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Review

Protein adducts: quantitative and qualitative aspects of their formation, analysis and applications

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Contents

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1. Introduction

The past few decades have seen an increasing use of protein adduct measurement, in parallel with an ongoing and rapid development of the analytical techniques and more ready availability of the instruments involved. The range of applications is broadening with respect to the types and numbers of adducts that can be measured, along with improvement of analytical sensitivity. An adduct has been defined as a complex that forms when a chemical binds to a biological molecule [1]. In the present context it has also been found practical to use the expression adduct as the moiety covalently bound to a protein as a consequence of a reaction with an electrophilic agent.

Adduct levels in humans have been determined primarily for the purpose of monitoring exposure, e.g. for hygienic surveillance. However, quantitation of adducts formed from carcinogenic and mutagenic compounds originated from a need to determine tissue doses as a parameter in risk estimation. Such information appeared to be particularly important, because there is no threshold dose for mutation and because unacceptable risks often occur even at doses much lower than those which give rise to biological effects detectable in epidemiological or experimental studies (cf. Refs. [2–4]). For these reasons it is important to have highly sensitive methodology for the detection, identification and quantitation of exposure to genotoxic chemicals. However, the usefulness of adduct studies is by no means restricted to mutagens and carcinogens. The chemical reactivity of compounds and/or of their metabolic intermediates may, even at very low concentrations, alter tissue constituents in such a way as to give rise to various harmful conditions or diseases.

Various aspects of protein adduct determination have been subject to several reviews, a few of which are quoted below, including quantitative aspects [5]; analytical procedures [6]; and general aspects on adduct measurements [7–9].

The present review gives a background to measurement of protein adducts focusing on methods and applications. It also describes an introduction to quantitative treatment of certain toxicological problems. Therefore, individual xenobiotics will not be discussed in detail and references for applications to specific compounds are not extensive. The purpose of this paper has been to emphasize certain general aspects of the problem area with examples, to a large extent using the authors' own work.

2. Historical background

The first observation that bioactivated chemicals bind cellular macromolecules was reported by Miller and Miller, in 1947. These investigators showed that exposure to a chemical carcinogen leads to the formation in vivo of intermediates that react with (form adducts to) cellular macromolecules [10].

Their subsequent work [11,12] led to the suggestion that most chemical carcinogens are mutagenic and that these compounds are themselves or are metabolised to electrophilic agents. The involvement of mutation in carcinogenesis had been suggested earlier by Bauer (1928, 1949) [13]. Equally important was the proposal by Brookes and Lawley (1964) [14] that DNA is the key target molecule in connection with chemical mutagenesis.

In his monograph entitled ''Biological Alkylating Agents'' published in 1962, Ross introduced quantitative reaction-kinetic relationships [15], based on, *inter alia*, the Swain–Scott relationship [16] into biological research. In 1969 Loveless [17] demonstrated that the ability to react with certain nucleophilic sites (e.g. guanine-O^6) in DNA is a major determinant of the mutagenic potency of alkylating agents. In agreement with this, it was demonstrated about the same time that the mutagenic effectiveness of simple alkylating agents at low doses was approximately proportional to their rates of reaction with compounds of low nucleophilic strength, corresponding to that of the oxygen atoms in DNA [18] (see also Vogel et al. [19]).

In the 1970s adducts to proteins were introduced for in vivo monitoring of exposure to potentially reactive compounds, particularly carcinogens, and for dose measurement. Groth and Neumann [20] realized the value of protein adduct determination in toxicology, proposing that hemoglobin (Hb) adducts derived from aromatic amines be employed as a measure of the bioavailability of reactive metabolites. Focusing on evaluation of cancer risks and hereditary damage, in 1974 Ehrenberg and coworkers [2,3] initiated quantitation of in vivo doses based on measurement of adducts to proteins, in particular Hb [21]. An early application of this approach to workers exposed to ethylene oxide was described by Calleman et al. in 1978 [22]. In this study alkylated cysteine and histidine were determined by gas chromatography-mass spectrometry (GC-MS) after complete hydrolysis of the globin and amino acid separation.

The tediousness of this procedure emphasized the need for a more rapid and reliable method for measuring specific amino acid adducts. An important step in this direction was the development of the ''*N*-alkyl Edman method'', by which N-alkylated N-terminal valine residues in Hb are specifically detached as derivatives of an Edman reagent that are suitable for mass spectrometric analysis [23,24].

The usefulness of procedures for monitoring of Hb adducts from aromatic amines was supported by further work, even if it was realized that the metabolites forming the Hb adduct, nitrosoarenes, were not identical with those involved in the genotoxic action [25,26]. This technique was early applied for monitoring of human exposure to aniline [27], which causes methemoglobinemia and anemia among workers in the chemical industry.

Today, there are a number of procedures for isolation and analysis of protein adducts, characterized by a high degree of sensitivity, particularly those based on GC-MS (cf. Section 5). In the 1980s and 90s there has been an increasing use of protein adduct measurement in animal experiments and in studies of smokers and of occupational exposures, as well as exposure to urban air pollutants. In the last 10 years detection of background adducts in persons without known exposure has given rise to interest in employing protein adducts to reveal the background load of reactive compounds/intermediates in the body, of both endogenous and exogenous origin. Although protein adducts are measured in a number of laboratories, applications have been more limited than the potential warrants. One limiting factor in these regards may be the requirement for advanced and expensive analytical instruments. In contrast, monitoring of DNA adducts is performed extensively, probably because the procedures involved are simpler and less expensive and because DNA is a primary target in carcinogenic and mutagenic action. Measurement of DNA and protein adducts are complementary in several respects, but in humans it is often difficult to obtain precise quantitative and qualitative data from DNA adduct measurements. Some of these considerations will be discussed further below.

3. Reactive compounds

3.1. Classification

In connection with many types of biological damage, reactivity was recognized at an early stage

as a major determinant of chemical toxicity. Thus, chemical substances that are reactive or give rise to reactive metabolites, whether of exogenous or endogenous origin, may lead to harmful effects in humans. Virtually all known ultimate initiators of chemical carcinogenesis are electrophilically reactive and most are alkylating agents. Table 1 depicts electrophilic compounds/intermediates of importance in this context.

3.2. Mechanisms of adduct formation

Electrophilically reactive compounds are characterized by low electron density centres and the associated ability to react with molecular centres of high electron density, i.e. nucleophiles (*Y*). Adduct formation in biomacromolecules is due to the presence of nucleophilic atoms (especially O, N and S). For most types of electrophilic reagents (*RX*) (see Table 1), the reaction mechanism underlying adduct formation is referred to as nucleophilic substitution (Formula 1). When an electrophile reacts with a nucleophilic atom, a new covalent bond is formed from an unshared pair of electrons (: or denoted by a minus sign) in the nucleophile. In the case of the mechanisms discussed below (Formula 1), the reacting centres of the nucleophile (*Y*) and the leaving group (*X*) can either be neutral or negatively charged, depending on the nature of the reacting species. (In order to avoid misunderstanding, it should be noted that for certain *RX*, e.g. oxiranes, the leaving group, :*X*, may be a part of *R*.)

$$RX + :Y \rightarrow RY + :X \quad (1)$$

The overall reaction occurs in principle via two different mechanisms which influence the kinetics. In the S_N2 reaction (substitution, nucleophilic, bimolecular), an intermediate activated complex of *RX* and *Y* is formed in the transition state (Formula 2) and the rate of reaction depends on the concentration of each of the reactants.

$$S_N2: \quad RX + Y^- \rightarrow [X{-}R{-}Y]^- \rightarrow RY + X^- \quad (2)$$

In S_N1 reactions (substitution, nucleophilic, unimolecular), a reactive cationic intermediate (R^+) is initially formed after dissociation of *RX* into R^+ and a leaving group (X^-). R^+ then reacts rapidly with Y^- (Formula 3). The first of these reactions is slow and rate-limiting.

$$S_N1: \quad RX \rightarrow R^+ + X^- \quad \text{(slow)} \quad (3a)$$

$$R^+ + Y^- \rightarrow RY \quad \text{(fast)} \quad (3b)$$

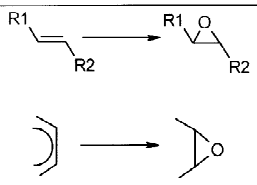
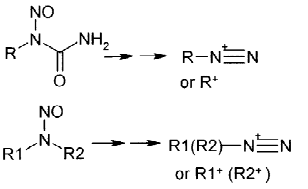
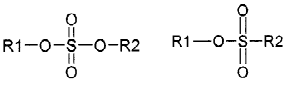
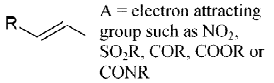
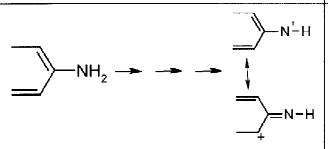
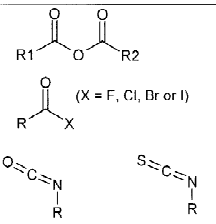
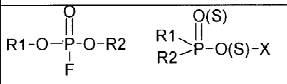
Many electrophilic compounds react by a combination of both mechanisms, with a predominance of S_N2 if the nucleophiles are strong. An important difference between these mechanisms is that when *R* is chiral, the configuration is inverted in S_N2 reactions, whereas racemization often occurs in S_N1 reactions.

In order to relate reaction mechanisms to some industrially important compounds, oxiranes and aldehydes will be discussed briefly (cf. Table 1). Under neutral and alkaline conditions oxiranes react generally by an S_N2 mechanism where substituents, e.g. alkyl groups, have been shown to direct the nucleophilic attack to the sterically least hindered oxirane carbon. Under acidic conditions, reaction rates may be enhanced through hydrogen ion or acid catalysis, protonation of the epoxide oxygen facilitating oxirane ring opening with formation of a reactive carbocation, with a tendency towards S_N1 character of the reaction [28,29]. However, electron withdrawing substituents, e.g. ethenyl or phenyl, weaken epoxide bonds, thus facilitating reaction on the substituted carbon, particularly following acid catalysis [30]. For related reasons several diol epoxides of polycyclic aromatic hydrocarbons (PAH) predominantly react by nucleophilic attack on the benzylic carbon [31–33].

Aldehydes react reversibly with nucleophiles, e.g. amines, to produce Schiff bases via formation of carbinolamines (Fig. 1). The electrophilic properties of the carbonyl carbon atom can be further increased by initial protonation of the carbonyl oxygen. This proton-activated carbonyl carbon atom is able to react with weaker nucleophiles as well. Thus many of these addition reactions, especially those involving weak nucleophiles, proceed more rapidly under acidic conditions.

