Haemoglobin adducts and specific immunoglobulin G in humans as biomarkers of exposure to hexahydrophthalic anhydride

Bo A. G. Jönsson, Christian H. Lindh and Hans Welinder

The aim of this study was to determine whether haemoglobin adducts (Hb) of hexahydrophthalic anhydride (HHPA) and HHPA-specific immunoglobulin G (IgG) can be used as biomarkers of exposure to HHPA. The exposures of HHPA in 10 workers were determined from the mean urinary hexahydrophthalic acid (HHP acid) levels (range 76–3300 nmol HHP acid mmol^{-1} creatinine) during a period of 4 weeks. Blood was collected at the end of the period and Hb–HHPA adducts were analysed by gas chromatography mass spectrometry. The Hb–HHPA adduct levels ranged from 0.45 to 24.7 pmol g^{-1} Hb. There was a close correlation between the urinary HHP acid levels and the amount of Hb–HHPA adducts (r = 0.87). One-day exposures to HHPA and methylhexahydrophthalic anhydride (MHHPA) in 142 workers were determined from analysis of urinary HHP acid (range 0–3300 nmol HHP acid mmol^{-1} creatinine) and methylhexahydrophthalic acid (MHHP acid; range 0–1700 nmol MHHP acid mmol^{-1} creatinine). HHPA-specific IgG were analysed in the 142 workers with an ELISA method. The optical density for HHPA-specific IgG varied between 0 and 1.25. There was no statistically significant correlation between the sum of the urinary HHP acid and MHHP acid and the HHPA-specific IgG (r = 0.12; p = 0.14). Thus, Hb–HHPA adducts seem to be applicable as biomarkers of exposure to HHPA while the possible role of HHPA-specific IgG as an indicator of exposure has to be further evaluated.

Keywords: *hexahydrophthalic anhydride, methylhexahydrophthalic anhydride, haemoglobin adducts, specific IgG, biological monitoring.*

Introduction

Adducts of haemoglobin (Hb) were suggested more than 30 years ago as biomarkers of exposure to carcinogens (Ehrenberg *et al.* 1974). Many papers have since then been published on this topic on a variety of carcinogens such as the aromatic amines, polyaromatic hydrocarbons, aflatoxins, and compounds with a double bond undergoing epoxidation *in vivo* (Van Welie *et al.* 1992, Skipper *et al.* 1994). However, the relationships between doses and levels of adducts are still in most cases based on animal studies. In man, most studies of relationships between exposures and adducts show correlations at group levels. Thus, these studies mainly show that exposure gives rise to an increased adduct level compared with controls or that heavy exposure gives higher adduct levels as compared with lower exposure. Studies describing the individual, cumulative exposure in relation to the adduct levels are to our knowledge missing, although efforts in this direction have been reported (Gan *et al.* 1988, Duus *et al.* 1989).

Specific immunoglobulins G (IgG) have been frequently analysed in studies of allergens but their role in the sensitization process is unclear. It has been suggested that specific IgG can be used as biomarkers of exposure to sensitizing compounds (Biagini *et al.* 1990.) This hypothesis is supported by findings in workers exposed to organic acid anhydrides (OAAs; Nielsen *et al.* 1988, Welinder *et al.* 1990, 1994). However, further studies are necessary.

OAAs are highly sensitizing compounds (Venables 1989, Welinder *et al.* 1994). Specific IgE and IgG have been reported in a large fraction of workers exposed to only a few μg OAA m^{-3} in air. Hexahydrophthalic anhydride (HHPA) and methylhexahydrophthalic anhydride (MHHPA) are two particularly sensitizing OAAs. The OAAs are known to react with proteins *in vivo* (Palacían 1990) and it is commonly believed that the formation of protein adducts *in vivo* is the patomechanism behind the sensitizing potential of these compounds. Thus, protein adducts of OAAs and other allergenic, low molecular weight compounds are interesting since these may be directly involved in the sensitization process. However, there are only a few reports of protein adducts of allergenic compounds (Jin *et al.* 1993, Jönsson *et al.* 1995, Sepai *et al.* 1995a, b, Day *et al.* 1996, Lind *et al.* 1997a, b).

In this paper we evaluate the use of Hb–HHPA adducts and HHPA-specific IgG as biomarkers of exposure to HHPA.

MATERIALS AND METHODS

Study subjects

The subjects were workers exposed to HHPA and/or MHHPA (Figure 1) in a plant manufacturing electrical capacitors. Most of the workers were mainly exposed to either HHPA or MHHPA. The HHPA (Merck, Darmstadt, Germany) used was the *cis*-isomer which is the industrially and toxicologically most important isomer. The MHHPA (Ciba-Geigy, Basel, Switzerland) was *cis* with regard to the carboxylic groups and a mixture of *cis* and *trans* (50:50) with regard to the methyl group. No other organic acid anhydride was used in the plant. Some of the workers wore protective devices. The participants gave an informed consent and the study was approved by the Ethics Committee of Lund University.

Collection of biological samples

Urine samples were collected during the last 4 h of the work-shift. From 10 subjects urine was sampled during a period of 4 weeks on 10–12 different occasions. In addition, urine was collected from 142 subjects (the 10 subjects included) on one single occasion. In 25 of these subjects urine was also collected on one occasion 7 months before the sampling; eight of the ten subjects in which frequent urine sampling was performed were included in these 25 subjects. In 15 of the 142 subjects urine was also collected on

Bo A. G. Jönsson *(author for correspondence),* **Christian H. Lindh** *and* **Hans Welinder** *are in the Department of Occupational and Environmental Medicine, Institute of Laboratory Medicine, University Hospital, S-221 85 Lund, Sweden.*

1354–750X/97 $12·00

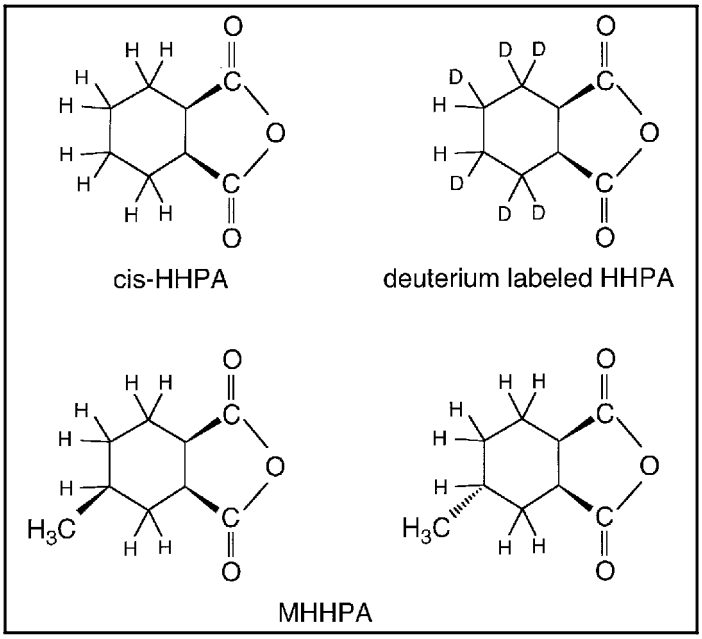

Figure 1. Chemical structures of the studied dicarboxylic acid anhydrides: HHPA, deuterium-labelled HHPA (internal standard) and the two MHHPA isomers.

sampling. The urine was stored in polyethylene test tubes at –20 °C until the analysis.

