[CANCER RESEARCH 47, 602–608, January 15, 1987]

Hemoglobin Adducts of 4-Aminobiphenyl in Smokers and Nonsmokers[1]

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ABSTRACT

A quantitative method has been developed for the analysis of 4-aminobiphenyl (4-ABP) covalently bound as the sulfinic acid amide to the 93β cysteine of human hemoglobin. The method uses mild basic hydrolysis of hemoglobin to release the parent amine, derivatization to form the pentafluoropropionamide, and capillary gas chromatography with detection by negative-ion chemical ionization mass spectrometry. The method is precise and gives reproducible results on multiple blood samples taken from individuals over 48 h. Application of this method to blood samples from cigarette smokers and nonsmokers revealed consistently higher adduct levels in smokers. The mean value for smokers was 154 pg 4-ABP per g Hb compared to 28 pg/g Hb for nonsmokers, with no overlap of adduct levels between the two groups. Studies on quitting smokers revealed that adduct levels declined over a period of 6–8 weeks to nonsmoker levels. The finding of 4-ABP adducts in all nonsmokers was not anticipated but is consistent with low-level ubiquitous contamination of air, food, or water. In other animals sampled, rats and dogs had measurable adduct levels, but monkeys and fish did not. The hemoglobin adduct of 4-ABP is the product of a series of reactions between the hemoprotein and *N*-hydroxy-4-ABP. The formation of hydroxylamines from carcinogenic aromatic amines and their subsequent reactions with DNA are generally thought to be critical events in the initiation of bladder tumors. We suggest that the observed hemoglobin adduct levels formed by this proximate carcinogen will reflect the extent to which these steps have occurred. This is the first report of 4-ABP adducts in human blood.

INTRODUCTION

A number of aromatic amines, including 4-ABP[3] have been detected in tobacco smoke (1). Since some of these amines are potent human bladder carcinogens (*e.g.*, 4-ABP and 2-naphthylamine), it is a reasonable hypothesis that increased exposure to these amines is a factor in the observed increase in the incidence of bladder cancer among cigarette smokers. Relative risks for bladder cancer in this group are generally in the range of 1.5 to 3.0 (2, 3), although other studies have reported somewhat higher relative risks (4). A dose-response relationship is also seen between the number of cigarettes smoked and the observed relative risk for bladder cancer (3).

N-Hydroxylation of the parent amine appears to be the critical step in the metabolic activation of these compounds to carcinogenic species (5). Transport of the hydroxylamine to the bladder in the more stable form of an *N*-glucuronide, followed by hydrolysis in the acidic environment of the human bladder, results in the formation of electrophilic nitrenium ions involved in critical macromolecular binding reactions (6, 7). A method of dosimetry which quantitates the *in vivo* reaction products of the hydroxylamine might therefore be useful in estimating the biologically effective dose of 4-aminobiphenyl.

This paper describes a method of biological dosimetry for 4-ABP which is based on the formation of acid-labile sulphinamide adducts arising from the interaction of *N*-hydroxy-4-aminobiphenyl with a single cysteine residue of hemoglobin. Previous work in a rat model (8) showed that the chemical and pharmacological properties of this adduct would allow it to be of potential use in estimating exposure to this carcinogen. The development of a highly sensitive GC-MS method for the quantification of this hemoglobin adduct has enabled measurement of erythrocyte doses at the levels necessary to observe exposure related to cigarette smoking.

MATERIALS AND METHODS

Chemicals and Reagents. All aqueous solutions were prepared with distilled deionized water (Milli-Q water purification system). Reagent-grade toluene was extracted with an equal volume of 1.0 N HCl, followed by three extractions with water. Hexane (Baker Resi-Analyzed grade) was redistilled twice and stored in KOH/ethanol-cleaned glass-stoppered bottles. Trimethylamine in hexane was prepared by adding 1 g reagent-grade trimethylamine hydrochloride to 2 ml water, neutralizing with NaOH and extracting into 5 ml hexane. The internal standard, 4′-F-ABP (Pfaltz and Bauer) was recrystallized from dichloromethane/hexane and used to prepare a stock solution of 10 μg/ml in 0.1 N HCl. The stock solution was stored at 4°C and diluted to 100 ng/ml with 0.1 N HCl on each day of use. PFPA (Pierce or Supelco) was stored in air-tight vials and replaced periodically. All other chemicals were reagent grade.

Analysis of Human Blood Samples. Ten-ml blood samples were obtained from human subjects classified according to smoking status [a smoker was classified as an individual currently smoking at least one pack per day (range, 20–50 cigarettes/day), while nonsmokers were classified as individuals who have never smoked]. The blood was centrifuged at 3,000 × *g* to generate packed red blood cells. After removal of the serum, the RBC were washed three times with 0.9% saline and lysed by the addition of 15 ml deionized water and 2 ml toluene and vigorous shaking. After approximately 30 min, the lysate was centrifuged at 10,000 × *g* (10–20 min) to remove the cell debris. The clear hemoglobin layer was transferred to dialysis tubing and dialyzed against two changes of distilled, deionized water at 4°C for 2 days. The dialysate was added to weighed Corex tubes (50 ml, with Teflon-lined caps, No. 8422A) and reweighed to determine the approximate volume. The hemoglobin concentration of each sample was determined by Drabkin's method (no. 525A; Sigma) and used to calculate the amount of hemoglobin in each sample. After spiking with 1.00 ng 4′-F-ABP, the dialysate was made 0.1 M in NaOH and incubated for 3 h at room temperature. The hydrolysate was extracted with 15 ml hexane. The resulting emulsion was broken by freezing and then thawing the samples. The extraction was then repeated with an additional 15 ml of hexane. The hexane extracts were combined, extracted with 10 ml 1.0 N HCl, and then discarded. After neutralization with NaOH, the aqueous phase was extracted twice with 15 ml hexane. The hexane extracts were combined and dried over anhydrous sodium sulfate and magnesium sulfate. Two μl of trimethylamine in hexane was added to the hexane extracts in pear-shaped flasks, followed by 2 μl of PFPA. After 10 min at room temperature, the hexane was concentrated to about 1 ml with a rotary evaporator. The hexane was then added to a ½-dram vial and evaporated to dryness. The residue was redissolved in 20 μl of hexane for analysis.