In comparison to aldehydes, carbonyl compounds with the ability to donate electrons to the electrophilic carbonyl carbon atom, e.g. by hyper-conjugation from adjacent alkyl groups, will become less

Table 1
Classes of electrophilic compounds

Electrophilic agents	Examples of reactive compounds or precursors	Structural formula (R is a variable group, mostly H, an alkyl or an arylalkyl group)	Mechanism of reaction
Alkylating and arylating	Alkyl halides	R—X [X = I, Br, Cl and (F)]	Nucleophilic substitution
	Oxiranes, formed also from alkenes or aromatic hydrocarbons (simple or polycyclic)		
	Alkylnitrosamides; Alkylnitrosamines (reactive via alkyldiazonium ion or carbocation)		
	Dialkyl sulfates; Alkyl alkanesulfonates		
	Activated ethene compounds, e.g. α,β-unsaturated aldehydes; *o*- or *p*-Quinones	 A = electron attracting group such as NO_2, SO_2R, COR, COOR or CONR	1,4-Addition
Compounds with electrophilic nitrogen	Aromatic amines (reactive via nitrenium ion)		Nucleophilic substitution
Carbonyl compounds	Aldehydes		Schiff base via carbinolamine
Acylating	Organic acid anhydrides; Organic acid halides; Isocyanates; Isothiocyanates	 (X = F, Cl, Br or I)	Nucleophilic substitution or addition
Phosphorylating	Organo phosphorous compounds		Nucleophilic substitution
Metal ions	CrO_4^{2-}/Cr^{3+}, Be^{2+}		Several mechanisms
Free radicals	OH, $\cdot CCl_3$		Radical-mediated reactions

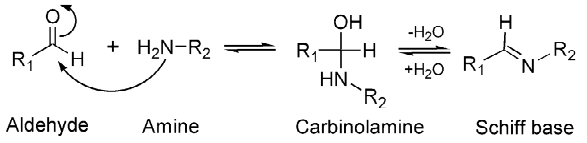


Fig. 1. Mechanism of adduct formation by saturated and unsaturated aldehydes with nucleophilic attack at the carbonyl carbon atom.

reactive. For this reason, ketones are in general less reactive than aldehydes. α,β-Unsaturated aldehydes are bifunctional and, because of their activated ethene group, can also react with nucleophilic groups via 1,4-addition (Fig. 2). Other compounds, such as acrylamide, also contain activated ethene moities and can thus react through 1,4-addition.

3.3. Relative reactivity

A useful approach for describing S_N2 reaction rates for simple alkylating agents with nucleophilic atoms in aqueous solution was presented at an early stage by Swain and Scott [16]:

$$\log\left(\frac{k_Y}{k_{H_2O}}\right) = s \times n \quad (4)$$

where the reactivity of an alkylating agent (rate constant k_Y) towards a certain nucleophilic site (Y) relative to its reactivity towards water (k_{H_2O}) is given by the antilog of the product of the selectivity (or substrate) constant (s) of the alkylating agent and the nucleophilic strength (n) of the nucleophile.

Ross [15] utilized the Swain–Scott empirical relationship (Eq. (4)) to compare the relative reactivities of typical nucleophiles (thiosulfate, pyridine and acetate) towards various alkylating agents. In general, with increasing s-value, nucleophilic reactivity decreases in the order S>N>O. The selectivity (or substrate) constant s of the electrophilic agent thus describes to what extent the reaction rate depends on the strength of the nucleophile (n-value). The two parameters k_Y and s in Eq. (4) above thereby indicate the relative and absolute degrees of alkylation of various cellular nucleophiles at equal concentrations. The n-values in biological macromolecules increase in the following order: $H_2O=0$ (by definition), oxygen atoms on the bases in DNA≈2, guanine–$N7$≈3.5, protein–NH_2≈4–5 and protein–SH≈5–6.

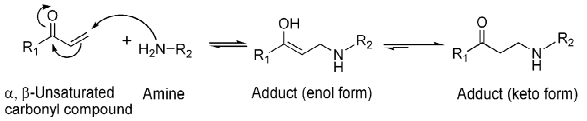


Fig. 2. Illustration of a 1,4-addition reaction involving an activated ethene compound. The ethene bond can be activated not only by a carbonyl group (as exemplified), but also by NO_2 or SO_2R.

This approach has been applied to the prediction of the mutagenic potencies of simple alkylating agents on the basis of their reactivities towards the nucleophilic oxygen atoms (n≈2) in DNA, to which adduct formation is considered to be a putative premutagenic event [18,19]. For instance, alkylnitrosamines and alkylnitrosoureas (with s-values around 0.25) are more efficient mutagens than ethylene oxide and other simple epoxides (with s-values around 0.9) (see for instance Ref. [34]).

Examples of the influence of s-values on reaction patterns are described by several authors [35–37]. Several aspects of reactivity towards different nucleophiles summarized in the literature, not only by Swain and Scott [16], but also by Ross [15], Koskikallio [38], Lawley [39] and Ehrenberg and Osterman-Golkar [5], seem to apply well to simple alkylating agents. It is not yet clear to what extent such considerations can explain the reactivities of more complex electrophilic compounds. Superimposed on the influence of s and n on the reaction rate are the effects of steric factors and of the electrical charges on the reactants. Furthermore, nucleophilic centers are normally reactive only when unprotonated, i.e. as free bases; the corresponding acids are much less reactive. Such influences on reactions with nucleophilic sites in biomacromolecules are discussed in more detail in Section 4.2 below.

4. Biomacromolecules as monitors for electrophilic compounds

4.1. Biomacromolecules available as monitors

For the purpose of monitoring exposure and/or risk assessment of xenobiotics, analysis of the com-

pounds which are biologically active in vivo is desirable. In this context electrophilically reactive compounds/intermediates, with half-lives in vivo varying from milliseconds to hours (depending on their reactivity and rates of enzymatic detoxification), constitute a special analytical problem. Furthermore, many reactive compounds or their precursors, e.g. chemicals of industrial importance (cf. Table 1) are low-molecular mass compounds without distinct moieties (''tags'') that could facilitate analysis of exposure. This situation can be compared to, e.g. analysis of polychlorinated biphenyls, compounds which exhibit a high degree of persistence both in vitro and in vivo and which contain aromatic ring systems and chlorine atoms useful for both isolation and analysis.

As mentioned above, the formation of stable reaction products involving nucleophilic centers in biomacromolecules (particularly DNA and proteins) offers possibilities for the sampling and analysis of electrophilic, short-lived compounds. Important determinants of the usefulness of a biomacromolecule as a monitor for electrophilic agents are the stabilities of the monitor molecule and of the adducts formed. Accumulation of adducts during chronic or intermittent exposure increases the analytical power. Furthermore, a well-defined life span of the biomacromolecule involved simplifies quantitative calculations of the dose (concentration over time; cf. Section 6.2) of the causative electrophile. Fig. 3 illustrates adduct formation and determinants of adduct levels.

From both humans and animals blood samples can be taken conveniently. One millilitre of blood contains approximately 150 mg Hb in red blood cells, about 30 mg serum albumin (SA) and 0.005–0.008 mg DNA in white blood cells. Hb and SA have properties that render them advantageous for use as monitor molecules including: accessibility in large amounts; known kinetics and rates of turnover; formation of adducts that are not removed by active repair systems and that are for the most part chemically stable (cf. Table 2). In comparison, enzymatic processes repair DNA adducts at varying rates, which often results in only a low level of accumulation and difficulties in defining life span. In Table 2 the levels and turnover rates of biomacromolecules in blood are summarized.

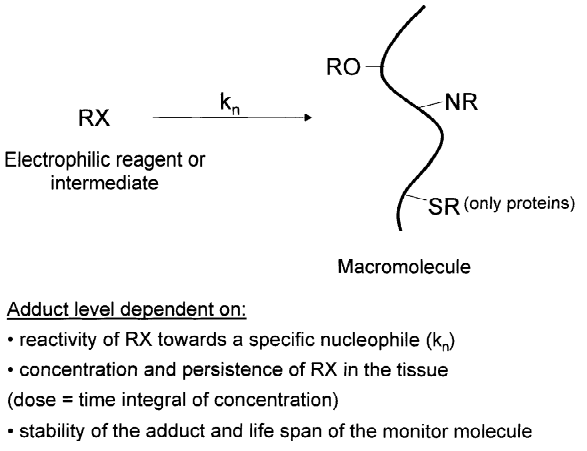


Fig. 3. Schematic illustration of adduct formation to nucleophilically reactive atoms in macromolecules (proteins and DNA). The electrophilic agent (*RX*) is a directly reactive compound and/or reactive metabolite from the precursor (*A*).

The relatively long and well-controlled life span of Hb (approximately 4 months in humans) was one important reason for choosing this protein as a dose monitor for electrophilically reactive compounds. Adducts of SA accumulate to a lesser extent, but might, in certain cases be preferable for monitoring purposes (cf. Refs. [41,44]). In general, the protein with the highest degree of alkylation by the compound being studied is to be preferred. Blood proteins are useful for monitoring exposure which is ongoing or occurred several months previously. However, the rapid turnover of certain DNA adducts may be advantageous for studying, e.g. background adducts in dietary intervention studies [45].

For tracing mutagenic factors which may be involved in the development of diagnosed tumors, the ability to monitor exposure several decades backwards in time would be of value. In this perspective the life spans of Hb and SA are far too short. Attempts have therefore been made to utilize more long-lived proteins for such dose monitoring [46].

Histones, i.e. DNA-binding lysine- and arginine-rich proteins (M = 11 000–21 000) among the nucleoproteins of eukaryotic cells, exhibit half-lives in mice which are about 20% of the life span of the animal [46]. Preliminary experiments have revealed the potential usefulness of histones for dose moni-

Table 2
Biomacromolecules in blood used as monitor molecules, their availability and turnover rates

Macromolecule	Type of sample	Amount available in blood	Turnover
Hemoglobin (Hb)	Red blood cells	~150 mg/ml M: 64 500	*Life span:* Humans 126 days [40] Rat 60 days [40] Mouse 40 days [40]
Serum albumin (SA)	Blood plasma	30–45 mg/ml M: 68 500	*Half-life:* Human 20 days [41] Rat: 2.5 days [42] Mouse:1.9 days [42]
DNA	White blood cells	~6 μg/ml [40,43] $M_{nucleotide}$: 309	Complex kinetics

toring. Binding of the mutagenic diolepoxide of benzo[*a*]pyrene (BaP) to histones, probably to lysine residues, has been reported.

Collagen, a structural protein in connective tissue and cartilage, is also of interest in this context because of its very slow turnover and its abundance (constituting about one-third of the total protein in the body) [46]. This protein (M = 295 000) has an unusual amino-acid composition, with large amounts of glycine and proline and the presence of hydroxyproline and hydroxylysine. Its usefulness, though, as a monitor is limited by the crosslinking of the protein which accompanies ageing and by the insolubility of mature collagen. However, solubilization by specific enzymatic degradation [47] might render collagen potentially useful as a monitor molecule for studies of specific problems.

