4. Other Data Relevant to an Evaluation of Carcinogenicity and its Mechanisms

4.1 Deposition, retention, clearance and metabolism

Several reviews of the toxicology of cobalt, including toxicokinetic aspects, are available (IARC, 1991; Midtgård & Binderup, 1994; Lauwerys & Lison, 1994; Lison, 1996; Barceloux, 1999). This section will focus on the toxicokinetic data published since the previous IARC evaluation (1991) and potentially relevant for cancer. Particular emphasis will be put on studies that examined the fate of inhaled hard-metal particles and related components, when available.

Solubilization of cobalt from tungsten carbide–cobalt powder

It has been shown that tungsten carbide–cobalt powder (WC–Co) is more toxic to murine macrophages *in vitro* than pure cobalt-metal particles, and that the cellular uptake of cobalt is enhanced when the metal is present in the form of WC–Co (Lison & Lauwerys, 1990). In a further study by the same authors, the solubilization of cobalt in the extracellular milieu was shown to increase in the presence of WC. This phenomenon, however, does not explain the greater toxicity of the WC–Co mixture, because increasing the amount of solubilized cobalt in the extracellular medium in the absence of WC did not

result in increased toxicity. Moreover, the amount of cobalt solubilized from a toxic dose of WC–Co was insufficient alone to affect macrophage viability. A toxic effect was only observed when the WC–Co mixture came into direct contact with the cells. These results indicate that the toxicity of the WC–Co mixture does not result simply from an enhanced bioavailability of its cobalt component and suggest that hard-metal dust behaves as a specific toxic entity (Lison & Lauwerys, 1992).

4.1.1 *Humans*

(*a*) *Deposition and retention*

Since the previous IARC (1991) evaluation, no additional relevant data concerning the deposition and/or retention of inhaled cobalt-containing particles in humans have been located.

In several studies conducted on lung tissue or bronchoalveolar lavage fluid (BALF) from patients with lung disease induced by hard-metal particles (hard-metal disease), the presence of tungsten, tantalum or titanium particles was detected, but no or insignificant amounts of cobalt were found (Lison, 1996).

Citizens in Catalonia, Spain, were found to have cobalt in their lungs at the limit of detection (Garcia *et al*., 2001). In contrast, citizens of Mexico City showed remarkably high concentrations of cobalt in their lungs over three decades, which was attributed to air pollution (Fortoul *et al*., 1996). In an autopsy study carried out in Japan, cobalt concentrations in the lung were reported to be related mainly to blood concentrations and were found to be lower in patients who had died from lung cancer than from other causes (Adachi *et al*., 1991; Takemoto *et al*., 1991). A study of uranium miners in Germany demonstrated by NAA that cobalt, associated with uranium, arsenic, chromium and antimony was present at high concentrations in the lungs, with or without concurrent lung tumours, even 20 years after cessation of mining (Wiethege *et al*., 1999).

(*b*) *Intake and absorption*

There are few data on the respiratory absorption of inhaled cobalt-containing materials in humans. The absorption rate is probably dependent on the solubility in biological fluids and in alveolar macrophages of the cobalt compounds under consideration. Increased excretion of the element in post-shift urine of workers exposed to soluble cobalt-containing particles (cobalt metal and salts, hard-metal particles) has been interpreted as an indirect indication of rapid absorption in the lung; in contrast, when workers were exposed to the less soluble cobalt oxide particles, the pattern of urinary excretion indicated a lower absorption rate and probably a longer retention time in the lung (Lison & Lauwerys, 1994; Lison *et al.*, 1994). The importance of speciation and solubility for respiratory absorption has also been highlighted by Christensen and Mikkelsen (1986). These authors found that cobalt concentrations in blood and urine increased (0.2–24 µg/L and 0.4–848 µg/L, respectively) in pottery plate painters using a soluble cobalt paint compared to the control group of painters without cobalt exposure (0.05–0.6 µg/L and 0.05–7.7 µg/L, respectively). The

pottery painters exposed to slightly soluble cobalt paint had only slightly increased cobalt concentrations compared to controls (see Section 1.3.2(*d*)).

The absorption of cobalt compounds has been estimated to vary from 5 to 45% of an orally-administered dose (Valberg *et al.*, 1969; Smith *et al.*, 1972; Elinder & Friberg, 1986). The mean urinary excretion within 24 h of radioactive cobalt (from cobalt chloride) given orally at 20 μmoles to 17 volunteers was estimated to be about 18% (Sorbie *et al*., 1971). In a short-term cross-over study in volunteers, the gastrointestinal uptake of soluble cobalt chloride measured as cobalt concentrations in urine was found to be considerably higher than that of insoluble cobalt oxide (urine ranges, < 0.17–4373 and < 0.17–14.6 nmol/mmol creatinine, respectively). It was also shown that ingestion of controlled amounts of soluble cobalt compounds resulted in significantly higher cobalt concentrations in urine ($p < 0.01$) in women (median, 109.7 nmol/mmol creatinine) than in men (median, 38.4 nmol/mmol creatinine), suggesting that the gastrointestinal uptake of cobalt is higher in women than men (Christensen *et al*., 1993).

Cobalt has been detected in pubic hair, toe nails and sperm of some but not all workers diagnosed with hard-metal disease (Rizzato *et al*., 1992, 1994; Sabbioni *et al*., 1994a).

It was found that absorption of cobalt through the skin and gastrointestinal tract also contributed to concentrations of cobalt in urine in occupationally-exposed individuals (Christensen *et al.*, 1993; Scansetti *et al*., 1994; Christensen, 1995; Linnainmaa & Kiilunen, 1997). Concentrations of cobalt in urine of smokers at a hard-metal factory were higher than those in nonsmokers (10.2 nmol/L [0.6 μg/L] versus 5.1 nmol/L [0.3 μg/L] on average), while no difference in concentrations of cobalt in blood was detected (Alexandersson, 1988). However, the cobalt excreted in urine was found not to be derived from cobalt contained in cigarettes nor from daily intake of vitamin B_{12}, but through eating and smoking with cobalt-contaminated hands at work (Linnainmaa & Kiilunen, 1997).

After absorption, cobalt is distributed systemically but does not accumulate in any specific organ, except the lung in the case of inhalation of insoluble particles. Normal cobalt concentrations in human lung have been reported to be 0.27 ± 0.40 (mean ± SD) μg/g dried lung based on tissue samples taken from 2274 autopsies in Japan (Takemoto *et al*., 1991). A majority of the autopsies were carried out on subjects with malignant neoplasms. There was no increase in cobalt concentration with age, no gender difference and no association with degree of emphysema nor degree of contamination (the grade of particle deposition in the lung).

The normal concentration of cobalt in blood is in the range of 0.1–0.5 μg/L and that in urine is below 2 μg/L in non-occupationally exposed persons. The concentrations of cobalt in blood, and particularly in urine, increase in proportion to the degree of occupational (inhalation) exposure and may be used for biological monitoring in order to assess individual exposure (Elinder & Friberg, 1986). As well as the high concentrations found in workers exposed to cobalt, increased concentrations of cobalt have been found in blood (serum) of uraemic patients (Curtis *et al*., 1976; Lins & Pehrsson, 1976) and in urine of individuals taking multivitamin preparations (as cyanocobalamin, a source of cobalt) (Reynolds, 1989) (see also IARC, 1991 and Section 1.1.5).

(*c*) *Excretion*

The major proportion of systemically-distributed cobalt is cleared rapidly (within days) from the body, mainly via urine, but a certain proportion (10%) has a longer biological half-life, in the range of 2–15 years (Newton & Rundo, 1970; Elinder & Friberg 1986). Of an oral dose of cobaltous chloride, 6–8% was eliminated within 1 week in normal healthy persons (Curtis *et al*., 1976). The elimination of cobalt is considerably slower in patients undergoing haemodialysis, which supports the importance of renal clearance (Curtis *et al*., 1976). In workers in the hard-metal industry, it has been shown that concentrations of cobalt in urine increase rapidly in the hours that follow cessation of exposure, with a peak of elimination about 2–4 h after exposure, and a subsequent decrease (more rapid in the first 24 h) in the following days (Apostoli *et al*., 1994).

4.1.2 *Experimental systems*

Following subcutaneous administration of cobalt chloride (250 µmol/kg bw) to rats, cobalt was found predominantly (> 95%) in plasma, from which it was rapidly eliminated (half-life ($t_{1/2}$), approximately 25 h) (Rosenberg, 1993). In-vitro studies (Merritt *et al*., 1984) have shown that cobalt ions bind strongly to circulating proteins, mainly albumin. Edel *et al*. (1990) reported the in-vitro interaction of hard metals with human lung and plasma components and identified three biochemical pools of cobalt with different molecular weights in the lung cytosol. It has been suggested that cobalt binding to proteins may be of significance for immunological reactions involving cobalt as a hapten (Sjögren *et al.*, 1980). Wetterhahn (1981) showed that oxyanions of chromium, vanadium, arsenic and tungsten enter cells using the normal active transport system for phosphate and sulfate and may inhibit enzymes involved in phosphoryl or sulfuryl transfert reactions. Similarly, the divalent ions of cobalt may complex small molecules such as enzymes and alter their normal activity.

While cobalt-metal particles are practically insoluble in water, the solubilization of these particles is greatly enhanced in biological fluids due to extensive binding to proteins (0.003 mg/L in physiological saline, but 152.5 mg/L in human plasma at 37 °C) (Harding, 1950) and is increased up to sevenfold in the presence of WC particles (in oxygenated phosphate buffer at 37 °C) (Lison *et al*., 1995).

(*a*) *In-vivo studies*

Gastrointestinal absorption of cobalt in rats is dependent on the dose, the ratio of iron to cobalt and the status of body iron stores (Schade *et al.*, 1970). It has been shown that following oral administration of cobalt chloride, 75% is eliminated in faeces and the highest accumulation of cobalt is found in liver, kidney, heart and spleen (Domingo *et al.*, 1984; Domingo, 1989; Ayala-Fierro *et al.*, 1999).

Following intravenous administration of cobalt chloride to rats, 10% of the dose was found to be excreted in faeces, indicating that cobalt can be secreted in the bile. Elimination was triphasic. During the first 4 h, cobalt was rapidly cleared from blood with a

half-life of 1.3 h. The second phase from 4 h to 12 h demonstrated a slower clearance rate with a half-life of 4.3 h. The final phase from 12 h to 36 h had a half-life of 19 h (Ayalu-Fierro *et al.*, 1999).

Kyono *et al*. (1992) exposed rats to ultrafine metallic cobalt particles (mean primary diameter, 20 nm) using a nebulizer producing droplets (MMAD, 0.76 μm; GSD, 2.1; concentration, 2.12 ± 0.55 mg/m^3) for 5 h per day for 4 days and induced reversible lung lesions. Clearance from the lung followed two phases: 75% of the cobalt was cleared within 3 days with a biological half-life of 53 h; the second phase from 3 days to 28 days had a slower clearance rate with a half-life of 156 h.

Kreyling *et al*. (1993) performed clearance studies using inhalation of monodisperse, porous cobalt oxide particles (MMAD, 1.4 and 2.7 μm) in Long-Evans rats. Of the small and large particles, 37% and 38%, respectively, were eliminated in the faeces within 3 days. The half-life for long-term thoracic retention was 25 and 53 days, respectively. After 6 months, large and small cobalt particles were still distributed in the bodies of the rats, mainly in the lung (91 and 52%), skeleton (6 and 22%) and in soft tissue (1.4 and 17%), respectively.

Lison and Lauwerys (1994) found that when non-toxic doses of cobalt metal were administered intratracheally to rats either alone (0.03 mg/100 g body weight) or mixed with tungsten carbide (0.5 mg/100 g body weight; WC–Co containing 6% of cobalt-metal particles), the retention time of the metal in the lung was longer in cobalt- than in WC–Co-treated animals. After 1 day, the lungs of animals instilled with cobalt alone contained twice as much cobalt as in those administered the same amount of cobalt as WC–Co (12 versus 5 μg cobalt/g lung after 24 h).

Slauson *et al*. (1989) induced patchy alveolitis, bronchiolitis and inflammation in the lungs of calves using parainfluenza-3 virus followed by a single inhalation exposure to an aerosol of submicronic cobalt oxide (total dose, about 80 mg). The virus-exposed calves retained 90% of initial cobalt lung burden at day 7 compared with 51% retention in controls. This difference was still present at day 21. Pneumonic calves also exhibited decreased translocation of particles to regional lymph nodes. The authors suggested impaired particulate clearance from acutely-inflamed lungs, which implicated decreased mucociliary clearance and interstitial sequestration within pulmonary alveolar macrophages as the major contributing factors.

(*b*) *In-vitro studies*

In-vitro dissolution of monodisperse, 2.7-μm cobalt oxide particles in baboon alveolar macrophage cell cultures was found to be three times higher than in a cell-free system; the daily dissolution rate was 0.25% versus 0.07% and 0.09% for beads containing particles only and particles combined with alveolar macrophages, respectively (Lirsac *et al*., 1989). Kreyling *et al*. (1990) studied in-vitro dissolution of cobalt oxide particles in human and canine alveolar macrophages and found that smaller particles had faster dissolution rates. In-vitro dissolution rates were found to be similar to in-vivo translocation rates previously

found for human and canine lung. Dissolution of ultrafine cobalt powder in artificial lung fluid was six times higher than that of standard cobalt powder (Kyono *et al*., 1992).

Collier *et al*. (1992) studied factors influencing in-vitro dissolution rates in a simple non-cellular system using 1.7 μm count median diameter (CMD) porous cobalt oxide particles and cobalt-labelled fused aluminosilicate (Co-FAP). Less than 0.5% of cobalt oxide and 1.8% of Co-FAP dissolved over 3 months. The difference in dissolution was much greater in the first week than in the following weeks, with Co-FAP being 20 times more soluble. The dissolution rate for cobalt oxide was higher at lower pH. Lundborg *et al*. (1992) measured and changed phagolysosomal pH within rabbit alveolar macrophages. No clear effect on cobalt dissolution rate was detected for 0.6-μm cobalt oxide particles at pH values ranging between 5.1 and 5.6. Lundborg *et al*. (1995) also studied the effect of phagolysosomal size on cobalt dissolution in rabbit alveolar macrophages incubated with sucrose and in human alveolar macrophages from smokers and non-smokers. The authors found no difference in cobalt dissolution in either rabbit or human cells in spite of large differences in morphological appearance of the macrophages.

Lison and Lauwerys (1994) found that cellular uptake of cobalt was greater when the metal was presented to mouse macrophages as WC–Co. This increased bioavailability of cobalt from hard-metal particles has been interpreted as the result of a physicochemical interaction between cobalt metal and tungsten carbide particles (Lison *et al*., 1995).

In-vitro exposure of HeLa (tumour) cells to cobalt chloride has been shown to result in the intracellular accumulation of cobalt (Hartwig *et al.*, 1990).

4.2 Toxic effects

4.2.1 *Humans*

The health effects resulting from exposure to metallic cobalt-containing particles may be subdivided into local and systemic effects. Local effects are those that occur at the points of contact or deposition of the particles, the skin and the respiratory tract; these effects may be due to the particles themselves (as a result of surface interactions between the particles and biological targets) and/or to cobalt ions solubilized from the particles. Toxic effects outside the respiratory tract are unlikely to be caused by the metallic particles themselves, but result from the release of cobalt ions from the particles and their subsequent absorption into the circulation. (Systemic effects may also be indirect consequences of the damage caused in the lungs).

(*a*) *Dermal effects*

The skin sensitizing properties of cobalt are well known, both from human experience and from animal testing (Veien & Svejgaard, 1978; Wahlberg & Boman, 1978; Fischer & Rystedt, 1983). Exposure to cobalt may lead to allergic contact dermatitis, sometimes having features of an airborne dermatitis, particularly in hard-metal workers (Dooms-Goossens *et al*., 1986). Urticarial reactions have also been described. Cross-reaction with

nickel (as well as co-sensitization) is frequent (Shirakawa *et al*., 1990). The dermal effects of cobalt may occur with all forms of cobalt, i.e. cobalt metal and other cobalt compounds, such as salts.