Received 6/9/86; revised 10/10/86; accepted 10/16/86.

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[1] This investigation was supported by Grant 5-P01-ES00597, awarded by the National Institute of Environmental Health Sciences; Toxicology Training Grant 2-T32-ES07020 from NIH; and Special Institutional Grant SIG-10-II from the American Cancer Society.

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[3] The abbreviations used are: 4-ABP, 4-aminobiphenyl; 4′-F-ABP, 4′-fluoro-4-aminobiphenyl; PFPA, pentafluoropropionic anhydride; Hb, hemoglobin; GC, gas chromatography; MS, mass spectrometry; RBC, red blood cells.

Gas Chromatography-Mass Spectrometry. GC-MS was performed on a Hewlett-Packard 5987 GC-MS equipped with a standard electron ionization/chemical ionization source. Analyses were carried out using negative-ion chemical ionization, with methane as the reagent gas. For maximum sensitivity, the instrument was manually tuned each day using perfluorotributylamine. The source pressure and temperature were typically 0.8 torr and 150°C, respectively. The electron energy varied from 60 to 150 eV and the emission current was 400–450 μA. The gas chromatograph was interfaced to the mass spectrometer with an open-split capillary interface, with the interface, injection port, and transfer lines kept at 240 to 250°C. The GC column was a 20 m × 0.25 mm I.D. fused silica capillary coated with Supelcowax 10 (film thickness, 0.25 μm). The carrier gas was helium (ultrahigh purity grade), with a flow rate of approximately 2 ml/min. Gas line purifiers (no. 6406; Matheson) were installed on both gas lines. Four-μl injections were made in the splitless mode (splitless valve open for 0.5 min) with an initial oven temperature of 60°C for 1 min, followed by a temperature ramp to 240°C at 20°C/min and a 5-min isothermal phase (240°C). The PFP derivatives of 4-ABP and 4′-F-ABP eluted during the isothermal phase. The following single ions were monitored with dwell times of 300 ms each: 295.2 for PFP-ABP and 313.2 for PFP-4′-F-ABP [corresponding to the respective $(M\text{-}HF)^-$ fragments]. Further characteristics of these compounds, under electron ionization and positive chemical ionization mass spectrometry, are discussed in greater detail elsewhere (9).

Calculations. The integrated peak area of the 295.2 ion was divided by the peak area of the 313.2 ion and then multiplied by the ratio of the molecular weights of the respective amines (169/187) to determine the amount (in nanograms) of 4-ABP in the sample. This value was divided by the grams of hemoglobin in the sample to calculate the adduct level expressed as nanograms 4-ABP per gram hemoglobin.

Source of Human Blood Samples. In precision studies blood samples (2–10 ml each) were obtained from the Massachusetts Institute of Technology Medical Department after completion of standard clinical assays. These were combined to form a single blood sample of about 100 ml. After stirring to achieve homogeneity, the blood was processed as described previously to isolate the washed RBC. These were again mixed to homogeneity and then aliquoted into seven or eight samples of 5 ml each.

Smoking Cessation Studies. Subjects who joined The Stop Smoking Clinic, Inc. (Danvers, MA) were recruited to donate blood samples before and at various time points after cessation of smoking. After completing questionnaires on their smoking history and other personal characteristics, the subjects donated 10-ml blood samples, obtained by puncture of an antecubital vein into evacuated containers coated with heparin or EDTA. The five subjects included four men (aged 23–78) and one woman (age 36), with reported smoking frequencies of one to two packs per day. Samples were collected from these five successful quitters at three time points up to 60 days following cessation of smoking. Serum cotinines were done to verify that the subjects had indeed quit smoking during the course of the study.

Smoking and Nonsmoking Subjects. Samples were obtained from F. Perera, R. M. Santella, and D. Brenner of the Columbia University School of Public Health in a coded blind fashion (samples were labeled 1000-1 to 1053-3 and the smoking status was not known to us at the time of analysis). Each sample consisted of about 5 ml of washed RBC from a total of 35 ml blood drawn in quantities of 7 ml into heparinized tubes. For some subjects, an additional 5 ml of RBC were obtained from a second 35-ml blood sample drawn either 15 or 48 h after the initial sample. These samples are further described and characterized elsewhere.[4]

RESULTS

Our initial work on developing a dosimeter for 4-ABP (8) revealed a stable, covalent adduct with hemoglobin which was formed in high yield. Information in the literature (10) suggested that this adduct may be a sulphinamide formed via interaction with a single cysteine residue (93β) of hemoglobin. We have crystallized human oxyhemoglobin after reaction with *N*-hydroxy-4-ABP *in vitro* and determined its structure to 4 Å resolution by X-ray crystallography.[5] At this resolution, the exact nature of the chemical bond between the biphenyl residue and the protein is not discernible, but it is clear that the 93β cysteine is the residue to which the biphenyl ring system is attached. Using a rat model, the adduct was shown to possess the following characteristics which make it suitable for use as a biological dosimeter: (*a*) The adduct is formed in a dose-dependent manner over the range tested (0.5–5000 μg/kg) at the exceptionally high level of over 5% of an administered dose. Further unpublished studies from our laboratory using d9-4-ABP have indicated that this dose-response relationship is unchanged at doses as low as 0.005 μg/kg. (*b*) The adduct appears to be stable *in vivo*, being removed at a rate similar to that of unadducted hemoglobin. (*c*) During chronic dosing, the adducts appear to accumulate over the lifetime of the erythrocyte to a steady state adduct level, as predicted (11). (*d*) The adduct can be broken down *in vitro*, by base- or acid-catalyzed hydrolysis, to the parent amine, thus simplifying its isolation from the rest of the protein molecule.