4.2. Nucleophilic sites in proteins and their reactivities

Several amino acids possess side-chains containing nucleophilic atoms with reactivity towards electrophiles (Fig. 4). This reactivity is mainly associated with the non-protonated form (Y^-), the relative amount of which depends on the pK_a of the group and pH according to the relationship

$$\frac{[Y^-]}{[Y^-]+[YH]} = \frac{1}{1+10^{pK_a-pH}} \quad (5)$$

The carboxylate moieties in glutamate and aspartate residues and at the C-termini of proteins have pK_a values in the range of 3–4.5 and may thus be considered to be fully non-protonated in the blood

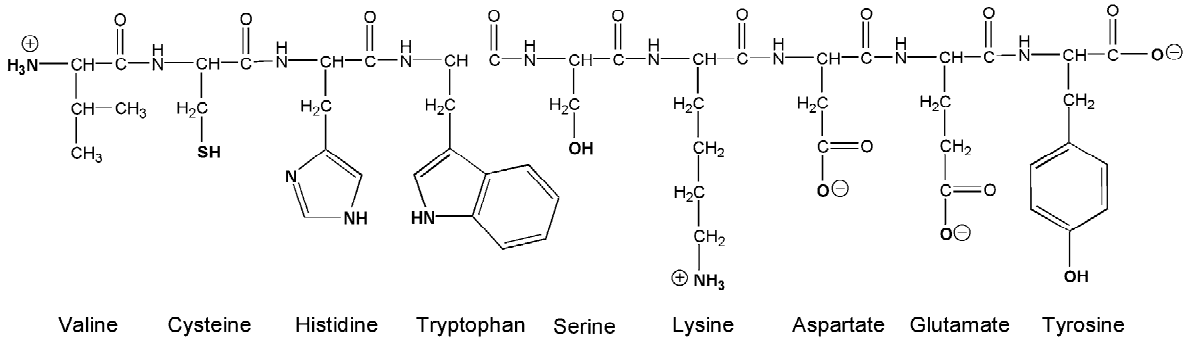


Fig. 4. An example of a peptide containing amino acids in their predominating form with regard to protonation or non-protonation at pH~7. The atoms that are in the form of free base possess nucleophilic reactivity are in bold letters. The amino acid valine exemplifies the nucleophilic N-terminal.

(pH 7.4). The pK_a of strongly basic nitrogen atoms in the side-chain amino group of lysine and the guanidine moiety in arginine are in the range of 9.5–12.5; at the pH of the blood these groups are thus nearly completely protonated.

In three amino acids in Hb, the pK_a's of the nucleophilic centers are in the neighbourhood of the blood pH. These centers are the nitrogen atoms of N-terminal residues (valine in many species), the imidazole nitrogens of histidine residues and the thiol sulfur of cysteines (pK_a values given in Table 3). To the extent that the reaction rate depends on the relative amount of free base (Table 3), the N-terminus of the β-chain in human Hb is expected to be alkylated more rapidly than the α-chain N-terminus. However, the correlation between basicity (as measured by pK_a) and nucleophilic strength would give an effect in the opposite direction [15].

Kinetic studies have exhibited some, but not a very large, difference between α- and β-chain N-termini in their rates of reaction with electrophiles such as simple epoxides (Törnqvist et al., unpublished data). Mass spectrometric measurement of adducts formed initially upon incubation of human Hb with diepoxybutane in vitro reveals that the N-termini are major sites of alkylation, with approximately equal reactivities of the α- and β-chain N-termini (however, pH regulation is not mentioned) [50].

Certain aspects of the pattern of adducts at nucleophilic sites in Hb and DNA have been shown to comply rather well with predictions based on the Swain–Scott relationship [35]. The patterns of alkylation of Hb by several alkylators with different selectivity constants *s* were compared, and by and large the findings were in agreement with expectations; more pronounced increase in adduct levels was observed with increasing *s* values in the series carboxyl–O < histidine ring–N < cysteine–S groups [35] (Fig. 5). Similarly, the ratio of alkylation at guanine–O^6/guanine–$N7$, in DNA is low at high *s* values, (e.g. 0.5% for ethylene oxide, for which $s = 0.96$) and high for compounds with low *s* values, such as nitrosamides, (e.g. 63% for *N*-(2-hydroxyethyl)-*N*-nitrosourea, with $s \approx 0.25$) [19,35,37,51,52].

There are, however, many exceptions to these simple rules, due to other factors which influence the rate of adduct formation. A higher degree of alkylation of cysteine residues in rat Hb compared to human Hb has been observed for simple alkylating agents (e.g. for ethylene oxide, this difference is approximately 150-fold) [35]. Two factors which contribute to the elevated reactivity of a specific cysteine residue ($Cys^{125\beta}$) in rat Hb have been discussed, i.e. location on the surface of the Hb molecule, and thus low steric hindrance [53]; and a low pK_a value, resulting in a relatively high level of unprotonated thiolate anion (Cys–S^-) [54]. The observation that following administration of fluoranthene to rats, only a cysteine adduct of the mutagenic diol epoxide was detectable probably has a related explanation [55]. In the case of electrically charged molecules, reaction rates may be enhanced when the electrophile and nucleophile are oppositely charged and, conversely, decreased when their charges are of the same sign [19,56].

Different aspects of the reactivities of alkylating

Table 3
Dissociation constants (pK_a) of certain nucleophilic groups in proteins, and calculated fractions available as free base at pH 7.4 (cf. Eq. (5))

Group	pK_a	% as free base
Cysteine–S	7.9–8.5	24–7
Histidine-ring–N	5.6–7.0	98–72
Terminal NH_2 in Hb (valines)[a]		
α-Chain	7.8	28
β-Chain	6.8	80

[a] Ref. [48] (The values are still controversial [49]).

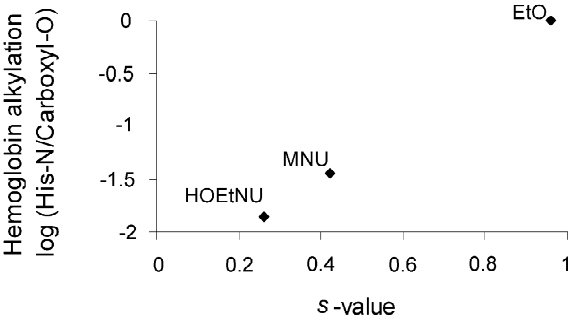


Fig. 5. Relationship between the alkylation of histidine–N^τ and carboxylic oxygen atoms in hemoglobin of mouse and the *s* values for ethylene oxide (EtO), *N*-hydroxyethyl-*N*-nitrosourea (HOEtNU) and *N*-methyl-*N*-nitrosourea (MNU) (adapted from Segerbäck [35]).

agents towards various nucleophiles have been summarized by several authors (see Refs. in Section 3.3). It is not yet clear to what extent such considerations can explain the reactivities of more complex electrophiles. Superimposed on the influences of *s* and *n* on the reaction rate are steric factors (so-called ''steric hindrance'') that usually tend to decrease reaction rates, particularly in the case of bulky compounds. It has been suggested that bulky compounds may ''dock'' to a protein molecule by protein–ligand interactions, followed by reaction with nucleophilic sites, thus giving rise to site-specific alkylation [57]. Examples of such behaviour are the predominant alkylation of human SA by diol epoxides of fluoranthene and BaP at histidine[146] [58].

4.3. Comparison of Hb and DNA for qualitative and quantitative monitoring

DNA in the cell nucleus is the target where genotoxic compounds or their metabolites exert mutagenicity and initiate the carcinogenic process. DNA would therefore seem to be the monitor of choice for the determination of adduct levels as an in vivo measure of exposure to genotoxic chemicals. However, in connection with the determination of in vivo doses in terms useful for estimation of cancer risk [2,3], the use of proteins as monitors seemed to offer several advantages over the use of DNA (for definition of in vivo dose, see Section 6.2). The use of DNA and protein adducts for exposure and dose monitoring has been compared earlier (e.g. in Refs. [7,59,60]).

As demonstrated in several studies (for instance see Refs. [25,35]) the initial levels of adducts formed in DNA and protein in animals administered different doses of a genotoxic agent are proportional to one another. The levels of DNA adducts in different organs are dependent on the amount of time which has elapsed since exposure, the efficiency of DNA repair and the pharmaco-kinetics of the substance (see Ref. [35]). Since adducts to DNA and adducts to Hb arising from a particular electrophilic agent disappear at different rates, the relative amounts of these adducts will differ at different times after acute exposure [61]. For a more meaningful comparison, the levels of DNA and Hb adducts a short time after exposure should be determined by extrapolation to time zero.

The rates of reaction of electrophiles with proteins and DNA (per gram of material), are usually of the same order of magnitude. Thus, since proteins such as Hb and SA are present in blood in much larger amounts than DNA (Table 2), measurement of protein adducts favours high power of detection. Furthermore, better methods for specific detection and identification of protein adducts by mass-spectrometric procedures are developed than is the case for DNA adducts (although sensitive methods for mass spectrometric analysis of DNA adducts are now being developed; cf. Sections 5.5.1 and 5.6.1).

It should be noted that determination of adducts to DNA in white blood cells will, like analysis of Hb adducts, provide information concerning the initial dose in the blood. Measurement of DNA adducts in various organs shortly after acute exposure or under conditions where DNA repair can be compensated for may yield the distribution of the total dose between target organs [35,62].

It should be noticed that there are exceptions to the rule that an agent that binds covalently to nucleophilic sites in proteins is also reactive towards nucleophilic sites in DNA and vice-versa. For instance, acrylamide forms adducts to amino and thiol groups in proteins by 1,4-addition [63–65], but does not form detectable stable adducts to DNA in vivo [66]. The genotoxicity of acrylamide is assumed to be exerted by its metabolite glycidamide, an oxirane that is reactive towards both Hb and DNA [65,66]. In addition, certain compounds give rise to different types of adducts in protein and DNA. For instance, unsaturated aldehydes, which are bifunctional, may give rise to exocyclic rings on DNA bases [67].

The use of adduct levels for in vivo dosimetry requires, particularly at low levels of exposure, that the adducts measured are formed by reaction with the protein and not by misincorporation of modified amino acids in connection with protein synthesis. Valine containing a substituted amino group cannot be utilized in protein synthesis, in contrast to, e.g. histidine and cysteine with modified side-chains [68]. Adducts to the N-termini of globin measured, e.g., by the N-alkyl Edman method are therefore evidently formed post-translationally. DNA adducts may also arise from analogous misincorporation of modified

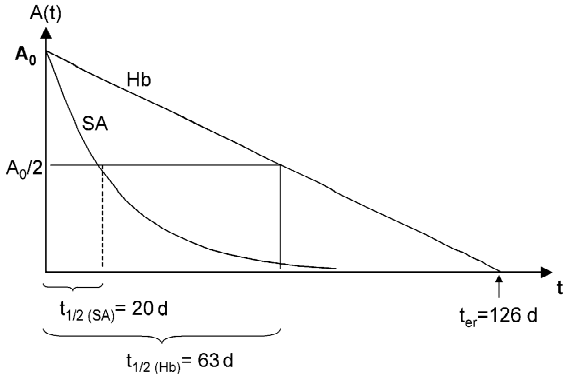


Fig. 6. The levels for chemically stable adducts to Hb and SA in humans as a function of time [$A(t)$] after acute exposure.

bases as indicated by the incorporation of base analogues [69,70].

4.4. Kinetics of formation and disappearance of adducts

For a careful quantitative treatment of results from adduct measurement, e.g. in toxicological evaluations, the kinetics of the variations of the adduct levels have to be considered. However, for work with qualitative or semi-quantitative purposes Figs. 6 and 7 may give sufficient ideas on the basic kinetics.