(*b*) *Respiratory effects*

The various respiratory disorders caused by the inhalation of metallic cobalt-containing particles have been extensively reviewed (Balmes, 1987; Cugell *et al*., 1990; Seghizzi *et al*., 1994; Lison, 1996; Barceloux, 1999; Nemery *et al*., 2001a). These particles may cause non-specific mucosal irritation of the upper and lower airways leading to rhinitis, sinusitis, pharyngitis, tracheitis or bronchitis, but the main diseases of concern are bronchial asthma and a fibrosing alveolitis known as hard-metal lung disease.

(i) *Bronchial asthma*

Bronchial asthma, which like contact dermatitis is presumably based on immunological sensitization to cobalt, has been described in workers exposed to various forms of cobalt, i.e. not only in workers exposed to hard-metal dust, but also in those exposed to 'pure' cobalt particles (Swennen *et al*., 1993; Linna *et al*., 2003), as well as in subjects exposed to other cobalt compounds, such as cobalt salts. Occupational asthma is more frequent than fibrosing alveolitis in hard-metal workers or workers exposed to cobalt dust, but occasionally the two conditions co-exist (Davison *et al*., 1983; Van Cutsem *et al*., 1987; Cugell *et al*., 1990). Chronic bronchitis is reported to be quite prevalent in hard-metal workers, particularly in older studies when dust exposure was considerable and smoking status was not well ascertained (Tolot *et al*., 1970). It is not clear whether those patients with airway changes (asthma or chronic obstructive lung disease) represent 'airway variants' of the same respiratory disease, or whether the pathogenesis of these airway changes is altogether different from that of parenchymal changes. Earlier autopsy studies frequently indicated the presence of emphysema in patients with hard-metal lung disease.

(ii) *Hard-metal lung disease*

Interstitial (or parenchymal) lung disease caused by metallic cobalt-containing particles is a rare occupational lung disease. Several reviews are available on this fibrosing alveolitis which is generally called hard-metal lung disease (Bech *et al*., 1962; Anthoine *et al*., 1982; Hartung, 1986; Balmes, 1987; Van Den Eeckhout *et al*., 1988; Cugell, 1992; Seghizzi *et al*., 1994; Lison, 1996; Newman *et al*., 1998; Nemery *et al*., 2001a,b). A discussion of the occurrence and features of interstitial lung disease caused by metallic cobalt-containing compounds is not only relevant in itself, but it may also have a bearing on the risk of lung cancer, because fibrosing alveolitis and lung cancer may be related mechanistically with regard to both oxidative damage and inflammatory events. Moreover, there is some evidence from observations in humans that lung fibrosis represents a risk for lung cancer, although this evidence is not unequivocal (Bouros *et al*., 2002).

Terminology of (interstitial) hard-metal lung disease

The terminology used to label this disease is complex and confusing. Especially in the earlier literature, hard-metal disease was mostly referred to as a pneumoconiosis (e.g. hard-metal pneumoconiosis or tungsten carbide pneumoconiosis). This term is justified inasmuch as pneumoconiosis is defined as "the non-neoplastic reaction of the lungs to inhaled mineral or organic dust and the resultant alteration in their structure, but excluding asthma, bronchitis and emphysema" (Parkes, 1994). However, it can be argued that the term pneumoconiosis is not entirely appropriate, because it suggests that the disease results from the accumulation of high quantities of dust in the lungs and this is not always the case in hard-metal workers. Indeed, like hypersensitivity pneumonitis and chronic beryllium disease, hard-metal lung disease differs from the common mineral pneumoconioses in that the occurrence of the disease is not clearly related to the cumulative dust burden, but is more probably due to individual susceptibility. Thus the term hard-metal pneumoconiosis has tended to be abandoned in favour of 'hard-metal lung disease'. An advantage, but also a drawback, of the latter term is that the respiratory effects of exposure to hard-metal dust include not only interstitial lung disease (pneumonitis, fibrosis), but also (and probably more frequently) airway disorders, such as bronchitis and occupational asthma. Therefore, the phrases 'hard-metal lung', 'hard-metal disease' or 'hard-metal lung disease' usually encompass more than just the parenchymal form of the disease (Nemery *et al.*, 2001a).

In its most typical pathological presentation, this interstitial lung disease consists of a giant-cell interstitial pneumonia (GIP), one of the five types of interstitial pneumonias originally described by Liebow (1975). GIP is now accepted as being pathognomonic of hard-metal lung disease. Ohori *et al*. (1989) reviewed the published literature and concluded that GIP is indeed highly specific for hard-metal lung, since they found only three published cases of GIP that had not had exposure to cobalt or hard metal. However, while there is no doubt that GIP should be considered to be hard-metal lung disease unless proven otherwise, not all patients with hard-metal lung disease have a 'textbook presentation' of GIP. Indeed, the lung pathology in hard-metal lung disease is variable depending on, among other factors, the stage of the disease and probably also on its pathogenesis in individual patients. The pathology in some patients may be more reminiscent of mixed dust pneumoconiosis (Bech, 1974). Moreover, a pathological diagnosis is not always available.

The most compelling argument against the term hard-metal lung disease is that the disease may also occur without exposure to hard-metal dust. This was established when GIP was found in diamond polishers in Belgium shortly after the introduction of a new technology to facet diamonds. These workers were exposed not to hard-metal dust, but to cobalt-containing dust that originated from the use of high-speed cobalt–diamond polishing discs (Demedts *et al*., 1984). This observation confirmed an earlier hypothesis that cobalt, rather than tungsten carbide, is responsible for hard-metal lung, and it led to the proposal that the interstitial lung disease should be called 'cobalt pneumopathy' or 'cobalt lung', rather than hard-metal lung (Lahaye *et al*., 1984). Nevertheless, the term cobalt lung is not entirely appropriate either, because not all types of exposure to cobalt appear to lead to interstitial lung disease.

Table 14 summarizes the various terms which have been used for the interstitial lung disease caused by hard-metal and cobalt dust. The most correct term is probably 'cobalt-related interstitial lung disease', but this would add more confusion and thus the term hard-metal lung disease will be used here.

Table 14. Terminology of hard-metal lung disease

Name of disease (term)	Features supporting use of term	Features not supporting use of term	Reference
Hard-metal pneumoconiosis	Lung disease is caused by exposure to hard-metal dust	Pathogenesis (hypersensitivity) differs from that of mineral pneumoconiosis.	Parkes (1994)
Tungsten carbide pneumoconiosis	Tungsten carbide is main component of hard metal	Tungsten carbide is not the actual causative agent.	
Hard-metal disease Hard-metal lung Hard-metal lung disease	Lung disease is caused by exposure to hard metal	Terms encompass interstitial lung disease as well as airway disease, such as asthma; similar disease may be caused by exposure to materials other than hard metal.	Nemery *et al.* (2001a)
Cobalt lung or cobalt pneumopathy	Cobalt is the most critical toxic agent	Not all types of exposure to cobalt lead to interstitial lung disease.	Lahaye *et al.* (1984)
Giant cell interstitial pneumonia (GIP)	Pathognomonic pathological feature	Pathology not always available and not always present in individual cases	Ohori *et al.* (1989)

Adapted from Nemery *et al.* (2001a)

Pathogenesis of hard-metal lung disease

There is little doubt that cobalt plays a critical role in the pathogenesis of hard-metal lung disease. Studies in experimental systems have demonstrated that WC–Co particles exhibit a unique pulmonary toxicity compared with cobalt particles (see Section 4.2.2). The toxicity is probably due, at least in part, to the production of toxic oxygen species. However, the hard-metal lung disease that occurs in humans has never been reproduced in experimental animals; neither the typical pattern of inflammation (GIP), nor the progressive nature of the fibrosis.

The basis of individual susceptibility to develop hard-metal lung disease is not known. Cobalt is known to elicit allergic reactions in the skin, probably via cell-mediated pathways (Veien & Svejgaard, 1978), but the relationship, if any, between this cell-mediated allergy and GIP is unknown. Occasionally, patients have been found to have both cobalt dermatitis and interstitial lung disease (Sjögren *et al*., 1980; Cassina *et al*., 1987; Demedts & Ceuppens, 1989). Immunological studies (Shirakawa *et al*., 1988; Kusaka *et al*., 1989;

Shirakawa *et al*., 1989, 1990; Kusaka *et al*., 1991; Shirakawa *et al*., 1992) have found both specific antibodies and positive lymphocyte transformation tests against cobalt (as well as nickel) in some patients with hard-metal asthma. However, to date, the immunopathogenesis of GIP is unknown. In one case of GIP, expression of intracellular transforming growth factor β1 (TGF-β1) was shown in alveolar macrophages, including multinucleate forms, and in hyperplastic alveolar epithelium (Corrin *et al*., 1994). In another case of GIP, immunolocalization of tumour necrosis factor α (TNF-α) was found to be highly associated with the infiltrating mononuclear cells within the interstitium and with cannibalistic multinucleated giant cells in the alveolar spaces (Rolfe *et al*., 1992). The involvement of autoimmune processes is suggested by the report of the recurrence of GIP in a transplanted lung (Frost *et al*., 1993) and in one case of hard-metal alveolitis accompanied by rheumatoid arthritis (Hahtola *et al.*, 2000).

Recent evidence also indicates that the susceptibility to develop cobalt-related interstitial (hard-metal) lung disease is associated with the HLA-DPB1*02 allele, i.e. with the presence of glutamate at position 69 in the HLA-DPB chain (Potolicchio *et al*., 1997), probably because of a high affinity of the HLA-DP molecule for cobalt (Potolicchio *et al*., 1999). It should be noted that the HLA-DPB1*02 allele is the same as that associated with susceptibility to chronic beryllium disease (Richeldi *et al*., 1993).

Clinical presentation

The clinical presentation of hard-metal lung disease is variable: some patients present with subacute alveolitis and others with chronic interstitial fibrosis (Balmes, 1987; Cugell *et al*., 1990; Cugell, 1992). In this respect, hard-metal lung disease is somewhat similar to hypersensitivity pneumonitis (extrinsic allergic alveolitis). Thus, the patient may experience work-related bouts of acute illness, which may lead progressively to pronounced disease with more persistent shortness of breath; but in other instances, the course of the disease is more insidious and the work-relatedness of the condition is not clearly apparent.

Most studies have found no relation between disease occurrence and length of occupational exposure. Subacute presentations may be found in young workers after only a few years exposure, but may also occur in older workers with very long careers. Chronic presentations are more likely in older subjects. The role of smoking in the susceptibility to hard-metal disease has not been evaluated thoroughly, but it is possible that non-smokers are slightly over-represented (Nemery *et al.*, 2001a).

Epidemiology

Descriptions of the epidemiology of hard-metal lung disease can be found in Lison (1996) and Newman *et al*. (1998). Precise incidence figures are not available. Clinical surveys and cross-sectional studies in the hard-metal industry have shown that typical hard-metal lung disease is a relatively rare occurrence, affecting a small percentage of the workforce at most (Miller *et al*., 1953; Bech *et al*., 1962; Dorsit *et al*., 1970; Coates & Watson, 1971; Sprince *et al*., 1984; Kusaka *et al*., 1986; Sprince *et al*., 1988; Meyer-Bisch *et al*., 1989; Tan *et al.*, 2000), unless conditions of hygiene are very poor (Auchincloss

et al., 1992; Fischbein *et al*., 1992). In diamond polishers in Belgium, the prevalence of cobalt-related occupational respiratory disease, including both airway and interstitial lung disease, has been estimated at about 1% of the total workforce (Van den Eeckhout *et al.*, 1988). A cross-sectional survey of 10 workshops, involving a total of 194 polishers, found no cases of overt lung disease, but there was a significant inverse relationship between spirometric indices of pulmonary function and mean levels of exposure to cobalt as assessed by ambient air or biological monitoring (Nemery *et al*., 1992).

Lung disease has been associated not only with the manufacture of cobalt–diamond tools (Migliori *et al*., 1994), but also with their use, at least in the case of high-speed cobalt–diamond discs used for diamond polishing (Demedts *et al*., 1984; Lahaye *et al*., 1984; Wilk-Rivard & Szeinuk, 2001; Harding, 2003). This could be explained by the fact that the projection of cobalt in bonded diamond tools is higher (up to 90%) than in hard metal.

Carbide coatings can now also be deposited by flame or plasma guns onto softer substrates to harden their surfaces, and this process also exposes workers to a risk of hard-metal lung disease (Rochat *et al*., 1987; Figueroa *et al*., 1992).

A detailed and comprehensive cross-sectional survey of 82 workers exposed to cobalt compounds in a plant in Belgium involved in cobalt refining and 82 sex- and age-matched controls from the same plant found no radiological or functional evidence of interstitial lung disease in spite of substantial exposure to cobalt (mean duration of exposure, 8 years; range, 0.3–39.4 years; mean cobalt concentration in air, 125 $\mu g/m^3$ with about a quarter of the workers having had exposures above 500 $\mu g/m^3$) and (subclinical) evidence for other effects of cobalt (thyroid metabolism and haematological parameters) (Swennen *et al*., 1993). The absence of interstitial lung disease in workers exposed to cobalt-metal particles in the absence of other compounds such as tungsten carbide has recently been confirmed in another cross-sectional survey of 110 current and former cobalt refinery workers and 140 control workers in Finland (Linna *et al*., 2003). These cross-sectional studies suggest (but do not prove) that exposure to even relatively high levels of cobalt-metal particles does not lead to interstitial lung disease (although such exposure does lead to asthma).

There is no published evidence for the occurrence of typical ‘hard-metal lung disease’ in workers exposed to cobalt-containing alloys, although adverse respiratory effects may be associated with the manufacture or maintenance of some cobalt-containing alloys (Deng *et al*., 1991; Kennedy *et al*., 1995). Dental technicians (who are exposed to a variety of agents, including cobalt) may also develop interstitial lung disease (Lob & Hugonnaud, 1977; De Vuyst *et al*., 1986; Sherson *et al*., 1988; Selden *et al*., 1995).

Interstitial lung disease has not been described in workers exposed to cobalt salts, except for a study describing four cases of pulmonary fibrosis in a cobalt carbonate factory that operated before the Second World War (Reinl *et al*., 1979).

It is conceivable that full-blown hard-metal lung represents a ‘tip of the iceberg phenomenon’ and that there is other less specific pulmonary damage in many more subjects. The relationship of overt or latent disease with exposure levels remains unknown. This is due, in

part, to the role of individual susceptibility factors, but also to the nature of the hard-metal industry, which is often composed of relatively small tool manufacturing plants or repair workshops, thus making large and comprehensive surveys of the industry rather difficult. In addition, epidemiological studies of a rare and specific condition, such as hard-metal lung, are also difficult because of the poor sensitivity of conventional epidemiological techniques such as questionnaire studies, pulmonary function testing and chest X-ray. Moreover, cross-sectional studies are not the best method to detect clinical cases of hard-metal lung disease, because of the healthy worker effect, and possibly also because of a 'healthy workshop effect' (Nemery *et al*., 1992). The latter refers to the frequently-experienced fact that the factories with the poorest occupational hygiene practice, and therefore probably those with the highest attack rates, are also the least likely to participate in health surveys (Auchincloss *et al*., 1992).

(*c*) *Extrapulmonary effects*

Cobalt exerts a number of toxic effects outside the respiratory system (IARC, 1991; Lison, 1996), which are not specific for metallic cobalt-containing particles. Cobalt stimulates erythropoiesis, thus possibly causing polycythaemia, and has been used in the past for the treatment of anaemia (Alexander, 1972; Curtis *et al.*, 1976). Cobalt is toxic to the thyroid (Kriss *et al.*, 1955; Little & Sunico, 1958) and it is cardiotoxic (see IARC, 1991). The occurrence of cardiomyopathy in occupationally-exposed workers has been investigated and there is some evidence that it may occur, although this is still debated (Horowitz *et al*., 1988; Jarvis *et al*,. 1992; Seghizzi *et al*., 1994).