Serum for the determinations of specific IgG was collected from the same 142 subjects as in which urine was sampled. In 134 of these the urine and serum were collected at the same time but in eight subjects the serum was collected up to 9 months before the urine sampling. The blood for the serum was obtained from an antecubital vein and collected in 4 ml Vacutainer Brand SST blood sampling tubes (Becton Dickinson, Meylan, France). After sampling, the blood was cooled to room temperature and then centrifuged at 1500 *g* for 10 min. The serum was separated and stored at –20 °C until the analysis.

Blood samples for the determination of Hb-adducts were collected from the 10 subjects in which frequent urine sampling was performed after the collection of the last urine sample. The blood was drawn from an antecubital vein and collected in 10 ml Venoject blood sampling tubes (Terumo Europe, Leuven, Belgium) containing sodium heparin. After sampling, the blood was allowed to cool to room temperature and then centrifuged at 1500 *g* for 10 min. The plasma was separated and the red blood cells were washed three times with a 0.9% sodium chloride solution and then transferred to polyethylene test tubes and frozen at –20 °C until the analysis.

Preparation and analysis of biological samples

Urinary HHP acid and MHHP acid

The levels of HHP acid and MHHP acid in urine were determined as previously described (Jönsson and Lindh 1996). To the urine samples (500 µl) were added 0.5 µg $^2H_6$-labelled HHP acid (Synthelec, Lund, Sweden) and 0.5 µg $^2H_8$-labelled MHHP acid (Synthelec), followed by acidification with 2 ml of 0.1 M HCl (Acros, Geel, Belgium). The samples were applied to C_{18} Bond Elut solid phase extraction columns (100 mg; Varian, Palo Alto, CA, USA) previously conditioned by 10 ml of methanol (LabScan, Dublin, Ireland) and 2 ml of 0.1 M HCl. The columns were washed with 2 ml of 0.1 M HCl, dried by suction of air, and subsequently the HHP acid and MHHP acid were eluted with 1 ml 0.1 M ammonium hydrogen carbonate (BDH, Poole, UK). The eluate was evaporated to dryness, 250 µl of an aqueous 0.1 M tetrabutylammonium hydrogen sulphate (TBA; Merck) solution (pH 7) and 250 µl 0.13 M pentafluorobenzyl bromide (PFBBr; Aldrich, Gillingham, UK) in dichloromethane (LabScan) added and then placed in an ultrasonic bath for 90 min. Two ml of hexane (LabScan) were added to the samples, which were placed in a freezer until the aqueous phase was frozen. The organic phase was evaporated and the dry residue was dissolved in 2 ml of toluene (LabScan). Two µl was injected splitless at 300 °C on a VG Trio 1000 quadrupole mass spectrometer (MS; Fisons, Manchester, UK) connected with a Carlo-Erba 8065 gas chromatograph (GC) equipped with an A200S auto-sampler (Carlo-Erba, Milan, Italy). The column was a DB-5 MS (J&W, Folsom, CA, USA; 30 m × 0.25 mm i.d.) with a film thickness of 0.25 µm. The column temperature programming was: (1) 100 °C for 1 min, (2) 15 °C min^{-1} to 320 °C. The MS was in the negative ion chemical ionization (NICI) mode with ammonia as moderating gas. Selected ion monitoring (SIM) of the ester of HHP acid was performed at *m/z* 153 while *m/z* 159 was chosen for the internal standard. For the ester of MHHP acid *m/z* 365 was chosen for SIM while *m/z* 373 was chosen for the internal standard. The between-day precision for HHP acid was 3% while that for MHHP acid was 3–8%.

Hb adducts of HHPA acid

The levels of HHPA bound to Hb were determined as follows. The frozen red cells were thawed, lysed with an equal volume of water, frozen and thawed again. The cell debris was removed by centrifugation at 18500 *g* for 30 min. The Hb samples were dialysed (cut off 12–14 kDa) against phosphate buffered saline three times during 3 days. Aliquots of the dialysed Hb samples were taken for determination of the Hb concentration using the Drabkin reagent method (Sigma, St Louis, MO, USA). To the Hb samples (3 ml containing 50–80 g l^{-1} Hb) were added 4 ml water and 1 ml 1.8 M HCl and and 100 µl aliquots of an internal standard solution containing 1 ng $^2H_6$-labelled HHP acid. The HHPA adducts were hydrolysed from the Hb at 100 °C for 18 h. After the hydrolysis, the samples were centrifuged at 3000 *g* for 30 min and the supernatants were extracted two times with 6 ml ethyl acetate (LabScan). The combined ethyl acetate phases were evaporated to dryness in a Speed Vac centrifuge (Savant, Farmingdale, NY, USA). The dry residues were dissolved in 6 ml 0.1 M HCl. C_{18} Bond Elut columns (100 mg; Varian) were conditioned by passing 10 ml of methanol (LabScan) followed by 5 ml of 0.1 M HCl through the columns. The samples were then added to the columns, which resulted in the trapping of HHP acid. As a washing step 2 ml of 0.1 M HCl was added. The columns were then dried by suction of air for 1 min. Thereafter, the HHP acid was eluted into test tubes by 1 ml 0.1 M ammonium hydrogen carbonate. The eluates were evaporated to dryness and the residues were derivatized by addition of 250 µl of 0.1 M TBA and 250 µl of 0.13 M PFBBr. The samples were vortexed and placed in an ultrasonic bath for 90 min. Then, 2 ml of hexane was added and the samples were vortexed and centrifuged for phase separation for 1 min at 1500 *g*. The test tubes were placed in a freezer at –20 °C until the aqueous phase was frozen. Thereafter the organic phases were poured into another test tube and evaporated in a nitrogen gas flow. The dry residues were dissolved in 0.5 ml of toluene and transferred to auto-sampler injection vials.

The samples were analysed by the VG-Carlo-Erba GC–MS equipment. Samples were injected with a splitless injection technique. The injector temperature was kept at 300 °C, the injection volume was 2 µl, and the split exit valve was kept closed for 0.5 min after the injection. The analytical column was a fused silica capillary column (30 m × 0.25 mm i.d.) with a DB-5 MS stationary phase and a film thickness of 0.25 µm (J&W). The initial column temperature was 100 °C for 1 min. The temperature was thereafter increased by 9 °C min^{-1} to 190 °C, then by 4 °C min^{-1} to 250 °C, and finally by 40 °C min^{-1} to 320 °C. The MS interface was at 320 °C and the ion source at 200 °C. The column carrier gas was helium at a pressure of 70 kPa. The MS was in the NICI mode with ammonia as moderating gas. SIM of the ester of HHP acid was performed at *m/z* 153 and 351 while *m/z* 159 and 357 were chosen for the internal standard. The samples were analysed in duplicate.