Analytical Method. Our initial efforts to develop methods for the analysis of 4-ABP-hemoblogin adducts utilized the standard HCl/acetone method (12) for the isolation of globin. In that procedure, hemoglobin purified by dialysis is added to an acidic solution of acetone. The globin precipitates, leaving the heme in solution. Free 4-ABP is also present in the acidic acetone solution, due to hydrolysis of the sulphinamide adducts. Evaporation of the acetone precipitates the heme, which is removed by filtration. Further purification by C18 chromatography produces a buffer/methanol solution containing the amine, which is quantified by reversed-phase high-performance liquid chromatography. This technique was not sufficiently sensitive to measure adduct levels at the subnanogram level expected to be found in human blood samples.

Reports in the literature indicated that capillary gas chromatography with electron capture detection could analyze amines in the nanogram to picogram range after derivatization with pentafluorobenzoyl chloride (13), pentafluoropropionic anhydride, or heptafluorobutyric anhydride (14). Based on this background information, a method was developed which consisted of extracting the amine from the buffer/methanol solution into hexane, derivatization with pentafluorobenzoyl chloride, clean-up by silica gel chromatography, and quantification by GC using electron capture detection. The above method was not suitable for adduct levels below a microgram due to a high background of electron capture-sensitive compounds in the solvents and other equipment. In some plastic materials (filter holders and air filters), trace amounts of 4-ABP were detected.

An alternative basic hydrolysis procedure was found to be more suitable for trace analysis since only glass and teflon materials are used and only two solvents (hexane and water) needed to be purified. In the method, illustrated in Fig. 1, the dialyzed hemoglobin is made 0.1 M in NaOH for a 2–3-h incubation period. The hydrolyzed amine is then extracted into hexane, derivatized with pentafluoropropionic anhydride, and then quantified by gas chromatography. Electron capture detection was initially used because of its enormous sensitivity, but was found to possess insufficient selectivity. The chromatograms from real samples contained enough EC-sensitive mate-

[4] F. P. Perera, personal communication.

[5] D. Ringe, R. J. Turesky, P. L. Skipper, and S. R. Tannenbaum, Structure of a single stable hemoglobin adduct formed by 4-aminobiphenyl, unpublished results.

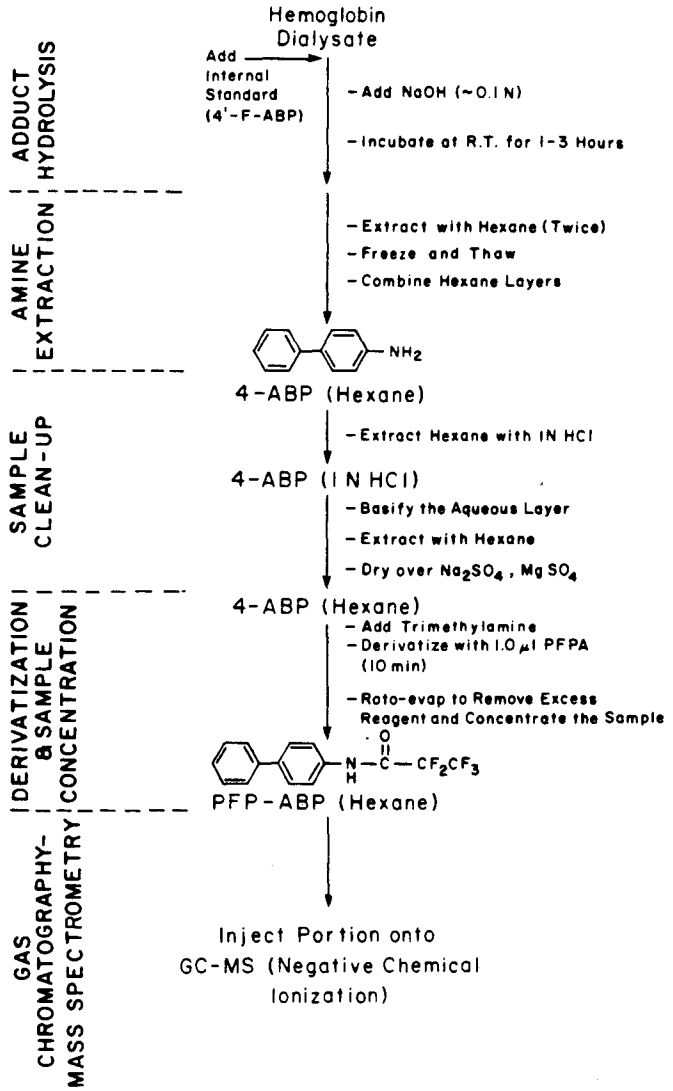

Fig. 1. Method for analysis of 4-aminobiphenyl adducted to hemoglobin.

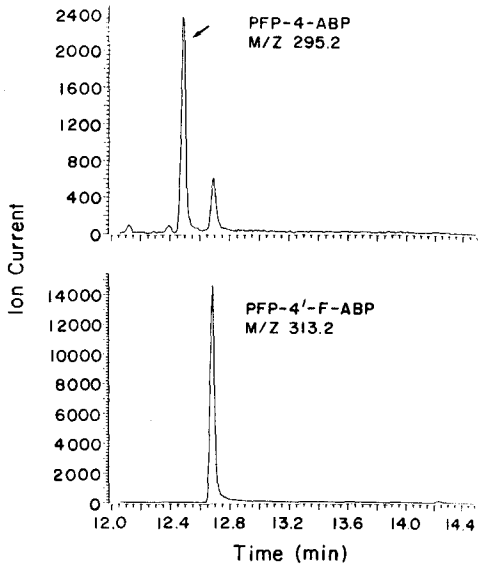

Fig. 2. Chromatographic analysis of 4-ABP. The *upper trace* was produced by monitoring at *m/z* 295.2. The PFP-ABP eluted at 12.5 min and is marked with an *arrow*. The peak at 12.7 min is a fragment ion of the internal standard. The *lower trace* (PFP-4'-F-ABP, internal standard) was produced by monitoring at *m/z* 313.2. The sample was obtained from a smoker and contained 135 pg 4-ABP/g Hb. The amount of 4-ABP injected was 14 pg.

