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COMMENTARY

Protein adducts in the molecular dosimetry of chemical carcinogens

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Genotoxic carcinogens form covalent bonds with proteins as well as with DNA. The adducts which result are useful for assessing exposure to the carcinogen, determining inter-individual differences in metabolism and other carcinogen processing, and perhaps in risk assessment. This commentary reviews the development of molecular dosimetry based on protein adducts and describes some of the principles involved. Also described are studies of the binding of bulky lipophilic carcinogens to proteins, which clearly indicate that a high degree of specificity is characteristic of many carcinogen–protein interactions. Studies which have been conducted with human populations are summarized and some proposals for future studies are made.

Introduction

The concept that human cancers can be induced by chemical substances has its origins in the last decade of the nineteenth century with the observations of Rehn (1) concerning the association of urinary bladder cancer with exposure to aromatic amines. By the 1930s, 2-naphthylamine had been shown to induce tumors of the urinary bladder in dogs (2) and the hepatocarcinogenic activities of *o*-aminoazotoluene (3) and *N,N*-dimethyl-4-aminoazobenzene (DAB*) (4) had been demonstrated. During studies designed to elucidate the mechanism of action of DAB, it was discovered that this carcinogen became covalently bound to proteins in the liver of rodents to which it was fed (5). A series of structure–activity studies provided data which formed the basis for a 'protein-deletion' theory of carcinogenesis (5). These studies were reported in the mid-1940s at about the time of Avery's studies on the ability of bacterial DNA to transform bacteria of one type to another type (6) and well before the seminal paper of Watson and Crick (7). With the growing realization that DNA was the informational macromolecule in cells, interest in the binding of carcinogens to proteins waned.

With the increasing availability of radiolabeled carcinogens [their use was first reported in 1948 (8)] it became possible to search for nucleic acid adducts. Between 1957 and 1964, binding to nucleic acids by *N*- and *S*-mustards (9,10), dimethylnitrosamine and ethionine (11), acetylaminofluorene (12), DAB (13) and several polycyclic aromatic hydrocarbons (PAH) (14,15) was reported. No adduct structures were reported at that time, but these studies were merely the forerunners of much more extensive investigations into the reactions of carcinogens with DNA. While many of these were designed to elucidate mechanisms of carcinogen action, there were also many structure–activity studies undertaken with the goal of learning what elements of a chemical structure were necessary for carcinogenic activity. The development of bacterial mutation assays (16) greatly enhanced the ability of researchers to search for potential carcinogens.

The growing understanding of the mechanisms of chemical carcinogenesis and a realization of the extent to which chemicals were implicated in the causation of cancer generated a new imperative–risk assessment. This in turn led to a renewed interest in the binding of chemical carcinogens to proteins because the products formed would have the potential for revealing the underlying chemical processes noninvasively, as well as for revealing precise information about exposure.

Risk evaluation is ultimately based on a quantitative estimate of the dose of the toxicologically relevant metabolites delivered to target tissues. At present, such data are the result of a multistage process which begins with environmental analysis. Ambient levels are translated into intake amounts through considerations such as route and time-dependency of exposure, and rates of consumption. Finally, dose–response relationships obtained from studies with experimental animals are used to estimate the responses of human populations. As a means for shortening this lengthy process, and for eliminating uncertainties in the conversion of ambient levels to doses and the extrapolation of test animal results to humans, a new form of dosimetry has been introduced. It is based on the quantitative analysis of the *in vivo* products of the reactions of chemical carcinogens with endogenous macromolecules. Two different yet complementary lines of research have evolved. One line focuses on the DNA adducts; the other takes proteins as the principal target molecules. We have largely adopted the latter approach. This commentary will address the reasons for that choice and provide a theoretical basis for the use of protein adducts in molecular dosimetry. In addition, it will sketch some of the historical background to the field and will summarize our studies in human populations using protein adducts formed by a variety of chemical carcinogens.

Principles of protein adduct dosimetry

The concept that ultimate carcinogens are electrophiles and that these exert their biological effects by reacting with cellular nucleophiles was first proposed by the Millers (17,18). A schematic illustrating this concept is shown in Figure 1, which also illustrates the multiple outcomes that are possible. Since the concentrations of carcinogens in real life are very low, pseudo-first-order reaction kinetics are to be expected. A comparison of protein and DNA adduction may be made, since under these conditions, product ratios are the same as the ratios of reaction rate constants, and the protein adducts will thus serve as surrogates for DNA adducts. Although a wide variety of proteins

*Abbreviations: DAB, *N,N*-dimethyl-4-aminoazobenzene; PAH, polycyclic aromatic hydrocarbons; Hb, hemoglobin; MMS, methylmethanesulfonate; B[*a*]P, benzo[*a*]pyrene; DMN, dimethylnitrosamine; DTIC, 5-(3,3-dimethyl-1-triazeno)-imidazo-4-carboxamide; 4-ABP, 4-aminobiphenyl; Glu-P-1, 2-amino-6-methyldipyrido[1,2-*a*:3′,2′-*d*]imidazole; SA, serum albumin; AFB_1, aflatoxin B_1; IQ, 2-amino-3-methylimidazo[4,5-*f*]quinoline; B[*a*]PDE, benzo[*a*]pyrene diol epoxide; CPD, cigarettes per day; 3-ABP, 3-aminobiphenyl; HOEtVal, *N*-hydroxyethylvaline; ETS, environmental tobacco smoke; RIA, radioimmunoassay.

might be considered for this purpose, only some proteins will provide useful information for chemical, biological or epidemiological studies. For example some proteins become labile as a result of adduct formation (19). A list of desirable properties of a protein adduct dosimeter might include the following: (i) chemical stability under biological conditions; (ii) does not influence stability of protein; (iii) easy accessibility for epidemiological studies; (iv) a relationship between protein dose and DNA dose; and (v) provide a record of exposure over a significant fraction of a lifetime.

No protein will meet all of the criteria perfectly, but hemoglobin and serum albumin are promising candidates and appear to meet at least the first four criteria for specific carcinogens. Since there is no perfect *a priori* basis for choosing one protein over another for dosimetry purposes, only an empirical approach to the choice of a protein is possible at the present time.

Hemoglobin

The individual who first recognized the potential of protein adducts to serve as surrogates for DNA adducts was Lars Ehrenberg (20). He and his associates both pioneered this area of research and have continued to make important contributions. His group specifically focused on hemoglobin (Hb) adducts and derived the mathematical description of steady-state adduct levels that result from continuous exposure to an Hb alkylating agent. Hb has the unique biological property of having a lifespan equivalent to the lifespan of the erythrocyte, which in humans is ~120 days. The age distribution of the protein is uniform; thus, if a carcinogen forms an adduct that is chemically stable, and if exposure to the carcinogen is chronic, then the amount of adduct will be proportional to Hb age: Hb molecules 120 days old will have 120 times as much adduct as Hb molecules that are only 1 day old. The average level of adduct will be halfway between or ~60 times the amount formed by the daily dose. Even if the daily dose is not constant, or if exposure is intermittent, the average level of adduct will still be 60 times the average dose over the past 120 days. The Hb dosimeter therefore reflects a 4 month record of the average exposure. Concomitantly, when exposure ceases the adduct level drops rapidly, since the initial class of erythrocytes that is lost is the oldest and thus has the highest level of adduct.