Following acute exposure (e.g. by injection or accidental release) to a genotoxic compound capable of reacting (often after metabolism) with proteins, to produce a stable adduct, disappearance of this adduct will be determined by the turnover kinetics of the protein. In the equations below, A denotes the adduct level, A_0 the initial adduct level after acute exposure and k the rate constant for disappearance. The level $A(t)$ at time t of an adduct to SA, the turnover of which follows regular first-order kinetics, will thus decrease according to

$$\frac{dA}{dt} = -kA, \quad \text{i.e.} \quad A(t) = A_0 \cdot e^{-kt} \tag{6}$$

The relationship of the rate constant to the half-life ($t_{1/2}$) is given by

$$k = \frac{\ln 2}{t_{1/2}} \tag{6a}$$

In contrast, in certain species (e.g. man and mouse, but possibly not strains of rat [71]) Hb has a predetermined life span equal to that of the red blood cells, which in humans is about 126 days. Since acute exposure leads to the formation of adducts in Hb molecules of all ages, 1/126 of the initial level of adducts to human Hb will be lost each day (see Fig. 6), i.e. in accordance with zero-order kinetics:

$$\frac{dA}{dt} = -\frac{1}{t_{er}} A_0$$

$$t_{er} = \text{life span of the red blood cells} \tag{7}$$

If the Hb adduct is unstable, the rate at which its level will decrease is expressed by the product of functions (6) and (7), where k is the rate constant for decay due to this instability [72].

Most human exposure is more-or-less continuous and adducts will therefore accumulate until a steady-state level is reached. Recurrent or intermittent exposure may also be considered constant in this respect if the time intervals between peak exposures are not too large. After onset of a relatively constant daily exposure, the adduct level will increase daily by the increment $\bar{a}$, following first-order kinetics (as in SA), to produce adduct level $A(t)$ at time t following onset of exposure:

$$A(t) = \frac{\bar{a}}{k}(1 - e^{-kt}) \tag{8}$$

This adduct level approaches a ''steady-state'' asymptotically (Fig. 7)

$$A(t = \infty) = \frac{\bar{a}}{k} \tag{9}$$

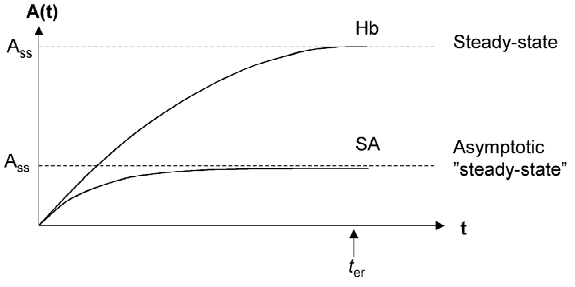


Fig. 7. Theoretical accumulation of adducts to Hb and SA as a function of time. Hb adducts reach a steady-state level, whereas SA adducts approach a ''steady-state'' level asymptotically.

The level of Hb adducts will correspondingly increase towards a true steady-state according to the relationship:

$$A(t) = \bar{a} \cdot t\left(1 - \frac{t}{2t_{er}}\right); \quad A(t \geq t_{er}) = \frac{\bar{a} \cdot t_{er}}{2} \qquad (10)$$

When $t = t_{er}$ this function reaches a steady state (Fig. 7):

$$A_{ss} = \frac{t_{er}}{2} \cdot \bar{a}, \quad \text{in man, } A_{ss} \approx 63\bar{a} \qquad (11)$$

If continuous or intermittent exposure is discontinued prior to or after A_{ss} has been reached, the Hb adduct level will subsequently decrease according to functions developed by Granath et al. [72]. Remnants of previous exposures in the form of accumulated Hb adducts make precautions necessary in attempting to establish relationships between exposure dose and in vivo dose (cf. Section 6.2) [73,74]. In this respect, the determination of SA adducts is simpler: according to first-order kinetics, (i.e. every adduct exhibits the same probability of disappearing, irrespective of its age), function (6) describes the rate of the decay from any point in time ($t = t_0$, $C = C_0$).

When temporal variations in exposure are so large that a daily (or weekly) average increment in adduct levels becomes misleading for dose assessment; the variations of adduct levels with time may be calculated as described by Ref. [72]; see also Ref. [75].

5. Procedures for measurement of protein adducts

5.1. Introduction

In principle, all procedures for chemical analysis of protein adducts (here adduct denotes the moiety bound to the macromolecule, cf. Section 1) consist of the following steps:

- Isolation of the protein
- Detachment of the adduct (or adduct–amino acid or adduct–peptide complex) from the protein
- Isolation of the detached adduct
- Derivatization of the adduct
- Analysis of the adduct

Adduct measurement is performed in one of two ways, depending on the structure of the adduct and the methodology available;

I analysis of the adduct after detachment from the amino acid in the protein; or
II analysis of the modified amino acid or modified peptide (adduct-amino acid/peptide complex) after detachment from the protein by cleavage of peptide bonds.

Analysis according to approach I using current methods requires in principle that the adduct be lipophilic in order to facilitate isolation from the protein. After detachment according to approach II the analyte consists of the adduct still bound to an amino acid or peptide, which facilitates the isolation of, in particular, hydrophilic and low-molecular-mass adducts. Below, the procedures and methodology employed for isolation and chemical analysis of adducts are reviewed. For detailed methodological descriptions, see the publications cited as examples of applications.

5.2. Methods for isolation of blood proteins

After collection of a blood sample, the plasma and red cells are separated by centrifugation and the red cells subsequently washed with saline, prior to further work-up or storage at ≤ 20 °C. Analysis of adducts is then performed using the protein isolated by precipitation (except most procedures for detachment of the adduct by mild hydrolysis according to approach I above).

One method commonly used for precipitation of globin from hemolysed red blood cells involves addition of the hemolysate to 50 m*M* HCl dissolved in 2-propanol, followed by pelleting by centrifugation (at $3000 \times g$), first of the cell membranes and then, following addition of ethyl acetate, of the globin [76], see also Ref. [77]. A procedure described in 1930 [78] for the precipitation of globin is based on precipitation of the hemolysate in 0.12 *M* (1%) HCl dissolved in acetone (see, e.g. Ref. [79]). In order to avoid too strongly acidic conditions, the use of 2 m*M* HCl dissolved in acetone has also been employed [80]. Precipitation with HCl–acetone requires that cell membranes are first removed (by centrifugation at 20 000 *g*). For routine analysis it

has not been considered necessary, except in special cases, to further purify the globin, since other proteins are present at relatively low concentrations.

For studies of adducts to serum albumin (SA), isolation of SA from blood plasma has been achieved by addition of saturated ammonium sulfate (1:1, v/v) to precipitate a protein fraction devoid SA (removed by centrifugation), followed by precipitation of SA by gradual addition of 1 *M* acetic acid to the supernatant (centrifugation at 9000×*g*) [81]. In order to reduce the water content of precipitated globin or SA, the precipitates are washed with, e.g. ethyl acetate or diethyl ether before drying.

When isolation of proteins by precipitation is not applicable, other purification procedures are employed. Application of methods for direct detachment of adducts from Hb in hemolysate (approach I above) requires prior dialysis of the hemolysate. For the purification of SA, affinity chromatography [41], ion-exchange chromatography (IEC) [82] or IEC in combination with gel filtration [83,84] have all been adopted. Measurement of total levels of plasma protein adducts after detachment of the adducts by mild hydrolysis without prior purification of plasma proteins, has also been employed (e.g. Refs. [85,86]).

5.3. Methods for detachment and isolation of adducts

5.3.1. Detachment of adducts from the protein

5.3.1.1. Hydrolysis under relatively mild conditions. Certain adducts, such as those bound as esters to carboxyl groups or as sulfinamides to cysteines, can be detached from the amino acid and the protein through mild alkaline or acidic hydrolysis. This approach involves careful and mild purification of protein samples, accomplished, e.g. by dialysis or IEC, prior to hydrolysis (which is usually performed using 0.1 *M* HCl or 0.1 *M* NaOH). The conditions (acid or base, time, temperature) for hydrolysis are optimized for the particular adduct being studied.

The detached adducts are isolated employing solvent extraction or solid-phase extraction (SPE). Enrichment by immunoaffinity chromatography has been applied in studies of hydrolysable adducts of BaP [87,88]. The adducts released and isolated by these methods can be analyzed with a high sensitivity, particularly if these adducts are further derivatized with fluorinated reagents for the detection upon analysis by gas chromatography-mass spectrometry (GC-MS), using chemical ionization in the negative-ion mode (NICI).

Sulfinamides in Hb originating from cysteine-SH in reaction with nitrosoarenes formed in the erythrocytes from *N*-hydroxylated aromatic amines could be used for analysis of the free amines formed in hydrolysis [26,89,90]. This is a frequently applied approach in connection with human biomonitoring studies (reviewed in Ref. [91]). The procedure is also applicable to nitroarene exposure [71].

Adducts formed from PAH and in particular from BaP [87,92,93], as well as from the tobacco-specific nitrosamine 4-(methylnitrosamino)-1-(3-pyridyl)-1-butanone (NNK) [94–96] are bound as esters to carboxyl groups and have also been analyzed after detachment by mild hydrolysis.

Adducts from organic acid anhydrides can be detached by mild hydrolysis and comprehensive studies on both Hb and plasma protein adducts originating from these compounds in exposed humans have been performed [97–99].

Isocyanates are capable of acylating thiol, hydroxy and amino groups in proteins to form *S* or *O*-alkyl carbamates and carbamoyl adducts, respectively. The reaction product with the thiol group in cysteine has been shown to be hydrolyzed under mild conditions (both acidic and alkaline); the thiol adducts are thus detached and can then be degraded to the corresponding free amines, which can be easily derivatized for subsequent measurement. These reactions have been utilized for biomonitoring of isocyanates through their reaction products with blood proteins [83,100]. In Section 5.3.2.5, a method for specific measurement of adducts from isocyanates as carbamoylated N-terminal valines in Hb is described.

5.3.1.2. Acetylation. For the analysis of protein adducts to cysteinyl residues formed from benzene oxide, stronger conditions are required for detachment [101], see also Ref. [102]. In this case, the cysteine-*S* bond is cleaved by reaction with trifluoroacetic anhydride and methanesulfonic acid (at 100 °C) to produce *S*-phenyltrifluorothioacetate. This reaction with trifluoroacetic anhydride has the advantage that fluorine atoms, allowing sensitive detection

during GC-MS analysis by NICI, are introduced into the adduct derivative in connection with the detachment step.

5.3.1.3. Cleavage with Raney nickel. An alternative method for determination of globin adducts, described by Ting et al. [103], is based on desulfuration of sulfur-containing compounds employing Raney nickel (Ra–Ni). In this reaction the carbon-sulfur bond between the alkyl group and cysteine-*S* is cleaved, the sulfur atom is removed as H_2S and at least one new carbon-hydrogen bond is formed [104,105]. This method has been used mainly for adducts of styrene oxide [106], but can be used to detach any adduct to cysteine thiol groups from proteins. When the cysteine adduct is unsubstituted, a simple hydrocarbon is formed (see Fig. 8). For analytical and chromatographic purposes, derivatization of the product formed by Ra-Ni treatment has been achieved with fluorinated reagents prior to analysis by GC-MS NICI [103].