Possible neuropsychological sequelae, consisting of deficits in encoding or slowed memory consolidation, have been reported in patients with hard-metal disease (Jordan *et al*., 1990, 1997).

4.2.2 *Experimental systems*

Cobalt and its various compounds and/or alloys have been shown in experimental systems to produce non-neoplastic toxicity in different organs including the respiratory tract, the thyroid gland, erythropoietic tissue, myocardium and reproductive organs (Lison, 1996; National Toxicology Program, 1998; Barceloux, 1999). This section focuses on effects that may contribute to the evaluation of the carcinogenicity of inhaled hard-metal dusts and their components and is therefore limited mainly to studies examining effects on the respiratory tract.

(*a*) *Cobalt metal, hard metals and other alloys*

(i) *Inflammation and fibrosis: in-vivo studies*

A series of early experimental studies, initiated in the 1950s, explored the potential mechanisms of the respiratory diseases observed in workers in plants producing hard metal in Germany, the United Kingdom and the USA (see IARC, 1991). These studies

were essentially designed to compare the effects of cobalt metal or oxide, tungsten, tungsten carbide and hard-metal mixtures.

Rats

Harding (1950) was probably the first to describe severe and fatal pulmonary oedema and haemorrhage in piebald rats administered cobalt-metal powder by intratracheal instillation (500 μg/rat), and suggested that this acute pulmonary toxicity might be related to the high solubility of cobalt metal in protein-containing fluids, presumably through some attachment of cobalt metal to protein.

Kaplun and Mezencewa (1960) found that the lung toxicity induced in rats by a single intratracheal instillation of cobalt-metal particles (5 or 10 mg/animal) was exacerbated by the simultaneous addition of tungsten or titanium (10 mg of a mixture containing 8–15% cobalt). Examination of the lungs after 4, 6 and 8 months revealed that pathological changes induced by the mixtures were identical to those produced by cobalt alone but more marked. The authors described a 'thickening' of the lung parenchyma with accumulation of lymphocytes, histiocytes and fibroblasts, hyperplasia of the walls of airways and blood vessels, and the presence of adenomas occurring several months after a single dose. The enhanced toxicity of the tungsten carbide–cobalt mixture was explained by the higher solubility of cobalt in the presence of tungsten (4–5-fold increase in 0.3% HCl during 24 h) (Kaplun & Mezencewa, 1960).

Kitamura *et al.* (1980) examined the pulmonary response of male Sprague-Dawley rats to a single administration of cemented tungsten carbide powder obtained after grinding pre-sintered alloy with diamond wheels. The powder was administered intratracheally at a dose of 23 mg/100 g bw. About 20% of the animals died during the first 3 days after exposure; histological examination of the lungs revealed marked haemorrhagic oedema with intense alveolar congestion. Among survivors, a transient reduction in body weight gain was also observed during the first week post-exposure. Six months after exposure, all sacrificed animals showed pulmonary lesions of patchy fibrosis in the vicinity of deposited dust (peribronchiolar and perivascular regions), occasionally associated with traction emphysema. There was no definitive inflammatory reaction nor interstitial pneumonitis (alveolitis). The lesions were suggested to result from condensation of collapsed alveoli without noticeable dense collagenization. In rats sacrificed at 12 months, the lesions had apparently regressed and two-thirds of the animals had neither fibrosis nor dust retention; the remaining animals showed changes similar to those observed at 6 months. The toxic effect on the lung was attributed, without experimental evidence, to the cytotoxic action of cobalt released from the particles. Neither cobalt metal nor tungsten carbide alone were tested.

Tozawa *et al*. (1981) examined the lung response to pre-sintered cemented carbides (WC:Co, 98:2 or WC:Co:TiC:TaC, 64:16:6:14) in male Sprague-Dawley rats, 6 and 12 months after a single intratracheal administration. They observed marked fibrotic foci after 6 months that were to some extent reversed 6 months later. They also noted that cobalt was eliminated more rapidly than tungsten from the lung. Neither cobalt metal nor tungsten carbide alone were tested.

Lasfargues *et al.* (1992) carried out studies in female Sprague-Dawley rats to compare the acute toxicity of hard-metal particles (WC–Co mixture containing 6% of cobalt-metal particles; d_{50}, 2 μm) with tungsten carbide particles (WC; cobalt content, 0.002%) and with an equivalent dose of cobalt-metal particles alone. After intratracheal instillation of a high dose of cobalt-metal particles (1 mg/100 g bw; median particle size d_{50}, 4 μm), a significantly increased lung weight was noted at 48 h. The lung weights of the animals exposed to WC (15.67 mg/100 g bw) were no different from those of control rats, but significant increases were noted in animals exposed to the hard metal (16.67 mg/100 g bw). These increases were much more substantial in the WC–Co group than in those animals instilled with an equivalent dose of cobalt particles alone. Increased mortality was observed in the group of animals exposed to WC–Co but not in those instilled with cobalt metal or WC alone. A second series of experiments with non-lethal doses (cobalt metal, 0.06 mg/100 g bw; tungsten carbide particles, 1 mg/100 g bw; hard-metal mixture, 1 mg/100 mg bw) was performed in order to analyse the cellular fraction of BALF and lung histology 24 h after dosing. While histological lung sections from rats instilled with cobalt alone or tungsten carbide particles were almost normal, an intense alveolitis was observed in rats exposed to the hard-metal mixture. In rats exposed to cobalt metal alone, no significant biochemical or cellular modifications in BALF were observed. Analysis of the cellular fraction of BALF from animals exposed to hard-metal particles showed a marked increase in the total cell number, similar to that induced by the same dose of crystalline silica; the increase in the neutrophil fraction was even more pronounced than that in the silica-treated group. Similarly, biochemical analyses of the cell-free fraction of BALF showed an increase in lactate dehydrogenase (LDH) activity, total protein and albumin concentration in the group instilled with hard metal, while exposure to the individual components of the mixture, i.e. Co or WC, did not produce any significant modification of these parameters (Lasfargues *et al*., 1992). No change in the ex-vivo production of the inflammatory mediators interleukin-1 (IL-1) and TNF-α, a growth factor fibronectin or a proteinase inhibitor cystatin-c by lung phagocytes was found 24 h after administration of cobalt metal (0.06 mg/100 g bw), WC (1 mg/100 g bw) and WC–Co (1 mg/100 g bw) (Huaux *et al*., 1995).

Lasfargues *et al.* (1995) also examined the delayed responses after single intratracheal administrations of tungsten carbide or hard-metal particles (WC or WC–Co, 1, 5 or 10 mg/100 g bw) or cobalt-metal particles (0.06, 0.3 or 0.6 mg/100 g bw) alone. The lung response to the hard-metal mixture was characterized by an immediate toxic response (increased cellularity and LDH, *N*-acetylglucosaminidase, total protein and albumin concentrations) in BALF followed by a subacute response after 28 days. The effects of cobalt or tungsten carbide alone were very modest, occurring at the highest doses only. Four months after instillation, fibrosis could not be identified histologically in the lungs of the animals treated with the hard-metal powder. This reversibility of the lesions was considered reminiscent of the natural history of hard-metal disease in humans. After repeated intratracheal administrations (once a month for 4 months) of the different particles (1 mg/100 g bw WC or WC–Co, or 0.06, 0.3 or 0.6 mg/100 g bw cobalt), no effect on the lung parenchymal architecture was observed in the groups treated with tungsten carbide or cobalt alone. In contrast,

clear fibrotic lesions were observed in the group instilled with hard metal. No giant multinucleated cells were observed in BALF nor lung tissue of animals treated with WC–Co.

Kyono *et al*. (1992) examined the effect of ultrafine cobalt-metal particles (mean diameter, 20 nm) on the lungs of Sprague-Dawley-Jcl rats exposed by inhalation (2 mg/m^3) for 5 h per day for 4 days. The rats were killed at 2 h, or at 3, 8 or 28 days after the end of exposure. Focal hypertrophy and proliferation of the lower airway epithelium, damaged macrophages and type I pneumocytes as well as proliferation of type II cells, fibroblasts and myofibroblasts were observed early after exposure. Morphological transformation of damaged type I cells to the 'juvenile' form (large nucleolus, abundant smooth endoplasmic reticulum, prominent Golgi apparatus and cytoplasm) was also reported, and interpreted as a sign of active biosynthesis and a capability of self-repair of this cell type. Cobalt was shown to be removed from the lung in two phases with estimated half-lives of 53 and 156 h, respectively. The morphological lesions caused by ultrafine cobalt under the presented conditions were reversible after 1 month: severe fibrosis was not detected in the lungs examined at 28 days. In a companion study, a single intratracheal instillation of ultrafine cobalt metal (0.5 or 2 mg) into rats caused alveolar septal fibrosis detectable 15 months after treatment. Therefore, the authors noted that the possibility that fibrosis can develop after prolonged exposure to ultrafine cobalt metal must be considered.

Adamis *et al*. (1997) examined the lung response in male Sprague-Dawley rats exposed to respirable dust samples collected at various stages of hard-metal production in a plant in Hungary. Samples included finished powder for pressing (8% cobalt content), heat-treated, pre-sintered material (8% cobalt) and wet grinding of sintered hard metal (3% cobalt). The animals were administered 1 and/or 3 mg of dust suspended in saline and were killed after 1, 4, 7 or 30 days. Analyses of BALF (LDH, acid phosphatase protein and phospholipids) indicated the occurrence of an inflammatory reaction, a damage of the cell membrane and an increase of capillary permeability which varied with the type of powder used, with the pre-sintered sample showing the greatest toxicity. Histological studies showed that the pathological changes induced by the three powders were essentially the same, consisting of oedema, neutrophil and lymphocyte infiltration, together with an accumulation of argyrophilic fibres in the interalveolar septa and in the lumina of alveoli and bronchioli.

Zhang *et al*. (1998) compared ultrafine cobalt-metal particles (mean diameter, 20 nm; 47.9 m^2/g surface area) with ultrafine nickel and titanium dioxide powders for their capacity to produce inflammation after a single intratracheal instillation into male Wistar rats (1 mg/animal). All indices measured in BALF indicated that ultrafine nickel was the most toxic material. In the group of animals treated with cobalt particles, the lung:body weight ratio was significantly increased at days 1, 3, 7 and 15 after exposure and returned to normal after 30 days; LDH activity, total protein, lipid peroxide concentrations and inflammatory cells in BALF were significantly increased for up to 30 days.

Guinea-pigs

A single intratracheal instillation of cobalt metal (10–50 mg) into guinea-pigs [strain not specified] was shown to result in the development of acute pneumonia with diffuse

eosinophilic infiltration and bronchiolitis obliterans. The subchronic response assessed 8–12 months after the dose was characterized by the presence of multinucleated cells and a lack of cellular reaction within the alveolar walls. It was concluded that cobalt metal is not fibrogenic and does not provoke a chronic lesion in the regional lymph nodes (Delahant, 1955; Schepers, 1955a).

In the comparative studies (Schepers, 1955b), instillation of cobalt metal mixed with tungsten carbide (150 mg in a 9:91 ratio, i.e. a dose of 13.5 mg cobalt metal) into guinea-pigs [strain not specified] induced a transient inflammatory reaction with residual papillary hypertrophy of bronchial mucosa and peribronchial and periarterial fibrosis in the vicinity of retained particles. In inhalation experiments, a similar mixture (ratio 1:3) caused severe inflammation with focal pneumonia and bronchial hyperplasia and metaplasia, but fibrosing alveolitis was not observed after treatment with the cobalt metal–tungsten carbide mixture. Multinucleated giant cells were also found in animals treated with a combination of tungsten carbide and carbon (without cobalt); tungsten carbide alone was not tested (Schepers, 1955c).

Rabbits

Exposure of rabbits by inhalation to cobalt metal (0.2 and 1.3 mg/m^3) for 4 weeks did not produce any inflammatory reaction; the particles were not taken up by macrophages and the phagocytic capacity of these cells was not impaired (Johansson *et al*., 1980).

Mini-pigs

Kerfoot *et al*. (1975) exposed mini-pigs for 3 months (6 h per day, 5 days per week) to aerosols of cobalt metal at concentrations of 0.1 and 1.0 mg/m^3. The animals were first submitted to a sensitization period of 5 days' exposure to cobalt followed by a 10-day removal from exposure before the 3 months of exposure. Post-exposure lung function studies demonstrated a dose-dependent and reversible reduction in lung compliance but no radiographic or histological signs of fibrosis, except some increased collagen deposition which could only be detected electron-microscopically. The authors interpreted these changes as demonstrating functional impairment. Functional alterations were no longer detectable 2 months after the end of cobalt exposure. [The Working Group noted that the collagen increase was not assessed quantitatively.]

(ii) *Cytotoxicity: in-vitro studies*

Mouse macrophage cells

Cytotoxic effects on mouse peritoneal macrophages of a range of metallic particles of orthopaedic interest have been examined. High doses of cobalt metal and cobalt–chromium alloy (0.5 mg metal/2 mL/3 × 10^6 cells) caused membrane damage as indicated by the increased release of cytoplasmic LDH. Decreased glucose-6-phosphate dehydrogenase (G6PD) activity was also observed after 10 h incubation with the same dose of metal (Rae, 1975).

In mouse peritoneal and alveolar macrophages, Lison and Lauwerys (1990) showed that the cytotoxicity of cobalt-metal particles, assessed by LDH release and morphological examination, was significantly increased in the presence of tungsten carbide particles either as industrial hard-metal powders or a WC–Co mixture reconstituted in the laboratory (6% cobalt in weight). Both particles (Co and WC) needed to be present simultaneously in order to exert their increased cytotoxic action. The interaction between tungsten carbide and cobalt particles was associated with an increased solubilization of cobalt in the culture medium. However, in the test system used, the toxicity of hard-metal particles could not be ascribed to solubilized cobalt ions, because the effects could not be reproduced with cobalt chloride or with cobalt ions solubilized from the WC–Co mixture (Lison & Lauwerys, 1992). In further studies, the cytotoxic potential of cobalt-metal particles, based on the measurement of glucose uptake, G6PD activity and superoxide anion production, was assessed *in vitro* in mouse peritoneal and alveolar macrophages incubated in a culture medium supplemented with 0.1% lactalbumin hydrolysate. Glucose uptake and superoxide anion production were significantly more depressed by a WC–Co mixture than by cobalt alone, while G6PD activity was decreased by both WC–Co and cobalt-metal particles alone (Lison & Lauwerys, 1991). Cobalt-metal particles (d_{50}, 4 μm and 12 μm; 10–100 μg/10^6 cells) affected cell integrity only marginally (Lison & Lauwerys, 1991).

Using the LDH release assay in mouse peritoneal macrophages, a similar toxic interaction between cobalt-metal particles and other metallic carbides (titanium, niobium and chromium carbides) but not latex beads (Lison & Lauwerys, 1992), crystalline silica, iron or diamond particles, was found. It was noted that the interaction between the carbides and the cobalt particles was dependent to some extent on the specific surface area of the particles suggesting the involvement of a surface chemistry (physicochemical) phenomenon (Lison & Lauwerys, 1995).

Lison *et al*. (1995) found that butylated hydroxytoluene protected macrophage cultures from the toxicity of a WC–Co (94:6) mixture, suggesting the involvement of lipid peroxidation in the cytotoxic activity of these particles. Lipid peroxidation was also demonstrated by the formation of thiobarbituric acid-reactive substances when arachidonic acid was incubated with WC–Co particles. Lison and Lauwerys (1993) had shown earlier that other enzymes that detoxify activated oxygen species such as catalase and superoxide dismutase (SOD), and scavengers such as sodium azide, benzoate, mannitol, taurine or methionine, did not protect against the cytotoxicity of WC–Co particles.