Since Ehrenberg first proposed the dosimeter, many studies

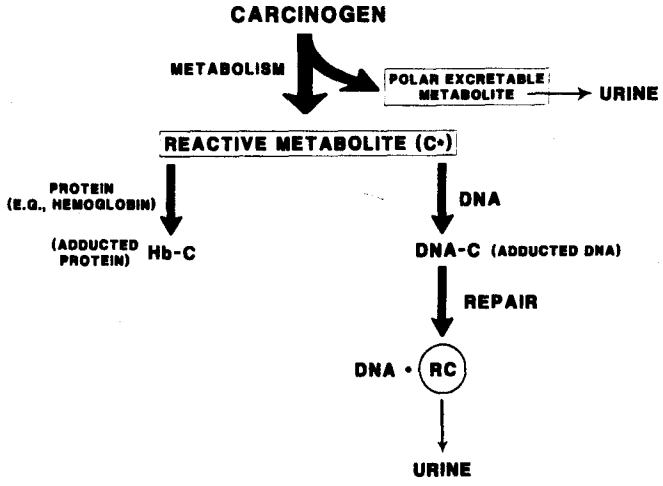

Fig. 1. Metabolism of genotoxic carcinogens leading to protein and DNA adducts.

in animals and humans have confirmed the theoretical description of adduct accumulation and removal and the linear relationship between adduct level and dose. Experimentally, these studies have been conducted by determination and measurement of specific chemical structures and/or by binding of radioactive carcinogens *in vitro* or *in vivo*. Osterman-Golkar *et al.* (21) established the stability of alkylated residues in Hb modified by ethylene oxide or dimethylnitrosamine, and the equivalence of the lifespan of the adduct produced by a single dose to the lifespan of Hb in the mouse. Alkylation of Hb in mice treated with vinyl chloride was reported by the same investigators (22). Segerbäck *et al.* (23) further characterized the experimental model in mice treated acutely and chronically with the direct-acting alkylating agent methyl methanesulfonate (MMS) in which they demonstrated the validity of the steady-state level of alkyl residues in Hb as a measure of chronic exposure. Later, Segerbäck (24) showed that bulkier carcinogens, e.g. benzene and benzo[*a*]pyrene (B[*a*]P), were also bound to Hb *in vivo* in a form that had the same lifetime as the erythrocyte. The binding of fluoranthene to rat Hb also yields adducts that have lifetimes equivalent to the erythrocyte lifespan (25,26).

Farmer *et al.* (27) have developed a high-resolution GC–MS method for estimating the production of *S*-methylcysteine in Hb following exposure to methylating agents. This method was used to study *in vivo* alkylation of Hb in rats dosed with MMS, dimethylnitrosamine (DMN) and the antitumor agent 5-(3,3-dimethyl-1-triazeno)imidazo-4-carboxamide (DTIC). A linear dose–response curve for MMS was observed over a 100-fold dose range, but the dose–response curve for DMN was nonlinear. No alkylation was observed with the DTIC, but it may have been overshadowed by a low level of naturally occurring *S*-methylcysteine which was found to be present in Hb of the rat and 13 other animal species. These findings emphasize the importance of careful dose–response studies in animals for each compound for which human exposure data are to be sought by this approach. Farmer *et al.* (28) have also devised a GC–MS method for the detection of hydroxypropyl histidine in Hb as a measure of exposure to propylene oxide.

The properties of the Hb dosimeter are illustrated by the results of experiments presented in Figures 2 and 3. Figure 2 summarizes the results of an animal experiment in which one rat was dosed with 4-aminobiphenyl (4-ABP) repeatedly for a period longer than the lifespan of rat erythrocytes. Both the experimental points and the theoretical curve for this type of experiment are shown. The agreement of the two is within the error of measurement. Additional experiments in the rat demonstrated that the response of this dosimeter is linear throughout a 5 log range of doses (29). Figure 3 shows the results of a 'quit-smoking' study of 4-ABP adducts in people. Again, the adduct level drops approximately according to theory, and in effect a human experiment has been conducted which mimics the rat experiment.

Not all carcinogens form adducts that are so stable that they persist in circulation until the erythrocytes are destroyed. Neumann (30) has reported several cases of unstable aromatic amine adducts in the rat. Carmella and Hecht (31) report adduct depletion rates for tobacco-specific nitrosamines in the rat which follow first-order kinetics with a half-life of 9.1 days. The loss of adduct by a first-order process suggests that the rate is governed by cleavage of the protein adduct bond rather than by depletion of the protein.

In addition to the above, there are carcinogens and other electrophilic compounds for which the structures of Hb adducts have been characterized but for which the long-term adduct

stability has not yet been determined: styrene oxide (32,33), urethane (34), diethylnitrosamine (35), acetaminophen (36) and Glu-P-1 (37).

Serum albumin

In order for an ultimate carcinogen to bind covalently to Hb the electrophile must be sufficiently stable to diffuse out of the cell in which the carcinogen is metabolized and into an erythrocyte. In the process the reactive species must cross two cell membranes. Adduct formation might be expected to be more efficient with serum proteins because these are not isolated by the erythrocyte membrane. An additional reason for considering serum proteins is that hepatocytes are not only the cells in which plasma proteins are synthesized, but also the cells in which most xenobiotic metabolism, including the microsomal oxidations which activate carcinogens, takes place.

Serum albumin (SA) has been chosen from among the serum proteins because of its abundance and because its role as a carrier of fatty acids, endobiotics and xenobiotics increases the probability that it will bind and form covalent adducts with ultimate carcinogens. The molecule consists of three distinct structural domains; these domains have a similar overall architecture but local variations in sequence influence the affinities for different ligands (38). SA is synthesized in hepatocytes on the endoplasmic reticulum as pre-proalbumin, cleaved in the Golgi apparatus to proalbumin, and then cleaved again to albumin during export through the cell membrane. Since microsomal oxidation and albumin synthesis occur within the same cell we reasoned that highly reactive electrophiles might preferentially react with SA compared to Hb, and that adduction of the pre-pro-SA or pro-SA would not influence its export.

SA has a relatively slow turnover in man (half-life of 20–25 days), and its mathematical properties as a dosimeter have been derived (39). Assuming SA is cleared with first-order kinetics and that no specific mechanism exists for accelerating the removal of adducted SA, then adduct clearance will be described by the function:

$$A(t) = A_c e^{-kt}$$

where $A(t)$ is the level at time t, and A_c is the chronic exposure level. After time t, the loss by clearance will be $A_c - A(t)$ or $A_c - A_c e^{-kt}$.

Under conditions of chronic exposure, the SA adduct level will stabilize at a value such that the amount of adducts cleared per unit of time is equal to the number of new adducts formed during the same interval.

Letting $t = 1$ day, and equating clearance with the daily production of new adducts yields:

$$A_c - A_c e^{-k} = \text{(daily increment)}$$

Solving for A_c:

$$A_c = \text{(daily increment)} \times (1 - e^{-k})^{-1}$$

for man, $k \simeq 0.035$ days^{-1} and $(1 - e^{-k})^{-1} = 29$.

Therefore, the chronic exposure SA adduct level is expected to be ~30-fold higher than the adduct level produced by a single dose.

SA, like Hb, has a variety of nucleophilic sites capable of reacting with electrophiles. Covalent bond formation by the

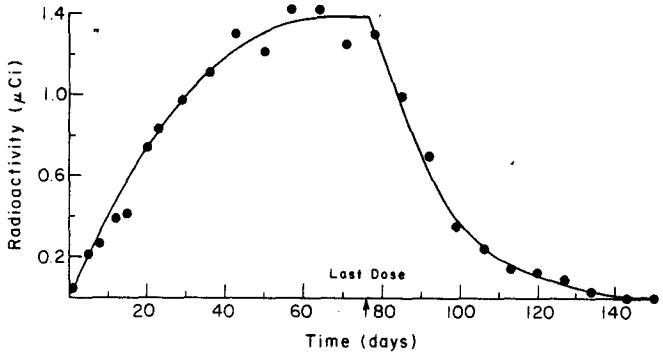

Fig. 2. Accumulation of radioactivity in the blood of a rat dosed repetitively with [^{3}H]4-ABP (from ref. 29).