5.3.2. Detachment of modified amino acids by cleavage of peptide bonds

5.3.2.1. Total acid hydrolysis of proteins. One of the first methods used for the isolation of adducts [21] was total hydrolysis of the protein in 6 *M* HCl (110 °C, overnight) and subsequent isolation of the amino acids carrying adducts (mainly histidines and cysteines) by ion-exchange chromatography. When applied to studies of individuals exposed to ethylene oxide, the modified amino acids thus isolated, i.e. 2-hydroxyethylated histidines, were derivatized with heptafluorobutanoic anhydride prior to analysis by GC-MS [22,107]. This approach is rather harsh and time-consuming and does not result in a high degree of sensitivity in the analysis. Nonetheless, this method is useful for the isolation of stable adducts for the purpose of identifying these adducts and their binding sites in the protein after in vitro or in vivo treatments, e.g. with radiolabelled compounds [37]. Total acid hydrolysis has, however, been employed successfully for the detection of low levels (pmol/g) of an adduct derived from a heterocyclic amine. A high degree of sensitivity was achieved in this case by fluorescence detection following comprehensive work-up procedures [108].

In the last few years a method based on total acid hydrolysis of blood proteins for measurement of a new biomarker for oxidative stress, has been developed and applied in studies on humans exposed to ambient air pollution and with varying dietary habits [109–111]. Oxidized amino acids (lysine, proline and arginine) in the intact protein are first derivatized with fluorescein amine. After total hydrolysis of the protein, amino acids are separated by HPLC and modified amino acids (adduct levels >1 nmol/g) quantitated on the basis of their UV absorption.

5.3.2.2. Hydrazinolysis of proteins. Quantitation of protein adducts that are unstable under acidic conditions and formed at sites other than aspartate, glutamate, cysteine and N-terminal residues in Hb has required the development of alternative methods. Digestion of proteins with hydrazine was recently shown to be suitable for the release of amino acids carrying adducts [112]. This procedure was first used

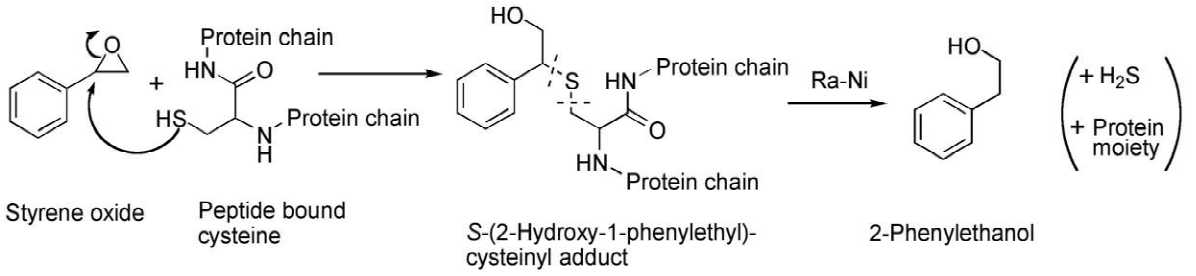


Fig. 8. The principle of analysis of cysteine adducts employing of Raney nickel. One of the two possible adducts formed from styrene oxide (i.e. cysteine substituted via the α-carbon) is shown.

by Akabori and coworkers [113] for the determination of C-terminal amino acids.

Hydrazinolysis has been successfully employed to analyze BaP diolepoxide adducts to histidine in SA and Hb. In this procedure degradation of precipitated protein is performed in hydrazine at 100 °C overnight. The amino acid hydrazides formed (Fig. 9) are isolated with C_{18} SPE and further derivatized with *tert*-carbobutoxy groups to facilitate chromatography and purification. These adduct derivatives are then further purified by cation-exchange SPE before analysis.

The advantage of hydrazinolysis (under alkaline conditions) is that acid-sensitive adducts, such as those from PAH diolepoxides, located in the interior of the protein, are released as alkylated amino acid hydrazides. To date, this method has been utilized for identification of adducts in Hb and SA treated in vitro with BaP diolepoxide by HPLC-MS/MS and for studies of adducts to these proteins in mice treated with radiolabelled BaP [44]. The procedure seems promising for a broad spectrum of applications.

5.3.2.3. Enzymatic hydrolysis of proteins. Development of electrospray ionization (ESI) techniques for LC-MS has simplified peptide sequencing and is now used for a broad range of applications in the analysis of enzymatic protein digests in protein characterization (proteomics). Digestion of proteins into peptides by proteases combined with ESI MS has also been shown to be useful in connection with adduct analysis. A major advantage of protease digestion is that these enzymes cleave at specific sites in the protein. The most commonly used protease, trypsin, hydrolyses peptide bonds whose carbonyl groups are contributed by lysine and arginine. To avoid the formation of peptides with overlapping sequences, the specificity of the protease is essential.

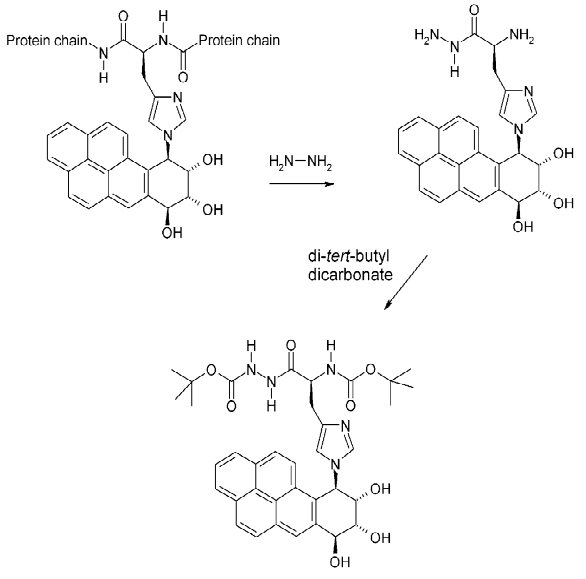


Fig. 9. The principle underlying analysis of benzo[*a*]pyrene diolepoxide adducts by hydrazinolysis.

Enzymatic hydrolysis combined with MS-techniques has successfully been applied for studies of adduct binding sites in Hb treated in vitro with methyl bromide, diepoxybutane, epichlorohydrin and styrene oxide. Analysis of modified tryptic peptides was performed by electrospray ionization-tandem mass spectrometry (ESI MS/MS) or matrix-assisted laser desorption ionization-tandem mass spectrometry (MALDI MS/MS) [50,114–116]. In addition, binding sites of adducts in human SA treated in vitro with PAH diolepoxides have been characterized by analysis of peptides released by proteinase K and pronase with HPLC-MS/MS (ESI) [58] and nanospray MS/MS [117].

To date there are only a few examples of in vivo studies on peptides modified by alkylating agents. Kautiainen et al. [118] utilized a ring-closed adduct formed from reaction of diepoxybutane with N-terminal valine in Hb as a specific biomarker, in studies of mice treated by i.p. injection with diepoxybutane. Trypsination of the α-chain of such globin yields an N-terminal heptapeptide which, after fractionation by HPLC-UV, can be analysed by HPLC-MS/MS (ESI). An exceptional example of studies on human exposure, using modified peptides, is the analysis of BaP diolepoxide adducts to the dipeptide His–Pro in pronase digests of SA by enrichment of the adducts with immunoaffinity columns followed by HPLC and laser-induced fluorescence (LIF) detection [119].

5.3.2.4. Detachment of modified N-terminal valine residues from hemoglobin. The N-termini of the globin chains in Hb are primary sites for reaction with several classes of electrophilic substances.

Methods for the detachment of adducts to N-terminal valine residues in Hb were explored in order to find a procedure that was milder, simpler and more sensitive than those based on total hydrolysis. It was observed that when the Edman degradation reaction was employed, the *N*-alkylated N-terminal valine detached spontaneously as a phenylthiohydantoin (PTH) even under the conditions (pH~7, see Fig. 10) utilized for the initial coupling of phenyl isothiocyanate to the protein [23]. In contrast, the classical Edman degradation for detachment of N-terminal residues requires an acidification step. These observations led to the development of the ''N-alkyl Edman'' procedure.

In this procedure the precipitated globin is dissolved in formamide and treated with a fluorinated Edman reagent (pentafluorophenyl isothiocyanate) at near-neutral pH. Thereafter, the derivatives of the detached *N*-alkylated N-terminal valines are isolated by extraction [24,77]. Thus, detachment and derivatization of the adduct–amino acid complex to a fluorinated derivative (i.e. a pentafluorophenylthiohydantoin, PFPTH) is achieved in a single step. Analysis can usually be performed after purification by extraction. If the adduct contains highly hydrophilic groups, e.g. several hydroxyl groups, the extraction procedure must be modified and derivatization designed to obtain more lipophilic character [79]. This analysis is carried out with GC-MS NICI in order to obtain maximal sensitivity.

Mechanistic studies on the N-alkyl Edman reaction have revealed the reason why rapid cyclization of the analytes occurs even under the conditions employed for the coupling reaction [120,121]. This type of accelerated intramolecular reaction is often observed when alkyl groups are situated on a chain between the two reacting centers. This effect, often called the *gem*-dialkyl effect, was first explained by Thorpe and Ingold [122]. The rates of this reaction for alkyl-substituted and unsubstituted chains often differ by several orders of magnitude [123]. The fact that the *gem*-dialkyl effect introduces a selectivity for N-substituted valines as compared to unsubstituted *N*-terminal valine residues in Hb contributes to the high degree of sensitivity of this method.

This procedure has been employed with a broad range of alkylating agents, e.g. simple epoxides (reviewed in Ref. [124]), and acrylonitrile and acrylamide forming adducts through addition reactions (see e.g. Refs. [64,125]). The method is also applicable to adducts derived from aldehydes, following stabilization by reduction of Schiff bases to secondary amines (and, at the same time, carbonyl to 2-hydroxyl moieties) [126,127].

5.3.2.5. Detachment of carbamoylated N-terminal valine residues from hemoglobin. Partly as a spin-off of the Edman degradation procedure, a method for detachment of adducts of isocyanates to N-terminal valines has attracted attention as a measure of exposure to isocyanates [128,129]. Carbamoylated N-terminal valines, derived from isocyanates are reasonably stable and can be detached as hydantoins upon acidification with 2 m*M* HCl or a mixture of HCl and acetic acid (2:1) at 100 °C for 1–8 h (Fig. 11A). The principle involved is the same as that in the original method for protein sequencing (published as early as 1930) of Abderhalden and Brockman [130], which was a precursor to the Edman degradation method.

Measurement of the hydantoins formed specifically from isocyanates offers several advantages over a

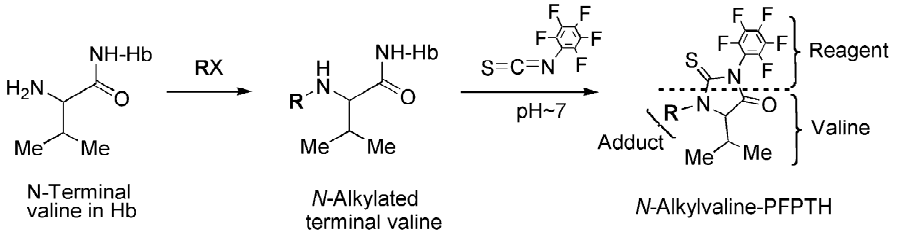


Fig. 10. The principle of the N-alkyl Edman procedure. Alkylation of N-terminal valine residues in Hb is followed by derivatization with pentafluorophenyl isothiocyanate (PFPITC) to form *N*-alkylvaline derivatives (pentafluorophenyl thiohydantoins, PFPTH) suitable for GC-MS analysis.