Table 1 *Background levels of 4-ABP-hemoglobin adducts in various animal species*

Species	Adduct level (pg 4-ABP/g Hb)
Rat (Sprague-Dawley, Fisher), 20 samples	500–3000
Dog (beagle), 3 samples	118–240
Monkey (rhesus), 3 samples	ND[a]
Fish (flounder), 8 samples	ND

[a] ND, none detected above the detection limit of 5–10 pg/g Hb.

rials to make adduct analysis below 100 pg/g hemoglobin unfeasible. A chromatogram from a human nonsmoker sample quantified by GC-electron capture detection was reported previously (8).

The use of negative-ion chemical ionization mass spectrometry, in the selected ion mode, provided both the sensitivity and selectivity needed for detection and quantification of adducts at levels found in human blood samples. At a minimum signal-to-noise ratio of 10 in the final chromatogram, the method is sensitive to levels below 10 pg 4-ABP/10 ml blood. The method has been used to quantify adduct levels up to six orders of magnitude above this level (µg/g of hemoglobin) and appears to be linear in this range and beyond. As can be seen from the chromatograms in Fig. 2, the background noise produced by other compounds in the final hexane solution is minimal.

The precision of the assay has been determined by analysis of aliquots of 100-ml blood samples. In one study, eight aliquots were analyzed, yielding values of 47, 49, 52, 52, 54, 55, 61, and 63 pg/g Hb (mean, 54; SD, 5.5) and in another study, seven aliquots were analyzed (values of 32, 34, 37, 38, 41, 42, and 44 pg/gHb; mean, 38; SD, 4.3). In each case, one standard deviation was equal to about 10% of the mean adduct level. Sources of this variation would also include errors in the assay for hemoglobin content.

Reproducibility was assessed by multiple sampling of 15 subjects and subsequent analyses of the samples in the same or in different batches. Samples were obtained 15 or 48 h apart. RBC from these samples were frozen and analyzed in three different batches over a 13-mo period. For seven subjects, the duplicates were done within one batch and for the rest the duplicates were analyzed in different batches. These experiments were designed to determine the magnitude of intraindividual variation relative to interindividual variation. The coefficient of variation due to sampling from the same individual at different times, 15–48 h later, was estimated to be 9%, meaning day-to-day biological variation in adduct levels was unmeasureable since, as indicated above, the within-batch coefficient of variation (or precision) was 10%.

Numerous studies were done to rule out the possibility of 4-ABP contamination of the various solvents (hexane, toluene, and water), reagents (PFPA, $MgSO_4$, Na_2SO_4, heparin, and EDTA), and equipment used in the analysis. No such contamination has been detected at levels above the subpicogram detection limit of the analysis. In addition, the absence of a detectable adduct level in fish and monkey blood (Table 1) eliminates other possible sources of laboratory contamination which were not tested.

The presence of a background adduct level in other nondosed animal species, such as the rat and dog (Table 1), must be due to differences in exposure to 4-ABP and/or the ability to form sulphinamide adducts with hemoglobin. The source of these exposures is not known, but presumably may include water, air, or food. Analysis of the drinking water given rats failed to detect any 4-ABP contamination. Attempts to analyze the air for 4-ABP were unsuccessful as commercially available air

sampling cartridges contained trace amounts of 4-ABP. The most likely source of 4-ABP exposure in the rats remains the rat chow, where a contamination of about 50 ppt would be sufficient to account for the observed background adduct level.

An additional problem of potential importance is that unadducted 4-ABP contained in a blood sample might be quantified as adduct. In situations where the daily exposure to 4-ABP may change dramatically, this could cause the measured adduct level to change significantly on a daily basis, thus negating one of the benefits of hemoglobin dosimetry, that of providing an averaged adduct level over the lifetime of the hemoglobin. Two steps in the method appeared to eliminate this potential problem. The use of toluene to help lyse the red blood cells should extract any free amine, as the pH is above the pKa of these compounds. In addition, the subsequent dialysis steps dilute to insignificant levels any free amine which was not extracted into the toluene. This was confirmed by spiking blood samples with 1 ng 4′-F-ABP before carrying out the rest of the procedure. The final chromatograms failed to show detectable levels of PFP-4′-F-ABP, indicating that the added free amine was removed prior to the hydrolysis and derivatization steps of the analytical procedure.

The amount of hemoglobin in 10-ml blood samples from various human subjects may vary by as much as 20–30%, due to both the range of hematocrits and recovery of hemoglobin in the analytical method. For this reason, another important consideration was whether the amount of hemoglobin in the sample affected the determination of the adduct level. This was studied by taking a sample of hemoglobin from a rat, and dividing it into two unequal amounts (0.44 and 0.73 g; see Table 2). The amount of 4-ABP released from these two samples during hydrolysis was proportional to the amount of hemoglobin in each. This was repeated with another rat hemoglobin sample, where the difference in hemoglobin was much greater (0.17 and 1.05 g). Again, the calculated adduct levels were the same for both aliquots. Thus, there did not appear to be a hemoglobin concentration effect in the analysis.

Attempts to simplify the method, either by altering or omitting certain steps, or by using materials which have not been cleaned as described, have generally resulted in significantly poorer performance of the assay. A number of these changes, such as insufficiently drying the hexane or omitting the trimethylamine catalyst, result in a lower yield of the PFP-amines. The use of plastic or insufficiently clean materials (glassware and solvents) results in a large background of interfering compounds in the final chromatogram. Although the use of the internal standard generally compensates for low yield, or high background levels, the uncertainty is greater than with high yield and low background. Recovery of internal standard is generally 20–40%.