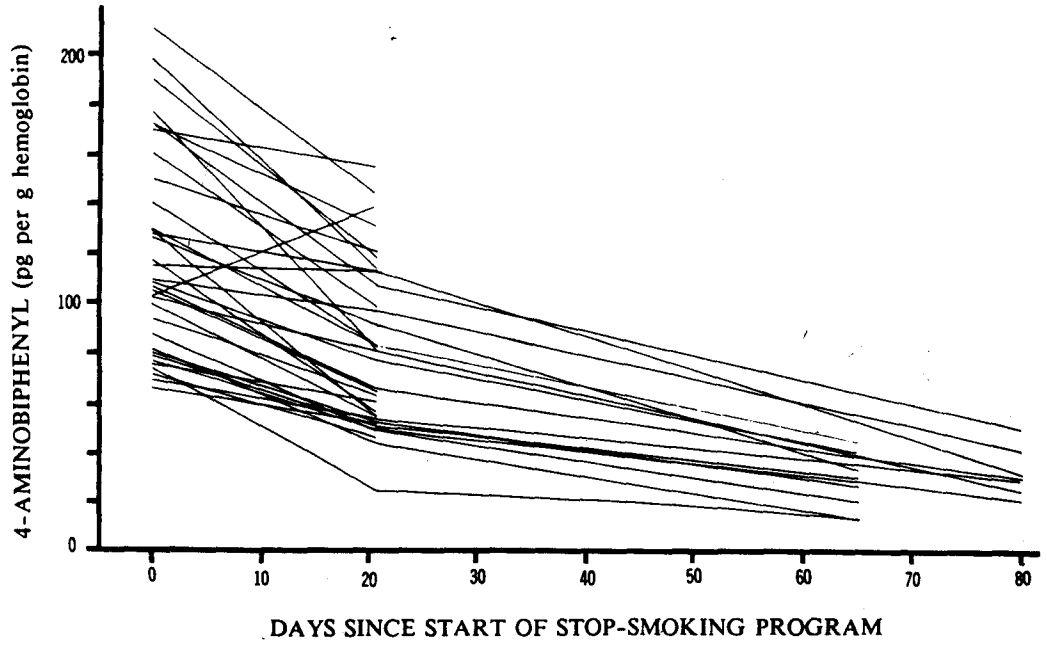

Fig. 3. Levels of 4-ABP Hb adducts over time among 34 participants in a smoking withdrawal program (from ref. 53).

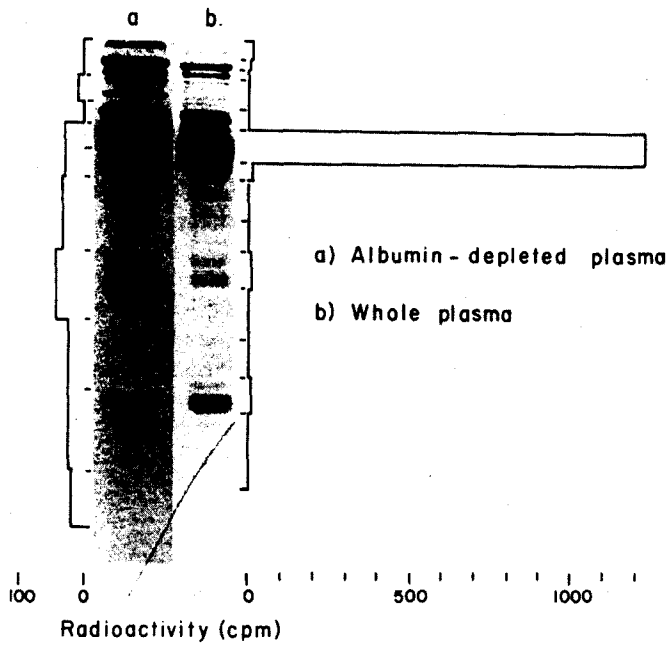

Fig. 4. SDS−PAGE analysis of rat plasma proteins after administration of [2-^{3}H]AFB$_1$.

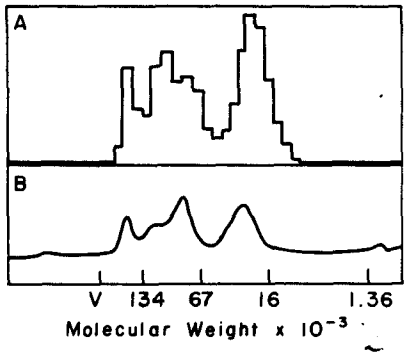

Fig. 5. Sephadex G-150 chromatography of albumin-free rat plasma proteins after administration of [^{3}H(G)]IQ. (**A**) Radioactivity; (**B**) absorbance at 280 nm (from ref. 41).

ultimate carcinogens derived from DMN (40), aflatoxin B$_1$ (AFB$_1$) (39), 2-amino-3-methylimidazo[4,5-*f*]quinoline (IQ) (41) and 4-acetamidobiphenyl (42) has been documented.

AFB$_1$ binds to SA with remarkable selectivity. Approximately 1−3% of a single dose is bound to plasma proteins in the rat, and the binding is almost exclusively to SA (Figure 4). Since all of the plasma proteins are synthesized in the hepatocytes one might expect an equivalent amount of binding to these proteins as well, but it is not observed. In contrast, binding of IQ in the rat occurs proportionately in all plasma proteins (Figure 5).

Studies on adduct stability and the relationship of adduct level to dose have not been nearly as extensive for SA as for Hb. A technical problem confronting this type of study is the relatively short lifetime of SA in rodents (half-life of 1−2 days in the mouse and 2−3 days in the rat). Nevertheless, AFB$_1$ has been shown to accumulate in rat SA as the result of chronic dosing, to be removed at the rate of turnover of albumin, and to have a linear adduct−dose relationship (43). Similar studies have been conducted with 4-ABP (42) and IQ (41).

The availability of SA in serum banks from clinical and epidemiological studies presents unique opportunities for molecular dosimetry which will probably stimulate further studies on SA adducts in future years.

Adduct formation as a chemical reaction

All of the reactions so far observed which result in the formation of a covalent bond between an activated carcinogen and a protein are formally either nucleophilic substitutions or additions to multiple bonds. Substitution or addition occurs at an electrophilic center of the carcinogen and the attacking nucleophile is a heteroatom in the protein. Two general cases may be distinguished. In one, the carcinogens are mostly low mol. wt compounds, and reaction with proteins takes place largely as if the constituent amino acids were free solutes rather than units of a polymer. The tertiary structure of proteins appears to play an increasingly important, and sometimes dominant, role when carcinogens are of a higher mol. wt and more lipophilic.

The first case has been extensively studied. The carcinogens or other similar electrophiles examined include: ethylene, propylene and styrene oxides; epoxides formed from urethane and vinyl chloride; *N*-nitroso compounds which ultimately yield diazohydroxides; acrylamide; alkylating agents such as MMS, methyl bromide and chloroacetaldehyde. The spectrum of products formed by these small reactants appears to be governed primarily by the relative rates of reaction with the different amino acids, which in turn has been shown to obey the Swain−Scott relationship fairly closely. Product structures are those anticipated from the formal reaction. No evidence has been presented for site-selectivity for any particular amino acid.

A second group of carcinogens is comprised of AFB$_1$, certain aromatic amines, and PAH. The metabolic activation products of these compounds exhibit reactivity toward proteins which have proven remarkably unpredictable. In some cases, too, it has been necessary to invoke further transformation of an unstable initial reaction product to account for the isolated product structure.