The detection limit for Hb–HHPA was about 0.3 pmol g^{-1} Hb and the between-day precision was 13% at 10 pmol g^{-1} Hb using

detection limit for Hb–MHHPA using the described method and *m/z* 365 was 5 pmol g^{-1} Hb.

Specific antibody determination

The levels of IgG specific to HHPA and MHHPA were determined as previously described by Welinder *et al.* (1994) by a non-competitive enzyme linked immunosorbent assay (ELISA) using human serum albumin (HSA) conjugates of the anhydrides. The principal steps were: (i) microtitreplates were coated with HSA conjugates of HHPA and MHHPA, respectively, (ii) serum was added, (iii) rabbit antihuman IgG conjugated with alkaline phosphatase was added after incubation, (iv) substrate solution was added after incubation, (v) the results (absorbance values; OD) were read after incubation. All samples were analysed in triplicate. The coefficient of variation (CV) for the method was 7%.

Statistics

For comparison between groups of determinations the paired *t*-test was used. Correlations between pairs of values were determined by linear regression. Statistically significant refers to $P < 0.05$.

Results

The urinary HHP acid levels in the samples from the 142 subjects collected during one single occasion ranged from 0 to 3300 nmol $mmol^{-1}$ creatinine with a mean of 160 nmol $mmol^{-1}$ creatinine. The urinary MHHP acid in the samples from the 142 subjects ranged from 0 to 1700 nmol $mmol^{-1}$ creatinine with a mean of 130 nmol $mmol^{-1}$ creatinine. If the urinary HHP acid and MHHP acid levels in 25 of the samples were compared with samples collected from the same subjects 7 months earlier there was no significant difference. When HHP acid and MHHP acid urinary levels from 15 subjects were compared with samples collected from the same subjects 1 year later there was also no significant difference.

In the 10 subjects in which frequent urine sampling was performed, the exposure levels of HHPA as determined by analysis of HHP acid in urine samples ranged from 28 to 7900 nmol HHP acid $mmol^{-1}$ creatinine (Table 1). The mean HHP acid levels for each individual ranged from 76 to 3300 nmol HHP acid $mmol^{-1}$ creatinine. The levels of MHHP acid ranged from 0 to 110 nmol MHHP acid $mmol^{-1}$ creatinine with means of each individual between 0 and 56 nmol MHHP acid $mmol^{-1}$ creatinine. In each subject there was a variation in the exposure levels of HHPA during the 4-week period. The CVs ranged between 27 and 99% (Table 1). In Figure 2 the HHP acid levels from subjects with CVs at 27, 29, 61, and 99% are shown. For MHHPA, the CVs ranged between 30 and 54% (three of the subjects were excluded because all or all but one sample were below the detection limit).

The Hb–HHPA adduct levels ranged from 0.45 to 24.7 pmol g^{-1} Hb. The levels of Hb–MHHPA adducts were below the detection limit. The correlation using the data on the urinary HHP acid levels at the last sampling occasion was $r = 0.68$. The results from one unexposed subject was included at zero exposure. The correlation was improved when adding data from the other determination of urinary HHP acid levels. When the mean urinary levels of HHP acid from all collected samples during 4 weeks were used, the correlation between the urinary level and the HHP–Hb adduct levels was excellent at $r = 0.87$ (Figure 3). However, in all but one case ($r = 0.5$; $p = 0.11$) there were significant correlations between the urinary levels at each collection occasion and the HHP–Hb adduct levels ($r = 0.50$–0.92).

The optical density (OD) for HHPA-specific IgG in the whole group varied between 0 and 1.25. The OD for MHHPA-specific IgG in the whole group varied between 0 and 1.24. There was a close correlation between the levels for HHPA-specific IgG and MHHPA-specific IgG ($r = 0.98$). There was no statistically significant correlation between the sum of urinary HHP acid and MHHP acid levels and the OD of HHPA-specific IgG ($r = 0.12$; $p = 0.14$; Figure 4). A similar pattern was obtained if the sum of the mean urinary HHP acid and MHHP acid levels of the 10 subjects in which frequent urine sampling was performed was correlated to the OD of specific IgG (Figure 5).

There was a statistically significant negative correlation between, on the one hand, the ratio between the amounts of Hb–HHPA adducts and the levels of urinary HHP acid (adduct levels normalized with regard to the exposure) and the OD of HHPA-specific IgG, on the other ($r = -0.63$; $p = 0.049$; Figure 6).

Subject	Urinary HHP acid Mean (range) (nmol $mmol^{-1}$)	Variation[a] in urinary HHP acid (%)	Urinary HHP acid 7 months earlier (nmol $mmol^{-1}$)	Urinary MHHP acid Mean (range) (nmol $mmol^{-1}$)	Variation[a] in urinary MHHP acid (%)	Work task
1	1400 (720–2900)	48	760	33 (17–74)	51	Mechanic
2	76 (28–220)	76	60	56 (29–110)	50	Founder
3	1200 (290–1900)	42	1200	0 (0[b]–0)	NA[c]	Founder
4	2100 (600–7900)	99	—	31 (15–56)	47	Founder
5	330 (120–750)	65	—	3 (0–37)	NA	Founder
6	270 (190–400)	27	290	34 (17–58)	41	Founder
7	820 (300–1300)	37	510	25 (10–56)	54	Mechanic
8	3300 (570–7500)	61	3100	43 (24–110)	54	Mechanic
9	770 (350–2000)	64	430	33 (20–51)	30	Founder
10	1000 (550–1500)	29	730	3 (0–28)	NA	Founder

Table 1. Urinary HHP acid and MHHP acid levels and work task in 10 subjects in which urine was sampled 10 times over a period of 4 weeks.

[a] Determined as coefficient of variation between urine samples collected on different occasions.
[b] Below the detection limit at 20 ng MHHP acid ml^{-1} urine.
[c] Not applicable.