Adduct Levels in Nonsmoking and Smoking Subjects. The distribution of 4-ABP-hemoglobin adduct levels in a population of smokers and nonsmokers is shown in Fig. 3 in which the adduct levels in the samples are plotted *versus* the number of samples of the same adduct level (frequency). Samples from smokers had consistently higher adduct levels than the samples

Table 2 *Effect of hemoglobin concentration on quantification of adduct levels*

	Hb analyzed (g)	4-ABP measured (ng)	Adduct level (ng 4-ABP/g Hb)
Rat sample A	0.44	0.76	1.73
	0.73	1.27	1.74
Rat sample B	0.17	0.34	2.00
	1.05	2.23	2.12

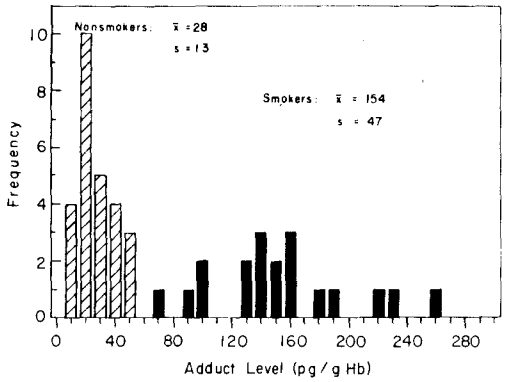

Fig. 3. 4-ABP-hemoglobin adduct levels in smokers (*solid bars*) and nonsmokers (*shaded bars*).

from nonsmokers. The mean adduct level of the smokers (154 pg 4-ABP per g Hb) was about 5.5 times higher than the mean of the nonsmoking subjects (28 pg/g Hb). The difference between means, with 95% confidence interval, was 126 ± 20 pg/g Hb, a highly significant difference ($P < .001$). Single determinations were done for 29 subjects, while the average of two or three determinations is reported for the remaining 16 subjects.

Absolute verification of the identity of the compound which we are quantifying under GC-negative-ion chemical ionization mass spectrometry as 4-ABP is not possible due to the small amounts which are contained in human blood samples. There is not enough, for example, to obtain a full mass spectrum under electron ionization mode. However, we have monitored the molecular ion of the PFP-derivatives of 4-ABP (m/z 315) and the internal standard (m/z 333) under electron ionization mode, using two different capillary GC columns (carbowax and dimethyl silicone). In each case, the compound isolated from human blood eluted at the retention time of genuine PFP-ABP. The amount quantified under electron ionization mode agreed with the value obtained under negative-ion chemical ionization mode.

Preliminary results have also been obtained from a study to determine the effect of smoking cessation on the levels of 4-ABP-hemoglobin adducts. In the five subjects tested thus far, the level of bound 4-ABP decreased dramatically during the first 6–8 weeks to levels seen in nonsmokers (Fig. 4). The mean difference between the first and third samples, with 95% confidence interval, was 76 ± 15 pg/g Hb. This study, in which each subject serves as his or her own control, confirms that the observed higher adduct levels in smokers is the direct result of their cigarette smoking.

DISCUSSION

The presence of a detectable adduct level in nonsmokers suggests that there may be sources of human exposure to 4-ABP other than active cigarette smoking, such as passive smoking, dietary contamination, or air pollution. As with most aromatic amines, the amount of 4-ABP in the sidestream tobacco smoke is much greater than that found in the mainstream smoke (146 *versus* 4.6 ng/cigarette) (1). This suggests that at least some of the 4-ABP in nonsmokers may result from involuntary inhalation of sidestream smoke. Adduct levels of other aromatic amines found in tobacco smoke (aniline, the 3 toluidines, and 2-naphthylamine) have been analyzed by a slightly modified method (9). In most cases, these amines have been detected in the blood of nonsmokers as well as smokers.

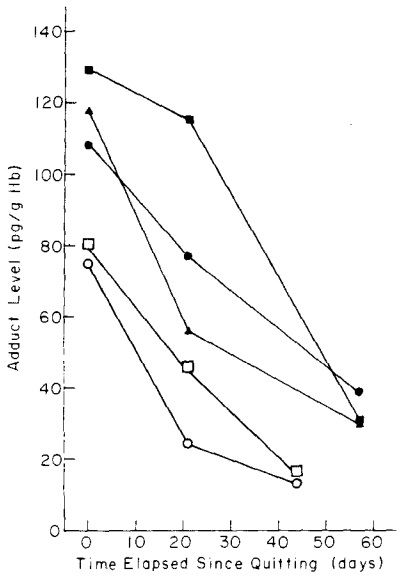

Fig. 4. Effect of smoking cessation on 4-ABP-hemoglobin adduct levels. Each symbol represents a different individual.

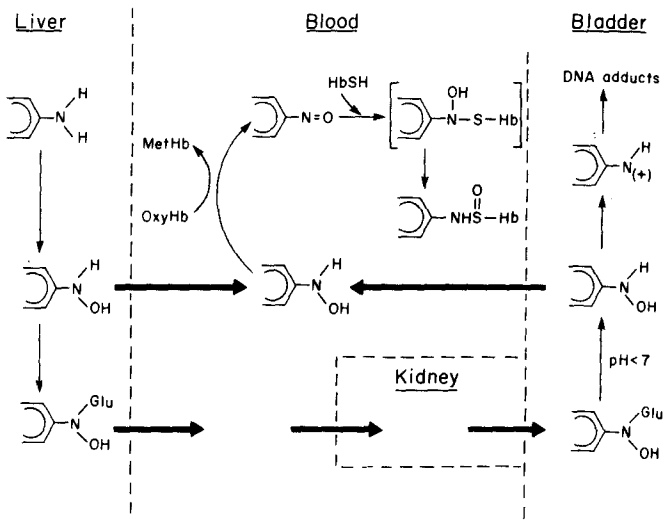

Fig. 5. Proposed relationship between hemoglobin adducts and bladder epithelial cell DNA adducts. *Heavy arrows*, transport between compartments.