Rat fibroblasts, alveolar macrophages and type II pneumocyte cells

Thomas and Evans (1986) observed no effect of a cobalt–chromium–molybdenum alloy (0.5–10 mg/mL) on the proliferation of rat fibroblasts in culture nor on production of collagen.

Roesems *et al*. (1997), using the same experimental model as Lison and Lauwerys (1990; see above) but incubating the preparations in the absence of lactalbumin hydrolysate, which was replaced by foetal calf serum, showed that rat alveolar type II pneumocytes were less sensitive than alveolar macrophages to cobalt-metal particles *in vitro*, and

that human type II pneumocytes were even less sensitive than rat type II pneumocytes. In contrast, using the dimethylthiazol diphenyl tetrazolium (MTT) assay, rat type II pneumocytes were found to be more sensitive than alveolar macrophages (25 μg/600 000 cells) and the toxicity of cobalt-metal particles could be reproduced by cobalt ions (Roesems *et al.*, 2000).

In the experimental system used by Roesems *et al*. (2000), an increased cytotoxicity of cobalt-metal particles associated with WC was confirmed in rat alveolar macrophage cell cultures, but not in type II pneumocytes. In contrast to the results presented by Lison and Lauwerys (1992), Roesems *et al.* (2000) found that cobalt ions played a role in the cytotoxic effect of cobalt-metal particles whether associated or not with WC. This difference was probably due to the presence of lactalbumin hydrolysate which was found to quench cobalt ions and may have masked their cytotoxicity in the experiments by Lison and Lauwerys (1992) and Lison (2000). *In vivo*, however, the bioavailability of cobalt ions is relatively limited because these cations precipitate in the presence of physiological concentrations of phosphates ($Co_3(PO_4)_2$; K_{sp}: 2.5×10^{-35} at 25 °C) (Lison *et al.*, 1995; 2001) and bind to proteins such as albumin (Merritt *et al*., 1984).

In rat type II pneumocytes, cobalt-metal particles (15–1200 μg/3 × 10^6 cells) were found to stimulate the hexose monophosphate shunt in a dose- and time-dependent manner, indicating that these particles caused oxidative stress (Hoet *et al*., 2002).

(iii) *Biochemical effects*

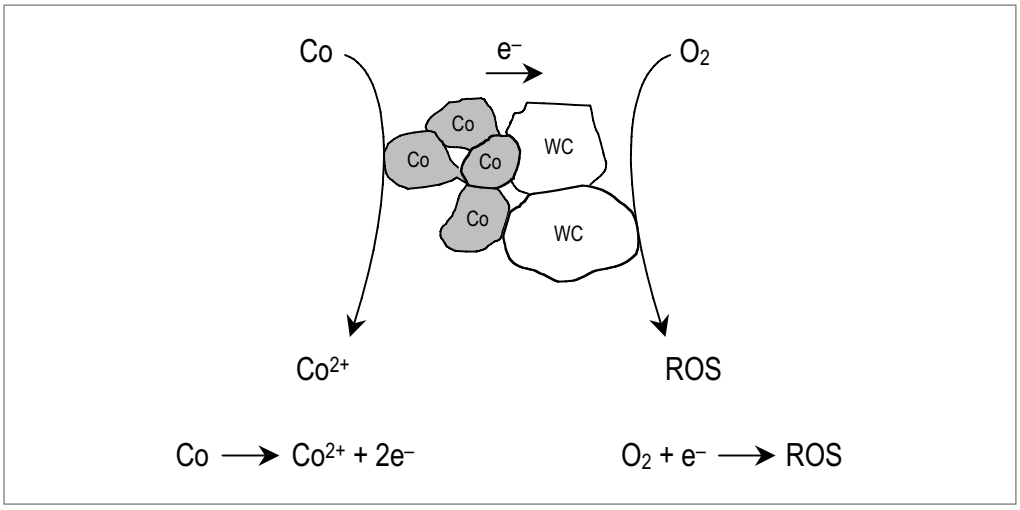
As a transition element, cobalt shares a number of chemical properties with iron and thus it has been suggested that it may catalyse the decomposition of hydrogen peroxide by a Fenton-like mechanism. While several studies have indeed indicated that reactive oxygen species (ROS) are formed in the presence of a mixture of cobalt(II) ions and hydrogen peroxide (Moorhouse *et al.*, 1985), the exact nature of the radicals formed is still a matter of speculation. These free radicals have been proposed to account for several toxic properties of cobalt compounds, including their genotoxic activity.

Lison and Lauwerys (1993) using a deoxyribose degradation assay reported a significant formation of hydroxyl radicals *in vitro* when cobalt-metal particles (d_{50}, 4 μm; 6 μg/mL) were incubated with hydrogen peroxide. However, this effect was less than that seen with an equivalent concentration of cobalt(II) ions. The activity of cobalt-metal particles was increased about threefold when associated with tungsten carbide particles (d_{50}, 2 μm). It was also noted that the latter behaved as a strong oxidizing compound, but the exact role of this activity in the interaction with cobalt metal or cobalt(II) ions could not be elucidated.

Using electron spin resonance (ESR) and 5,5-dimethyl-1-pyrroline *N*-oxide (DMPO) spin trapping in a cell-free system, Lison *et al*. (1995) reported that cobalt-metal particles (d_{50}, 4 μm; 1 mg/mL phosphate buffer) produced small amounts of activated oxygen species, presumed to be hydroxyl radicals. This activity was observed in the absence of hydrogen peroxide and could not be reproduced with cobalt(II) ions, indicating that a Fenton-like mechanism was not involved. The production of activated oxygen species by

cobalt-metal particles was markedly increased in the presence of tungsten carbide particles (Co:WC, 6:94). It was proposed that this reaction could be the consequence of a solid-solid interaction between particles whereby molecular oxygen is reduced at the surface of WC particles by electrons migrating from cobalt-metal particles, which are consequently oxidized and solubilized. The resulting Co(II) did not drive the production of ROS (see Figure 2). Further investigations of the surface interaction between cobalt metal and tungsten carbide particles (Zanetti & Fubini, 1997) indicated that the association of the two solids behaves like a new chemical entity, with physico-chemical properties different from those of the individual components, and which provides a lasting source of ROS as long as metallic cobalt is present. Radical generation originates from reactive oxygen formed at the carbide surface. When compared to other metals (iron, nickel), cobalt metal was the most active in the above reaction (Fenoglio *et al*., 2000). In the presence of hydrogen peroxide, the WC–Co mixture exhibits a peroxidase-like activity (Fenoglio *et al.*, 2000; Prandi, 2002).

Figure 2. Mechanism proposed for release of reactive oxygen species (ROS) from buffered aqueous suspensions of cobalt/tungsten carbide (Co/WC) mixtures (hard metals)



Adapted from Zanetti & Fubini (1997)
Cobalt is progressively oxidized and solubilized; oxygen is activated at the carbide surface.
e^-, electron

Leonard *et al*. (1998), using ESR, confirmed that cobalt-metal particles in aqueous suspension reduced molecular oxygen. The authors proposed that the species generated is likely to be a cobalt(I)-bound superoxide anion (Co(I)-$OO^{\bullet}$) which exhibits strong oxidizing properties. This product was further shown in the presence of SOD, to generate hydrogen peroxide which reacts with Co(I) to produce a hydroxyl radical and Co(II) via a Co(I)-

mediated Fenton-like reaction. In the presence of proper chelators, such as glutathione (GSH), Gly-Gly-His and anserine, the cobalt(II) ions formed by the molecular oxygen oxidation of cobalt produce hydroxyl radicals and Co(III) through a Co(II)-mediated Fenton-like mechanism.

Keane *et al*. (2002) confirmed the generation of hydroxyl radicals by hard-metal materials in aqueous suspension by examining the properties of detonation coating, a hard metal-related material made of a WC and cobalt mixture (6.7 and 5.4% of cobalt in pre- and post-detonation powders, respectively). The post-detonation powder was a much stronger generator of hydroxyl radicals than the pre-detonation material.

(*b*) *Other relevant cobalt compounds*

(i) *Inflammation and fibrosis: in-vivo studies*

In a dose-finding study for a carcinogenicity assay (Bucher *et al*., 1990), male and female Fischer 344/N rats and $B6C3F_1$ mice were exposed to cobalt sulfate heptahydrate aerosols of 0, 0.3, 1.0, 3.0, 10 or 30 mg/m³ for 6 h per day on 5 days per week for 13 weeks. The main histopathological effects in both species were limited to the respiratory tract. Lesions included degeneration of the olfactory epithelium, squamous metaplasia of the respiratory epithelium, inflammation in the nose, and fibrosis, histiocytic infiltrates, bronchiolar epithelial regeneration and epithelial hyperplasia in the alveoli of the lungs. In rats, inflammation, necrosis, squamous metaplasia, ulcers and inflammatory polyps of the larynx were observed; mice developed metaplasia of the trachea. The most sensitive tissue in rats was the larynx: squamous metaplasia was observed with the lowest exposure concentration (0.3 mg/m³). Degeneration of the olfactory epithelium was noted at the two highest doses tested (10 and 30 mg/m³). A no-observed-adverse-effect level was not reached in these studies.

In the subsequent carcinogenicity study (0, 0.3, 1.0 or 3.0 mg/m³ cobalt sulfate heptahydrate, 6 h per day, 5 days per week for 104 weeks) conducted in the same rodent strains (National Toxicology Program, 1998; Bucher *et al.*, 1999) (see also Section 3.1.1), similar non-neoplastic effects were noted. Degeneration of olfactory epithelium was more pronounced than in the 13-week dose-finding study and was observed at the lowest dose tested (0.3 mg/m³). In rats, proteinosis, alveolar epithelial metaplasia, granulomatous alveolar inflammation and interstitial fibrosis were observed at all dose levels. The non-neoplastic lesions were less severe in mice and mainly consisted of cytoplasmic vacuolization of the bronchi. Diffuse and focal histiocytic cell infiltrations were also observed in lungs of mice with neoplasms and were therefore considered to be a consequence of the neoplasms rather than a primary effect of cobalt sulfate.

Wehner *et al*. (1977) examined the influence of lifetime inhalation of cobalt oxide (10 µg/L, 7 h/day, 5 days/week) alone or in combination with cigarette smoke in male Syrian golden hamsters with the aim of detecting a carcinogenic effect. No tumorigenic action of cobalt oxide was observed and there was no additive effect of exposure to smoke.

Some pulmonary changes consisting of focal interstitial fibrosis, granulomas, hyperplasia of alveolar cells and emphysema were observed in animals exposed to cobalt oxide alone.

Lewis *et al.* (1991) showed that the intratracheal instillation of cobalt chloride (1–1000 μg/kg) into Syrian golden hamster lungs induced biochemical changes compatible with the development of oxidative stress (i.e. decreased concentrations of reduced glutathione, increased concentrations of oxidized glutathione, and stimulation of the pentose phosphate pathway). Similar changes were also observed *in vitro* after incubation of lung slices with cobalt chloride (0.1–10 mM), and preceded the detection of cellular toxicity indicating their possible early involvement in the pulmonary toxicity of cobalt (II) ions. It was later shown in the same in-vitro model that simultaneous treatment with hydrogen peroxide or 1,3-bis(2-chloroethyl)-1-nitrosourea, a glutathione reductase inhibitor, potentiated the oxidative stress induced by cobalt chloride; however, this effect was not associated with an enhancement of cell dysfunction observed with cobalt chloride alone or cobalt chloride and hydrogen peroxide together. Furthermore, on the basis of comparative analysis of the results with the known oxidant tert-butyl hydroperoxide, glutathione oxidation did not appear to be the cause of the cellular dysfunction caused by cobalt chloride (Lewis *et al.*, 1992).

In a series of studies on the effects of various cobalt compounds on rabbit lung morphology (Johansson *et al.*, 1983, 1986), exposure to 0.4, 0.5 and 2.0 mg/m^3 soluble cobalt chloride for 1 and 4 months increased the number of alveolar macrophages and their oxidative metabolic activity. Exposure to 0.4 and 2 mg/m^3 cobalt chloride for 14–16 weeks (6 h/day, 5 days/week) induced a combination of lesions characterized by nodular aggregation of type II pneumocytes, abnormal accumulation of enlarged, vacuolated alveolar macrophages and interstitial inflammation (Johansson *et al.*, 1987). The effect of cobalt chloride (0.5 mg/m^3 for 4 months) on rabbit lung, i.e. formation of noduli of type II cells, was potentiated by simultaneous exposure to nickel chloride administered at the same dose (Johansson *et al.*, 1991). Camner *et al.* (1993) reported that the inflammatory reaction (as indicated by the presence of neutrophils and eosinophils in BALF) induced by the inhalation of cobalt chloride (2.4 mg/m^3, 6 h/day for 2 weeks) was more pronounced in guinea-pigs that had been pre-sensitized to cobalt by repeated application of cobalt chloride.

(ii) *Cytotoxicity: in-vitro studies*

At relatively high doses (0.1–1 mM), cobalt(II) ions have been shown to inhibit exocytosis and respiratory burst in rabbit neutrophils through an interaction with a calcium-dependent intracellular mechanism (Elferink & Deierkauf, 1989).

In U-937 cells and human alveolar macrophages, cobalt ions (0.5–1 mM as cobalt chloride) induced apoptosis and accumulation of ubiquitinated proteins. It was suggested that cobalt-induced apoptosis contributed to cobalt-induced lung injury (Araya *et al.*, 2002). In neuronal PC12 cells cobalt chloride triggered apoptosis in a dose- and time-dependent manner, presumably via the production of ROS and the increase of the DNA-binding activity of transcriptor factor AP-1 (Zou *et al.*, 2001). A subsequent study showed

that caspase-3 and p38 mitogen-activated protein kinase-mediated apoptosis was induced by cobalt chloride in PC12 cells (Zou *et al*., 2002).

Microtubule disorganization has been reported in 3T3 cells exposed to high concentrations of cobalt sulfate (100 μM) for 16 h (Chou, 1989).

Soluble cobalt compounds (40 μM [5 μg/mL] as cobalt chloride), but not particulate materials, have been reported to induce cytotoxicity and neoplastic transformation in the C3H10T½ assay (Doran *et al*., 1998) (see Section 4.4).

(iii) *Biochemical effects*

Using two assays to detect hydroxyl radicals ($HO^{\bullet}$), based either on the degradation of deoxyribose or the hydroxylation of phenol or salicylate, Moorhouse *et al*. (1985) found that, in an acellular system at physiological pH, cobalt(II) ions promoted the formation of hydroxyl-like radicals in the presence of hydrogen peroxide (H_2O_2, 1.44 mM); the formation of the radicals was decreased by catalase, but not by SOD or ascorbic acid. Ethylenediaminetetraacetic acid (EDTA) in excess of Co(II) accelerated the formation of ROS, and hydroxyl radical scavengers such as mannitol, sodium formate, ethanol or urea, blocked deoxyribose degradation by the cobalt(II)–H_2O_2 mixture. Lison and Lauwerys (1993) reported similar findings, i.e. a significant degradation of deoxyribose in the presence of cobalt(II) (0.1 mM) mixed with hydrogen peroxide (1.44 mM), suggesting the formation of hydroxyl radicals.

Using an ESR spin-trapping technique (with DMPO), Kadiiska *et al*. (1989) found that cobalt(II) ions, unlike iron(II) ions, did not react with hydrogen peroxide by the classic Fenton reaction at physiological pH, either in a chemical system or in rat liver microsomes. They suggested that superoxide anions, not hydroxy radicals, were primarily formed. In a subsequent study using the same technique, Hanna *et al*. (1992) used several ligands to complex cobalt(II) ions and further documented the formation of superoxide anions, but not hydroxyl radicals, in the presence of hydrogen peroxide.

