39]. All the preparation work was carried out at room temperature (rt) [12].

In order to determine the contents of AA in whole blood, plasma or Eryc it is necessary to prepare calibration curves. These are constructed by spiking the appropriate biological samples with predetermined concentrations of AA. The following instruction for the preparation of standards (Table 1) and for the spiking and preparation of samples (Table 2) are also suitable for the assessment of internal stress of AA, e.g. of aniline and chloroanilines. The corresponding steps are the same.

To the starting solution, 10 mg each of the AA under investigation are added in a mixture of acetonitrile/0.9% saline (1:1/V:V) and topped up to 50 ml (content: 200 mg/l). To the stock solution, 1 ml each of the above starting solution with 0.9% saline are diluted to 20 ml (contents: 10 mg/l). After spiking the biologic materials to 5 ml, comparable standards are obtained in a range of concentration between 5 and 1000 µg/l. The spiked biological samples are incubated for each further step for 10 min at rt. For certain AA, particularly AA with two aromatic bodies, it is advisable to readjust the physiological conditions by incubating the spiked blood samples for at least 2 h at 37°C.

The separate steps of sample preparation of whole blood, plasma and Eryc and the corresponding spiking for the construction of the corresponding calibration curve are summarized in Table 2.

To investigate whether AA become bound to Eryc under in- vitro conditions, the whole blood was spiked and incubated. It was then diluted with 2 ml 0.9% NaCl solution and the plasma was removed. In a second rinsing stage, this plasma mixture was adjusted to 5 ml with the 0.9% NaCl solution, which helps in drawing up the plasma calibration curve and plasma AA determination.

To wash out the unfixed AA from the plasma-free Eryc, these were dialysed against portions of 5 ml 0.9% NaCl solution (incubation time at each stage: 10 min, at rt). After being centrifuged five times, the Eryc were adjusted to a total volume of 5 ml with 0.9% NaCl solution and the PCV was taken.

To draw up the Eryc-calibration curves, the purified Eryc at this stage had to be re-spiked with reference to the initial quantity of blood (usually 5 ml). For this, they should be dissolved in conc. hydrochloric acid, although they may be used in undissolved form as well. For spiking, solutions of AA in 0.9% saline are preferable.

The count for Eryc-bound AA was not compared with the PCV until evaluation of the samples was complete. The plasma and Eryc results were then set against the initial volume of the whole blood sample from which these had been separated. This stage serves to correct any

Table 2. Pipetting schedule: Determination of the aromatic amine concentration in blood and the partition of the aromatic amines in the plasma and Eryc directly or after spiking with aromatic amine solution respectively after 5 dialysis steps

Starting material	Stabilized blood for			
Sequence of stages	Blood-analysis		Plasma-analysis	Eryc-analysis
1. Starting blood volume (ml)	5.0		5.0	
2. Hematocrit determination (HC_1)	.....%		.....%	
3a. Spiking in µg/l aromatic amines	.....		.....	
3b. Spiking volume/5.0 ml blood[a]	.....		.....	
4. Incubation period (min) at RT	10		⁄	
5. Dosage of 0.9% NaCl-solution (ml)	⁄		2.0	
6. Incubation period (min) at RT	⁄		10	
7. Centrifugation, 5 min at	⁄		1200 *g*	
8. Partition in (ml)	⁄		~4.5	~2.5
9. Dosage of 0.9% NaCl-solution until to (ml)	⁄		⁄	5.0
10. Incubation period (min) at RT	⁄		⁄	10
11. Centrifugation, 5 min at	⁄		⁄	1200 *g*
12. Upper phase to the plasma until to (ml), residual discarded	⁄		5.0	⁄
13. Dosage of 0.9% NaCl-solution until to (ml)	⁄		⁄	5.0
14. Incubation period (min) at RT	⁄		⁄	10
15. Centrifugation, 5 min at	⁄		⁄	1200 *g*
16. Upper phase discarded, number of replications (Pos. 13–15)	⁄		⁄	4
17. Upper phase discarded	⁄		⁄	yes
18. Lower phase with 0.9% NaCl-solution up to (ml)	⁄		⁄	5.0
19. Hematocrit determination (HC_2)	⁄		⁄	.....%
20. Centrifugation, 5 min at	⁄		⁄	1200 *g*
21. Upper phase discarded	⁄		⁄	yes
22. Lower phase with 0.9% NaCl-solution up to (ml)	10.0		10.0	5.0
23. Partition in (ml)	5.0	5.0	⁄	⁄
24. Hydrolysis with (ml) conc. HCl	⁄	10.0	⁄	10.0
25. For calibration				
a. Spiking in µg/l aromatic amines	⁄	⁄	⁄	.....
b. Spiking volume/5.0 ml blood[a]	⁄	⁄	⁄	.....
26. To aromatic amine determination	yes	yes	yes	yes
27. a. Preparation: without hydrolysis	yes	⁄	yes	⁄
b. Preparation: with hydrolysis	⁄	2 h 80°C resp. 2 h 100°C	⁄	2 h 80°C resp. 5 h 100°C