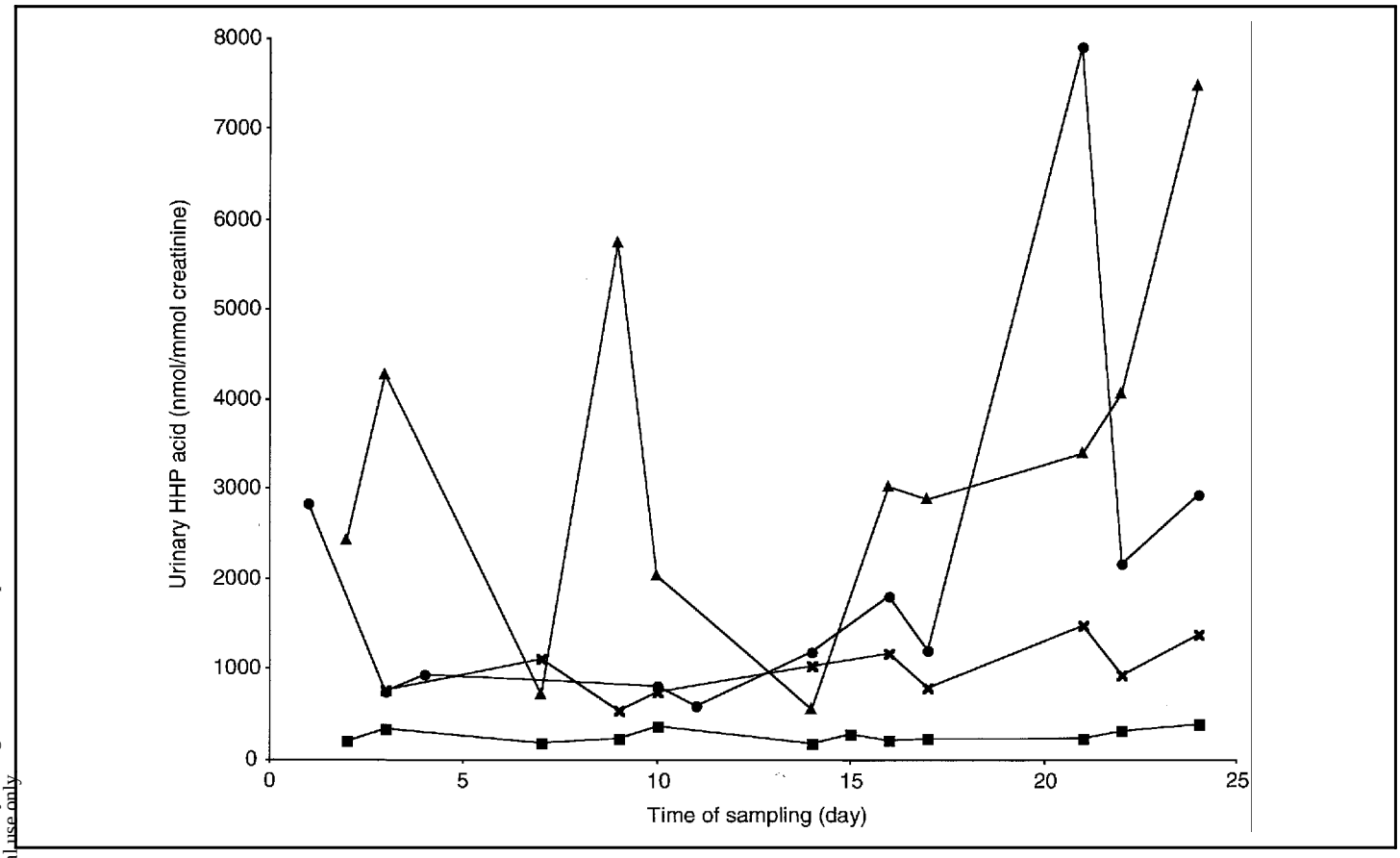

Figure 2. Urinary levels of HHP acid during a period of 4 weeks in four workers exposed to HHPA. The coefficients of variation between the HHP acid levels in the different urine samples was for the worker indicated by squares 27%, the worker indicated by crosses 29%, the worker indicated by circles 99%, and for the worker indicated by triangles 61%. The worker indicated by triangles was a mechanic and the others were founders.

Discussion

The present results show that there was a close correlation between the cumulative exposures to HHPA during 4 weeks and the levels of Hb–HHPA adducts. It has also been shown that there was no correlation between the sum of exposure to HHPA and MHHPA and the levels of HHPA-specific IgG.

Biological monitoring may be a valuable alternative to laborious air sampling for the estimation of cumulative exposures over long time periods or estimation of exposures to many subjects. Moreover, if the workers are using protective devices, as in the studied plant, biological monitoring is often superior to air monitoring. The close correlation between air levels of HHPA and the urinary HHP acid has been confirmed in several reports (Jönsson *et al.* 1991, 1993, Jönsson and Skerfving 1993). Thus, HHP acid in urine seems to be a good biomarker for exposure to HHPA. Recently, a similar correlation has been established for MHHPA (Lindh *et al.* 1997). Both HHP acid and MHHP acid have a short half-time in the body and the levels in urine reflect therefore only the exposure during 1 day. Percutaneous absorption seems to be of minor importance (Jönsson *et al.* 1993).

Ten subjects were chosen for the collection of urine during 4 weeks. The highest exposed subjects were among the most heavily HHPA-exposed workers in the plant. The exposure to MHHPA was much lower. The exposure pattern was quite different for the 10 subjects. Some of the subjects seemed to have a rather stable exposure over the different days while for the others the exposure varied greatly. However, there seemed not to be any major change in the exposure levels either when analysing urine samples collected 7 months before the sampling or when using urine collected 1 year after the sampling. Thus, the HHP acid and MHHP acid levels in urine can be assumed to be rather representative for the exposure at least for the 7 months prior to the collection of the samples.

The chemical nature of the Hb adducts has not been investigated in this study. However, it has previously been reported that acid anhydrides mainly react with the lysine residues in proteins (Palacián *et al.* 1990). Moreover, lysine adducts between collagen and another anhydride, methyltetrahydrophthalic anhydride, have been found in guinea pigs (Jönsson *et al.* 1995). We have therefore assumed that lysine was the main binding amino acid for HHPA. *N*-ε-hexahydrophthaloyl-L-lysine has been shown to be efficiently hydrolysed to free HHP acid by 0.1 M HCl at 100 °C for 18 h (unpublished results). Thus, to avoid addition of noice to the analytical matrix we chose to use rather mild hydrolysis conditions in our procedure.