Using ESR, Wang *et al*. (1993) detected the ascorbic acid radical *in vivo* in circulating blood after intravenous administration of ascorbic acid (100 mM) and cobalt(II) at two separate sites into male Sprague-Dawley rats. Similar but less intense signals were also observed with nickel(II) and iron(II) ions. The formation of the ascorbic acid radical was interpreted as the in-vivo formation of free radicals in animals overloaded with cobalt(II) ions; the mechanism of this radical formation was, however, not addressed. The authors suggested that their findings might explain the mechanism of the toxicity observed in workers exposed to cobalt-containing materials.

The in-vitro generation of ROS by cobalt(II) from hydrogen peroxide and related DNA damage have also been examined by Mao *et al*. (1996). The formation of hydroxyl radicals and/or singlet oxygen ($^1O_2$) showed that the oxidation potential of cobalt(II) could be modulated by several chelators such as anserine or 1,10-phenanthroline. Shi *et al*. (1993) examined the modulation of ROS production from cobalt(II) ions and hydroperoxides and showed that several chelating agents, including endogenous compounds such as reduced GSH, facilitated the production of these species.

Sarkar (1995) hypothesized that oligopeptides or proteins represent other ligands that can modulate the redox potential of cobalt(II) ions. The presence of such proteins (histones) in the nucleus might allow the production of ROS in close proximity to biologically-relevant targets such as DNA. It has also been suggested that the ability of cobalt(II) to substitute for zinc(II) in the DNA-binding domain of nuclear (transcription factor) proteins might allow the in-situ formation of free radicals that may damage genetic regulatory/response elements and may explain the mutagenic potential of these metals.

(iv) *Other effects*

Cobalt also interferes with cellular mechanisms that control the degradation of regulatory proteins such as p53, which is involved in the control of the cell cycle, genome maintenance and apoptosis. An *et al.* (1998) reported that, in mammalian cells, cobalt chloride (100 μM) activates hypoxia-inducible factor-1α which in turn induces accumulation of p53 through direct association of the two proteins. Cobalt sulfate (50 μg/mL [178 μM]) has been shown to induce p53 proteins in mouse cells treated *in vitro* (Duerksen-Hughes *et al.*, 1999). Inhibition of proteasome activity by cobalt (1 mM), subsequent accumulation of ubiquitinated proteins and increased apoptosis have been reported in human alveolar macrophages and U-937 cells (Araya *et al*., 2002) (See Section 4.4.2). Whether these biochemical mechanisms are involved in the carcinogenic responses observed with some cobalt compounds remains, however, to be examined.

4.3 Reproductive and developmental effects

Only a few studies have been conducted with soluble cobalt compounds to explore their potential effects on development.

Wide (1984) reported that a single intravenous injection of cobalt chloride hexahydrate into pregnant NMRI mice (5 mM per animal in the tail vein; [120 μg/animal]) on day 8 of gestation significantly affected fetal development (71% of skeletal malformations versus 30% in controls); in animals injected at day 3 of gestation, no interference with implantation was noted. In the same experiment but replacing cobalt chloride by tungstate (25 mM of W per animal; [460 μg/animal]) a significant increase in the number of resorptions was observed (19% versus 7% in controls), but no skeletal malformations.

In a study undertaken by Pedigo and colleagues (1988), following 13 weeks of chronic exposure to 100 to 400 ppm [100–400 μg/mL] cobalt chloride in drinking water, male CD-1 mice showed marked dose-related decreases in fertility, testicular weight, sperm concentration and motility, and increases in circulating levels of testosterone. Pedigo and Vernon (1993) reported that cobalt chloride (400 ppm in drinking-water for 10 weeks) increased pre-implantation losses per pregnant female in the dominant lethal assay by compromising the fertility of treated male mice.

Paksy *et al.* (1999) found that in-vitro incubation of postblastocyst mouse embryos with cobalt(II) ions (as cobalt sulfate) adversely affected the development stages at a concentration of 100 μM and decreased the trophoblast area (at a concentration of 10 μM).

In pregnant Wistar rats, oral administration of cobalt(II) ions as cobalt chloride (12, 24 or 48 mg/kg bw per day from day 14 of gestation through to day 21 of lactation) significantly affected the late period of gestation as well as postnatal survival and development of the pups. Signs of maternal toxicity were apparently also noted but the details are not reported (Domingo *et al*., 1985).

A study conducted in pregnant Sprague-Dawley rats (Paternain *et al*., 1988) concluded that the administration of cobalt chloride (up to a dose of 100 mg/kg by gavage, from day 6–15 of gestation) was not embryotoxic nor teratogenic, despite signs of maternal toxicity.

Sprague-Dawley rats maintained on diets (15 g per day) containing 265 ppm [31.8 mg/kg bw per day] cobalt for up to 98 days showed degenerative changes in the testis from day 70 to the end of the treatment; given that cobalt was not detected in testis, these changes were considered secondary to hypoxia due to blockage of veins and arteries by red blood cells and changes in permeability of the vasculature and seminiferous tubules (Mollenhaur *et al.*, 1985). Decreased sperm motility and/or increased numbers of abnormal sperm were noted in mice, but not in rats, exposed to 3 mg/m^3 or higher concentrations (30 mg/m^3) in 13-week inhalation studies with cobalt sulfate (National Toxicology Program, 1991).

The fetal and postnatal developmental effects of cobalt sulfate have been compared in C57BL mice, Sprague-Dawley rats and/or New Zealand rabbits (Szakmáry *et al*., 2001). Several developmental alterations (elevated frequency of fetuses with body weight or skeletal retardation, embryolethality, increased anomalies in several organs) were observed in pregnant mice and rats treated with cobalt sulfate by gavage on days 1–20 of gestation (25, 50 or 100 mg/kg bw per day, respectively). In rabbits, cobalt sulfate at 20 mg/kg bw was embryotoxic with inhibition of skeletal development. No teratogenic effects were noted in rabbits treated with up to 200 mg/kg per day during days 6–20 of gestation. Postnatal developmental parameters were transiently altered in the pups of rats treated daily with 25 mg/kg cobalt sulfate. [The Working Group noted that the doses used in these studies were relatively high and produced maternal toxicity. The interpretation of these data should, therefore, be considered with caution].

4.4 Genetic and related effects

4.4.1 *Humans*

(*a*) *Sister chromatid exchange*

Five studies have been conducted to date on the possible cytogenetic effects induced by cobalt compounds in lymphocytes (or leukocytes) of individuals exposed to metals.

Results of sister chromatid exchange have been obtained in two studies in which exposure was to a mixture of metals. Occupational exposure to metals was studied by Gennart *et al*. (1993) who determined sister chromatid exchange in 26 male workers (aged 23–59 years) exposed to cobalt, chromium, nickel and iron dust in a factory produ-

cing metal powder and in 25 controls (aged 24–59 years), who were clerical workers, matched for age, smoking habits and alcohol consumption. The metal particle sizes ranged from 2 to 100 μm. Slight exposure to nickel or chromium oxides could not be excluded, since, at one stage of the production process, the metals are melted in an oven. The workers had been employed for at least 2 years (range, 2–20 years). The atmospheric concentrations of cobalt were measured at two different work areas in 1986 and in 1989, at the time of the cytogenetic survey. An improvement in the local exhaust ventilation system took place between the two sampling times. At the work area where the ovens were located, the (geometric) mean cobalt concentration in the air (based on 4–8 values) was 92 $\mu g/m^3$ in 1986 and 40 $\mu g/m^3$ in 1989. At the second work area, the individual values ranged from 110 to 164 $\mu g/m^3$ in 1986 and from 10 to 12 $\mu g/m^3$ in 1989. The differences in the concentrations of cobalt in the urine in exposed persons (cobalt geometric mean, 23.6 μg/g creatinine; range, 6.4–173.1) and controls (cobalt geometric mean, 1.1 μg/g creatinine; range, 0.2–3.2) were statistically significant. Analysis of variance revealed that both exposure status (exposed versus controls) and smoking habits (smokers and former smokers versus never smokers) had statistically-significant effects on the sister chromatid exchange or high-frequency cell (HFC) rank values. These effects may not be attributable to cobalt alone.

Stea *et al*. (2000) compared sister chromatid exchange in patients who had chrome–cobalt alloy prostheses and in those with other metal alloys. The study population consisted of 30 patients (11 men and 19 women; mean age, 63.8 years; range, 33–78) with joint (28 hip and two knee) prostheses and 17 control subjects (11 men and six women; mean age, 58.65 years; range, 40–71) matched for age, sex, and exposure to occupational and environmental risk factors such as chemicals, antineoplastic drugs and traffic smog. Ten subjects (mean age, 65.1 years; range, 51–76) had prostheses made of titanium–aluminium–vanadium alloys, 14 subjects (mean age, 61.9 years; range, 33–75) had prostheses made of chrome–cobalt alloys and five (mean age, 65 years; range, 57–78) had mixed prostheses. Of the prostheses, 13 were cemented (in some cases only one component was cemented) and 17 were cementless. The average duration of the implant was 7.5 years (range, 0.5–25) for the hip prostheses and 2.5 years for the two knee prostheses. The mean sister chromatid exchange rate in subjects with prostheses (5.2 ± 1.5) was not statistically different from that in subjects without prostheses (4.4 ± 1.3). Subjects with titanium–aluminium–vanadium alloy prostheses had a significantly higher sister chromatid exchange frequency (6.3 ± 2.3) than the controls (4.4 ± 1.3) whereas subjects with prostheses made of chrome–cobalt alloys or mixed prostheses had a higher, but not significantly, sister chromatid exchange frequency (4.7 ± 1.1 and 5.0 ± 2.1, respectively) than the controls. The number of sister chromatid exchanges was not affected by the presence of bone-cement used in prosthesis fixation nor by duration of the implant. There was no difference in the incidence of sister chromatid exchange between the two populations (those with prostheses and controls) considered globally and the considered risk factors, including smoking. The HFC values (> 9 exchanges per cell) were also recorded. Among the cases studied, three patients with implants (one with a prosthesis made of chrome–cobalt alloy and two with

mixed prostheses) showed markedly elevated percentages of HFCs (> 10%). It was concluded that the indication of possible cytogenetic damage in the patient populations should be considered with caution, since the sample population was small.

(*b*) *Micronuclei, DNA damage*

Burgaz *et al*. (2002) applied the micronucleus test to assess the effect of occupational exposure to metal alloys in both exfoliated nasal cells, and *in vitro* in lymphocytes, with the cytochalasin-B technology which allows discrimination between lymphocytes that have divided once during the in-vitro culture periods (binucleates) and those that have not (mononucleates). The groups studied consisted of 27 male dental laboratory technicians (mean age, 29.2 ± 10.8 years) exposed to metal alloys (35–65% cobalt, 20–30% chromium, 0–30% nickel) in dental laboratories during the production of skeletal prostheses, and 15 male controls (mean age, 28.4 ± 9.5 years) from the faculty of pharmacy. The differences in concentrations of cobalt in urine of technicians and controls were statistically significant (urinary cobalt, 0.12 ± 0.24 μg/g creatinine in controls and 24.8 ± 24.1 μg/g creatinine in technicians). The mean frequencies of micronucleated binucleates among peripheral lymphocytes were significantly higher (4.00 ± 2.98) in the dental technicians than in controls (1.40 ± 1.30). A statistically-significant difference was also found between the mean frequencies of micronuclei in nasal cells among the dental technicians (3.5 ± 1.80) and the controls (1.19 ± 0.53). The correlation between duration of exposure (13.1 ± 9.1 years) and frequencies of micronuclei was statistically significant in lymphocytes, but not in nasal cells of technicians. The results of multifactorial variance analysis revealed that occupational exposure was the only factor that significantly influenced the induction of micronuclei. In the exposed group, a significant correlation was found between urinary cobalt concentrations and frequencies of micronuclei in nasal cells, but not in lymphocytes.

The possible genotoxic effects of occupational exposure to cobalt alone or to hard-metal dust (WC–Co) was explored in a study using the in-vitro cytochalasin-B micronucleus test in lymphocytes as end-point for mutations (De Boeck *et al*., 2000). Micronuclei were scored both as binucleates and as mononucleates to discriminate between micronuclei accumulated during chronic exposure *in vivo* (mononucleates) and additional micronuclei expressed during the culture period *in vitro* (binucleates). The authors aimed to assess genotoxic effects in workers from cobalt refineries and hard-metal plants who were exposed at the time of the study to the TLV/time-weighted average (TWA) of cobalt-containing dust. The study comprised three groups of male workers: 35 workers (mean age, 38.5 ± 7.7 years; range, 27.7–55.3) exposed to cobalt dust from three refineries, 29 workers (mean age, 40.7 ± 12.4 years; range, 20.7–63.6) exposed to hard-metal dust (WC–Co) from two production plants and 27 matched control subjects (mean age, 38.0 ± 8.8 years; range, 23.3–56.4) recruited from the respective plants. In these three groups, the (geometric) mean concentration of cobalt in urine was 21.5 μg/g creatinine (range, 5.0–82.5) in workers exposed to cobalt, 19.9 μg/g creatinine (range, 4.0–129.9) in workers exposed to hard-metal dust and 1.7 μg/g creatinine (range, 0.6–5.5) in controls. The study design integrated additional complementary biomarkers of DNA damage: 8-hydroxy-

deoxyguanosine (8-OHdG) in urine, DNA single-strand breaks and formamido-pyrimidine DNA glycosylase (FPG)-sensitive sites with the alkaline Comet assay in mononuclear leukocytes. No significant increase in genotoxic effects was detected in workers exposed to cobalt-containing dust compared with controls. No difference in any genotoxicity biomarker was found between workers exposed to cobalt and to hard-metal dusts. The only statistically-significant difference observed was a higher frequency of micronucleated binucleate cytokinesis-blocked lymphocytes in workers exposed to cobalt compared to workers exposed to hard-metal dusts, but not in comparison with their concurrent controls. The frequency of micronucleated mononucleates did not vary among the different worker groups. Multiple regression analysis indicated that workers who smoked and were exposed to hard-metal dusts had elevated 8-OHdG and micronucleated mononucleate values. The authors concluded that workers exposed solely to cobalt-containing dust at TLV/TWA (20 μg cobalt/g creatinine in urine, equivalent to TWA exposure to 20 $\mu g/m^3$) did not show increased genotoxic effects but that workers who smoked and were exposed to hard-metal dusts form a specific occupational group which needs closer medical surveillance.

Hengstler *et al*. (2003) concluded from a study of workers co-exposed to cadmium, cobalt, lead and other heavy metals, that such mixed exposure may have genotoxic effects. The authors determined DNA single-strand break induction by the alkaline elution method in cryopreserved mononuclear blood cells of 78 individuals co-exposed to cadmium (range of concentrations in air, 0.05–138 $\mu g/m^3$), cobalt (range, 0–10 $\mu g/m^3$) and lead (range, 0–125 $\mu g/m^3$) and of 22 subjects without occupational exposure to heavy metals (control group). Non-parametric correlation analysis showed significant correlations between DNA single-strand breaks and cobalt ($p < 0.001$; $r = 0.401$) and cadmium ($p = 0.001$; $r = 0.371$) concentrations in air, but not lead concentrations. They elaborated a model with a logistic regression analysis and concluded from it that more than multiplicative effects existed for co-exposure to cadmium, cobalt and lead. Some concerns about the study were addressed by Kirsch-Volders and Lison (2003) who concluded that it did not provide convincing evidence to support the alarming conclusion of Hengstler *et al*. (2003).

4.4.2 *Experimental systems* (see Table 15 for references)

(*a*) *Metallic cobalt*

The results of tests for genetic and related effects of metallic cobalt, cobalt-metal alloys and cobalt (II) and (III) salts, with references, are given in Table 15.

Cobalt metal is active not only as a solid particle but also as a soluble compound.