[a] Content of the calibration solution

Remarks to the manipulations in step:

12. The plasma obtained from step 8 is made up to 5.0 ml with the 0.9% NaCl-washing solution separated in step 11
16. The washing procedures described in positions 13 to 15 are repeated four times

loss of Eryc during the isolation and purification stages, by means of PCV on initial and final solutions of equal volume.

Procedure of determination

Aniline determination in the prepared samples is performed by means of gas chromatography designed for nitrogen specific detection (GC Varian 3700) following concentration.

For the concentration stage, 50 ml diethylether are added to the hydrolysates adjusted under cooled conditions to alkaline with 32% sodium hydroxide; the mixture is shaken mechanically for 10 min. The aqueous phase is discarded, and the organic phase is extracted by 20 ml 2 *M* hydrochloric acid, with 10 min of mechanical shaking. The aqueous phase is then transferred to a second separating funnel and 5 ml 32% aqueous sodium hydroxide are added. Any heating of the sample should be carefully avoided at this stage. The pH should be clearly higher than 9. This basic aqueous phase is then re-extracted with 5 ml diethylether, shaking mechanically for 10 min. The aqueous phase should be discarded and the organic phase collected in a 10-ml test tube. The ether phase is dried with anhydrous sodium sulfate and decanted off. The sodium sulfate is washed with 2 ml diethylether. The organic phases should be brought together in a 10-ml glass tube. The dissolving liquid is removed at rt in a vacuum centrifuge with 10 µl of glacial acetic acid added. The residue is absorbed into a total of 50 µl diisopropylether.

Setting up		
Separation column:	Material:	glass capillary
	length:	50 m
	internal diameter:	0.3 mm
Separation phase:	PS 255, immobilised SE 30	
Detector:	N-FID	
Temperature:	Oven:	5 min at 95°C, then at 5°C/min to 220°C, taking 0–5 min
	Injector:	280°C
	Detector:	300°C
Pre-pressure:	1.05 bar	
Make-up gas:	30 ml	
Partition rinsing:	—	
Volume of sample:	1 µl	
Injection pace:	Continuous injection for 6 s, then at a rate of 120 ml per min (split)	

The measurements were evaluated using a standard curve constructed in the relevant matrix. The blank measurements for all the reagents used were below the limit of detection of 5 µg/l. The calibration curves are constructed by marking the measured peak areas against the AA concentrations (AAC) used. For the calculation of the AA content in µg AAC/l of the biological samples to be assessed, the measured peak areas of the various AA are determined by using the corresponding calibration curves. Taking into consideration possible losses due to preparation, the AA content in Eryc (µg AA-Eryc) is therefore calculated in relation to Eryc or whole blood volume as follows:

$$\mu g\ AA\text{-}Eryc/l\ Eryc = \frac{\mu g\ AAC \times 100}{HC_{Eryc}}$$

$$\mu g\ AA\text{-}Eryc/l\ blood = \frac{\mu g\ AAC \times HC_{whole\ blood}}{HC_{Eryc}}.$$

The determination of Met-Hb and glucose-6-phosphate-dehydrogenase was carried out according to clinico-chemical standard methods [39]. For the differentiation between free and acetylated aniline in urine corresponding samples are worked up and analysed with and without hydrolysis as described above. Furthermore the acetanilide was also evaluated directly together with p-aminophenol and p-acetaminophenol after an enrichment in the solid phase by high pressure liquid chromatography [26].

By our modification of the somewhat gentler conditions of hydrolysis as described in animal experiments [32, 42], up to 50% more aniline and AA could be released from Eryc of persons exposed to aniline. Differently structured conjugates of AA with proteins or polysaccharides possibly exist in human Eryc along with the described sulfinic acid amides. Their splitting off may require more aggressive condition hydrolysis [16, 17]. Furthermore, with unexposed humans we could recognize an ubiquitous basis-stress on traces of aniline conjugates.