It seems reasonable to assume that the HHP acid analysed after the hydrolysis of the Hb reflect HHPA bound to Hb since the low molecular weight chemicals had been removed by dialysis and Hb is by far the most abundant protein in the erythrocytes. However, this remains

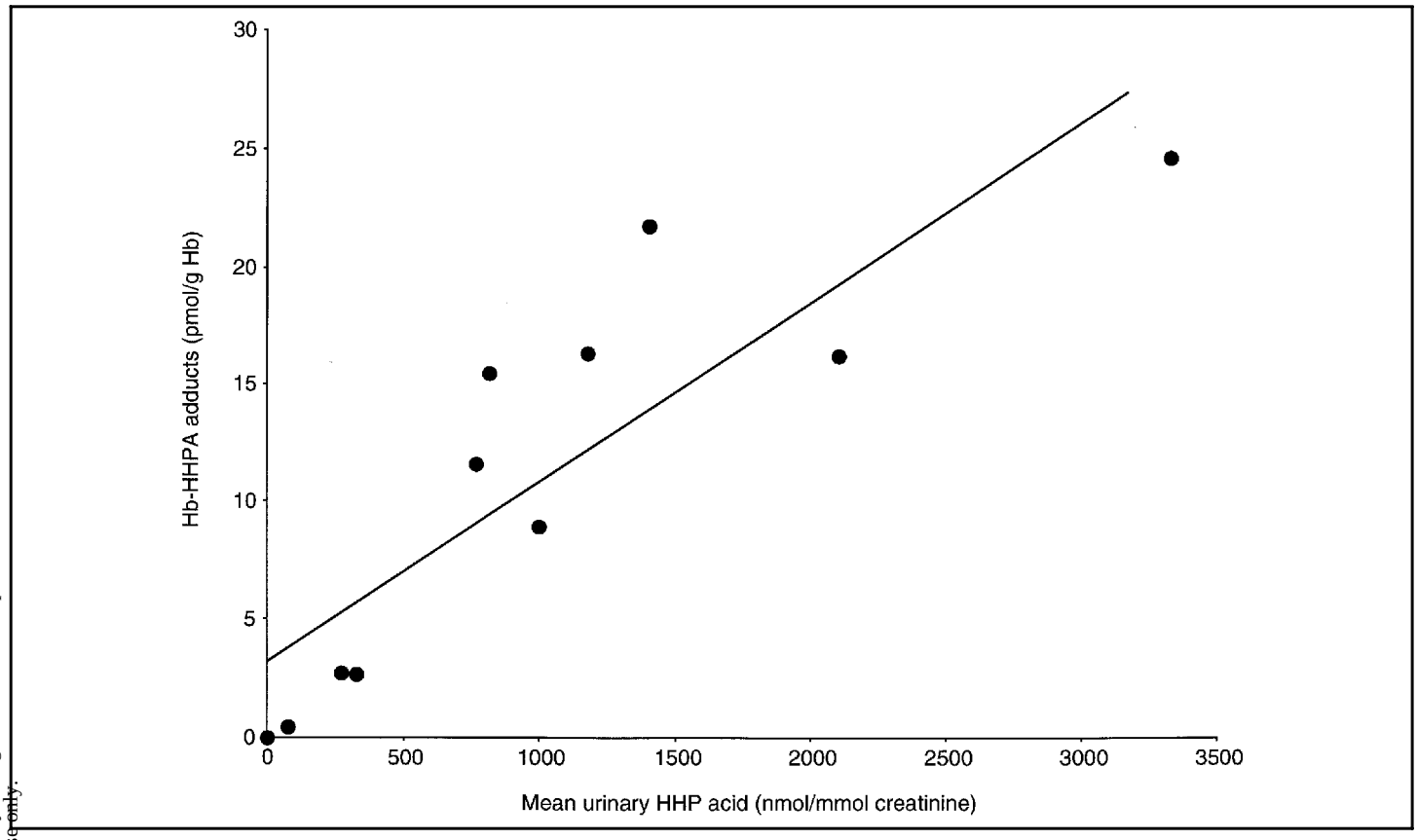

Figure 3. Correlation between the mean urinary HHP acid levels during 4 weeks and the amount of Hb–HHPA adducts from 10 workers exposed to HHPA. One unexposed subject was included at zero exposure.

The life-time of the erythrocytes is about 120 days in man and Hb adducts have therefore a potential to monitor exposure during this time. The half-lives of the Hb–HHPA adducts have not been evaluated, but the fact that the correlation between the exposure and the adduct levels was increased when exposure data for longer time periods were added indicates that the adducts are rather stable.

The levels of Hb adducts were in a similar range as Hb adducts described for other compounds (Sepai *et al.* 1995*a*, Lind *et al.* 1997*a*). It seems clear that the exposure was the single most important factor for the levels of Hb–HHPA adducts (r^2 = 0.75). From our results, it seems possible that the correlation would be even better if more data were included in the assessment of the exposure; we only monitored the exposure during about 10% of the life-time of the erythrocytes. However, some other factors, such as the specific IgG, may have some small impact (see discussion below). The urinary HHP acid level for the lowest exposed worker in which Hb–HHPA adducts were analysed corresponded to an HHPA air level of less than 10 μg m^{-3}. Thus, it seems possible to use the Hb–HHPA adducts for monitoring of very low exposures of HHPA.

There was a close association between the HHPA-specific IgG and the MHHPA-specific IgG, which is in agreement with earlier findings (Welinder and Nielsen 1991). This indicates a major cross-reactivity between the two antibodies. It is therefore irrelevant to deal with each chemical individually. We have chosen to correlate the sum of the urinary HHP acid and MHHP acid with the specific IgG to HHPA. A correlation between the sum of the urinary levels and the mean of HHPA- and MHHPA-specific IgG gave almost entirely the same results.

We found no significant correlation between the after-shift values of HHP acid and MHHP acid levels and the HHPA-specific IgG in the 142 subjects (Figure 4). This may be expected, as the urine levels represent exposures during only one work shift, while the specific IgG have an estimated biological half-time of about 0.4 year (unpublished results). However, the same pattern was observed when IgG was correlated to the data from 10 subjects over 4 weeks (Figure 5). Also, exposure assessments from 1 day spot samples gave a good correlation with Hb–HHPA adducts. These results would indicate that the specific IgG is a poor biomarker of exposure to HHPA or MHHPA. However, a false non-correlation must be considered if, for example, the subjects with low exposures but with high specific IgG levels previously have had a higher exposure. However, there seemed not to be any major changes in the exposure levels in the plant during the last 7 months before sampling. Also, several of the workers were also employed only a year or less before the study was performed and these may not have developed IgG antibodies at the time the samples were collected. On the other hand, the correlation was not improved by using the product between the urinary HHP acid and MHHP acid levels and the employment time (not shown). Thus, the exposure estimations may not explain the lack of correlation.

The patterns shown in Figures 4 and 5 indicate different kinds of IgG response to exposure; o