The genetic toxicology of cobalt compounds has been reviewed by Domingo (1989), Jensen and Tüchsen (1990), Léonard and Lauwerys (1990), Beyersmann and Hartwig (1992), Hartwig (1995), Lison *et al.* (2001), National Institute of Environmental Health Sciences (2002) and De Boeck *et al.* (2003a). A report of the European Congress on Cobalt and Hard Metal Disease, summarizing the state of the art was published by Sabbioni *et al.* (1994b). The interactions of cobalt compounds with DNA repair processes (Hartwig, 1998;

Table 15. Genetic and related effects of cobalt

Test system	Result[a]		Dose[b] (LED/HID)	Reference
	Without exogenous metabolic system	With exogenous metabolic system		
Cobalt				
DNA breaks, alkaline elution, purified DNA (3T3 mouse cells)	+[c]		1 μg/mL (d_{50} = 4 μm)	Anard *et al.* (1997)
DNA breaks, alkaline elution, purified DNA (3T3 mouse cells)	r[c,d]		1 μg/mL + Na formate (d_{50} = 4 μm)	Anard *et al.* (1997)
Cell transformation, C3H10T1/2 mouse fibroblast cells, *in vitro*	–[c]		500 μg/mL (d_{50} ≤ 5 μm)	Doran *et al.* (1998)
Induction of FPG-sensitive sites, alkaline Comet assay, human mononuclear leukocytes, *in vitro*	–[c]		6 μg/mL (d_{50} = 4 μm)	De Boeck *et al.* (1998)
DNA breaks, alkaline elution, human lymphocytes, *in vitro*	+[c]		3 μg/mL (d_{50} = 4 μm)	Anard *et al.* (1997)
DNA single-strand breaks and alkali-labile sites, alkaline Comet assay, human mononuclear leukocytes, *in vitro*	+[c]		4.5 μg/mL (d_{50} = 4 μm)	Anard *et al.* (1997)
DNA single-strand breaks and alkali-labile sites, alkaline Comet assay, human mononuclear leukocytes, *in vitro*	+[c]		0.6 μg/mL (d_{50} = 4 μm)	Van Goethem *et al.* (1997)
DNA single-strand breaks and alkali labile sites, alkaline Comet assay, human mononuclear leukocytes, *in vitro*	+[c]		0.3 μg/mL (d_{50} = 4 μm)	De Boeck *et al.* (1998)
DNA single-strand breaks and alkali labile sites, human mononuclear leukocytes, *in vitro*	+		0.6 μg/mL (d_{50} = 4 μm)	De Boeck *et al.* (2003b)
DNA repair inhibition, alkaline Comet Assay, human mononuclear leukocytes, *in vitro*	+		5.5 μg/mL MMS, post-treatment 1.2 μg/mL Co (d_{50} = 4 μm)	De Boeck *et al.* (1998)
DNA repair inhibition, alkaline Comet Assay, human mononuclear leukocytes, *in vitro*	+		co-exposure 5.5 μg/mL MMS, 1.2 μg/mL Co (d_{50} = 4 μm)	De Boeck *et al.* (1998)
Micronucleus formation, binucleates, cytochalasin-B assay, human lymphocytes, *in vitro*	+[c]		0.6 μg/mL (d_{50} = 4 μm)	Van Goethem *et al.* (1997)
Micronucleus formation, binucleates, cytochalasin-B assay, human lymphocytes, *in vitro*	+		3 μg/mL (d_{50} = 4 μm)	De Boeck *et al.* (2003b)
Cell transformation (foci), human non-tumorigenic osteosarcoma osteoblast-like *in vitro*	–		3 μg/mL (d_{50} = 1–4 μm)	Miller *et al.* (2001)

Table 15 (contd)

Test system	Result[a]		Dose[b] (LED/HID)	Reference
	Without exogenous metabolic system	With exogenous metabolic system		
Cobalt alloys				
Co-Cr alloy				
Cell transformation, C3H10T1/2 mouse fibroblast cells, *in vitro*	–[c]		500 µg/mL ($d_5 \leq 5$ µm)	Doran *et al.* (1998)
rW-Ni-Co alloy				
DNA single-strand breaks, alkaline elution, human non-tumorigenic osteosarcoma cells, *in vitro*	+		5 mg/mL (d_{50} = 1–5 µm)	Miller *et al.* (2002)
Sister chromatid exchange, human non-tumorigenic osteosarcoma cells, *in vitro*	+		5 mg/mL (d_{50} = 1–5 µm)	Miller *et al.* (2002)
Micronucleus formation, human non-tumorigenic osteosarcoma osteoblast-like cells, *in vitro*	+		25 µg/mL (d_{50} = 1.5 µm)	Miller *et al.* (2001)
Micronucleus formation, human non-tumorigenic osteosarcoma osteoblast-like cells, *in vitro*	+		5 mg/mL (d_{50} = 1–5 µm)	Miller *et al.* (2002)
Cell transformation (foci), human non-tumorigenic osteosarcoma osteoblast-like cells, *in vitro*	+		50 µg/mL (d_{50} = 1–5 µm)	Miller *et al.* (2001)
Cell transformation (foci), human non-tumorigenic osteosarcoma osteoblast-like cells, *in vitro*	+		10 mg/mL (d_{50} = 1–5 µm)	Miller *et al.* (2002)
Cobalt-containing metal carbides				
Cr_3C_2-Co				
DNA single-strand breaks and alkali-labile sites, alkaline Comet assay, human mononuclear leukocytes *in vitro*	?		0.6 µg Co eq./mL	De Boeck *et al.* (2003b)
DNA single-strand breaks and alkali-labile sites, alkaline Comet assay, human mononuclear leukocytes, *in vitro*	e[d]		6 µg Co eq./mL	De Boeck *et al.* (2003b)
Micronucleus formation, binucleates, cytochalasin-B assay, human lymphocytes, *in vitro*	+		3 µg Co eq./mL	De Boeck *et al.* (2003b)
Micronucleus formation, binucleates, cytochalasin-B assay, human lymphocytes, *in vitro*	e[d]		6 µg Co eq./mL	De Boeck *et al.* (2003b)

Table 15 (contd)

Test system	Result[a]		Dose[b] (LED/HID)	Reference
	Without exogenous metabolic system	With exogenous metabolic system		
Mo_2C-Co				
DNA single-strand breaks and alkali-labile sites, alkaline Comet assay, human mononuclear leukocytes, *in vitro*	?		0.6 µg Co eq./mL	De Boeck *et al.* (2003b)
DNA single-strand breaks and alkali-labile sites, alkaline Comet assay, human mononuclear leukocytes, *in vitro*	s[d]		6 µg Co eq./mL	De Boeck *et al.* (2003b)
Micronucleus formation, binucleates, cytochalasin-B assay, human lymphocytes, *in vitro*	–		6 µg Co eq./mL	De Boeck *et al.* (2003b)
Micronucleus formation, binucleates, cytochalasin-B assay, human lymphocytes, *in vitro*	s[d]		6 µg Co eq./mL	De Boeck *et al.* (2003b)
NbC-Co				
DNA single-strand breaks and alkali-labile sites, alkaline Comet assay, human mononuclear leukocytes, *in vitro*	?		0.6 µg Co eq./mL	De Boeck *et al.* (2003b)
DNA single-strand breaks and alkali-labile sites, alkaline Comet assay, human mononuclear leukocytes, *in vitro*	s[d]		6 µg Co eq./mL	De Boeck *et al.* (2003b)
Micronucleus formation, binucleates, cytochalasin-B assay, human lymphocytes, *in vitro*	+		3 µg Co eq./mL	De Boeck *et al.* (2003b)
Micronucleus formation, binucleates, cytochalasin-B assay, human lymphocytes, *in vitro*	e[d]		6 µg Co eq./mL	De Boeck *et al.* (2003b)
WC-Co				
Induction of FPG-sensitive sites, alkaline Comet assay, human mononuclear leukocytes, *in vitro*	–[c]		6 µg Co eq./mL (Co d_{50} = 4 µm) (WC d_{50} < 1 µm)	De Boeck *et al.* (1998)
DNA breaks, alkaline elution, alkaline Comet assay, human lymphocytes, *in vitro*	+[c] s[e]		1.5 µg Co eq/mL (Co d_{50} = 4 µm) (WC d_{50} = 2 µm)	Anard *et al.* (1997)
DNA single-strand breaks and alkali-labile sites, alkaline Comet assay, human lymphocytes, *in vitro*	+[c]		3 µg Co eq./mL (Co d_{50} = 4 µm) (WC d_{50} = 2 µm)	Anard *et al.* (1997)
DNA single-strand breaks and alkali-labile sites, alkaline Comet assay, human mononuclear leukocytes, *in* vitro	+[c]		0.6 µg Co eq./mL (Co d_{50} = 4 µm) (WC d_{50} = < 1 µm)	Van Goethem *et al.* (1997)

Table 15 (contd)

Test system	Result[a]		Dose[b] (LED/HID)	Reference
	Without exogenous metabolic system	With exogenous metabolic system		
DNA single-strand breaks and alkali-labile sites, alkaline Comet assay, human mononuclear leukocytes, *in vitro*	e[d]		6 µg Co eq./mL (Co d_{50} = 4 µm) (WC d_{50} = < 1 µm)	Van Goethem *et al.* (1997)
DNA single-strand breaks and alkali-labile sites, alkaline Comet assay, human mononuclear leukocytes, *in vitro*	+[c]		0.3 µg Co eq./mL (Co d_{50} = 4 µm) (WC d_{50} = < 1 µm)	De Boeck *et al.* (1998)
DNA single-strand breaks and alkali-labile sites, alkaline Comet assay, human mononuclear leukocytes, *in vitro*	e[d]		6 µg Co eq./mL (Co d_{50} = 4 µm) (WC d_{50} = < 1 µm)	De Boeck *et al.* (1998)
DNA single-strand breaks and alkali-labile sites, alkaline Comet assay, human mononuclear leukocytes, *in vitro*	?		0.6 µg Co eq./mL (Co d_{50} = 4 µm) (WC d_{50} = < 1 µm)	De Boeck *et al.* (2003b)
DNA single-strand breaks and alkali-labile sites, alkaline Comet assay, human mononuclear leukocytes, *in vitro*	e[d]		6 µg Co eq./mL (Co d_{50} = 4 µm) (WC d_{50} = < 1 µm)	De Boeck *et al.* (2003b)
Micronucleus formation, binucleates, cytochalasin-B assay, human lymphocytes, *in vitro*	+[c]		0.6 µg Co eq./mL (Co d_{50} = 4 µm) (WC d_{50} = < 1 µm)	Van Goethem *et al.* (1997)
Micronucleus formation, binucleates, cytochalasin-B assay, human lymphocytes, *in vitro*	e[d]		3 µg Co eq./mL (Co d_{50} = 4 µm) (WC d_{50} = < 1 µm)	Van Goethem *et al.* (1997)
Micronucleus formation, binucleates, cytochalasin-B assay, human lymphocytes, *in vitro*	+		0.6 µg Co eq./mL (Co d_{50} = 4 µm) (WC d_{50} < 1 µm)	De Boeck *et al.* (2003b)
Micronucleus formation, binucleates, cytochalasin-B assay, human lymphocytes, *in vitro*	e[d]		6 µg Co eq./mL(Co d_{50} = 4 µm) (WC d_{50} < 1 µm)	De Boeck *et al.* (2003b)
DNA single-strand breaks or alkali-labile sites, alkaline Comet assay, male Wistar rat type II pneumocytes, *in vivo*	+		16.6 mg/kg, i.t. (Co d_{50} = 4 µm) (WC d_{50} < 1 µm)	De Boeck *et al.* (2003c)
DNA single-strand breaks or alkali-labile sites, alkaline Comet assay, male Wistar rat BALF cells, *in vivo*	–		16.6 mg/kg i.t. (Co d_{50} = 4 µm) (WC d_{50} < 1 µm)	De Boeck *et al.* (2003c)
DNA single-strand breaks or alkali-labile sites, alkaline Comet assay, Wistar male rat mononuclear leukocytes, *in vivo*	–		16.6 mg/kg i.t. (Co d_{50} = 4 µm) (WC d_{50} < 1 µm)	De Boeck *et al.* (2003c)
Micronucleus formation, male Wistar rat type II pneumocytes, *in vivo*	+		16.6 mg/kg i.t. (Co d_{50} = 4 µm) (WC d_{50} < 1 µm)	De Boeck *et al.* (2003c)
Micronucleus formation, cytochalasin-B assay, male Wistar rat lymphocytes, *in vivo*	–		49.8 mg/kg i.t. (Co d_{50} = 4 µm) (WC d_{50} < 1 µm)	De Boeck *et al.* (2003c)

Table 15 (contd)

Test system	Result[a]		Dose[b] (LED/HID)	Reference
	Without exogenous metabolic system	With exogenous metabolic system		
rWC-Co particles				
DNA breaks, alkaline elution, purified DNA (3T3 mouse cells)	+[c] e[d]		1 μg Co eq./mL (Co d_{50} = 4 μm) (WC d_{50} = 2 μm)	Anard *et al.* (1997)
DNA breaks, alkaline elution, purified DNA (3T3 mouse cells)	r[e]		1 μg Co eq./mL + Na formate (Co d_{50} = 4 μm) (WC d_{50} = 2 μm)	Anard *et al.* (1997)
DNA breaks, alkaline elution, human mononuclear lymphocytes, *in vitro*	+[c]		1.5 μg Co eq/mL (Co d_{50} = 4 μm) (WC d_{50} = 2 μm)	Anard *et al.* (1997)
Cobalt compounds				
Co(II) salts				
Cobalt(II) acetate				
Inhibition of repair of UV-induced pyrimidine dimers, nucleoid sedimentation, human HeLa S-3 cells, *in vitro*	+		100 μM	Snyder *et al.* (1989)
Enhancement of cell transformation by simian adenovirus SA7, Syrian hamster embryo cells, *in vitro*	+		0.2 mM	Casto *et al.* (1979)
DNA base damage (products of hydroxyl radical attack), female and male Fischer 344/NCr rats, *in vivo*	+ (kidney > liver > lung)		ip, single, 50 μM/kg	Kasprzak *et al.* (1994)
Cobalt(II) chloride				
Reduction of fidelity of DNA replication by substitution of Mg^{2+} *Escherichia coli* DNA polymerase, sea-urchin nuclear DNA polymerase, avian myeloblastosis virus DNA polymerase	+		1 mM [130 μg/mL]	Sirover & Loeb (1976)
Prophage induction, *Escherichia coli*	–		~ 320 μM[f] [415 μg/mL]	Rossman *et al.* (1984)
Escherichia coli $WP2_s$ inhibition of protein synthesis	+		6.25 μg/mL	Leitão *et al.* (1993)
Escherichia coli ABI886, inhibition of protein synthesis	+		6.25 μg/mL	Leitão *et al.* (1993)
Salmonella typhimurium TA100, reverse mutation	–		NG	Ogawa *et al.* (1986)
Salmonella typhimurium TA102, reverse mutation	–	–	40 ppm [40 μg/mL]	Wong (1988)

Table 15 (contd)