Results

The metabolic processes relevant to evaluation in the context of industrial medicine should, with regard to the AA group of substances, comment upon the example of acute or chronic aniline (AN) exposure.

The preliminary studies, which aimed at ascertaining the best methods of analysis for the detection of Hb-bound AA conjugates, in fact revealed that these conjugates did not form in vitro when blood samples were spiked with corresponding substances. This means that the process of Hb conjugation only takes place in the presence of active metabolic processes in vivo and that detection of Hb-AA conjugates in the blood demands corresponding exposure.

Analogous in vitro spiking of stabilized blood samples with AA led to comparatively low absorption of AA into the Eryc. It may be assumed that the AA added in vitro are capable of permeating the Eryc membrane, but do not bind with intracellular substrates, e.g. Hb. In fact, these unbound AA can be removed from inside the Eryc by consecutive dialysis stages using 0.9% NaCl solution. This is proved by the results given in Table 3, which lists the concentrations of AA in the Eryc following in vivo exposure to AN, respectively in vitro spiking with various AA, measured at each of the dialysis stages 1–5.

Compared with the concentration in Eryc given in [31], losses during preparation are compensated for in the present dialysis method by the taking of PCV.

When blood samples were analysed following exposure to AA, it was found that relevant quantities of conjugates were only detectable in approximately

Table 3. Comparison of aromatic amine content of erythrocytes under in vivo and in vitro conditions. A: aniline in µg/l; C: p-chloroaniline in µg/l; N: α-naphthylamine in µg/l; T: o-toluidine in µg/l

	Aniline accident (7.5% Met-Hb)				Pooled blood							
					Unspiked				Spiking at 500 µg/l of the following:			
µg/l industrial material in Eryc	A	C	N	T	A	C	N	T	A	C	N	T
Dialysis stage 1	260	<10	<10	<10	<10	<10	<10	<10	200	300	<10	100
Dialysis stage 2	210	<10	<10	<10	<10	<10	<10	<10	200	200	<10	100
Dialysis stage 3	190	<10	<10	<10	<10	<10	<10	<10	50	100	<10	<10
Dialysis stage 4	200	<10	<10	<10	<10	<10	<10	<10	<10	50	<10	<10
Dialysis stage 5	200	<10	<10	<10	<10	<10	<10	<10	<10	<10	<10	<10

Table 4. Correlation between urine-analysis results and Eryc findings following acute aniline and p-chloroaniline exposure. Measured parameters: Met-Hb, urine excretion of the parent amines and amines released from the Hb-conjugates, both by hydrolysis

	Met-Hb (%)	Mean results based on 3 determinations for:			
		p-Chloroaniline		Aniline	
		Urine (μg/g)[b]	Eryc[a] (μg/l)	Urine (μg/g)[b]	Eryc[a] (μg/l)
Accident + 30 min	36.2	1500	100	4000	300
Accident + 3 h	43.9	500	300	700	400
Accident + 7 h	15.9	200	200	200	800
Accident + 16 h	1.2	50	100	100	1000
Accident + 3 d	0.3	<10	100	<10	600
Accident + 7 d	1.0	<10	50	<10	100
Accident + 12 d	0.9	<10	<10	<10	<10

[a] Taken from Hb-conjugates, [b] creatinine

50% of the subjects thus exposed. Any connection between this result and individual differences in N-acetyltransferase activity will be considered in more detail later in this study.

In Table 4, the usual urinalysis findings are correlated with the Hb-AA conjugate results, using as an example the effects of accidental exposure to AN and p-chloroaniline (PClA). The absolute values differ in relation to time, and there is a marked difference in relation to the formation and degradation rates with the Eryc.

Whereas urinary excretion falls from a high initial peak to below the limit of detection within 3 d, Hb conjugation for PClA reaches its peak after 3 h, and for AN after approx. 16 h. Total degradation is not completed until Day 12.

According to these results, detection of Hb-bound AA conjugates is possible not only immediately following intoxication, but even after several days. Conclusions may not be drawn as to the relationship between these results and individual acetylation potential.

In Tables 5 and 6 differences in acetylation rate are shown for subjects undergoing chronic exposure and cases of acute intoxication. It is thus possible to differentiate "fast" and "slow" acetylators among the 14 probationers.

This differing potential for acetylation is in inverse proportion to the Met-Hb level and the degree of Hb-AN conjugation, as shown by the results in the tables. Accordingly, slow acetylators tend to have higher Met-Hb and Hb/AN conjugate findings than fast acetylators when exposure to industrial material for the two types is comparable.