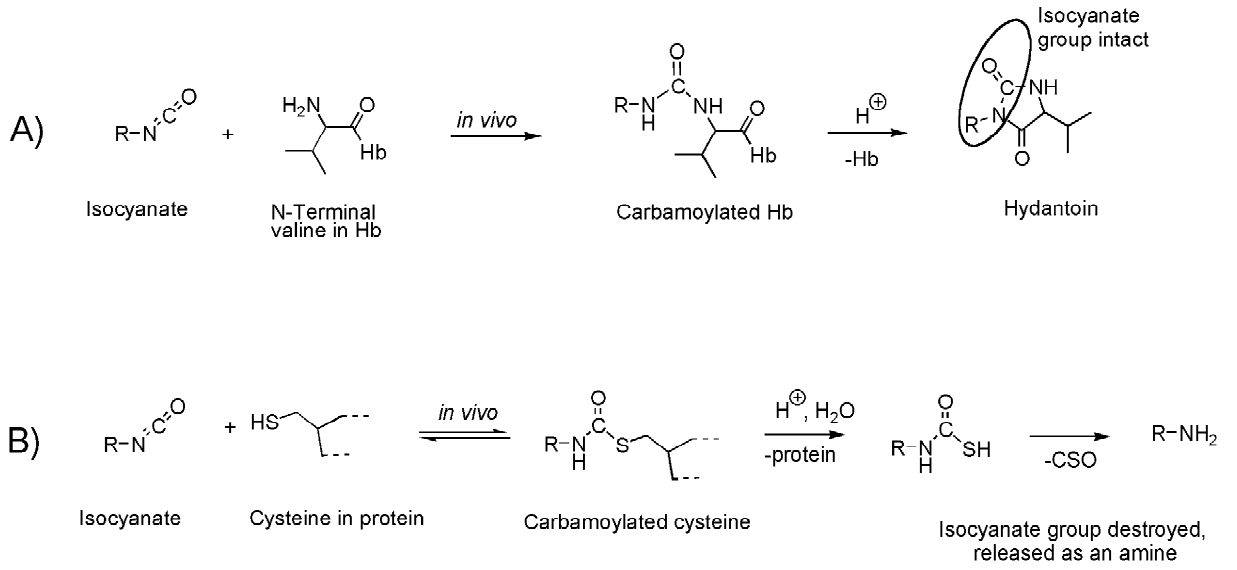


Fig. 11. (A) Reaction between isocyanate and N-terminal valine in hemoglobin and detachment of the adduct as a hydantoin after acidification. (B) Reaction between isocyanate and cysteine in proteins and detachment of the adduct by mild acid hydrolysis, leading to formation of a free amine (suggested mechanism).

measurement of amines after detachment of adducts derived from isocyanates by mild hydrolysis (cf. Section 5.3.1.1 above; see Fig. 11B). Formation of a lipophilic hydantoin, facilitates analysis of adducts from low-molecular-mass aliphatic isocyanates, e.g. methyl isocyanate (MIC). Hydantoins formed from diisocyanates can, after derivatization with, e.g. heptafluorobutyric anhydride, be analyzed by GC-MS NICI [129]. In the case of hydantoins derived from monoisocyanates, chemical ionization in the positive-ion mode (PICI) is more sensitive than NICI [131]. The usefulness of this approach has already been demonstrated in connection with measurement of exposure of victims of the Bhopal accident to MIC (as 3-methyl-5-isopropyl hydantoins in Hb) [132]. The method has also been shown to be suitable for biomonitoring of exposure to dimethylformamide, following metabolism to MIC [133,134].

5.4. Derivatization of detached adducts

Detached adducts or modified amino acids are usually derivatized with reagents designed to improve the chromatographic properties, volatility and/or stability of the adduct, as well as the sensitivity of the analysis. For measurement by GC-MS, detached adducts or modified amino acids are most commonly derivatized with fluorinated reagents in order to achieve a high degree of sensitivity in connection with analysis in the negative ion mode.

For studies of adducts originating from aromatic amines and isocyanates, the free amines obtained have been derivatized with fluorinated anhydrides [83,90,129,135,136]. In the case of studies of the tobacco-specific nitrosamine NNK, pentafluorobenzoate has been used [95,135]. Adducts to Hb and plasma proteins derived from organic acid anhydrides have been derivatized with pentafluorobenzylbromide after detachment by mild hydrolysis [82,97].

Pentafluorobenzoyl chloride has been used in the analysis of cysteine-*S* adducts of styrene oxide to Hb or SA, following cleavage by Raney nickel [137,138]. Use of trifluoroacetic anhydride for detachment of adducts from benzene oxide or benzoquinones to cysteine residues in Hb and SA [102,139,140] and of pentafluorophenyl isothiocyanate for the detachment of modified N-terminal valine residues [24] allows derivatization in the same step as detachment.

In the case of certain adducts, e.g. those containing several hydrophilic groups, low solubility in organic solvents in combination with undesirable

chromatographic properties and adsorption to glass, make further derivatization necessary. For this reason, hydroxyl groups in adducts from PAH diolepoxides, detached as tetrols by mild hydrolysis, have been silylated [93,141]. Several simple and bifunctional epoxides give rise to adducts containing two or three hydrophilic groups, e.g. epichlorohydrin and dihydroxyethyloxirane. These low-molecular-mass adducts are usually examined using the N-alkyl Edman method, which leads to the formation of PFPTHs of modified N-terminal valines. Hydrophilic groups in PFPTHs have been derivatized by silylation [142,143] or acetylation [79,143,144] prior to analysis. Recently, acetonization has been introduced as a useful derivatization of the adduct of glycidamide to N-terminal valine in Hb [145].

5.5. Methods employed for the detection of adducts

5.5.1. Mass spectrometric procedures

5.5.1.1. Gas chromatography–mass spectrometry. GC-MS continues to be the most commonly used method for identification and quantitation of protein adducts. For compounds to be analysable by GC-MS, they must be volatile, i.e. of relatively low-molecular mass. It is therefore necessary to isolate the adducts or the modified amino acids from the protein prior to analysis. As discussed in Section 5.4 derivatization might be required to increase the sensitivity, as well as to improve the chromatographic properties of molecules containing several polar groups.

The fragmentation pattern obtained using electron ionization (EI) often provides valuable structural information, but because of its low sensitivity, this procedure is rarely used. In general, the highest sensitivity has been attained by NICI-MS analysis of fluorine-containing derivatives of adducts or adduct–amino acid complexes. Positive-ion chemical ionization (PICI MS) is often used for, e.g. more accurate determination of the molecular mass of standards, but can also be more sensitive than NICI for some analytes (cf. Section 5.3.2.5).

In connection with chemical ionization (CI), secondary ions or electrons possessing low energy (thermal electrons) are formed from electron bombardment of the reagent (or buffer) gas, e.g. methane. These ions or electrons can then react further with the analyte to produce specific ions for each particular compound. Such reactions with the secondary ions involving proton transfer, charge exchange, addition, and abstraction/displacement are highly influenced by the nature of the analyte and the reagent gas. The formation of negative ions by trapping of thermal electrons (electron capture) occurs extensively in the case of electronegative compounds, in particular analytes that contain halogen atoms. Detection limits are in the range of 0.1–10 fmol for fluorinated derivatives of adducts [77,95], cf. Section 5.6.1.

5.5.1.2. Liquid chromatography–mass spectrometry. Analysis of modified peptides and proteins and of certain modified amino acids by GC-MS is rendered difficult by the polarity, low volatility and thermal lability of such compounds. In such cases, LC-MS and atmospheric pressure ionization (API) provides a better alternative, involving conditions that result in low thermal input and mild ionization. ESI and atmospheric pressure chemical ionization (APCI) are the most commonly employed API techniques (for further details see e.g. Ref. [146]).

In the case of ESI, ionization occurs already in the liquid phases, by protonation, deprotonation and addition of, e.g. sodium or potassium ions. At the ESI MS interface, the sample solution is introduced into the mass spectrometer as a fine spray of charged droplets generated in an electrical field applied between the inlet capillary and the mass spectrometer. These droplets are dispersed by repelling charges to produce smaller and smaller droplets and, finally, individual charged molecules exhibiting limited fragmentation [147].

For identification of the site of adduct formation in a protein, it is convenient to utilize tandem MS to determine the sequences of the peptides formed by protease treatment. MS/MS with collision-induced dissociation (CID) is a well-established technique for sequencing proteins [148]. Peptide ions most frequently dissociate at the peptide bond. The ions in one series of fragments differ in mass by the neutral loss of a single amino acid and, consequently, CID data can be used to determine the entire amino acid sequence.

For quantitation of modified peptides and amino acids HPLC-MS/MS analysis with APCI and ESI

has provided good results (cf. Sections 5.3.2.2 and 5.3.2.3). In these analyses the limit of detection can be as low as 50–500 fmol [112,118]. Even more sensitive detection has been achieved in connection with analysis of DNA adducts by HPLC/MS/MS (ESI) [149].

5.5.2. Other methods for detection

5.5.2.1. Laser-induced fluorescence. Laser-induced fluorescence (LIF) can be used to detect fluorescent compounds with a high sensitivity, and is employed routinely in connection with capillary LC or electrophoresis. Detection by LIF has also been applied to the analysis of background levels of BaP diolepoxide adducts in human SA [119]. Utilizing a monochromatic and unidirectional laser, an excitation wavelength of high energy is focused onto a small flow cell (usually I.D. <300 μm). Using such laser excitation together with detection of a narrow emission band emanating from the fluorescent chromophore, with minimized Raman and Rayleigh scattering, low background noise and, consequently, a high signal-to-noise ratio can be obtained [150,151].

5.5.2.2. Immunochemical approaches. Immunoassays are based on mostly specific interactions between antibodies and antigens. Originally, immunoassays have been employed to detect and quantitate biological macromolecules. However, a new generation of antibody-based assays allows analysis of small, modified biomolecules. As a result, immunoassays specific for biomarkers of carcinogen exposure and other effects of chemicals were developed [9,152,153]. Immunochemical detection of parent compounds in the blood and tissues, of metabolites in the urine and faeces, and of adducts to DNA and proteins has been successfully performed by a number of investigators. Antibody-based assays involve the use of polyclonal or monoclonal antibodies. Often immunotechniques are combined with other procedures for enrichment, isolation and detection in order to achieve optimal analysis.

One of the first reports in this area dealt with antibodies to proteins modified by acetaldehyde [154]. At the same time, Santella et al. [155] determined adduct levels in human globin using antibodies directed against a BaP diolepoxide–guanosine adduct. Adducts in SA originating from BaP have been determined by competitive ELISA (enzyme-linked immunosorbent assay) [109,156, 157]. Furthermore, quantitation of aflatoxin–albumin adduct levels has been achieved by three complementary immunochemical approaches [158]. Another examples is adducts of ethylene oxide to the N-terminal valine residue of the α-chain of human Hb, which have been analyzed in the form of adducted heptapeptides by a radioimmunoassay, a method shown to provide results similar to those obtained by GC-MS analysis [159].

The different immunoassays vary in sensitivity and specificity. Cross-reaction of antibodies with similar chemical structures occurs to some extent, resulting in detection of a broad spectrum of adducts. For instance, in the case of analysis of BaP adducts to SA, it is difficult to confirm covalent binding to the protein, or the structure of the adducts due to cross-reactivity with other PAH. Although immunoassays are useful for biological monitoring, the limitations of these methods must be taken into consideration for correct interpretation of results.