Test system	Result[a]		Dose[b] (LED/HID)	Reference
	Without exogenous metabolic system	With exogenous metabolic system		
Salmonella typhimurium TA1535, reverse mutation	–		NG	Arlauskas *et al*. (1985)
Salmonella typhimurium TA1535, reverse mutation	–	–	40 ppm [40 µg/mL]	Wong (1988)
Salmonella typhimurium TA1537, reverse mutation	–		NG	Arlauskas *et al*. (1985)
Salmonella typhimurium TA1537, reverse mutation	–		1000 µmol/plate [130 000 µg/plate]	Ogawa *et al.* (1986)
Salmonella typhimurium TA1537, reverse mutation	+	–	40 ppm [40 µg/mL]	Wong (1988)
Salmonella typhimurium TA1538, reverse mutation	–		NG	Arlauskas *et al*. (1985)
Salmonella typhimurium TA98, reverse mutation	–		NG	Arlauskas *et al*. (1985)
Salmonella typhimurium TA98, reverse mutation	–		NG	Ogawa *et al.* (1986)
Salmonella typhimurium TA98, reverse mutation	+	–	40 ppm [40 µg/mL]	Wong (1988)
Salmonella typhimurium TA2637, reverse mutation	–		1000 µmol/plate [130 000 µg/plate]	Ogawa *et al.* (1986)
Salmonella typhymurium, TA97 preincubation assay	+		100 µM [13 µg/mL]	Pagano & Zeiger (1992)
Salmonella typhimurium, TA97 preincubation assay	r[d]		100 µM [13 µg/mL] + DEDTC 420 µM	Pagano & Zeiger (1992)
Escherichia coli SY1032/pKY241 transfected with pUB_3, *supF* tRNA locus, mutation	+		20 µM [2.6 µg/mL]	Ogawa *et al.* (1999)
Bacillus subtilis rec strains H17/M45, growth inhibition	–		[325 µg/plate]	Nishioka (1975)
Bacillus subtilis rec strain H17, growth inhibition	+		[325 µg/plate]	Kanematsu *et al.* (1980)
Saccharomyces cerevisiae SBTD-2B, 'petite' mutation, respiratory deficiency	+		2 mM [260 µg/mL]	Prazmo *et al*. (1975)
Saccharomyces cerevisiae, strain 197/2d, 'petite' mutation	+		4 mM [520 µg/mL]	Putrament *et al.* (1977)
Saccharomyces cerevisiae, strain 197/2d, erythromycin-resistant mutation	–		4 mM [520 µg/mL]	Putrament *et al.* (1977)
Saccharomyces cerevisiae, 'petite' mutation, respiratory deficiency	(+)		640 µg/mL	Egilsson *et al.* (1979)
Saccharomyces cerevisiae D7, *ilv* gene mutation	–		10 mM [1300 µg/mL]	Fukunaga *et al.* (1982)
Saccharomyces cerevisiae D7, trp gene conversion	+		10 mM [1300 µg/mL]	Fukunaga *et al.* (1982)
Saccharomyces cerevisiae D7, *ilv* gene mutation	–		100 mM [13 000 µg/mL]	Singh (1983)
Saccharomyces cerevisiae D7, trp gene conversion	(+)		100 mM [13 000 µg/mL]	Singh (1983)

Table 15 (contd)

Test system	Result[a]		Dose[b] (LED/HID)	Reference
	Without exogenous metabolic system	With exogenous metabolic system		
Saccharomyces cerevisiae D7, trp gene conversion	(+)		1500 µg/mL [11.5 mM]	Kharab & Singh (1985)
Saccharomyces cerevisiae D7, *ilv* gene mutation	(+)		3000 µg/mL [23 mM]	Kharab & Singh (1985)
Saccharomyces cerevisiae D7, 'petite' mutation, respiratory deficiency	+		750 µg/mL [5.76 mM]	Kharab & Singh (1987)
Drosophila melanogaster, gene mutation or mitotic recombination, wing spot test mwh/flr	+		2 mM [260 µg/mL]	Ogawa *et al.* (1994)
Drosophila melanogaster, gene mutation or reduced mitotic recombination, wing spot test mwh/TM3	–		8 mM [1040 µg/mL]	Ogawa *et al.* (1994)
DNA strand breaks, alkaline sucrose gradient, Chinese hamster ovary cells, *in vitro*	+		2 mM [260 µg/mL]	Hamilton-Koch *et al.* (1986)
DNA strand breaks, nucleoid sedimentation assay, Chinese hamster ovary cells, *in vitro*	–		10 mM [1300 µg/mL]	Hamilton-Koch *et al.* (1986)
DNA strand breaks, nucleoid sedimentation, human HeLa cells, *in vitro*	+		50 µM [65 µg/mL]	Hartwig *et al.* (1990)
DNA-protein cross links, rat Novikoff ascites hepatoma cells, *in vitro*	+		1 mM [130 µg/mL]	Wedrychowski *et al.* (1986)
Gene mutation, Chinese hamster V79 cells, *Hprt* locus, *in vitro*	(+)		0.2 mM [26 µg/mL]	Miyaki *et al.* (1979)
Gene mutation, Chinese hamster V79 cells, *Hprt* locus, *in vitro*	+		100 µM [13 µg/mL]	Hartwig *et al.* (1990)
Gene mutation, Chinese hamster V79 cell line, *Gpt* locus, *in vitro*	–		100 µM [13 µg/mL]	Kitahara *et al.* (1996)
Gene mutation, Chinese hamster transgenic cell line G12, *Gpt* locus, *in vitro*	+		50 µM [6.5 µg/mL]	Kitahara *et al.* (1996)
Sister chromatid exchanges, mouse macrophage-like cells P388D$_1$, *in vitro*	+		100 µM [13 µg/mL]	Andersen (1983)
Cell transformation, C3H10T1/2 mouse fibroblast cells, *in vitro*	+[c]		38 µM [5 µg/mL]	Doran *et al.* (1998)
Reduction in colony forming, V79 Chinese hamster cells, *in vitro*	+ (42%)		180 µM [24 µg/mL]	Kasten *et al.* (1992)
Reduction of cloning efficiency, Chinese hamster ovary cells, *in vitro*	+ (50%)		4 mM [520 µg/mL]	Hamilton-Koch *et al.* (1986)
Displacement of acridine orange from DNA, calf thymus DNA and *Micrococcus luteus* DNA	+		0.33 mM [43 µg/mL]	Richardson *et al.* (1981)
Formation of metal–DNA complex, calf thymus B-DNA	+		NG	Aich *et al.* (1999)

Table 15 (contd)

Test system	Result[a]		Dose[b] (LED/HID)	Reference
	Without exogenous metabolic system	With exogenous metabolic system		
Induction of reporter gene expression under the control of the promoter region of the metallothionein gene, chick embryo liver cells transfected with luciferase or chloramphenicol acetyl transferase, *in vitro*	+		112 µM [15 µg/mL]	Lu *et al.* (1996)
Production of reactive oxygen species (fluorescence from oxidation of DCFH-DA), A549 cells, human lung cells, *in vitro*	+		200 µM [26 µg/ml]	Salnikow *et al.* (2000)
Production of reactive oxygen species (fluorescence from oxidation of DCFH-DA), A549 cells, human lung cells, *in vitro*	r[d]		300 µM [39 µg/mL] + 2-mercapto-ethanol	Salnikow *et al.* (2000)
Production of reactive oxygen species (fluorescence from oxidation of DCFH-DA), A549 cells, human lung cells, *in vitro*	r[d]		300 µM [39 µg/mL] + vitamin E	Salnikow *et al.* (2000)
DNA strand breaks, fluorescence analysis of DNA unwinding, human white blood cells, *in vitro*	+		50 µM [6.5 µg/mL]	McLean *et al.* (1982)
DNA strand breaks, alkaline sucrose gradient, human diploid fibroblasts, *in vitro*	+		5 mM	Hamilton-Koch *et al.* (1986)
DNA strand breaks, nick translation, human diploid fibroblasts, *in vitro*	+		10 mM [1300 µg/mL]	Hamilton-Koch *et al.* (1986)
DNA strand breaks, nucleoid sedimentation, human diploid fibroblasts, *in vitro*	–		10 mM [1300 µg/mL]	Hamilton-Koch *et al.* (1986)
DNA strand breaks and alkali-labile sites, alkaline Comet assay, human mononuclear leukocytes, *in vitro*	+[c]		0.3 µg/mL	De Boeck *et al.* (1998)
Induction of gene expression (*Cap43*), A549 cells, human lung cells, *in vitro*	+		100 µM [13 µg/mL]	Salnikow *et al.* (2000)
Induction of gene expression (*Cap43*), A549 cells, human lung cells, *in vitro*	s[d]		100 µM [13 µg/mL] + 2-mercapto-ethanol	Salnikow *et al.* (2000)
Induction of gene expression (*Cap43*), A549 cells, human lung cells, *in vitro*	s[d]		100 µM [13 µg/mL] + H_2O_2	Salnikow *et al.* (2000)
Sister chromatid exchange, human lymphocytes, *in vitro*	+		10 µM [1.3 µg/mL]	Andersen (1983)
Aneuploidy, human lymphocytes, *in vitro*	+		3.7 µg/mL	Resende de Souza-Nazareth (1976)

Table 15 (contd)

Test system	Result[a]		Dose[b] (LED/HID)	Reference
	Without exogenous metabolic system	With exogenous metabolic system		
Aneuploidy, pseudodiploidy and hyperploidy, bone marrow of male hamsters, *in vivo*	+		400 mg/kg bw ip[g]	Farah (1983)
Aneuploidy, pseudodiploidy and hyperploidy, testes of hamsters, meiosis 1, *in vivo*	+		400 mg/kg bw ip[g]	Farah (1983)
Inhibition of binding of p53 protein to p53 consensus sequence on linear DNA fragment	+ (full)		> 100 µM (300 µM)	Palecek *et al.* (1999)
Inhibition of binding of p53 protein to supercoiled DNA	+		600 µM	Palecek *et al.* (1999)
Affinity of reconstituted apopolypeptide (Zn finger protein) with estrogen response element consensus oligonucleotide	r		NG (K_D 0.720 µM)[h]	Sarkar (1995)
Inactivation of bacterial Fpg protein (with Zn finger domain), conversion of supercoiled bacteriophage PM2 DNA into open circular form, electrophoresis	–		1000 µM	Asmuss *et al.* (2000)
Inhibition of XPA (with Zn finger domain) binding to UV-irradiated oligonucleotide, gel mobility shift analysis	+		50 µM [6.5 µg/mL]	Asmuss *et al.* (2000)
Cobalt(II) chloride hexahydrate				
Lysogenic induction, *Escherichia coli* $WP2_s$ (λ)	r[i]		(10 µg /mL)[f] + UV	Leitão *et al.* (1993)
Lysogenic induction, *Escherichia coli* K12 ABI886 (λ)	r[i]		(10 µg/mL)[k] + UV	Leitão *et al.* (1993)
Lysogenic induction, *Escherichia coli* ABI157 (λ)	+		100 µg/mL – Mg	Leitão *et al.* (1993)
Phage reactivation, *Escherichia coli* ABI157 (λ)	–		250 µg/mL – UV	Leitão *et al.* (1993)
Phage reactivation, *Escherichia coli* ABI157 (λ)	e[i]		62.5 µg/mL + UV	Leitão *et al.* (1993)
Escherichia coli WP2, reverse mutation	–[l1]		20 µg/mL [84 µM]	Kada & Kanematsu (1978)
Escherichia coli WP2 *uvrA*, reverse mutation	–		NG	Arlauskas *et al.* (1985)
Escherichia coli $WP2_s$ gene mutation	–[l1]		50 µg/mL [210 µM]	Leitão *et al.* (1993)
Escherichia coli $WP2_s$ gene muation	r[i]		50 µg/mL + UV	Leitão *et al.* (1993)
Saccharomyces cerevisiae D7, 'petite' mutation, respiratory deficiency	+		[130 µg/mL]	Lindegren *et al.* (1958)
Salmonella typhimurium TA100, reverse mutation	–		100 mM [23 800 µg/mL]	Tso & Fung (1981)
Salmonella typhimurium TA100, reverse mutation	–		NG	Arlauskas *et al.* (1985)

Table 15 (contd)

Test system	Result[a]		Dose[b] (LED/HID)	Reference
	Without exogenous metabolic system	With exogenous metabolic system		
Salmonella typhimurium TA1538, reverse mutation	–[i2]		20 µg/mL [84 µM]	Mochizuki & Kada (1982)
Salmonella typhimurium TA98, reverse mutation	–[i2]		20 µg/mL [84 µM]	Mochizuki & Kada (1982)
Bacillus subtilis strain NIG 1125, reverse mutation	–[i3]		30 µg/mL [126 µM]	Inoue *et al.* (1981)
Gene mutation, mouse lymphoma L5178Y cells, *Tk* locus, *in vitro*	–		57.11 µg/mL	Amacher & Paillet (1980)
Gene mutation, Chinese hamster V79 cells, *8AG* locus, *in vitro*	–		9 µM [2 µg/mL]	Yokoiyama *et al.* (1990)
Gene mutation, Chinese hamster V79 cells, *8AG* locus, *in vitro*	r[i]		3 µM [0.7 µg/mL] + γ rays	Yokoiyama *et al.* (1990)
Micronucleus formation, BALB/c mouse bone marrow, *in vitro*	–	–	50 µg/mL [385 µM]	Suzuki *et al.* (1993)
DNA strand breaks, alkaline elution, human lymphocytes, *in vitro*	–		102 µM [25 µg/mL]	Anard *et al.* (1997)
Inhibition of nucleotide excision repair (incision and polymerization steps) of UV-induced DNA damage, alkaline unwinding, VH16 human fibroblasts	+		50 µM [12 µg/mL]	Kasten *et al.* (1997)
Inhibition of nucleotide excision repair (ligation step) of UV-induced DNA damage, alkaline unwinding, VH16 human fibroblasts	–		200 µM [48 µg/mL]	Kasten *et al.* (1997)
Inhibition of UV-induced cyclobutane pyrimidine dimers (incision step), alkaline unwinding + T4 endonuclease V, VH16 human fibroblasts	+		150 µM [86 µg/mL]	Kasten *et al.* (1997)
Micronucleus formation, male BALB/c AnNCrj mouse bone marrow, *in vivo*	+		50 mg/kg bw	Suzuki *et al.* (1993)
Micronucleus formation, male BALB/c AnNCrj mouse bone marrow, *in vivo*	e[i]		50 mg/kg + DMH 20 mg/kg	Suzuki *et al.* (1993)
Micronucleus formation, male BALB/c AnNCrj mouse bone marrow, *in vivo*	e[i]		50 mg/kg + benzo(a)pyrene 50 mg/kg	Suzuki *et al.* (1993)
Micronucleus formation, male BALB/c AnNCrj mouse bone marrow, *in vivo*	e[i]		50 mg/kg + 2-naphthylamine 200 mg/kg	Suzuki *et al.* (1993)

Table 15 (contd)

Test system	Result[a]		Dose[b] (LED/HID)	Reference
	Without exogenous metabolic system	With exogenous metabolic system		
Cobalt(II) molybdenum(VI) oxide				
Enhancement of cell transformation by simian adenovirus SA7, Syrian hamster embryo cells, *in vitro*	+		250 µM [55 µg/mL]	Casto *et al.* (1979)
Cobalt(II) nitrate				
Chromosome aberrations (numerical), human diploid fibroblasts WI.38 and MRC_5, *in vitro*	–		0.08 µM[g] [0.015 µg/mL]	Paton & Allison (1972)
Chromosome aberrations (numerical), human mononuclear leucocytes, *in vitro*	–		0.8 µM[g] [0.15 µg/mL]	Paton & Allison (1972)
Cobalt(II) nitrate hexahydrate				
Drosophila melanogaster (*flr^3/In(3LR)TM3, $r^i p^P$Sep $bx^{34e} e^5$Ser*) × (*mwh*).*mwh* and *fbr^3*, gene mutations, chromosomal deletion, non disjunction or mitotic recombination (small single spots and large single spots), SMART test	+		1 mM [291 µg/mL]	Ye°ilada (2001)
Drosophila melanogaster (*flr^3/In(ELR)TM3, $r^i p^P$Sep $bx^{34e} e^5$Ser)* × *(mwh).mwh and fbr^3*, mitotic recombination (twin spots), SMART test	+		10 mM [2910 µg/mL]	Ye°ilada (2001)
Cobalt(II) sulfate				
Allium cepa, chromosomal aberrations	+		20 µM [3 µg/mL]	Gori & Zucconi (1957)
Allium cepa, aneuploidy	+		100 µM [15 µg/mL] for 5 days + H_20 for 3 days	Gori & Zucconi (1957)
Production of reactive oxygen species (degradation of 2-deoxyribose), malondialdehyde assay	+		1 µM [0.155 µg/mL]	Ball *et al.* (2000)
Production of reactive oxygen species (degradation of 2-deoxyribose), malondialdehyde assay	r[d]		50 µM [7.8 µg/mL] + desferrioxamine 1 mM	Ball *et al.* (2000)
Chemical changes in DNA bases GC/MS-SIM, calf thymus DNA	–		25 µM [4 µg/mL]	Nackerdien *et al.* (1991)
Chemical changes in DNA bases GC/MS-SIM, calf thymus DNA	+[d]		25 µM [4 µg/mL] + H_2O_2 208 mM	Nackerdien *et al.* (1991)