Table 6 contrasts the individual metabolic patterns of these two genetic variables. The results confirm that:

(1) a high degree of acetylation involves low Met-Hb levels and negligible AN-Hb conjugation, and
(2) a low degree of acetylation, in spite of comparably low exposure to AN, is associated with high Met-Hb levels and corresponding AN-Hb conjugation.

Table 5. Measurement of Met-Hb, total aniline and aminophenol and acetanilide and acetaminophenol in urine, plus aniline from Hb-conjugates following normal industrial exposure to aniline (air concentrations below the MAK-value)

	Subject No.	Met-Hb (%)	Urine				Eryc
			a	b	c	d	e
Fast acetylators 1–7	1	0.8	3.1	3.0	450	400	<10
	2	0.7	2.0	2.0	200	200	<10
	3	0.8	6.7	6.5	500	400	<10
	4	1.2	5.5	5.5	580	500	<10
	5	1.1	1.8	1.5	210	200	<10
	6	0.9	2.0	1.9	300	200	<10
	7	0.7	3.8	3.6	400	400	<10
Means from	1–7	0.9	3.6	3.4	377	340	<10
Slow acetylators 8–14	8	1.3	1.8	<1.0	250	<10	40
	9	1.5	2.5	<1.0	300	220	100
	10	1.0	5.9	1.5	600	40	200
	11	1.1	7.1	1.5	520	40	200
	12	1.4	3.0	<1.0	350	10	100
	13	1.3	2.7	<1.0	280	10	<10
	14	1.3	4.5	1.5	450	40	100
Mean from	8–14	1.4	3.9	<1.2	393	27	123

a p-aminophenol total in mg/g creatinine (following total hydrolysis); *b* p-acetaminophenol in mg/g creatinine (partitial quantity of a); *c* aniline total in µg/g creatinine (following total hydrolysis); *d* acetanilide in µg/g creatinine (partitial quantity of c); *e* aniline from hemoglobin conjugate in µg/l

An important fact emerging is that in a "fast acetylator", even high, accidental exposure to AA leads only to relatively low levels of Hb-conjugates, in our cases around the limit of detection. In a "slow acetylator" exposed to the same quantities, there is definite Hb conjugation and the levels remain detectable within the biological degradation time of Eryc. In this connection, it can no longer be regarded as coincidental that all of the cases of AN intoxication observed by us since 1980 with Met-Hb levels of >30% and normal G-6PDH levels were, without exception, slow acetylators.

The N-acetyltransferase activity derived from the acetylation rate can be measured directly by a provocation test with the sulfonamide sulfamethazine [28]. For ethical reasons this test may not be used in industrial medicine.

No other methods for the detection of the individual human potential for acetylation have been described. The tables only show the results of investigations of such persons, who after industrial handling of AA below MAK-values, were recognized as definitely fast or slow acetylators after at least three routine screening examinations. Prior knowledge about the acetylation potential could furthermore be verified in cases of accidentally elevated exposure.

Table 6. Measurement of Met-Hb, G-6PDH, aniline and acetanilide in urine, and aniline from Hb-conjugates: under normal industrial conditions of exposure below the MAK-values (blood and urine sampling to the shift end) and after operational accident (blood and urine sampling 30 min later)

				Mean aniline findings based on 3 determinations from:				
		Blood		Urine				Eryc
		Met-Hb (%)	G-6PDH	A	B	C	D (%)	E
Case 1 (fast acetylator)	Normal ind. exposure	—	—	550	550	1.05	100	—
	Normal ind. exposure	—	—	200	200	1.0	100	—
	Accident	7.0	132	98000	11000	8.9	11	10
	Normal ind. exposure	—	—	350	300	1.16	86	—
Case 2 (slow acetylator)	Normal ind. exposure	—	—	400	20	20.0	5	—
	Normal ind. exposure	—	—	150	10	15.0	7	—
	Normal ind. exposure	—	—	220	10	22.0	5	—
	Accident	45	133	80000	100	800	0.1	3000

A aniline total (AN + acetanilide) in µg/g creatinine (following total hydrolysis); *B* acetanilide in µg/g creatinine (partial quantity of A); *C* ratio of AN + acetanilide to acetanilide in urine; *D* percentage of acetylation; *E* AN from Hb-conjugates in µg/l

Table 7. Correlation of stress and strain results at a Met-Hb value of approximately 5% following normal contact with aniline only (blood and urine sampling at shift end)