5.6. General considerations concerning the methodology for analysis of protein adducts

5.6.1. Specificity and sensitivity

It is well-known to analytical chemists that samples prepared for analysis of adducts by detachment and purification still contain impurities that give rise to so-called ‘‘background noise’’, which reduces sensitivity. In order to circumvent this problem, detection based on specific properties of the adducts is required.

Mass spectrometry is advantageous in this respect, since the analyte is identified on the basis of its retention time and specific fragment pattern. By using MS/MS even higher specificity can be achieved and the limit of detection can also be improved (by a factor of >10; e.g. Ref. [160]), since discrimination of background noise is more efficient, allowing analysis of larger amounts of sample material. Fluorescence detection is sensitive and specific when applied to certain compounds, e.g. adducts from diolepoxides of PAH, resulting in a low level of background noise [119,141,161,162]. Another way of lowering background noise is to remove im-

purities in the sample by SPE, e.g. ion-exchange chromatography [112], phenylboronate chromatography [163,164] or immunoaffinity chromatography [119,165,166].

In connection with analysis by conventional LC-MS (using columns with an I.D. of ~4 mm), ESI is not sensitive enough to permit detection of adducts in human blood samples. In such cases miniaturized ESI LC-MS (nano- or capillary-LC with an I.D. of 75–300 μm) can offer more sensitive detection [167,168]. The volume of the sample solution injected is 20 μl or 3 nl on a conventional or nano-column, respectively. Since ESI MS involves concentration-dependent detection the MS response is relatively independent of the volume injected. The low flow-rate used in miniaturized ESI LC-MS has the advantage that it diminishes the level of background noise, which, together with the good response, makes this procedure more sensitive than conventional ESI LC-MS.

A technique which has been shown to further improve sensitivity is column switching (or on-column focusing). With this technique a compound can be analyzed on a miniaturized column after injection of a sample volume which is 10^3 times larger than would otherwise be possible [167,168]. Nanospray LC-MS with column switching has been applied to the measurement of adducts from melphalan to DNA with a detection limit of <1 fmol adduct [169]. This technique seems promising, but has not to date been applied to the analysis of protein adducts.

5.6.2. Validity, accuracy, precision and reproducibility

It is desirable to compare and validate a new analytical procedure with other, independent procedures. In a few studies of individuals exposed to ethylene oxide, it has been possible to compare adduct levels to N-terminal valine measured by the N-alkyl Edman procedure to levels of adducts to histidine residues determined after total hydrolysis of the globin [170] or adduct levels to N-terminal valine monitored by a radioimmunoassay [159,171]. In addition to a good correlation between these methods at high-to-intermediate adduct levels, this comparison revealed that at low adduct levels the N-alkyl Edman method appears to be more reliable.

Detection of low levels of adducts, particularly in studies of background exposure in unexposed persons, raises the question of the relevance of the measurement. Is a true adduct being measured? This problem has been dealt with in several studies. For instance, quantitative considerations have provided evidence that the background level of an adduct derived from ethylene oxide (metabolite of ethene) reflects a true adduct with endogenously produced ethene (measured in exhaled air) as the major source [172]. In another study MS/MS was used to confirm the structure of a background adduct to N-terminal valine in Hb shown to originate from acrylamide [173] (cf. Section 6.5).

The choice of standards (internal or reference) is of great importance for the precision, reproducibility and accuracy in measurement of adducts. For analysis by mass spectrometric techniques, internal standards containing stable isotopes are most commonly used. The most appropriate internal standard is the protein itself (Hb or SA) alkylated with the compound under study (with stable isotopes). For exact quantification, a reference standard, consisting of the adduct being studied, preferably in a protein alkylated in vitro, is used for preparation of calibration samples. Determination of adduct levels by independent methods in a protein alkylated in vitro to be used as a standard often requires comprehensive work (cf. Ref. [170]). Therefore, internal standards corresponding to the detached adduct, modified amino acids or modified peptides are often used. If a reference standard providing good accuracy is employed, any internal standard giving good precision and reproducibility can in principle be used. Isotope-substituted internal standards sometimes are used for direct quantitation without calibration curves.

The importance of the choice of standards was demonstrated in a ring test designed to evaluate analyses performed according to the N-alkyl Edman method. When the modified amino acid containing deuterium was used as internal standard, good precision, but low accuracy was obtained [174]. The low accuracy was due to a difference in yield of analyte, from the free, adduct-modified amino acid used as reference standard in calibration samples, and from the adducted protein in studied samples.

Preparation of calibration curves for studies of very low levels of adducts to N-termini in Hb presents a special problem, i.e. difficulties in obtain-

ing zero levels, which has been dealt with [77,175]. Another problem associated with analysis by GC-MS NICI is that the response factors often exhibit extensive variation over short periods of time. Thus, in order to ascertain reproducibility under such conditions, calibration samples should be inserted frequently into a series of analyses.

When working with trace amounts of analytes, contamination is a well-known problem, which can be reduced by separate handling of standards, working with high-to-medium adduct levels, and work with low to background adduct levels with regard to equipment as well as laboratory environment. Another general problem encountered during work-up of trace amounts of an analyte is adsorption to glass and other materials, which lowers reproducibility and sensitivity. Precautions designed to minimize this latter problem include derivatization of hydrophilic groups in the compound to be analysed (cf. Section 5.4) and silanization of glass ware (cf. Ref. [79]).

Artefactual formation of adducts during storage of protein samples or during work-up procedures is a problem that has also been encountered. The formation of 2-hydroxyethyl adducts (e.g. from ethylene oxide) in vitro has been carefully investigated in order to clarify the origin of background levels of such adducts in samples from unexposed individuals. It was found that the 2-hydroxyethyl adduct could be formed under the conditions employed for acid hydrolysis of proteins [176], as well as during storage, particularly of Hb [177]. In addition, it has been demonstrated, that Hb in the form of HbO_2 catalyzes the formation of styrene oxide which in this case could be a source of artefacts in blood samples from styrene-exposed persons [178,179].

It is known that Hb exhibits several enzymatic activities [180]. For instance, the sulfinamides used for monitoring exposure to aromatic amines can be formed via heme-mediated oxidation to nitrosoarenes [89]. Procedures for storage of proteins have not yet been optimized with respect to prevention of oxidative and other changes. However, with regards to studies on stable adducts in Hb, it is recommended that the protein be stored as precipitated globin [177]. This problem should always be considered, particularly when studying low levels of low-molecular-mass compounds.

Furthermore, it should be noted that storage of standards in solution can lead to degradation of adducts (cf., e.g. Ref. [77]). In mechanistic studies of the N-alkyl Edman procedure it has been demonstrated that degradation of analytes may also occur during the detachment reaction. Especially under more alkaline conditions, base-catalysed oxidative side-reactions have been observed [120,121].

Another possible source of artefacts is misincorporation of modified amino acids occurring, e.g. in diet in connection with the in vivo synthesis of the protein. Thus, Kautiainen et al. [68] demonstrated misincorporation of 2-hydroxyethylated cysteines and histidines in vivo. In contrast, valine substituted at the amino group was, as expected, not incorporated. This latter observation indicates that this problem does not arise in studies of adducts to N-termini.

In order to obtain good reproducibility it is necessary to synthesize stable derivatives of the adducts by derivatization of functional groups. It can be noted in this connection that most adduct studies have been performed with GC-MS, i.e. the derivatives studied must be thermostable. Improvement of LC-MS techniques to obtain higher sensitivity paves the way for analysis of adducts that give rise to thermolabile derivatives.

6. Applications of protein adduct measurement

6.1. Monitoring of exposure

As mentioned above measurement of adducts to biomacromolecules was originally developed for the purpose of dose determination [2,3] as a basis for cancer risk estimation. However, such measurements have been used, primarily for monitoring exposure. In a large number of studies on exposed humans (occupational exposure, tobacco use, accidents involving chemical exposure, background exposure) measurement of adducts has been performed with the aim of monitoring exposure to genotoxic, allergenic or neurotoxic compounds (see reviews in this volume [91,102,124]). Even if procedures for quantitative risk assessment are not aimed at, exposure monitoring will provide information of value for the

identification, quantitation and reduction of exposures to harmful compounds.

In studies of populations or groups subjected to occupational exposure to genotoxic agents, measurement of both protein adducts and cytogenetic endpoints have demonstrated the superiority of the former with respect to sensitivity [181,182]. A study of the levels of adducts and neurotoxic effects of acrylamide in occupationally exposed groups revealed a dose–response relationship between adduct levels and neurotoxic symptoms [183,184]. In the case of organic acid anhydrides, protein adduct levels have been shown to be correlated to circulating levels of immunoglobulins [185].

6.2. In vivo dose monitoring

The formation and disappearance of electrophilic reagents *RX* as a consequence of enzymatic and chemical reactions must be considered in connection with determination of in vivo dose (reflecting the bioavailability of *RX*) (Fig. 12). For this purpose the dose is most accurately defined as the concentration of *RX* ([*RX*]) integrated over a period of time [186], i.e. the ''Area Under the Curve'' (AUC) [187], with the units being, e.g. m$M\times$h (mmol$\times$kg$^{-1}\times$h) (Eq. (12)). (For a chemical, concentration is an intensity parameter, expressing the dose rate [186]). Calculation of in vivo dose renders adduct measurement useful for the treatment of toxicological problems (cf. Sections 6.3–6.5).

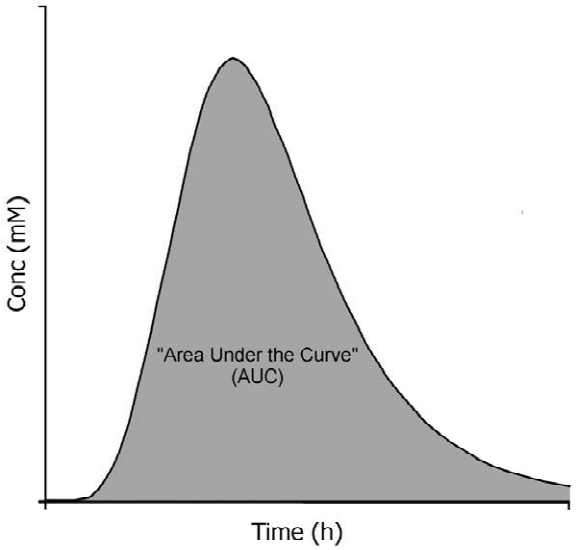


Fig. 12. Illustration of the in vivo dose concept. The concentration in vivo of an electrophilically reactive species integrated over time, i.e. the area under the curve (AUC), is defined as the dose expressed in m$M\times$h.

$$\text{Dose} = \int_0^t [RX](t)\,\mathrm{d}t \tag{12}$$

The reaction between *RX* and a nucleophilic site *Y* can be written

$$[RX] + [Y] \xrightarrow{k_Y} [RY] + [X] \tag{13}$$

where *RY* is the adduct and k_Y is the second-order rate constant for the reaction. In the case of acute exposure, the dose can be calculated from the adduct level, $[RY]/[Y]$ and the rate constant (Eq. (14)). If the adduct level is expressed as mol per gram globin, e.g. 10^{-9} mol$\times$g^{-1} (nmol/g), the unit for the rate constant will then be [L$\times$g$^{-1}\times$h^{-1}].

$$\text{Dose} = \frac{1}{k_Y}\frac{[RY]}{[Y]} \tag{14}$$

When the biomacromolecule employed as a monitor has a long life span and the adduct is stable, chronic or long-term exposure can be monitored. In the case of long-term exposure, a correction for the life-span of the macromolecule must be included in the calculation of dose from Eq. (14) (cf. Section 4.4 where $[RY]/[Y]$ is denoted *A*).