The best-characterized example of a carcinogen−protein reaction from this latter group is that of adduct formation by *N*-sulfonyloxy-*N*-acetyl-4-aminobiphenyl with SA (42). The major product isolated from rat SA following administration of 4-ABP to rats was a peptide containing a tryptophan which had reacted with the hydroxamic acid. While the exact structure was not reported, it was determined that substitution had occurred at the carbon *ortho* to the acetamido group. The most striking aspect of the adduct structure is the involvement of tryptophan. Rat and human SA contain only one tryptophan residue. Further more, tryptophan is generally considered as having one of, if not the least, nucleophilic side chains. It was inferred, therefore, that the primary event in adduct formation is noncovalent binding driven by hydrophobic interactions, and that covalent bond formation only occurred after such complex formation. The function of SA as a carrier protein for a wide variety of endo- and xenobiotics lends support to this inference. Further *in vitro* studies (unpublished) with human SA and 4-ABP, benzidine and 2-aminofluorene and an activating system of rat hepatocytes have indicated a certain generality to the reaction which may well be strongly dependent on the shape of the aromatic amine.

In contrast to the preceding, the structure of the major AFB$_1$ adduct formed with SA has been fully determined, but the site-specificity is unknown (39). Adduct formation is believed to occur initially by condensation of the open-ring form of AFB$_1$-8,9-dihydro-8,9-diol with a lysine residue to form a Schiff base. The same product, it should be noted, could arise from alkylation of lysine by the 8,9-epoxide as well. The Schiff base subsequently undergoes a series of prototropic rearrangements to yield the final structure indicated in Figure 6. The coumarin

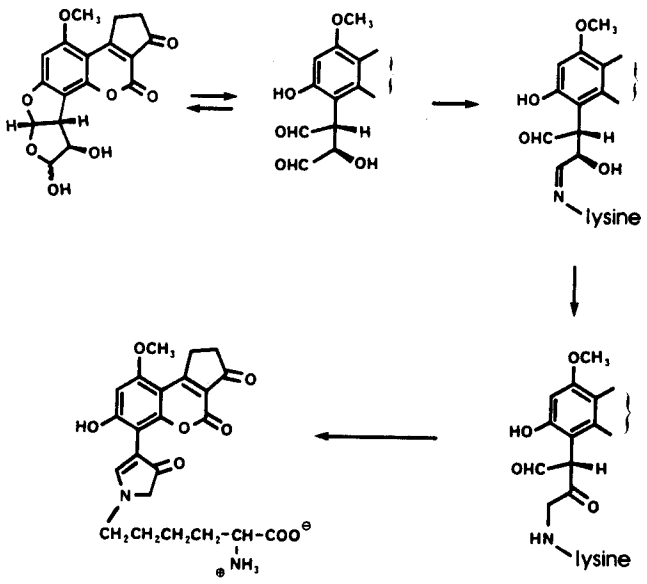

Fig. 6. Proposed mechanism for the formation of the lysine−AFB_1 adduct (from ref. 39).

ring system in the adduct remains intact. As a consequence, the spectral properties of the adduct are quite similar to those of AFB_1, so it is not readily apparent that the structure is so different from that of a simple alkylation product.

The site-specificity for AFB_1 adduct formation is unknown because the isolated adduct contained only a lysine residue with its attached aflatoxin moiety. There is circumstantial evidence, though, that AFB_1 does bind in a selective manner. When SA was treated with activated AFB_1 to yield a ligand:protein ratio of < 1, the modified protein was not recognized by a monoclonal antibody which recognizes the adduct. Raising the ratio to 6 produced a protein which was readily recognized. Thus, it may be inferred that there exists one or, perhaps, several sites in the protein where the carcinogen may find a lysine residue to react with, and also be unavailable for antibody binding. Adduct formation occurs at buried site(s) before it occurs at a site at which the aflatoxin residue is accessible to the antibody.

Two PAH have now been investigated in considerable detail. Fluoranthene (25) was shown to bind to rat hemoglobin *in vivo* largely through the two isomeric 1,2,3-trihydro-10b-*H*-2,3-*trans*-dihydroxy-1,10b-epoxides. The major adducts resulted from epoxide ring opening by the sulfhydryl group of the β-125 cysteine residue. Reaction was localized to this site from the amino acid sequence determined for the isolated adducts, which were obtained as a mixture of tetra- and pentapeptides. The exceptional reactivity of this cysteine probably is the basis for the strong site-selectivity, which is apparent from inspection of the chromatograms of enzymatic digests of the protein after reaction with synthetic diol epoxides. In each case there is no indication that any other product is formed.

The β-125 cysteine residue is peculiar to rat, guinea pig, and, apparently, some (44) but not all (45) mouse Hbs. Thus, adduct formation in the rat was of little use in predicting the reaction products of the diol epoxide with human Hb. *In vitro* reactions of both *syn* and *anti* diol epoxides of fluoranthene, as well as microsomally activated fluoranthene, with human Hb were conducted to characterize the binding to this protein. For technical reasons none of the products was structurally characterized, but chromatographic peaks with retention times corresponding to those of tetrols were always observed. For reasons to be discussed next, these suggest a similarity in the reaction products of fluoranthene with those of benzo[*a*]pyrene diol expoxide (B[*a*]PDE), which yields primarily carboxylic esters.

Attempts to characterize adducts of *anti*-B[*a*]PDE with human Hb by the process of chromatographically purifying enzymatic digests of the treated protein proved unsuccessful. Most of the material isolated consisted of the two 7,8,9,10-tetrahydrotetrols, which were eventually shown to arise from decomposition of adducts. It was also possible, through ^{18}O incorporation experiments, to prove that the tetrols arose from hydrolysis of one or more carboxylic esters (46). The sensitivity of the adduct arises from the nature of the alcohol component of the ester. Cleavage of the alkyl−oxygen bond (pathway 1 in Figure 7) is particularly facile since C10 is a benzylic carbon and forms a relatively stable carbocation. When hydrolysis occurs by this pathway, the resultant alcohol incorporates oxygen from solvent water. If pathway 2 is operative, oxygen is incorporated into the carboxylic acid. Only ester hydrolysis could lead to this dual outcome. Thus, by manipulation of pH, incorporation of solvent into B[*a*]P tetrols either occurred or did not, proving that the tetrols arose from carboxylic esters. The question of whether there exists one particularly reactive carboxylate, perhaps by virtue of its location in a binding site, is now being addressed.

A third, specifically defined, class of carcinogens may also be distinguished, consisting of aromatic amines activated through *N*-hydroxylamine formation. These electrophilic intermediates undergo oxidation to nitrosoarenes (47) which then react rather specifically with the thiol group of cysteines. The distinguishing characteristic of this reactant is that the nucleophilic attack occurs, not at a carbon atom as in all the preceding instances, but at the nitrogen atom. The final structure results from a rearrangement of the initially formed product and contains a protein−carcinogen bond which is a heteroatom−heteroatom bond (48). As such, it is inherently susceptible to cleavage.

Hb binds aromatic amines particularly well because the heme greatly accelerates the rate of nitrosoarene formation from hydroxylamine. The binding occurs at the β-93 cysteine residue in human Hb. This has been inferred from titration and competition experiments with *p*-mercuribenzoate (49) and, in the case of 4-ABP, demonstrated by X-ray crystallography (50). The reaction is quite general for one- and two-ring aromatic amines. It appears to occur as well with amines having a larger ring system, at least *in vitro*, but these adducts do not appear to be important *in vivo*, probably because alternate metabolic pathways become dominant.

SA can also bind nitrosoarenes, since it contains a free sulfhydryl group in its cysteine-34 residue. Not a great deal is known concerning the generality of this reaction. In one case involving the food pyrolysis product IQ, the full structure was determined (41). There is circumstantial evidence that sulfinamide formation also occurs with 4-ABP *in vivo*. Free 4-ABP was always present in enzymatic digests of SA isolated from rats given 4-ABP, despite efforts to remove noncovalently bound 4-ABP before proteolysis. Hb sulfinamide adducts of 4-ABP cannot be isolated by enzymatic digestion; the free amine is always the product of this type of treatment, so it is clear that the sulfinamide is unstable under these conditions. Thus, the free 4-ABP observed in digests of SA probably arises from an adduct. No other amines have been investigated with respect to their ability to form a sulfinamide adduct with the free cysteine of SA.