Subject no.	Met-Hb approx. %	G-6PDH	Mean aniline readings based on 3 determinations from:				Acetylator status +: fast ⁄: slow
			Blood		Urine		
			R	S	T	U	
1	5	190	710	<10	9500	8500	+
2	5	160	500	10	6200	5000	+
3	5	150	950	20	10000	9300	+
4	5	135	200	120	1900	100	⁄
5	5	130	370	10	3100	2000	+
6	5	128	410	20	2600	1700	+
7	5	120	250	150	2100	80	⁄
8	5	50	150	150	400	20	⁄
9	5	40	70	70	100	<10	⁄
10	5	20	20	20	100	<10	⁄

R AN after acidic treatment of total blood in µg/l; *S* AN from hemoglobin conjugate in µg/l; *T* AN total (AN + acetanilide) after acidic treatment in µg/g creatinine; *U* acetanilide in µg/g creatinine (partial quantity of T)

The effects of predisposed fluctuations from the G-6PDH and acetylation norms are already noticeable at levels of AA exposure not exceeding the tolerance level currently specified in the field of industrial medicine in relation to internal strain of AA at 5% Met-Hb.

In Table 7, a number of cases of AN exposure are listed; in each case an AN strain in the range of approximately 5% Met-Hb was the result. Even from this admittedly random tolerance limit for which no thorough, firm foundation has been established, it can be seen that the formation of Eryc-conjugates is significantly affected by the acetylator type, within a spectrum of internal AN stress.

Whether and, if so, to what extent acetylator type is directly linked to G-6PDH activity cannot be established with any certainty from the present data. There is, however, a surprising cluster of slow acetylators with reduced G-6PDH activity.

Discussion

In our studies we were able to reveal two essential new aspects of Biological Monitoring of AA.

(1) The conjugation of AA with Hb is a biochemical mechanism which gives a greater insight into the metabolisation of these substances. As this conjugation cannot be reproduced in vitro, it is clearly the result of an active metabolic process only taking place in vivo, whereby conjugates are formed. Demonstration of this mechanism means that, in addition to biological monitoring, the consequences of intoxication can be more critically assessed and the elimination of the industrial substances involved can be monitored.

It is therefore possible to comment on the degree of exposure and body burden not only for the period limited to elimination in the urine, but also in relation to the time scale of Eryc life. A method of this kind gives more of a longitudinal view which is extremely important for industrial physicians.

In order to test this on a larger population, thorough studies are being carried out in conjunction with OME on workers exposed to AA. When the results are thereby consolidated, the time-consuming analysis of urine samples may be replaced by the examination of stabilised whole blood samples and AA/Hb conjugate count which would be more informative.

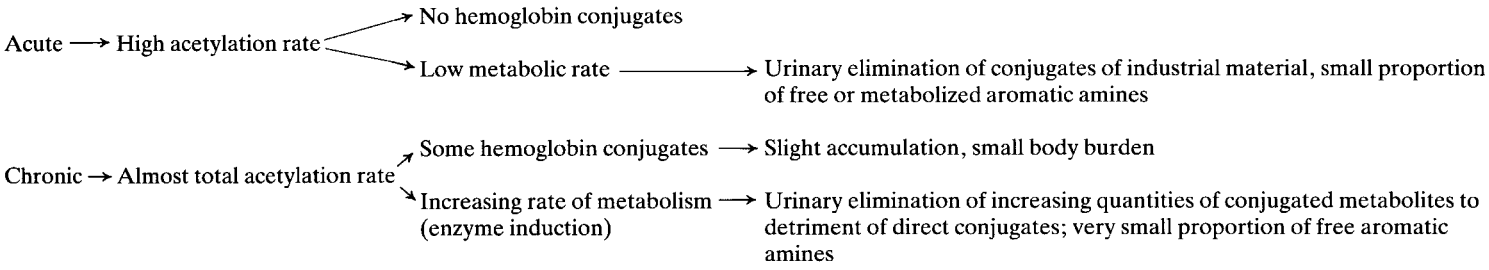
If it is found that this conjugate formation or metabolisation also applies to other substances in the AA group—perhaps even to their corresponding nitro compounds—this would open up whole new dimensions of preventive industrial medicine from merely specific to highly product-related diagnosis.

The quantifiable information on a case of exposure which can be derived from the degree of Hb-binding not only includes the extent of conjugation by these substances, but also gives more specific information than urine and blood analyses as to the actual internal stress of a substance and the effect these levels have with regard to the BAT value.