There are two kinds of dietary contamination which might contribute to the 4-ABP levels of nonsmokers. The first is the cooking of meat, which is known to produce a number of heterocyclic aromatic amines (15). One of these compounds, 5-phenyl-2-pyridinamine is structurally very similar to 4-ABP and has been identified in phenylalanine pyrolysates and broiled sardines (16, 17). 4-ABP has not been identified in any of these sources to our knowledge, nor is it known whether the researchers attempted to isolate this compound. If the technique of following the strongly mutagenic fractions was employed to isolate the amines, 4-ABP would probably be overlooked as it is comparatively less mutagenic than the heterocyclic amines. Trace amounts of 4-ABP have been detected in three azo dyes, FD&C yellow no. 6 (tartrazine), FD&C yellow no. 5 (sunset yellow),[6] and D&C red #33 (18, 19). Tartrazine and sunset yellow are currently used in a number of food, drug, and cosmetic products (20–22). Although the estimated intake of either of these dyes is too low to account directly for the 4-ABP levels in nonsmokers, the possibility exists that these dyes may contain larger quantities of 4-ABP "subsidiary dyes" (18), which would release greater amounts of 4-ABP during metabolism.

A third possibility is that the 4-ABP in nonsmokers may originate from air pollution, either 4-aminobiphenyl itself or 4-nitrobiphenyl. The latter compound, when administered i.v. or i.g. to rats or dogs results in the formation of the same sulphinamide adducts as seen when 4-ABP is administered, although the yields are some 20–40 times lower. Nitroaromatics have been detected in a number of combustion products (23–25), with 4-NBP specifically identified in extracts of carbon black (26) and diesel particulate (27). In principle, it is possible that a portion of the adducts observed in nonsmoking individuals may arise via exposure to 4-NBP. Other possibilities of contamination of food, air, and industrial products with 4-ABP exist, but are outside the scope of this discussion.

As discussed previously (8), the 4-ABP-Hb adducts are believed to be formed *in vivo* by a sequence of reactions which are shown in Fig. 5 as occurring in the liver and blood compartments. The hydroxylamine, which is formed in the liver in a cytochrome P-450 mediated oxidation, undergoes a subsequent cooxidation with oxyhemoglobin, yielding *N*-nitrosobiphenyl and methemoglobin. This reaction sequence has been well characterized by Kiese and others (28, 29) with regard to the ability of aromatic amines to induce methemoglobinemia. The nitrosoarene can either be converted back to the hydroxylamine (by diaphorase and the pentose phosphate pathway, for example) and induce another round of methemoglobin formation, or it can react with a nucleophile such as a cysteine residue on hemoglobin to form an adduct. The intermediate steps involving the transport of the hydroxylamine from the liver to blood are discussed in more detail below.

It is evident from the present study that cigarette smoking is highly correlated with an increased level of 4-ABP-hemoglobin adducts. This difference between smokers and nonsmokers is expected due to the fact that cigarette smoke contains significant amounts of 4-ABP (1). The observed distribution of adduct levels within both groups, however indicates that the adduct level is subject to variability even among individuals of like smoking status. This may reflect differences in intake of 4-ABP, but may also reflect differences in erythrocyte dose from similar intakes. Undoubtedly differences in brand of cigarette smoked, and in individual smoking habits such as quantity smoked, frequency, and degree of inhalation of the smoke contribute to variations in the amount of 4-ABP actually inhaled. In the case of nonsmokers, speculations as to differences in exposure are difficult since a definite source has not been established. In both groups, there is reason to expect variability due to the fact that the adduct level measured is a function of the amount of *N*-hydroxylamine which reaches the erythrocytes, and this is dependent on the yield of *N*-hydroxylamine. The yield of *N*-hydroxylamine to the erythrocytes is affected not only by two key metabolic steps, but perhaps, also by the mechanism of delivery of the hydroxylamine to the blood.

Current understanding of the mechanism of bladder cancer induction by aromatic amines implicates *N*-hydroxylation by hepatic cytochrome P-450 as the first critical step (5, 30). The presence or absence of various inducers to this enzyme system may introduce differences in the manner in which the amine is metabolized. A large number of chemicals, both synthetic and naturally occurring, have been shown to induce monooxygenase activity (31). In particular, cigarette smoking has been shown to have a stimulatory effect on a number of enzyme activities. In many cases, the inducers are specific for certain forms of the cytochrome P-450, which may result in a wide variation in the

[6] J. E. Bailey, Jr., personal communication.

P-450 profiles within individuals of a certain population. A higher level of C-hydroxylation, for example, which is thought to be a detoxifying process, could result in decreased formation of the critical *N*-hydroxylamine.

Likewise, an individual's capacity for *N*-acetylation is also expected to alter the amount of free *N*-hydroxylamine available for subsequent transport to the erythrocyte. In humans, there appears to be an "acetylator polymorphism" in which there are at least two phenotypes, "slow" and "fast," referring to the relative ability to *N*-acetylate aromatic amines (32). *N*-Acetylation appears to be necessary for the induction of tumors of the liver, mammary gland, and intestine (33, 34). It has been speculated that those individuals who are fast acetylators might be at increased risk for tumors at these sites, while slow acetylators might be at increased risk for bladder tumors (35). Consistent with the latter hypothesis is the evidence from several studies (35–37) which revealed an excess of slow acetylators among bladder cancer patients, as compared to control.

These variables could result in a situation in which the dose to the erythrocytes would be different in two individuals, even if the actual intake or exposure is the same. This obviously presents difficulties if one seeks to use the adduct levels or erythrocyte dose as a means of estimating the amount of 4-ABP to which an individual is exposed. A calculation of this sort must assume that a certain intake of 4-ABP will result in a defined percentage of the dose reacting with hemoglobin to form sulphinamide adducts, and will not vary among individuals. Such a calculation may be useful, however to estimate the range of exposures within a certain group of subjects as we have done (38).