Applications

Quantitative analysis of protein adducts serves two related but distinct goals, exposure assessment and risk assessment. At

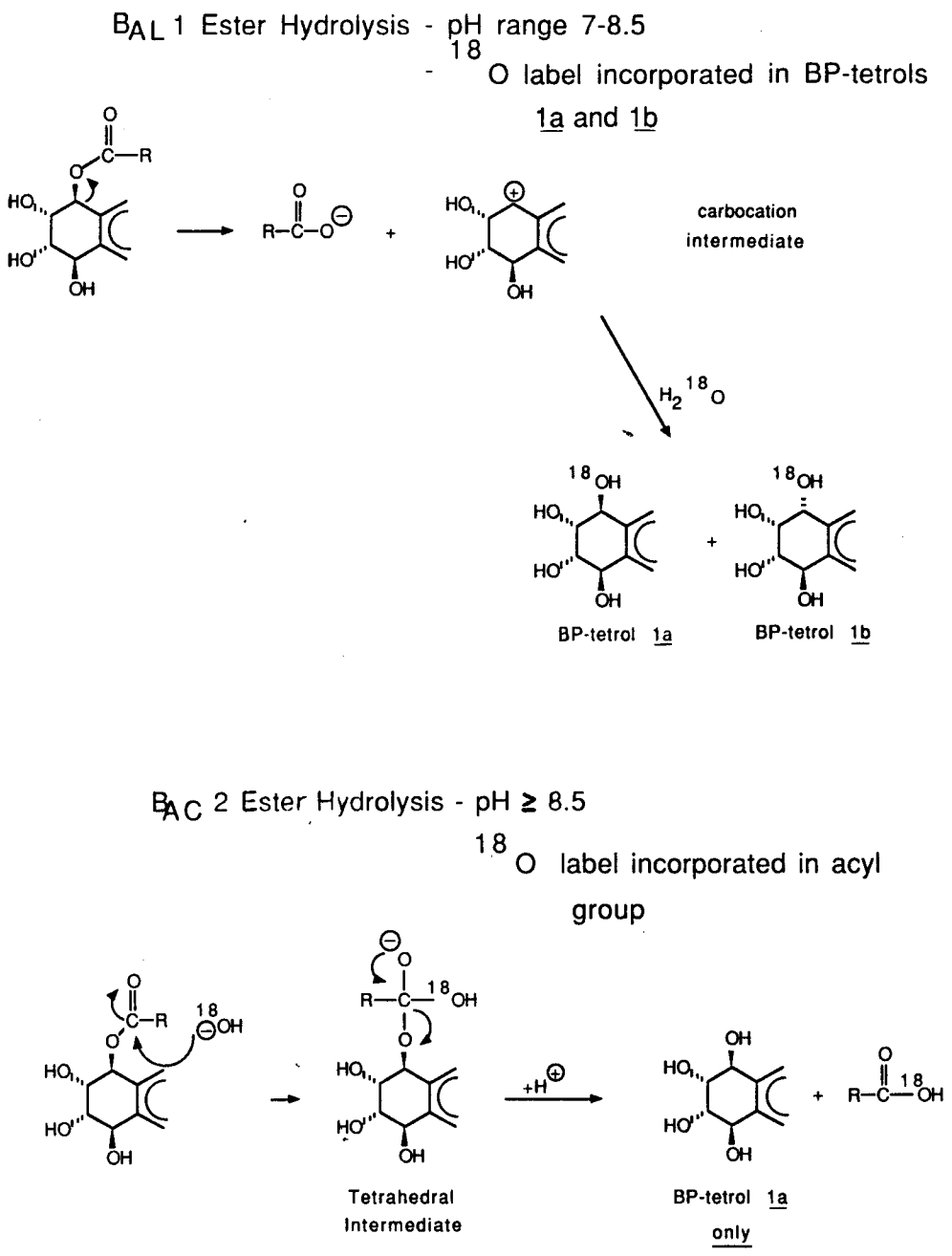

Fig. 7. Alternate mechanisms of benzylic ester hydrolysis under neutral to basic conditions. Under acidic conditions, a third mechanism is observed, which, like the $B_{AC}2$ mechanism, leads to incorporation of oxygen from solvent water into the carboxylic acid that is produced.

present, risk assessment based on protein adduct quantitation is more a hope than a reality. It is based on the premise that a proportionality exists between protein and DNA adduct levels; that protein adducts are a measure of the biologically effective dose. It is difficult to generalize concerning this premise; it is likely that each combination of chemical/target organ will need to be evaluated separately. In the event that a good correspondence is obtained between protein and target DNA adducts, it seems fair to assert that higher protein adduct levels will be associated with an increased risk for tumor development, at least with regard to mean values measured in groups. To test the proposition on individuals will require either a prospective or a nonconcurrent prospective epidemiological study, neither of which has yet been undertaken.

Exposure assessment is a simpler proposition. It is a combination of the identification of individuals or population subgroups that have been exposed to a chemical and a quantitative calculation of the actual dose received. Techniques currently being used require the assembly of a large array of diverse inputs. In contrast, levels of stable protein adducts can be converted directly to exposure if the dose–response relationship is known. The values obtained will be temporal moving averages and episodes of intense exposure will not be detected. The range and distribution of inter-individual differences in dose–response will govern both the confidence limit for quantitation of individual exposure and the minimum size of groups needed in studies designed to detect environmental or other factors.

Studies involving exposure assessment have been directed

Table I. Mean 4-ABP Hb adducts in populations of smokers and nonsmokers

Population	Nonsmokers[a]	Smokers[a]
New York	32 ± 13 (26)	154 ± 47 (19)
Turin	51 ± 22 (25)	178 ± 74 (40)
Stop Smoking Clinic		120 ± 41 (34)
Pregnant women	24 ± 11 (47)	135 ± 95 (34)

[a]Values in parentheses are the number of persons in each group.

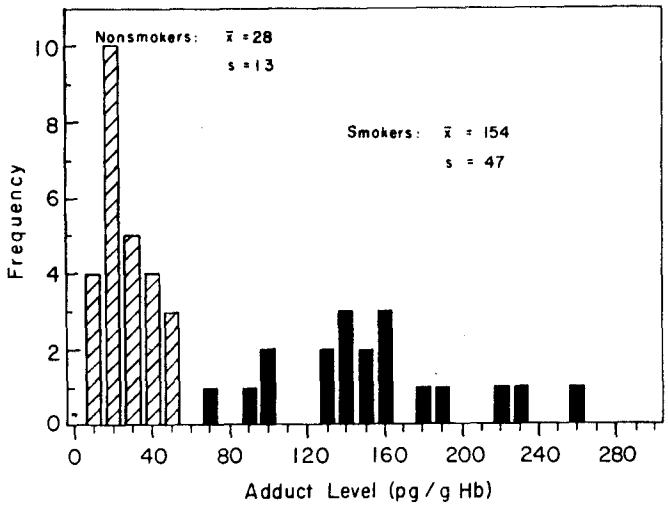

Fig. 8. 4-ABP–Hb adducts in smokers (solid bars) and nonsmokers (shaded bars) (from ref. 52).

toward several ends. These include the identification of environmental sources and exposed populations, intake quantitation, and exposure control. Carcinogens which have been studied include aromatic amines, with particular emphasis on aminobiphenyls, AFB_1, B[*a*]P and ethylene oxide.

Identification of environmental sources and exposed populations

Active smoking. Tobacco smoking is known to expose the smoker to a panoply of toxic compounds, many of which are carcinogenic. With regard to most of the carcinogens, it is an open question whether smoking is a minor or major contributor to the total exposure of an individual to that particular carcinogen. Several studies have now been reported which address this question.