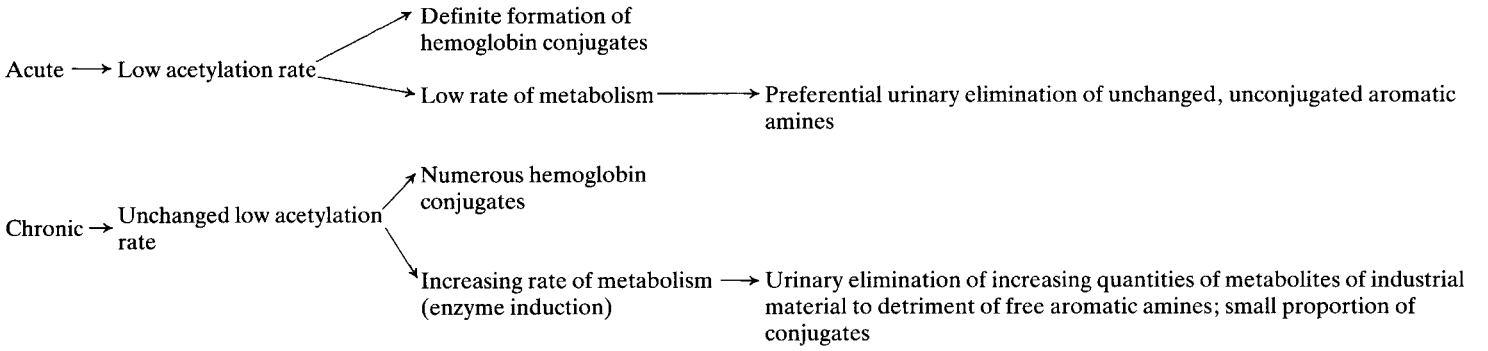
This includes the fact that in the event of accidents, AA loading can still be detected over a relatively long period by the measurement of AA/Hb con-

Table 8. Influence of individual N-acetyltransferase activity on the metabolism of aromatic amines. Aromatic amine stress in:

Fast acetylators



Slow acetylators



jugates. This answers the demand from industrial medicine for an assessment of unlimited usability to work with possible renewed effects of AA.

(2) Acetylation is one, perhaps even the most important, way of metabolisation of foreign substances in the body [20, 45]. Its pharmacological significance is well known [1, 10, 14, 36, 41, 44]. The measurement of acetylation, including its relation to industrial medicine, is well documented in comparable animal experiments.

Table 8 summarizes the differences in the extent and rate of AA metabolism, to provide a clear trend of the possible repercussions for evaluation in the field of industrial medicine.

We have been able to substantiate the observations of other authors regarding what is presumably a genetically determined individual difference in acetylation potential within a population [28, 45]. Fast acetylation does, however, entail slight Hb-conjugation in conjunction with improved elimination of a foreign substance.

Whether and, if so, in what way these findings are of clinical relevance can only be partially answered at this stage.

This applies for instance to the tendency of slow acetylators to yield relatively high Met-Hb figures compared with correspondingly low figures for fast acetylators when both are exposed to the same degree of loading. In this respect the findings can already be of some diagnostic use. However, more detailed analysis with a larger number of cases is called for before binding assessment criteria or, possibly, classifications for other findings relevant in the field of industrial medicine can be set out.

Lower and co-workers noted possible connections between the question of carcinogenicity of certain AA and the degree of acetylation [28, 29]. According to their studies, tumors occurred after contact with 2-naphthylamine, benzidine and 4-aminodiphenyl predominantly in subjects with a low acetylation rate for metabolising the AA. It is not possible to say at this stage whether or not this particular finding constitutes a fundamental pathogenetic principle.

Conclusion

Our studies illustrate the importance of Eryc in the detection and differentiation of internal aromatic amine stress. It is possible that all AA are capable of diffusing through the Eryc membrane, but it is only free, unbound AA that are stored in the Eryc during their lifetime and cannot be removed, even by repeated dialysis measures.

As a possible cause of the differences in individual conjugation of AA with Eryc proteins, we have noted a definite correlation between these differences and the individual acetylation type. By biological monitoring, particularly after acute exposure, we found practically no conjugation in fast acetylators, but quite marked AA-protein conjugation in slow acetylators. The degree of Met-Hb formation correlated with this is the same.

By this method BM can be carried out at any time on workers exposed to AA; fixed times for taking and examining blood and urine samples have thereby been rendered superfluous. These procedures initially only apply to pure AN, but may possibly be transferred to other AA.