Bladder cancer induction by aromatic amines such as 4-ABP is believed to occur by reaction of free *N*-hydroxylamine with DNA in the target cells of the bladder. Since oxidation of the amine to the hydroxylamine occurs in the liver, some form of transport is necessary to deliver this proximate carcinogen to the bladder epithelium. One mechanism is simply the export of free hydroxylamine from the liver into the blood. Normal circulation would then carry the hydroxylamine to the target tissue, assuming its lifetime in blood is sufficiently long. Studies on the oxidation of aniline in the isolated perfused rat liver (39) suggest that this is not an important pathway. On the other hand, this may be a mechanism whereby the hydroxylamine reaches the erythrocytes to form the hemoglobin adduct, as illustrated in Fig. 5.

The most likely mechanism for exposure of the bladder epithelium is also illustrated in Fig. 5 and involves transport of the *N*-glucuronide of the hydroxylamine to the bladder lumen and subsequent hydrolysis catalyzed by the low pH of urine. It has been demonstrated that the bladder is permeable to aromatic hydroxylamines and that when introduced into the lumen, the hydroxylamines are readily absorbed and enter the bloodstream (40). This pathway could thus be a route whereby the hydroxylamine reaches the erythrocyte to form the hemoglobin adduct. In this latter transport mechanism, it is expected that the concentration of hydroxylamine in the blood, and consequently, the amount of hemoglobin adduct, would be proportional to the dose of hydroxylamine to the DNA of the bladder epithelial cell.

The relative importance of the two pathways for transport of the hydroxylamine to the erythrocyte will determine how useful the hemoglobin adducts are in estimating exposure of the target tissue. Although proportionality between hemoglobin binding and DNA binding has already been established for another aromatic amine, trans-4-dimethylaminostilbene (41, 42), experiments to do the same for 4-ABP have not been completed.

In conclusion, we have developed a method for the quantitative determination of 4-ABP adducts in human hemoglobin. Thus far, all individuals examined have some level of adduct, but it is significantly higher in cigarette smokers than in nonsmokers. Levels in quitting smokers decline to those in nonsmokers. To our knowledge this is the first report of 4-ABP adducts in human blood. It appears likely that the erythrocyte dose, as measured by our procedure, will be a useful parameter in estimating exposure to one of the proximate carcinogenic forms of 4-ABP.

ACKNOWLEDGMENTS

We would like to thank Dr. Fred Kadlubar of the National Center for Toxicological Research for continuing helpful discussion.

REFERENCES

1. Patrianakos, C., and Hoffman, D. Chemical studies on tobacco smoke LXIV. On the analysis of aromatic amines in cigarette smoke. J. Anal. Chem., *3:* 150–154, 1979.
2. Mommsen, S., and Aagard, J. Tobacco as a risk factor in bladder cancer. Carcinogenesis (Lond.), *4:* 335–338, 1983.
3. Vineis, P., Esteve, J., and Terracini, B. Bladder cancer and smoking in males: types of cigarettes, age at start, effect of stopping and interaction with occupation. Int. J. Cancer, *34:* 165–170, 1984.
4. Vineis, P., Segnan, N., Costa, G., and Terracini, B. Evidence of a multiplicative effect between cigarette smoking and occupational exposures in the aetology of bladder cancer. Cancer Lett., *14:* 285–290, 1981.
5. Miller, J. A., Wyatt, C. S., Miller, E. C., and Hartman, H. A. The *N*-hydroxylation of 4-acetylaminobiphenyl by the rat and dog and the strong carcinogenicity of *N*-hydroxy-4-acetyl-aminobiphenyl in the rat. Cancer Res., *21:* 1465, 1961.
6. Kadlubar, F. F., Miller, J. A., and Miller, E. C. Hepatic microsomal *N*-glucuronidation and nucleic acid binding of *N*-hydroxy arylamines in relation to urinary bladder carcinogenesis. Cancer Res., *37:* 805–814, 1977.
7. Poupko, J. M., Hearn, W. L., and Radomski, J. L. *N*-glucuronidation of *N*-hydroxy aromatic amines: a mechanism for their transport and bladder-specific carcinogenicity. Toxicol. Appl. Pharmacol., *50:* 479–484, 1979.
8. Green, L. C., Skipper, P. L., Turesky, R. J., Bryant, M. S., and Tannenbaum, S. R. *In vivo* dosimetry of 4-aminobiphenyl in rats via a cysteine adduct in hemoglobin. Cancer Res., *44:* 4254–4259, 1984.
9. Stillwell, W. G., Bryant, M. S., and Wishnok, J. S. Gas chromatographic/mass spectrometric analysis of biologically important aromatic amines. Applications in human dosimetry. Biomed. Environ. Mass Spectrom., in press, 1986.
10. Kiese, M., and Taeger, K. The fate of phenylhydroxylamine in human red cells. Arch. Pharmacol., *292:* 59–66, 1976.
11. Segerback, S., Calleman, C. J., Ehrenberg, L., Lofroth, G., and Osterman-Golkar, L. Evaluation of genetic risks of alkylating agents. V. Quantitative determination of alkylated amino acids in haemoglobin as a measure of the dose after treatment of mice with methyl methanesulfonate. Mutat. Res., *49:* 71–82, 1978.
12. Anson, M. L., and Mirsky, A. E. Protein coagulation and its reversal: the preparation of insoluble globin, soluble globin, and heme. J. Gen. Physiol., *13:* 469–476, 1930.
13. Moffat, A. C., Horning, E. C., Matin, S. B., and Rowland, M. Perfluorobenzene derivatives as derivatizing agents for the gas chromatography of primary and secondary amines using electron capture detection. J. Chromatogr., *66:* 255–260, 1972.
14. Nony, D. R. Aromatic amines and azo compounds. *In:* M. C. Bowman (ed.), Handbook of Carcinogens and Hazardous Substances. Chemical and Trace Analysis, Chap. 3. New York: Marcel Dekker, 1982.
15. Sugimura, T. A view of a cancer researcher on environmental mutagens. *In:* T. Sugimura *et al.* (eds.), Environmental Mutagens and Carcinogens, pp. 3–20. New York: Alan R. Liss, 1982.
16. Stavenuiter, J. F. C., Kroon-Verrips, M., Bos, E. J., and Westra, J. G. Synthesis of 5-phenyl-2-pyridinamine, a possibly carcinogenic pyrolysis product of phenylalanine, and some of its putative metabolites. Carcinogenesis (Lond.), *6:* 13–19, 1985.
17. Kosuge, T., *et al.* Isolation and structure studies of mutagenic principles in amino acid pyrolysates. Chem. Pharm. Bull., *26:* 611–619, 1978.
18. Bailey, J. E., Jr. Determination of unsulfonated aromatic amines in D&C red no. 33 by the diazotization and coupling procedure followed by reversed-phase liquid chromatographic analysis. Anal. Chem., *57:* 189–196, 1985.
19. Bailey, J. E., Jr. Determination of the lower sulfonated subsidiary colors in FD&C no. 6 by reversed-phase high-performance liquid chromatography. J. Chromatogr., *347:* 163–172, 1985.
20. Tse, C. S. Food products containing tartrazine. N. Engl. J. Med., *306:* 681–682, 1982.