It has, for example, been demonstrated that 4-ABP exposure is predominantly the result of cigarette smoking. This conclusion may be reached from examination of the results of several studies of quite different populations. Smokers recruited from an all male cohort in Turin, Italy (51); from students and employees, Columbia University, NY (52); from smoking cessation classes conducted by the Stop Smoking Clinic (Danvers, MA) at seven suburban hospitals in eastern Massachusetts (53); and from pregnant women at term (unpublished results) have all exhibited remarkably similar adduct levels of this carcinogen (Table I). With the exception of the Stop Smoking Clinic patients, nonsmokers were also drawn from these populations. As in the smokers, there is little difference in the mean adduct levels between the different control groups.

It is also of interest to compare the distribution of adduct levels in smokers and nonsmokers (Figure 8). It is clear from the comparison that overlap of the two populations is almost nonexistent. Extremes of cigarette-related exposure are the cause in most cases of apparent misclassification. Very low adduct levels in a smoker has been associated with unusually low cigarette consumption [e.g. 2 cigarettes per day (CPD)]. Nonsmokers exhibiting exceptionally high adducts report high passive smoke exposure.

It is also evident that cigarette smokers are exposed to far more 3-aminobiphenyl (3-ABP) than are nonsmokers. The data to support this conclusion were acquired primarily from the same Turin population used to investigate 4-ABP adducts, as well as from the Stop Smoking Clinic study. Adducts of 3-ABP were found to be 12-fold higher in smokers than in nonsmokers, a much higher difference than was observed for 4-ABP adducts.

Initially, it was suggested that the difference in the ratios of 3- and 4-ABP in smokers to nonsmokers was evidence for a greater tobacco specificity for 3-ABP exposure, that there were significant non-tobacco-related environmental sources of 4-ABP. More recent studies, in which nonsmoker values are from one-third to one-half that observed previously and smoker values are little changed, have failed to support this suggestion. Aminobiphenyl adducts are quantitated by capillary gas chromatography combined with selected ion monitoring mass spectrometry. While this is a highly selective technique, it is not guaranteed to be free of artifactual results. This is particularly true in cases in which the substance being detected possesses an unexceptional structure such as an aminobiphenyl. Thus, it must be recognized that the adduct levels as measured represent only an upper limit, and not necessarily a true value.

Other aromatic amine Hb adduct levels were also found to be associated with tobacco smoking. These included 2-naphthylamine, *o*- and *p*-toluidine, and 2-ethyl- and 2,4-dimethylaniline (51,54). Of these, 2-naphthylamine is accepted to be a human carcinogen, and *o*-toluidine has, in one epidemiological study, been implicated (55). These associations need to be interpreted cautiously. While they are statistically significant, supporting evidence such as dose–response relationships or adduct decline in quitting smokers has not been observed. It is possible to find equally significant differences between the mean values determined for different batches of specimens, which suggests that laboratory variation contributes noticeably to the overall variation.

Cigarette smoking has also been strongly associated with exposure to ethylene oxide, measured as *N*-hydroxyethylvaline (HOEtVal) levels in Hb. Two studies have been reported. In the first (56), two relatively uniform groups were established, which differed in smoking status. One consisted of nonsmokers, and the other was comprised of smokers who consumed >20 CPD. Adduct levels in the nonsmokers ranged from 27 to 106 pmol/g Hb (mean = 58) and in the smokers from 217 to 690 (mean = 389). The lack of overlap in the ranges is noteworthy. In the first study of 4-ABP adducts, in which only smokers who consumed >20 CPD were included, overlap was also not observed. Further evidence that the relationship of HOEtVal levels to smoking status is a causal one was obtained in a second study, described later, in which a good relationship between adducts and the number of cigarettes consumed daily was observed (57).

The detection of HOEtVal in nonsmokers is less surprising than the detection of 4-ABP. Each might be expected as the result of involuntary exposure to environmental tobacco smoke (ETS). But, while 4-ABP is not known as an environmental contaminant except in ETS, there are many sources of ethylene, a metabolic precursor of ethylene oxide. Recent studies with experimental animals have provided quantitative data for the formation of hydroxyethyl adducts as the result of exposure to ethylene (58,59).

Tobacco type. Most cigarettes sold in the United States are manufactured from so-called 'blond tobacco', which is the product of flue-curing. In other countries, including Italy, cigarettes made with air-cured tobacco are also popular. Air curing results in a darker product, known as black tobacco, which yields higher concentrations of aromatic amines when smoked (60).

Whether the increased concentrations of amines in smoke would be reflected in increased Hb adduct levels in black-tobacco smokers was tested by comparing mean adduct levels in 43 blond-tobacco smokers and 18 black-tobacco smokers selected from a relatively homogeneous population (51). On balance, the results support the hypothesis. A statistically significant association of adduct level with type of tobacco type was observed in four cases: 4-ABP, *o*-toluidine, *p*-toluidine and 2,4-dimethylaniline. No association with tobacco type was observed unless an association with smoking status was also observed.

In this study, occupation was also considered. None of the subjects was involved in occupations known to be associated with aromatic amine exposure. Furthermore, no single occupation was associated with any of the three comparison groups (nonsmokers, blond- or black-tobacco smokers). Thus, occupation is unlikely to be a confounding factor.

Passive smoking. Involuntary exposure to tobacco smoke has been found weakly associated with lung cancer in some epidemiological studies, but several others have found no association (61,62). It has been argued that the relative risk of passive smoking is too low to be detected by epidemiological studies (63). If this conclusion is correct, then other means will be required to evaluate the risk of passive smoking.

In a recently concluded study (64), Hb adducts of 3- and 4-ABP were measured in subjects, who could be categorized according to their degree of exposure to ETS, to determine if an increase in these adducts could be detected in those more heavily exposed.

Nonsmokers were recruited by a variety of methods to cover a range of passive exposures. Nonsmoking bartenders were chosen to represent the greatest exposure group. Individuals with more normal, but quantitatively uncertain, exposure were chosen according to the criterion of self-reported exposure to the environmental smoke of one or more packs of cigarettes per day. Other individuals who reported negligible exposure to ETS, as well as the nonsmokers in the Turin study, were also included.

The subjects were divided into two groups (1 and 2) according to whether there was detectable cotinine in their plasma. The two groups were then subdivided according to the level of exposure reported in their interviews to create a total of five groups (1a, 1b, 1c and 2a, 2b). Levels of 4-ABP were marginally higher in group 2 and the difference was of borderline statistical significance: the median two-sample test statistic was 1.64 ($P = 0.05$) and the Wilcoxon two-sample test statistic was 1.08 ($P = 0.14$). A more significant difference in the hypothesized direction was found for 3-ABP levels (Figure 9). Group 2 had significantly higher levels than group 1. The median and Wilcoxon tests resulted in $P = 0.027$ and $P = 0.11$, respectively, using the lowest detectable level for subjects with undetectable adduct levels. When undetectable levels were omitted, P values were reduced to 0.017 and 0.05.

From this study it may be concluded that ETS is an identifiable source of 3- and 4-ABP and that persons with significant exposure to it constitute a distinguishable population. It should also be noted that the increases in adduct level in exposed compared with non-exposed individuals is sufficiently small to be consistent with the claim that questionnaire-based epidemiological studies cannot detect the relative risk associated with passive smoking.

Diet. Contamination of dietary components with AFB_1 varies greatly depending on geographical and geopolitical influences. In certain regions of the world the AFB_1 concentration in staple foods is often extremely great. Elsewhere the climatic conditions necessary for the growth of *Aspergillus flavus* are not prevalent and governments enforce strict regulation of the food supply. Thus, it is to be expected that these environmental differences will be reflected in adduct levels of AFB_1.