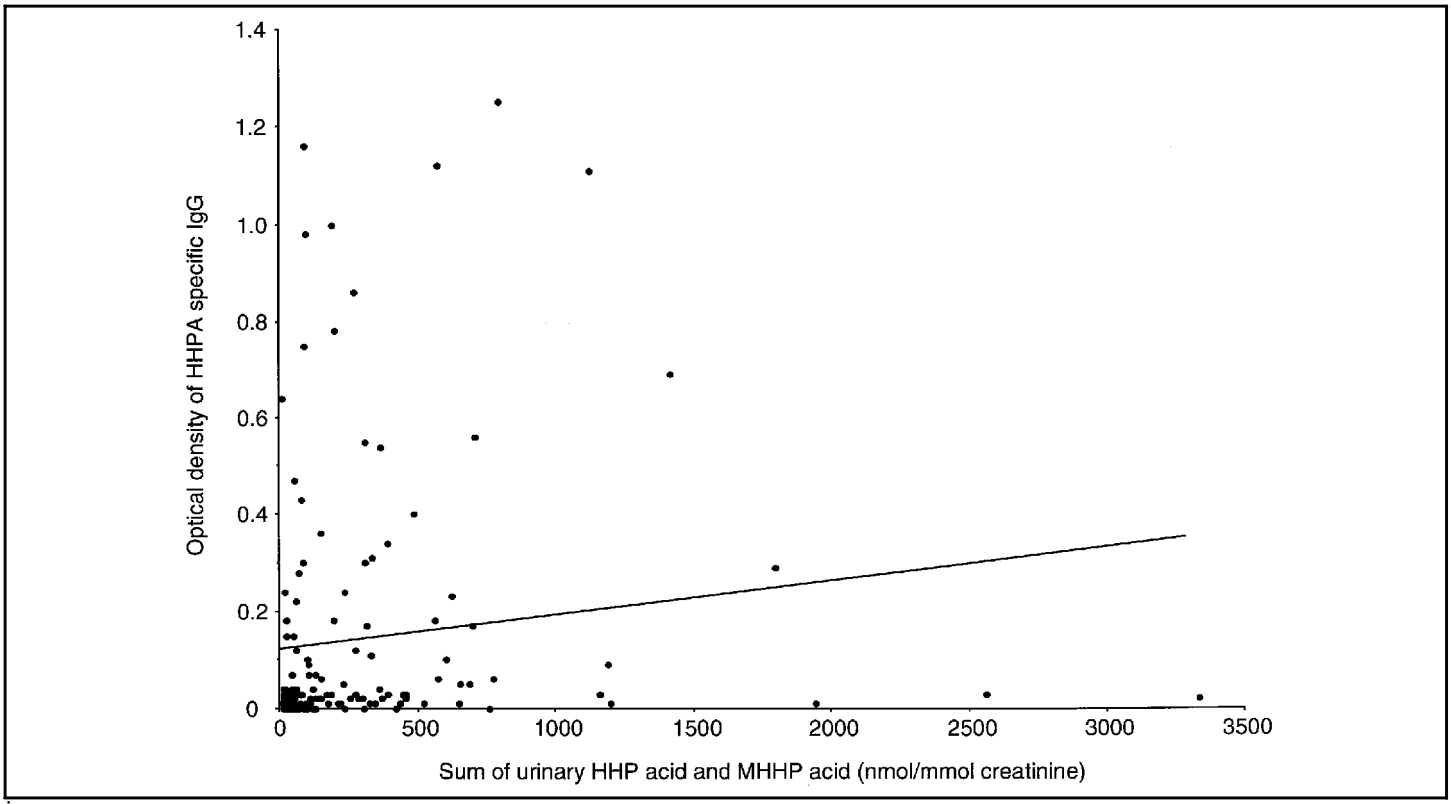

Figure 4. Correlation between the sum of HHP acid and MHHP acid in 1-day urine samples and the optical density of HHPA-specific IgG in 142 workers exposed to HHPA and MHHPA.

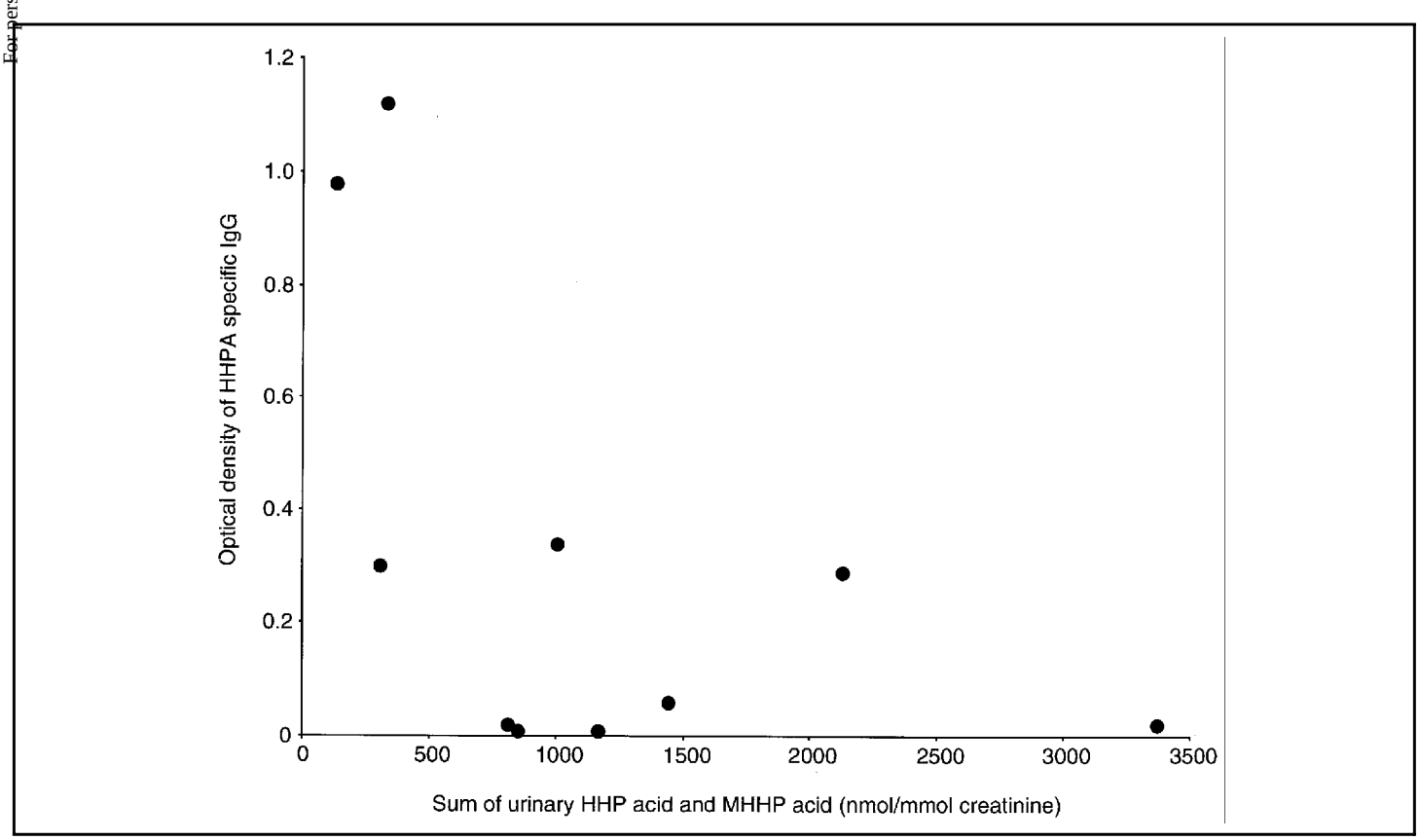

Figure 5. Plot of the optical density of HHPA-specific IgG versus the mean of the sum of the urinary HHP acid and MHHP acid levels during 4 weeks in 10 workers exposed to HHPA and MHHPA.