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References

1. Alarcon-Segovia D, Fishbein E, Alcala H (1971) Isoniazid acetylation rate and development of antinuclear antibodies upon isoniazid treatment. Arthritis Rheum 14:748–752
2. Ariëns EJ, Mutschler E, Simoniss AM (1978) Allgemeine Toxikologie. Thieme, Stuttgart, pp 32–120
3. Atlas SA, Nebert DW (1976) Pharmacogenetics and human disease. In: Parke DV, Smith RL (eds) Drug metabolism from microbe to man. Taylor and Francis, London, pp 393–430
4. Baur J, Neumann HG (1980) Correlation of nucleic acid binding by metabolites of trans-4-aminostilbene derivatives with tissue specific acute toxicity and carcinogenicity in rats. Carcinogenesis 1:877–886
5. Beckett AH, Bélanger PM (1976) Metabolic N-oxidation of secondary and primary aromatic amines as a route to ring hydroxylation, to various N-oxygenated products, and to dealkylation of secondary amines. Biochem Pharmacol 25:211–214
6. Barry EJ, Gutmann HR (1970) Further evidence for two types of adducts of N-hydroxy-2-fluorenyl-acetamide with rat liver proteins. Chem Biol Interact 2:158–159
7. Caldwell J (1982) Conjugation reactions of nitrogen centers (acetylation reactions). In: Jakoby WB, Bend JB, Caldwell J (eds) Metabolic basis of detoxication. Academic Press, New York, pp 151–170, 291–306
8. Cartwright GE (1977) Methemoglobinemia and sulfhemoglobinemia. In: Thorn GW, Adams RD, Braunwald E, Isselbacher KJ, Petersdorf RG (eds) Harrison's principles of internal medicine, 8th edn. McGraw-Hill, New York, pp 1710–1713
9. Damani LA (1982) Oxidation at nitrogen centers. In: Jakoby WB, Bend JB, Caldwell J (eds) Metabolic basis of detoxication. Academic Press, New York, pp 137 (127–149)
10. Devadatta S, Gangadharam PRJ, Andrews RH, Fox W, Ramakrishnan CV, Selkon JB, Velu S (1960) Peripheral neuritis due to isoniazid. Bull WHO 23:587–598
11. Doelle B, Toepner W, Neumann HG (1980) Reaction of arylnitroso compounds with mercaptanes. Xenobiotica 10:527–536
12. Ebaugh FG, Emerson CP, Ross JF (1953) The use of radioactive 51-chromium as an erythrocyte tagging agent for the determination of red cell survival in vivo. J Clin Invest 32:1260
13. Ehrenberg L, Osterman-Golkar S, Segerbaeck D, Haellstroem T (1976) Evaluation of genetic risks of alkylating agents. II. Hemoglobin as a dose monitor. Mutat Res 34:1–10
14. Evans DAP, Manley KA, McKusick VA (1960) Genetic control of isoniazid metabolism in man. Br Med J 2:485–491
15. Evans EE, Dinman BD, Leech AL (1958) AMA Arch Ind Health 18:422 (cited in Patty's Industrial Hygiene and Toxicology, 2nd edn)
16. Eyer P (1983) The red cell as a sensitive target for activated toxic arylamines. Arch Toxicol [Suppl] 6:3–12
17. Eyer P, Lierheimer E (1980) Biotransformation of nitrosobenzene in the red cell and the role of glutathione. Xenobiotica 10:517–526
18. Forth W (1983) Sind Sie ein Langsam-Azetylierer? Münch Med Wochenschr 125:18–19
19. Gaugler BJM, Neumann HG (1979) The binding of metabolites formed from aminostilbene derivates to nucleic acids in the liver of rats. Chem Biol Interact 24:355–372
20. Glowinski IB, Radtke HE, Weber WW (1978) Genetic variations in N-acetylation of carcinogenic arylamines by human and rabbit liver. Mol Pharmacol 14:940–949