The expectation has been realized. In a comparison of SA adduct levels in sera obtained from The Gambia and France, it was found that adducts were markedly higher in the first group (65). As determined by ELISA, the mean level was 3.5 ± 2.7 pg AFB_1 equivalent per mg plasma protein in 20 Gambian sera specimens. The French sera were lower, with a mean value of 0.25 ± 0.39 pg/mg for 16 specimens. When purified albumin was used in the assay, levels up to 40 pg/mg albumin were measured in the Gambian specimens. On the basis of animal experiments using [^{14}C]AFB_1 the authors concluded that these values actually underestimate the true levels.

A separate study was conducted in Guangxi province, China (66). In 42 subjects drawn from this population, the mean adduct level was 106 ± 64 pg/mg SA, which is considerably higher than observed in the Gambian population. The differences may, in part, be explained by differences in the assays used; no inter-

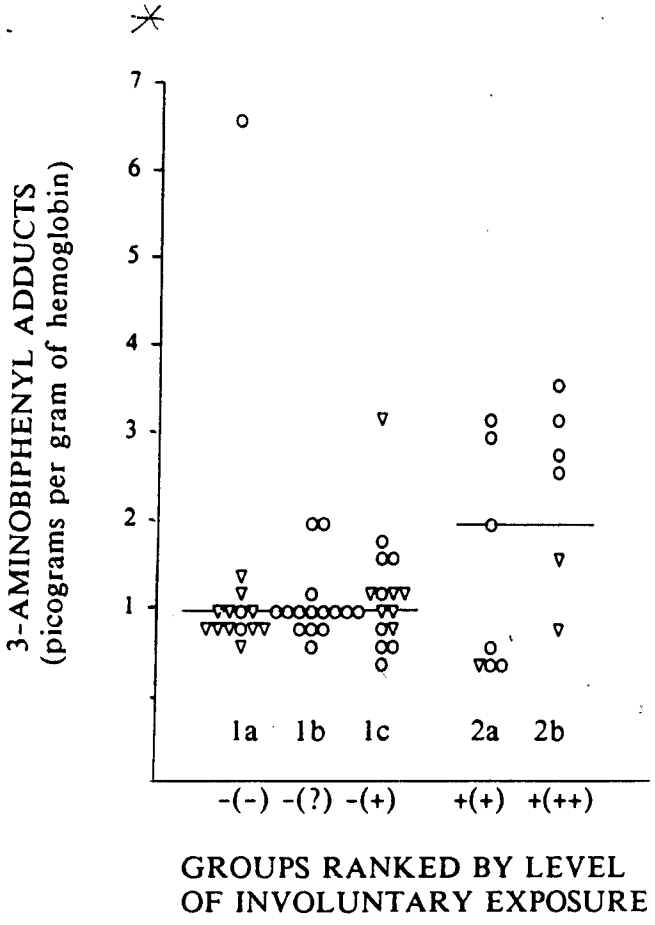

Fig. 9. 3-ABP–Hb adducts in nonsmokers without (1a, 1b and 1c) and with (2a, 2b) detectable serum cotinine. Symbols in parentheses refer to self-reported exposure to ETS. Triangles indicate undetectable adduct levels and are plotted at the limit of detection (from ref. 64).

laboratory comparisons of the same samples have been conducted. A more likely explanation, though, is that these subjects suffered a much greater exposure. As part of the study, food was collected daily for the week preceding blood collection and analyzed for AFB_1 content. When the measured concentrations were combined with the amount of food eaten, it was calculated that the average intake was 58 μg per day.

Intake quantitation

4-ABP and cigarette smoking. In one study of the effects of smoking on 4-ABP–Hb adducts, a significant correlation was found between the adduct level and the self-reported number of cigarettes smoked per day. In other studies the relationship exhibits the expected trend, but without statistical significance. When all the data obtained thus far are combined, a striking pattern emerges as illustrated in Figure 10.

A sharp increase in adduct levels occurs in individuals smoking 1–10 CPD relative to nonsmokers. Another considerable increase is observed among those smoking 11–19 CPD. Beyond that level of cigarette consumption there is no further significant increase in adduct levels. This pattern is in contrast to that observed for AFB_1 and ethylene/ethylene oxide, and is open to various interpretations.

The simplest explanation is that smoking patterns change as the number of cigarettes consumed increases so that actual intake of 4-ABP remains relatively constant beyond a certain point. This is not unreasonable, but it is not supported by measurements of plasma cotinine, which appear to increase steadily with increasing cigarette consumption. It is also not supported by measurements of HOEtVal levels, as discussed below.

An alternative explanation is that changes in metabolic profiles occur as the result of heavy cigarette consumption. That such changes do occur is quite clear (67), but how these would influence the overall disposition of 4-ABP is not yet known. What would be required would be a reduction in the relative yield of *N*-hydroxylamine available for reaction with Hb. There are many ways to achieve this outcome, and at present it is only possible to speculate as to which are likely.

Estimation of the dose–response from adduct levels in smokers of <20 CPD yields a value of ~5 pg adduct/g Hb/CPD. This figure agrees with an earlier estimate based on the difference in adduct levels between smokers and nonsmokers. In conjunction with reported concentrations of 4-ABP in smoke, the measured adduct levels were used to calculate that between 5 and 10% of inhaled 4-ABP is converted to Hb adducts. That fraction is essentially the same as had been determined in an animal model (29), lending support to the concept of using laboratory animals to characterize protein binding of carcinogens in man.

Hydroxyethyl adducts. Hydroxyethylation of the N-terminal valine of Hb has been used to monitor the degree of exposure to cigarette smoke (57). When the adduct levels were analyzed as a function of cigarette consumption, a very good correlation was observed ($P < 0.01$). The smoking-related increase in HOEtVal adduct in Hb was 7 pmol/g Hb/CPD. It has previously been calculated that the ethylene in cigarette smoke should give rise to ~80 pmol/g Hb (56). This figure was arrived at using the mouse as an animal model combined with rate constants for the reaction of ethylene oxide with Hb and for Hb turnover. Again, there appears to be very good correspondence between the dose–response observed in man and that predicted from an animal model.

There is little in the HOEtVal data to indicate a plateau in the dose–response curve, although more determinations in heavy smokers would be necessary to be certain. If true, though, the apparently linear relationship would indicate that HOEtVal adducts are a good dosimeter for tobacco smoke exposure in the same manner as serum cotinine. It would also mean that the processes leading to the formation of HOEtVal from inhaled smoke are relatively insensitive to the other effects of cigarette smoking such as enzyme induction.

Aflatoxin B_1. A third study of protein adducts in man, in which a dose–response relationship was determined, has been reported (66). The carcinogen in this case was AFB_1. The intake was determined by sampling of individual diets, quantitative analysis of the AFB_1 contamination in each sample, and measurement of the individual food consumption. Although more accurate than the estimation of the intake of a cigarette smoke component, this process was also subject to some degree of uncertainty arising from temporal considerations, as it was only conducted for 1 week. Nevertheless, agreement with an animal model within a factor of 2 was observed.

Forty-two subjects participated in this study. Aflatoxin adducts formed with SA were determined in serum specimens obtained during the week that food contamination was measured. Quantitation was performed by radioimmunoassay (RIA) and by direct measurement of the fluorescence of isolated SA. Both techniques yielded highly significant correlations between adduct level and intake, but produced different slopes for the dose–response curve. A likely explanation for this difference is that in the RIA approach, recovery was a factor and it may not have been correctly estimated. In any event, the two approaches correlated well with each other ($r = 0.72$, $P < 0.000001$).