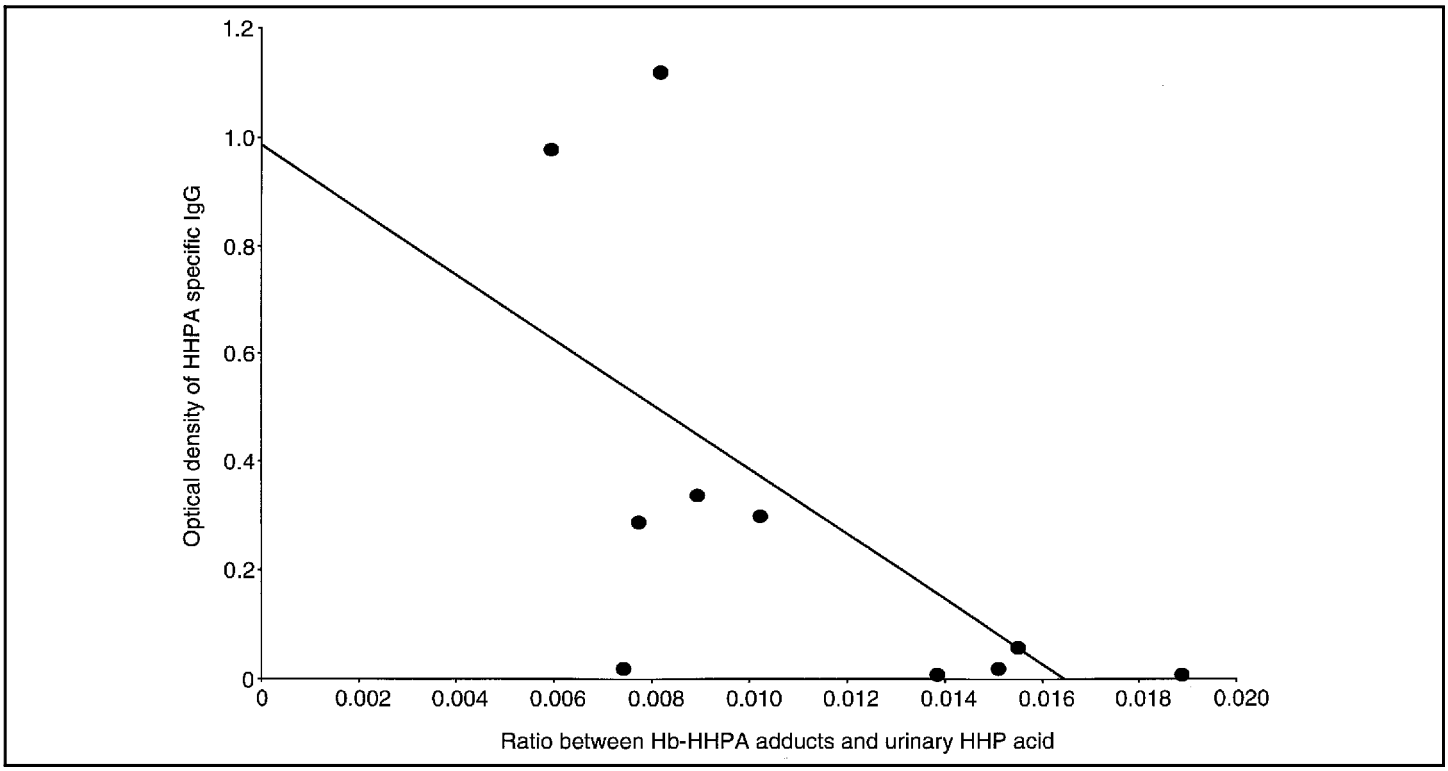

Figure 6. The optical density of HHPA-specific IgG versus the ratio between the amount of Hb–HHPA adducts and mean urinary HHP acid and MHHP acid levels in 10 workers during 4 weeks of exposure to HHPA and MHHPA.

with IgG even at low exposures, and another one with a low response or non-responding. This should be further evaluated.

There appears to be a conflict between the present findings and earlier findings in workers exposed to phthalic anhydride, MTHPA, HHPA, and MHHPA (Nielsen *et al.* 1988, Welinder *et al.* 1990, 1994). In these studies, exposure–response relationships of IgG formation were observed (trend test). However, the correlations were found on a group level. A plot of the individual results may reveal a more complex pattern. Thus, the possible role of IgG as an indicator of exposure has to be further studied. Using a similar analogy, more studies on the relationships between individual exposures and protein adduct levels have to be performed.

Since anhydrides must react with endogenous proteins to become antigenic, we wanted to study the impact of the Hb–HHPA adducts on the IgG levels. However, due to the strong correlation between exposure and Hb–HHPA adducts, we have to normalize the Hb–HHPA adducts with regard to the exposure (i.e. by dividing the Hb–HHPA adduct levels with the urinary HHP acid levels) to be able to study this. The significant negative correlation between the adduct–urinary HHP acid ratio and the OD of HHPA-specific IgG was unexpected; if there was a correlation we would rather expect a positive one. However, there might be several explanations for the obtained result, e.g., since many of the specific IgG are directed against the hapten as well as new antigenic determinants these might prevent HHPA or a masked HHPA-derivative from reaching the Hb. Another explanation would be if the HHPA in the subjects with a low ratio preferentially reacted with another protein, forming a more antigenic conjugate. However, the number of observations are few and further studies are needed.

Pien *et al.* (1988) have described specific IgG to a conjugate between trimellitic anhydride (TMA) and Hb. However, evidence for the *in vivo* antigenicity of the Hb–TMA conjugate remains to be established since it is possible that the affinity between the Hb-TMA and the IgG could have been a cross-reactivity with other TMA-conjugates such as the TMA–albumin conjugate. We have found similar results using HHPA-conjugates of fibrinogen and HSA (unpublished results). If the Hb–HHPA conjugate was found to be antigenic there would be reason to investigate if these adducts could be used as biomarkers of risk of allergy. However, until further studies on the sensitizing potential of the Hb–HHPA conjugate have been performed, the adduct levels should preferably be considered as a biomarker of exposure. In fact, the results with a negative correlation between the Hb–HHPA adduct-exposure ratio and the IgG rather suggest an opposite relationship between Hb–HHPA adducts and sensitization.

In conclusion, Hb–HHPA adducts seem to be applicable as biomarkers for exposure to HHPA. The possible role of HHPA-specific IgG as an indicator of exposure has to be further evaluated.

Acknowledgements

The excellent technical assistance given by Ms P. Aprea, Ms I. Bensryd, Ms B. Björk, Ms K. Diab, Ms K. Olsson, Ms H. Ottosson, and Mr K. O. Lennartsson is gratefully appreciated. This work was supported by grants from the Swedish Medical Research Council, the Swedish Council for Work Life Research, and the Medical Faculty at Lund University.