21. Jenkins FP, Robinson JA, Gellatly JBM, Salmond GWA (1972) The no-effect dose of aniline in human subjects and a comparison of aniline toxicity in man and the rat. Food Cosmet Toxicol 1:671–679
22. Kao J, Faulhaber J, Bridge JL (1978) The metabolism of aniline in rats, pigs and sheeps. Drug Metab Disp 6:549
23. Kiese M (1966) The biochemical production of ferrihemoglobin-forming derivatives from aromatic amines, and mechanisms of ferrihemoglobin-formation. Pharmacol Rev 18:1091
24. Kiese M, Taeger K (1976) The fate of phenylhydroxylamine in human red cells. Naunyn-Schmiedeberg's Arch Pharmacol 292:59–66
25. Lehnert G (1980) Biologische Arbeitsstoff-Toleranz-Werte: ein Konzept zur Individualprävention bei Exposition gegenüber gesundheitsschädlichen Arbeitsstoffen. Arbeitsmed Sozialmed Präventivmed 15:266–270
26. Lewalter J, Biedermann P, Schucht Th (1985) In: Analysen in biologischem Material. Deutsche Forschungsgemeinschaft, Verlag Chemie, Weinheim
27. Lewalter J, Korallus U (1985) Evaluation of chromium in separated erythrocytes as a new aspect for biological monitoring of exposition to hexavalent chromium. Int Arch Occup Environ Health 55:305–318
28. Lower GM (1979) N-acetyltransferase phenotype and risk in industrial urinary bladder cancer: approaches to high risk groups. In: Deichmann (ed) Toxicology and occupational medicine. Elsevier, North Holland, Amsterdam, pp 209–219
29. Lower GM, Nilson T, Bryan GT (1976) N-acetyltransferase phenotype of patients with "spontaneous urinary bladder cancer". Proc Am Assoc Cancer Res 17:203
30. Neumann HG (1980) Biochemical effects and early lesions in regard to dose-response studies. Oncology 37:255–258
31. Neumann HG (1981) Bestimmung Hämoglobin-gebundener Metaboliten als Biomonitoring-Verfahren für aromatische Amine. – Arbeitspapier des Arbeitskreises Analysen in biologischem Material der Senatskommission zur Prüfung gesundheitsschädlicher Arbeitsstoffe.
32. Neumann HG (1983) Dosimetry and dose-response relationships. International Seminar on Methods of Monitoring Human Exposure to Carcinogenic and Mutagenic Agents, Finland
33. Neumann HG, Metzler M, Toepner W (1977) Metabolic activation of diethylstilbestrol and aminostilbene derivatives. Arch Toxicol 39:21–30
34. Parke DV (1960) Studies in detoxication, 84. The metabolism of [^{14}C]-aniline in the rabbit and other animals. Biochem J 77:493–503
35. Pereira MA, Lin LHC, Chang LW (1981) Doses-depending of 2-acetylaminofluorene binding to liver DNA and hemoglobin in mice and rats. Toxicol Appl Pharmacol 60:472–478
36. Perry HM Jr, Tan EM, Carmody S, Sakamoto A (1970) Relationship of acetyltransferase activity to antinuclear antibodies and toxic symptoms in hypertensive patients treated with hydralazine. J Lab Clin Med 76:114–125
37. Piotrowski J (1961) Metabolizm niskich dawek aniliny u krolikow. (Metabolism of small doses of aniline in rabbits.) Med Pracy 12:309–317
38. Piotrowski JK (1977) Exposure tests for organic compounds in industrial toxicology. US Government Printing Office, Washington, DC:70–75
39. Richterich R, Colombo JP (1978) Klinische Chemie, Theorie, Praxis, Interpretation. S Karger, Basel, pp 435–454
40. Smith JN, Williams RT (1959) The fate of aniline in the rabbit. Biochem J 44:242–250
41. Strandberg I, Boman G, Hassler L, Sjöqvist F (1976) Acetylator phenotype in patients with hydralazine-induced lupoid syndrome. Acta Med Scand 200:367–371
42. Tannenbaum SR, Skipper PL, Green LC, Turewski RJ, Kadlubar FF (1983) Monitoring exposure to 4-aminobiphenyl via blood protein adducts. International Seminar on Methods of Monitoring Human Exposure to Carcinogenic and Mutagenic Agents, Finland
43. Uehleke H (1973) Mechanisms of methemoglobin formation by therapeutic and environmental agents. Proc 5th Int Congr Pharmacol, vol 2:124

44. Weber WW (1973) Acetylation of drugs. In: Fishman WH (ed) Metabolic conjugation and metabolic hydrolysis, vol 3. Academic Press, New York, pp 249–296
45. Weber WW, Glowinski IB (1980) Acetylation. In: Jakoby WB (ed) Enzymatic basis of detoxication, vol II. Academic Press, New York, pp 169–186
46. Wieland E, Neumann HG (1978) Methemoglobin formation and binding to blood constituents as indicators for the formation, availability, reactivity of activated metabolites derived from trans-4-aminostilbene and related aromatic amines. Arch Toxicol 40:17–35

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