The fraction of ingested aflatoxin which binds to SA was estimated as either 4.2% (fluorescence) or 2.3% (RIA). Estimates of the binding in rats are ~2%. Thus, it appears that adduct formation by aflatoxin in man can be reasonably modeled with experimental animals, just as 4-ABP and ethylene oxide adduct formation can be modeled.

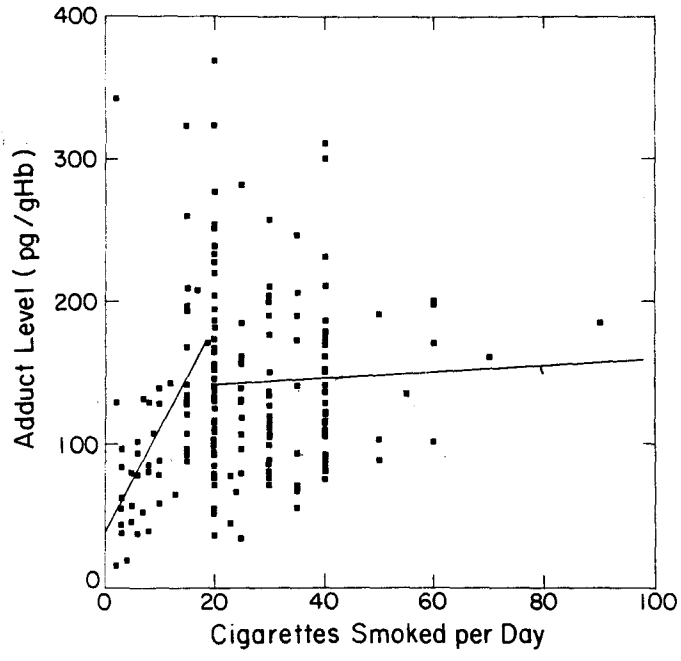

Fig. 10. 4-ABP–Hb adducts in smokers. The data were grouped into two sets (1–19 CPD and 20–90 CPD) for linear regression analysis.

Exposure control

Exposure control may be thought of as an auditing process, operating independently of the quantitative aspects of adduct formation or its biological effects. It requires only that within the range of exposures under study, the dose–response is a linear function. Then, an adduct level measurement made at one point in time can be used as a reference value for the subject being monitored. Subsequent determinations will indicate whether exposure increased or decreased, and more importantly, to what degree.

There are obvious applications for exposure control in occupational settings, but very little has been published on the subject. In the research context, exposure control has been used to determine the effect of quitting cigarette smoking on 3- and 4-ABP exposure (53,64). A study involving participants at the Stop Smoking Clinic (Danvers, MA) was undertaken with two general goals. The first was to verify the applicability of protein adduct determinations as a means of exposure control. This particular context was considered nearly ideal since the predominant source of the two amines, cigarette smoke, could itself be closely controlled. Secondly, this study would provide important information regarding how closely the kinetics of adduct decline would correspond to predictions based on erythrocyte turnover.

The 4-ABP results are presented graphically in Figure 3, in which only data obtained for the 34 participants who successfully quit for at least 3 weeks are shown. Fifteen of these also remained ex-smokers for another 9–11 weeks. The mean adduct levels declined from 120 pg/g Hb at the start of the program to 82 pg/g Hb at 3 weeks to 34 pg/g Hb at 9–11 weeks. No further reduction was observed in nine subjects who were tested at 17–23 weeks.

Besides clearly reflecting the effect of the elimination of exposure, the results also provide the first quantitative information concerning Hb adduct stability in human subjects. The observed decline in adducts was more rapid than predicted, the actual rate being about one-third faster. The difference is not sufficiently great to compromise the usefulness of this particular adduct as a dosimeter, but it does suggest a slight instability of the adduct *in vivo* or a shortened lifetime of Hb in smokers.

Adducts of 3-ABP in seven of the subjects were also measured at the beginning of the study and at 9 weeks. The means declined from 16 pg/g Hb to 1.7 pg/g Hb over the period. This decline, too, is somewhat greater than expected. Again there is the suggestion of some slight instability of the adduct or of Hb, but it is clear that 3-ABP adducts of hemoglobin can be used to monitor the effectiveness of efforts to eliminate exposure to this compound.

Future perspectives

Blood protein adducts have already proved useful in developing information concerning human exposure to the biologically effective form of environmental carcinogens. There is so little information on actual human exposure that merely collecting more data on more people and more compounds will provide a quantum leap in understanding the role of environmental chemicals in cancer risk. However, the potential of this tool can be further expanded through future developments in improved analytical chemistry (qualitative and quantitative), by extending the length of the temporal record back in time, by developing approaches which allow multicomponent analysis of the same biological sample, and by developing techniques which will allow the search for unknown exposures, e.g. cryptic or endogenously formed carcinogens.

The nature of epidemiological studies is such that the quality of these studies greatly improves with increasing numbers of observations. The total number of useful measurements of protein adducts to date is probably < 1000. This is because the methods are highly technical, labor intensive, and require expensive equipment available in few laboratories. Pharmaceutical companies routinely monitor tens of thousands of human samples from advanced stages of testing a new drug. The same could be accomplished for protein adducts by developing automated procedures for sample preparation and by investment in dedicated laboratories containing the most advanced analytical chemical equipment.

The nature of cancer is that exposures over a period of decades determines the relative lifetime risk of individuals. Hb and SA provide only a 3–4 month record of recent exposure. It would be extremely useful to have a long-term, perhaps even a lifetime protein dosimeter. There are proteins in the body that turn over much more slowly than Hb or SA, e.g. crystallin in the eye lens, and collagen and elastin in a variety of internal organs. Some portion of collagen is cross-linked and deposited and may last the lifetime of the animal. In addition, these structural proteins might yield information about specific target organ dose for some carcinogens, not just the overall systemic dose revealed by blood proteins. One of our current goals is to determine whether collagen is capable of covalently binding carcinogens of different structures and to estimate the lifetimes of potential adducts. If successful this will prove useful for examining long-past exposures, but due to the indeterminate lifetime of these proteins there seems little hope of obtaining quantitative lifetime dosimetry information. This is an area in need of new ideas and approaches.

Up to this time almost all protein adduct studies in humans have centered on measurement of one specific adduct. We have little to no information on the internal dose of more than one type of compound in the same individual at the same time. Since the result of living in a complex environment is constant exposure to many chemicals, it is essential to understand the collective exposure to all classes of carcinogens. We might think of each individual as having a unique carcinogen exposure spectrum, and perhaps those with the highest multiple exposures are at greatest risk for disease. The technical difficulties of adduct analysis make it unlikely that any one laboratory will be able to perform multi-component analyses on large numbers of samples. The solution appears to be either collaborative efforts between many laboratories on shared samples or construction of dedicated laboratories capable of multicomponent analyses.

The usual assumption of environmental toxicology is that measurement of concentrations of compounds in the environment provides the essential information for estimation of increased risk due to chemical exposure. However, it is now generally accepted that there may also be various classes of endogenous exposures, e.g. oxidative stress, products of intestinal microbial metabolism, and endogenously formed *N*-nitroso compounds. It is also possible that there are cryptic environmental exposures, e.g. compounds whose presence is undetected or compounds whose structure is unknown. There is a need for methods to detect such exposures, and one approach would be to search for adducts that bind to a specific nucleophilic site in a protein, e.g. all classes of adducts to the β-93 cysteine of Hb or to other specific side-chains, N-terminal amino acids or C-terminal amino acids. One could envision analysis, in the near future, of a large pooled blood

sample from a high-risk population using the most sophisticated chemical instrumentation.

Finally, we will have a better understanding of the nature of the risk for chemical exposures if we can relate external exposure to internal exposure by characterization of the various metabolic phenotypes responsible for carcinogen activation and detoxification. Also, it is clear that information on protein adducts will prove most useful if parallel measurements on the same individuals will be made on other biomarkers, particularly those relating to altered genetic structure and function.

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