References

Biagini, R. E., Klincewicz, S. L., Henningsen, G. M., Mackenzie, B. A., Gallagher, J. S., Bernstein, D. I. and Bernstein, I. L. (1990) Antibodies to morphine in workers exposed to opiates at a narcotics manufacturing facility and evidence for similar antibodies in heroin abusers. *Life Sciences*, **47**, 897–908.

Day, B. W., Jin, R. and Karol, M. H. (1996) In vivo and in vitro reactions of toluene diisocyanate isomers with guinea pig hemoglobin. *Chemical Research in Toxicology*, **9**, 568–573.

Duus, U., Osterman-Golkar, S., Törnqvist, M., Mowrer, J., Holm, S. and Ehrenberg, L. (1989) Studies of determinants of tissue dose and cancer risk from ethylene oxide exposure. In *Management of Risk from Genotoxic Substances in the Environment*, L. Freij, ed. (Swedish National Chemical Inspectorate, Solna), pp. 141–153.

Ehrenberg, L., Hiesche, K. D., Osterman-Golkar, S. and Wennberg, I. (1974) Evaluation of the genetic risk of alkylating agents: tissue doses in the mouse from air contaminated with ethylene oxide. *Mutation Research*, **24**, 83–102.

Gan, L. S., Skipper, P. L., Peng, X., Groopman, J. D., Chen, J. S., Wogan, G. N. and Tannenbaum, S. R. (1988) Serum albumin adducts in the molecular epidemiology of aflatoxin carcinogenesis: correlation with aflatoxin B_1 intake and urinary excretion of aflatoxin M_1. *Carcinogenesis*, **9**, 1323–1325.

Jin, R., Day, B. W. and Karol, M. H. (1993) Toluene diisocyanate protein adducts in the brochoalveolar lavage of guinea pigs exposed to vapors of the chemical. *Chemical Research in Toxicology*, **6**, 906–912.

Jönsson, B. A. G. and Lindh, C. H. (1996) Determination of hexahydrophthalic acid and methylhexahydrophthalic acid in urine using gas chromatography and negative ion chemical ionization mass spectrometry. *Chromatographia*, **42**, 647–652.

Jönsson, B. A. G. and Skerfving, S. (1993) Toxicokinetics and biological monitoring in experimental exposure of humans to gaseous hexahydrophthalic anhydride. *Scandinavian Journal of Work, Environment and Health*, **19**, 183–190.

Jönsson, B. A. G., Welinder, H. and Skarping, G. (1991) Hexahydrophthalic acid in urine as an index of exposure to hexahydrophthalic anhydride. *International Archives of Occupational and Environmental Health*, **63**, 77–79.

Jönsson, B. A. G., Welinder, H., Hansson, C. and Ståhlbom, B. (1993) Occupational exposure to hexahydrophthalic anhydride. Air analysis, percutaneous absorption, and biological monitoring. *International Archives of Occupational and Environmental Health*, **65**, 43–47.

Jönsson, B. A. G., Wishnok, J. S., Skipper, P. L., Stillwell, W. S. and Tannenbaum, S. R. (1995) Lysine adducts between methyltetrahydrophthalic anhydride and collagen in guinea-pig lung. *Toxicology and Applied Pharmacology*, **135**, 156–162.

Lind, P., Dalene, M., Tinnerberg, H. and Skarping, G. (1997a) Biomarkers in hydrolysed urine, plasma, and erythrocytes among workers exposed to thermal degradation products of toluene diisocyanate foam. *Analyst*, **122**, 51–56.

Lind, P., Dalene, M., Lindström, V., Grubb, A. and Skarping, G. (1997b) Albumin adducts in plasma from workers exposed to toluene diisocyanate. *Analyst*, **122**, 151–154.

Lindh, C. H., Jönsson, B. A. G. and Welinder, H. (1997) Biological monitoring of methylhexahydrophthalic anhydride by determination of methylhexahydrophthalic acid in urine and plasma from exposed workers. *International Archives of Occupational and Environmental Health* (in press).

Nielsen, J., Welinder, H., Schütz, A. and Skerfving, S. (1988) Specific serum antibodies against phthalic anhydride in occupationally exposed subjects. *Journal of Allergy and Clinical Immunology*, **82**, 126–133.

Palacián, E., Gonzalez, M. P., Pineiro, M. and Hernandes, F. (1990) Dicarboxylic acid anhydrides as dissociating agents of protein-containing structures. *Molecular and Cellular Biochemistry*, **97**, 101–111.

Pien, L. C., Zeiss, C. R., Leach, C. L., Hatoum, N. S., Levitz, D., Garvin, P. J. and Patterson, R. (1988) Antibody response to trimellityl hemoglobin in trimellitic anhydride-induced lung injury. *Journal of Allergy and Clinical Immunology*, **82**, 1098–1103.

Sepai, O., Henschler, D. and Sabbioni, G. (1995a) Albumin adducts, hemoglobin adducts and urinary metabolites in workers exposed to 4,4′-methylenediphenyl diiocyanate. *Carcinogenesis*, **16**, 2583–2587.

Sepai, O., Schütze, D., Heinrich, U., Hoymann, H. G., Henschler, D. and Sabbioni, G. (1995b) Hemoglobin adducts and urine metabolites of 4,4′-methylenedianiline after 4,4′-methylenediphenyl diisocyanate exposure of rats. *Chemico-Biological Interactions*, **97**, 185–198.

Skipper, P. L., Peng, X., Soohoo, C. K. and Tannenbaum, S. R. (1994) Protein adducts as biomarkers of human carcinogen exposure. *Drug Metabolism Reviews*, **26**, 111–124.

Van Welie, R. T. H., Van Dijck, R. G. J. M., Vermeulen, N. P. E. and Van Sittert, N. J. (1992) Mercapturic acids, protein adducts, and DNA adducts as biomarkers of electrophilic chemicals. *Critical Reviews in Toxicology*, **22**, 271–306.

Venables, K. M. (1989) Low molecular weight chemicals, hypersensitivity, and direct toxicity: the acid anhydrides. *British Journal of Industrial Medicine*, **46**, 222–232.

Welinder, H. and Nielsen, J. (1991) Immunologic tests of specific antibodies to organic acid anhydrides. *Allergy*, **46**, 601–609.

Welinder, H., Nielsen, J., Gustavsson, C., Bensryd, I. and Skerfving, S. (1990) Specific antibodies to methyltetrahydrophthalic anhydride in exposed workers. *Clinical and Experimental Allergy*, **20**, 639–645.

Welinder, H., Jönsson, B. A. G., Nielsen, J., Ottosson, H. and Gustavsson, C. (1994) Exposure–response relationships in the formation of specific antibodies to hexahydrophthalic anhydride in exposed workers. *Scandinavian Journal of Work, Environment and Health*, **20**, 459–465.

Received 6 January 1997, revised form accepted 13 April 1997