21. Drug and Therapeutics Bulletin. Tartrazine: a yellow hazard. *18:* 53–55, 1980.
22. International Agency for Research on Cancer. Monographs on the Evaluation of the Carcinogenic Risk of Chemicals to Man: Some Aromatic Azo Compounds, Vol. 8. Lyon, France: International Agency for Research on Cancer, 1974.
23. Wei, E. T., and Shu, H. P. Nitroaromatic carcinogens in diesel soot: a review of the laboratory findings. Am. J. Public Health, *73:* 1085–1088, 1983.
24. McCoy, E. C. Role of metabolism on the mutagenicity of nitroarenes. *In:* H. Greim, R. Jung, M. Kramer, H. Marquardt, and F. Oesch (eds.), Biochemical Basis of Chemical Carcinogenesis, pp. 57–67. New York: Raven Press, 1984.
25. Gibson, T. L. Sources of direct-acting nitroarene mutagens in airborne particulate matter. Mutat. Res., *122:* 115–121, 1983.
26. Fitch, W. L., Everhart, E. T., and Smith, D. H. Characterization of carbon black adsorbates and artifacts formed during extraction. Anal. Chem., *50:* 2122–2125, 1978.
27. Tomkins, B. A., Brazell, R. S., Roth, M. E., and Ostrum, V. H. Isolation of mononitrated polycyclic aromatic hydrocarbons in particulate matter by liquid chromatography and determination by gas chromatography with the thermal energy analyzer. Anal. Chem., *56:* 781–786, 1984.
28. Kiese, M. Methemoglobinemia: A Comprehensive Treatise. Cleveland, OH: CRC Press, Inc., 1974.
29. Radomski, J. L. The primary aromatic amines: their biological properties and structure-activity relationships. Annu. Rev. Pharmacol. Toxicol., *19:* 129–157, 1979.
30. Cramer, J. W., Miller, J. A., and Miller, E. C. *N*-Hydroxylation: a new metabolic reaction observed in the rat with the carcinogen 2-acetylaminofluorene. J. Biol. Chem., *235:* 885, 1960.
31. Conney, A. H. Induction of microsomal enzymes by foreign chemicals and carcinogenesis by polycyclic aromatic hydrocarbons: G. H. A. Clowes memorial lecture. Cancer Res., *42:* 4875–4917, 1982.
32. Glowinski, I. B., Radtke, H. E., and Weber, W. W. Genetic variation in *N*-acetylation of carcinogenic arylamines by human and rabbit liver. Mol. Pharmacol., *14:* 940–949, 1978.
33. Poupko, J. M., Radomski, T., Santella, R. M., and Radomski, R. M. Organ, species and compound specificity in the metabolic activation of primary aromatic amines. J. Natl. Cancer Inst., *70:* 1077–1080, 1983.
34. Garner, R. C., Martin, C. N., and Clayson, D. B. Carcinogenic aromatic amines and related compounds. *In:* C. E. Searle (ed.), Chemical Carcinogens, Ed. 2, ACS Monograph 182, Chap. 4, American Chemical Society, Washington, DC, 1984.
35. Lower, G. M., Jr., Nilsson, T., Nelson, C. E., Wolf, H., Gamsky, T. E., and Bryan, G. T. *N*-Acetyltransferase phenotype and risk in urinary bladder cancer: approaches in molecular epidemiology. Environ. Health Perspect., *29:* 71–79, 1979.
36. Mommsen, S., Barfod, N. M., and Aagard, J. *N*-Acetyltransferase phenotypes in the urinary bladder carcinogenesis of a low-risk population. Carcinogenesis (Lond.), *6:* 199–201, 1985.
37. Cartwright, R. A., *et al.* Role of *N*-acetyltransferase phenotypes in bladder carcinogenesis: a pharmacogenetic epidemiological approach to bladder cancer. Lancet, *2:* 842–846, 1982.
38. Tannenbaum, S. R., Bryant, M. S., Skipper, P. L., and Maclure, M. Hemoglobin adducts of tobacco-related aromatic amines: application to molecular epidemiology. Banbury Rep., *23:* 63–75, 1986.
39. Eyer, P., and Lierheimer, E. Biotransformation of nitrosobenzene in the red cell and the role of glutathione. Xenobiotica, *10:* 517–526, 1980.
40. Oglesby, L. A., Flammang, T. J., Tullis, D. L., and Kadlubar, F. F. Rapid absorption, distribution, and excretion of carcinogeneic *N*-hydroxyarylamines after direct urethral installation into the rat urinary bladder. Carcinogenesis (Lond.), *2:* 15–20, 1981.
41. Neumann, HG. Analysis of hemoglobin as a dose monitor for alkylating and arylating agents. Arch. Toxicol., *56:* 1–6, 1984.
42. Neumann, H-G. Dosimetry and dose-response relationships. *In:* A. Berlin, M. Draper, K. Hemminki, and H. Vaino (eds.), Monitoring Human Exposure to Carcinogenic and Mutagenic Agents, IARC Scientific Publication No. 59, pp. 115–126. Lyon, France: International Agency for Research on Cancer, 1984.