In the case of very short-lived reactive compounds it might be suitable to express the dose in terms of an adduct level (cf. Ref. [62]).

For the calculation of in vivo dose from measured adduct levels, the rate constant (k_Y) for the reaction with the nucleophilic site (*Y*) in the protein must be known. This rate constant is obtained in vitro employing different procedures. Hemolysate or erythrocytes are incubated at 37 °C with the electrophilic agent, and the dose and degree of alkylation at the nucleophilic site are measured as has been described previously, e.g. Ref. [188]. Approximate or relative rate constants may be obtained by following the reaction of *RX* in vitro with a model compound resembling the nucleophilic site in the macromolecule, by trapping remaining *RX* at differ-

ent time points using a strong nucleophile. A super-nucleophile, cob(I)alamin, has been shown to provide an accurate measure of the concentration of *RX* in such studies [189].

6.3. Studies of metabolism

Metabolism leading to the formation of reactive intermediates can be examined by measurement of protein adducts in both animals and humans. This approach allows both identification and quantitation of the reactive metabolites. Furthermore, the fraction of the absorbed amount that is activated to reactive species and the rate of elimination of reactive species can be deduced from measured adduct levels using in vivo dose calculations [187]. A few examples of this are presented below.

The rate of elimination has, for instance, been investigated in monkeys exposed to ethylene oxide and propylene oxide [188]. The simplest alkene, ethene, was shown in animal experiments at an early stage to give rise to 2-hydroxyethyl adducts in Hb which are identical to the adduct formed from ethylene oxide [190]. It has later been shown, again in animal experiments, that the whole range of C_2-C_8 alkenes are epoxidized to the corresponding oxiranes, with indications that longer 1-alkenes are epoxidized to a lesser extent than the C_2 and C_3 compounds [191].

Quantitation of metabolism in humans is more difficult, because assessment of exposure poses problems. The proportions of inhaled ethene which are metabolized to ethylene oxide in cigarette smokers [74] and in occupational settings [73] have been deduced from Hb adduct levels determined repeatedly before and after periods with and without smoking or exposure. By measurement of Hb adducts, the metabolism of acrylamide to glycidamide and the in vivo persistence of both these compounds have been investigated in animals and also, in available materials, in humans [192]. Metabolism of benzene has also been examined by measurement of protein adducts [102].

The identification of metabolites by characterization of adducts can be illustrated by studies on methylenedianiline. A stable Hb adduct, which might possibly reflect the mutagenic intermediate was looked for in rodents [193]. A large fraction of the Hb adducts were shown to be an oxidized imino compound bound via an azo bond to valine-*N*.

Various research groups have questioned the use of Hb adduct measurement for assessment of exposure or in vivo dose. For instance, it has been reported that following addition of ethylene oxide to human blood, Hb adduct levels were higher in blood from persons lacking the ability to conjugate ethylene oxide with GSH (later shown to lack GST-T). In this case, fitting the data to a kinetic model clearly showed that the dose, as defined above, in blood is lower in the presence of the conjugating enzyme in the erythrocytes [194]. This experience supports the view that the Hb adduct level reflects the in vivo dose, allowing for variations caused by differences in metabolite formation and detoxification, etc. Correlations between protein adduct levels and metabolic polymorphisms have been studied for a number of compounds (as recently reviewed by Pavanello and Clonfero [195]), and in particular aromatic amines, for which significant correlations have been found (reviewed by Richter and Branner in this volume [91]).

6.4. Assessment of health risks

The risk (i.e. probability of developing cancer) associated with exposure to genotoxic chemicals is considered to increase linearly with dose, exhibiting no safe threshold [196]. Thus, below some value (which varies for different compounds and situations and must be considered in relationship to the benefit from the activity that results in the exposure) this risk may be considered acceptably small. It is therefore advantageous to know the sensitivity of various analytical procedures for detecting causative factors. In the case of ethylene oxide and compounds with a similar reaction pattern, an adduct level to N-terminal valine residues above the range of 1–10 pmol/g Hb is associated with a cancer risk that could be of concern, an adduct level that can be quantitated using the N-alkyl Edman method [4,160,175].

In connection with cancer initiation, a key factor is the mutation frequency. Doses (defined in Eq. (12) above) in target organs are proportional to the cumulative level of premutagenic adducts to nucleophilic sites in DNA [62,186]. At low doses and

dose rates, the frequency of mutation is thus expected to be proportional to the dose.

A cancer risk model has been developed and preliminarily validated [65,196]. This risk model is linear and multiplicative (relative), i.e. based on the observation that at a given dose, the increment in risk is approximately proportional to the background cancer incidence or mortality (used as an implicit measure of background promotive conditions) [197].

In the case of neurotoxic and immunotoxic effects, disturbances in oxidative metabolism which lead to different diseases, etc., there is usually a no-effect threshold in the dose-response (or rather concentration-effect) relationship. For example, in the case of neurotoxic effects of acrylamide, it has been estimated that for a few months of exposure, this threshold dose corresponds to a level of 0.3–1 nmol of adducts of acrylamide to N-terminal valine residues per gram of Hb [184,192,198].

6.5. Measurement of background adducts as a tool for detecting background carcinogens

By and large, it may be assumed that the occurrence of reactive compounds in vivo above a certain level has a negative impact on health. Thus, observation of background adducts in knowingly unexposed persons could contribute to the disclosure and identification of causative factors in carcinogenesis. A relatively small portion (<20%) of the mutations required to explain the present cancer incidence in Western countries, can be attributed to known mutagenic factors, primarily background ionizing and UV radiation [175]. To a large extent, background mutation is expected to be due to errors in coding and proofreading in connection with DNA synthesis. However, an as-yet-unknown fraction of background mutagenesis could be due to reactive compounds/intermediates in the environment and in the body.

Background reactive agents may be searched for in different ways:

(a) The occurrence in control material of adducts that are identical with the adducts originating from specific exposures or sources.

With respect to low-molecular-mass chemicals and aromatic amines monitored in work environments, it is the rule rather than an exception that similar adducts are detected in unexposed persons, often at higher levels in smokers than in non-smokers (see Table 4 and for aromatic amines Ref. [91]). Such adducts are, among others, (2-hydroxyethyl)valine and (2-carbamoylethyl)valine in Hb (cf. Section 5.6.2). The former has been shown to originate from ethene [172], which is mainly produced endogenously by intestinal bacteria [203–205] and metabolized to ethylene oxide while the latter adduct to a large extent seems to originate from acrylamide generated by cooking various foodstuffs [173]. The daily formation and uptake of these two adducts seem rather small, but considering that exposure is lifelong, they are expected to make non-acceptable contributions to the background cancer incidence/mortality [173].

Table 4
Background adduct levels to N-terminal valine residues in Hb determined with the N-alkyl Edman method, with an observed difference in smokers and non-smokers

Adduct to valine–*N*	Non-smokers (pmol/g globin)	Smokers-10 cig./day (pmol/g globin)	Reactive compound	Refs.
2-Hydroxyethyl	20	100	Ethylene oxide	[24,175]
2-Hydroxypropyl	2	4	Propylene oxide	[175,199]
2-Carbamoylethyl	30	100	Acrylamide	[64]
2,3-Dihydroxypropyl	2–7	~10	Glycidol	[79,200]
2-Cyanoethyl	<2	80	Acrylonitrile	[64,125]
Methyl	220	250	*S*-Adenosyl-methionine and methylating agents in tobacco smoke	[201,202]

(b) Compounds/metabolites known to be produced endogenously or to be present in the general environment.

One example of such compounds is isoprene, a major component in exhaled air [206].

(c) Unknown adducts.

A strategy for searching, identifying, and quantitating previously unknown adducts to Hb N-termini has been suggested [120,207]. In this search tandem mass spectrometric techniques can be used to explore for fragments that are characteristic of the analyte. In the identification of adducts as risk factors, correlation to genotype and phenotype regarding metabolism may be helpful [208].

6.6. Other applications of protein adducts for biomonitoring in humans

Protein adduct measurement is also being used in areas other than monitoring of xenobiotics, e.g. for diagnostic purposes. Urea levels, primarily in diabetics and patients subjected to hemodialysis, have been measured as the level of isocyanic acid (a dissociation product of urea) in the blood. The isocyanic acid that binds to the N-terminal valine in Hb has been measured as hydantoin after release by acid hydrolysis [209,210].

In attempt to monitor long-term glycemia, primarily in diabetic patients, the accumulated level of Hb adducts from glucose (attached through a ketoamine linkage) has been analysed [211]. These adducts have been analysed, e.g. after reduction of the Schiff base (with incorporation of ^{3}H), acid hydrolysis and enrichment of the glycosylated amino acids employing phenylboronate chromatography [163]. The majority of the adducts are present on lysine residues, followed by N-terminal valine.

Acetaldehyde exposure in alcoholics has been analysed as Hb adducts utilizing the ELISA approach involving antibodies directed against acetaldehyde adducts [212]. Acetaldehyde adducts are formed at lysine residues and the N-terminal valines [213].

As discussed in Section 5.3.2.1 a method for studies of oxidized amino acids has been applied for studies of in vivo effects of oxidative stress. Measurement of nitrotyrosine in blood proteins has been suggested for measurement of exposure to nitrating and nitrosating agents and for studies of endogenous processes, e.g. inflammation [214,215]. Methods for monitoring of nitrotyrosine based on analysis of peptides is under development [214].

See also review by Frantzen [216].

6.7. Environmental monitoring

In connection with studies on leakage of acrylamides into the environment during construction of a tunnel [184], blood from cattle, wild animals and fish were utilized for monitoring ([217] and Törnqvist et al., to be published). This could be achieved readily using the N-alkyl Edman method, since many animal species have two or four N-terminal valine residues in their Hb. Other methods for protein adduct measurement are certainly also applicable in this context [218].

6.8. Summary and future perspectives

Protein adduct analysis has already been applied to a large number of compounds and development of new analytical approaches and techniques will surely allow application to an even broader range of compounds. The power of the methodology resides in the specificity and the possibilities of quantifying and identifying the causative xenobiotics. As described in the Introduction, adduct measurement has mostly been applied for monitoring exposure, e.g. occupational exposure to genotoxic, neurotoxic and allergenic compounds. The number of quantitative studies on, e.g. metabolism and investigations of toxicological problems are more limited. However, the translation of adduct measurements into in vivo doses is useful in risk assessment and offers a link between toxicological and epidemiological data, e.g. by detecting and understanding variations in individual sensitivity. Furthermore, the possibility of identifying the background load of reactive compounds in vivo and its dependence on dietary factors, life-style, etc. is far from being fully explored. Finally, analysis of protein adducts in blood samples from wild animals and fish could be a useful approach for environmental monitoring.

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