Table 15 (contd)

Test system	Result[a]		Dose[b] (LED/HID)	Reference
	Without exogenous metabolic system	With exogenous metabolic system		
Bacillus subtilis rec strain H17, growth inhibition	(+)		388 µg/plate	Kanematsu *et al.* (1980)
Cytoskeletal perturbation of microtubules and microfilaments, mouse cells SWISS 3T3, *in vitro*	+		100 µM [15.5 µg/mL]	Chou (1989)
Chemical changes in DNA bases GC/MS-SIM, isolated human chromatin, K562 cells	–		25 µM [4 µg/mL]	Nackerdien *et al.* (1991)
Chemical changes in DNA bases GC/MS-SIM, isolated human chromatin, K562 cells	+[d]		25 µM + H_2O_2 208 mM	Nackerdien *et al.* (1991)
Chemical changes in DNA bases GC/MS-SIM, isolated human chromatin, K562 cells	r[m]		25 µM [4 µg/mL] + H_2O_2 208 mM + EDTA 120 µM	Nackerdien *et al.* (1991)
Chemical changes in DNA bases GC/MS-SIM, isolated human chromatin, K562 cells	r[m]		25 µM + H_2O_2 208 mM + mannitol 50 mM	Nackerdien *et al.* (1991)
Chemical changes in DNA bases GC/MS-SIM, isolated human chromatin, K562 cells	r[m]		25 µM + H_2O_2 208 mM + DMSO 50 mM	Nackerdien *et al.* (1991)
Chemical changes in DNA bases GC/MS-SIM, isolated human chromatin, K562 cells	r[m], e[m]		25 µM + H_2O_2 208 mM + glutathione 1 mM	Nackerdien *et al.* (1991)
Chemical changes in DNA bases GC/MS-SIM, isolated human chromatin, K562 cells	e[m]		25 µM + H_2O_2 208 mM + SOD 200 units/mL	Nackerdien *et al.* (1991)
Induction of human metal-inducible genes (*MT-IIA*, *hsp70*, *c-fos*), HeLa human cervical carcinoma cells, *in vitro*	+[n]		500 µM	Murata *et al.* (1999)
Metal responsive element (MRE)-DNA binding activity, HeLa human cervical carcinoma cells, *in vitro*	–		500 µM	Murata *et al.* (1999)
Heat shock element (HSE)-DNA binding activity, HeLa human cervical carcinoma cells, *in vitro*	?		500 µM	Murata *et al.* (1999)
Cobalt(II) sulfate monohydrate				
Cell transformation, Syrian hamster embryo cells, *in vitro*	+		0.125 µg/mL [0.75 µM]	Kerckaert *et al.* (1996)

Table 15 (contd)

Test system	Result[a]		Dose[b] (LED/HID)	Reference
	Without exogenous metabolic system	With exogenous metabolic system		
Cobalt(II) sulfate heptahydrate				
Salmonella typhimurium TA100, reverse mutation	+	–[o]	100 µg/plate	Zeiger *et al.* (1992)
Salmonella typhimurium TA98, TA1535, reverse mutation	–	–	10 000 µg/plate	Zeiger *et al.* (1992)
Induction of p53, ELISA assay, NCTC929 mouse fibroblasts, *in vitro*	+		50 µg/mL [178 µM]	Duerksen-Hughes *et al.* (1999)
CO(II)acetate tetrahydrate				
Chromosomal aberrations, human lymphocytes, *in vitro*	–		0.6 µg/mL [2.4 µM]	Voroshilin *et al.* (1978)
Co(III)hexaamine ions and Co(III) amine complexes				
Conformational changes of DNA oligonucleotides, circular dichroism and NMR spectroscopy	+		µM range (< 24 µM)	Bauer & Wang (1997)
Co(III) complexes				
Escherichia coli, strains AB1157 (wild type), AB1886 *uvr*A6, GW801 *rec*A56, GW802 *rec*56 *uvr*A6, GW803 *rec*A56 *lex*A$^-$, PAM 5717 *lex*A and AB1899 *lon*, DNA repair assay	+ (8/15)[p] (+) (7/15)[p]		NG NG	Schultz *et al.* (1982)
Salmonella typhimurium, strain TA100, TA98, TA92, reverse mutation	+ (4/15)		0.1–0.5 µmol/plate	Schultz *et al.* (1982)
Salmonella typhimurium, strain TA1535, 1537, 1538. reverse mutation	–		2 µmol/plate	Schultz *et al.* (1982)
Co(III) salts				
$CoN0_3$				
Pisum abyssinicum chlorophyll mutation	+[q]		0.1–1 mM [18.3–183 µg/mL]	von Rosen (1964)
Co(III) complexes with desferal				
Plasmid PBR322, scission of double-stranded DNA	+		≤ 42.5 µM[r] + H_2O_2 4 mM	Joshi & Ganesh (1992)
$Co(OH)_3$				
Bacillus subtilis rec strain H17, growth inhibition	(+)		[2750 µg/plate]	Kanematsu *et al.* (1980)

Table 15 (contd)

Test system	Result[a]		Dose[b] (LED/HID)	Reference
	Without exogenous metabolic system	With exogenous metabolic system		
Co(III) Schiff–base complex				
Inhibition of Zn-finger transcription factor, HNMR spectroscopy	+		0.5 mM	Louie & Meade (1998)
Inhibition of Zn-finger transcription factor, Sp1, gel shift, filter binding assay	+		10 µM	Louie & Meade (1998)
Cobalt sulfides (2^+) and (4^+)				
CoS particles				
DNA strand breaks, alkaline sucrose gradient, Chinese hamster CHO cells, *in vitro*	+		10 µg/mL	Robison *et al.* (1982)
Gene mutation, Chinese hamster transgenic cell line G10, *Gpt* locus, *in vitro*	–		1 µg/cm^2	Kitahara *et al.* (1996)
Gene mutation, Chinese hamster transgenic cell line G10, *Gpt* locus, *in vitro*	s[d]		1 µg/cm^2 + H_2O_2 10 µM	Kitahara *et al.* (1996)
Gene mutation, Chinese hamster transgenic cell line G12, *Gpt* locus, *in vitro*	+		0.5 µg/cm^2	Kitahara *et al.* (1996)
Gene mutation, Chinese hamster transgenic cell line G12, *Gpt* locus, *in vitro*	s[d]		0.5 µg/cm^2 + H_2O_2 10 µM	Kitahara *et al.* (1996)
CoS (amorphous)				
Cell transformation, Syrian hamster embryo cells, *in vitro*	(+)		10 µg/mL (d_{50} = 2.0 µm)	Abbracchio *et al.* (1982); Costa *et al.* (1982)
CoS_2 (crystalline)				
Cell transformation, Syrian hamster embryo cells, *in vitro*	+[g]		1 µg/mL (d_{50} = 1.25 µm)	Abbracchio *et al.* (1982); Costa *et al.* (1982)

Table 15 (contd)

rW-Ni-Co alloy, reconstituted mixture of W (92%), Ni (5%) and Co (3%) particles; rWC-Co, reconstituted mixture of WC (94%) and Co (6%) particles; HNMR, proton nuclear magnetic resonance

[a] ?, inconclusive; +, positive; (+), weak positive; –, negative; r = reduction; e = enhancement; s = stable

[b] LED, lowest effective dose; HID, highest ineffective dose; ip, intraperitoneally; po, orally; i.t., intratracheal instillation; MMS, methylmethane sulfonate; DMSO, dimethylsulfoxide; EDTA, ethyldiaminetetraacetate; DEDTC, diethyldithiocarbamate; NG, not given; DCFH-D, 2′,7′-dichlorofluorescine diacetate; DMH, 1,1-dimethylhydrazine; 8AG, 8-azaguanine; H_2O_2, hydrogen peroxide; SOD, superoxide dismutase; UV, ultraviolet irradiation

[c] Refer to the same experiment where Co and WC–Co were compared

[d] as compared to CO

[e] as compared to rWC-Co

[f] Estimated from a graph in the paper

[g] Total dose given to each animal over nine days

[h] This value corresponds to the dissociation constant (K_D) for cobalt-reconstituted polypeptide binding with estrogen response element consensus oligonucleotide

[i] as compared to the other mutagen used

[j] toxic dose; highest ineffective subtoxic dose was not given.

[k] Similar effect to strain *E. coli* WP2s(λ), but data not shown in the paper

[l1, 2 or 3] antimutagenic effect; [l1], inhibition of mutagenesis induced by *N*-methyl-*N*′-nitrosoguanidine (MNNG); [l2], inhibition of mutagenesis induced by 3-amino-1,4-dimethyl-5*H*-pyrido[4,3-*b*]indole (Trp-P-1) or [l3], inhibition of spontaneous mutability

[m] as compared to Co + H_2O_2

[n] metallothionein (*MT-IIA*) and heat shock protein (*hsp70*) genes were induced but not *c-fos* gene.

[o] Tested at doses up to 10 000 μg/plate

[p] The ratio corresponds to the number of Co(III) complexes positive for DNA repair assay on the total number of Co(III) complexes tested

[q] Co as EDTA chelate (Co-EDTA) was also positive.

[r] Optimal concentration for 100% DNA cleavage; slight increase in concentration over this value lead to extensive degradation.

[s] more than corresponding amorphous salt

Hartwig & Schwerdtle, 2002) and with zinc finger proteins (Hartwig, 2001) and their effect on gene expression (Beyersman, 2002) have been reviewed. An evaluation of carcinogenic risks of cobalt and cobalt compounds was published in 1991 (IARC, 1991).

Metallic cobalt particles (median diameter (d_{50}) = 4 μm) have been shown with alkaline elution technology to induce DNA breakage and/or alkali-labile sites in DNA purified from 3T3 mouse cells. Similar changes have been demonstrated *in vitro* in human mononuclear leukocytes by both the alkaline elution and the Comet assay methods. Oxidative DNA damage was not detected at FPG-sensitive sites with the Comet methodology. In experiments run in parallel, a statistically-significant induction of micronuclei in binucleated human lymphocytes was obtained with the cytochalasin-B method. In-vitro cell transformation was not induced in mouse fibroblast cells by cobalt particles ($d_{50} \leq 5$ μm) nor in human osteoblast-like cells by approximately same size (d_{50} = 1–4 μm) cobalt particles.

Metallic cobalt (d_{50} = 1–5 μm) has been tested in combination with tungsten and nickel particles. *In vitro*, the mixture induced DNA single-strand breaks as shown by alkaline elution methodology, micronuclei, and cell transformation in human non-tumorigenic osteosarcoma osteoblast-like cell line (TE85, clone F-5).

(*b*) *Hard-metal particles*

When tested *in vitro* over a range of cobalt equivalent concentrations, a mixture of tungsten carbide and cobalt metal (WC–Co), caused significantly more (on average threefold more) DNA breaks than cobalt particles alone, both in isolated human DNA and in cultured human lymphocytes (alkaline elution and Comet assays); this DNA damage was inhibited by scavenging activated oxygen species. In the same assay run in parallel, cobalt chloride did not cause DNA breaks. Dose-dependency and time-dependency of DNA breakage and of induction of alkali-labile sites were shown for hard-metal particles in the Comet assay (De Boeck *et al.*, 2003b). A similarly greater genotoxic activity of hard metal compared with cobalt-metal particles alone has been found with the cytokinesis-blocked micronucleus test when applied *in vitro* to human lymphocytes. The data demonstrate clearly that interaction of cobalt with tungsten carbide particles leads to enhanced mutagenicity. Recently, this observation has been extended to other carbides. In the in-vitro cytokinesis-blocked micronucleus test, while the metal carbides alone did not increase the micronucleus frequency, cobalt alone and the four tested carbide–cobalt mixtures induced statistically-significant concentration-dependent increases in micronucleated binucleates. As with the tungsten carbide–cobalt metal mixture, nobium carbide and chromium carbide particles were able to interact with cobalt, producing greater mutagenic effects than those produced by the particles of the individual metals. Molybdenum carbide particles did not display interactive mutagenicity with cobalt in the micronucleus test, possibly because of their small specific surface area, compactness and/or spherical shape (De Boeck *et al.*, 2003b). However, with the Comet assay, when also performed directly at the end of the treatment, no firm conclusion could be made.

From a mechanistic point of view, the in-vitro studies comparing the effects of cobalt metal alone and the hard-metal mixture (WC–Co) provide convincing evidence that the

mutagenic activity of metallic cobalt is not exclusively mediated by the ionic form dissolved in biological media (Anard *et al.*, 1997). However, the dissolved cations do play an important role through direct or indirect mutagenic effects as reviewed separately for the soluble Co(II) and Co(III) compounds.

In-vivo experimental data on the mutagenicity of cobalt particles alone are lacking. Evidence of the in-vivo mutagenic potential of hard-metal dust was obtained recently in type II pneumocytes of rats (De Boeck, 2003c). DNA breaks/alkali-labile sites (alkaline Comet assay) and chromosome/genome mutations (micronucleus test) were assessed after a single intratracheal instillation of hard metal (WC–Co), and dose–effect and time trend relationships were examined. In addition, the alkaline Comet assay was performed on cells obtained from BALF and on peripheral blood mononucleated cells (PBMC). Protein content, LDH activity, total and differential cell counts of BALF were evaluated in parallel as parameters of pulmonary toxicity. In type II pneumocytes, WC–Co induced a statistically-significant increase in tail DNA (12-h time point) and in micronuclei (72 h) after a single instillation in rats at a dose which produced mild pulmonary toxicity. In PBMC, no increase in DNA damage nor in micronuclei was observed.

Cobalt compounds, like other metallic compounds, are known to be relatively inactive in prokaryotic systems (Rossman, 1981; Swierenga *et al.*, 1987).

(*c*) *Cobalt(II) chloride*

Cobalt(II) chloride was found to be inactive in the λ prophage induction assay, and gave conflicting results in the *Bacillus subtilis rec*$^{+/-}$ growth inhibition assay; when a cold preincubation procedure was used, positive results were observed (Kanematsu *et al.*, 1980). Lysogenic induction and phage reactivation was found in *Escherichia coli* in the absence of magnesium. Also in *E. coli*, reduction of fidelity of DNA replication by substitution of magnesium and inhibition of protein synthesis were observed. Cobalt(II) chloride was inactive in all but two bacterial mutagenicity tests. One study gave positive results in the absence, but not in the presence, of an exogenous metabolic system, and in the second study, a preincubation procedure was used.

In bacteria, cobalt(II) chloride has been reported to reduce the incidence of spontaneous mutations and to inhibit mutations induced by *N*-methyl-*N*′-nitrosoguanidine and 3-amino-1,4-dimethyl-5*H*-pyrido[4,3-*b*]indole. It was found to be comutagenic with several heteroaromatic compounds such as benzo(a)pyrene and naphthylamine.

In *Saccharomyces cerevisiae*, cobalt(II) chloride induced gene conversion and petite ρ^- mutation in mitochondrial DNA but not other types of mutation.

In *Drosophila melanogaster*, mitotic recombination was found.

In mammalian cells cultured *in vitro*, positive results were obtained for induction of DNA–protein cross-linkage, DNA strand breakage and sister chromatid exchange in most studies. Cobalt(II) chloride induced mutations at the *Hprt* locus in Chinese hamster V79 cells, but not at the *8AG* and the *Gpt* loci. At the same *Gpt* locus in a transgenic Chinese hamster V79 G12 cell line, lower concentrations of cobalt(II) chloride did induce gene

mutations. In a single study, at the *Tk* locus in mouse lymphoma L5178Y cells, the results were negative.

In most studies, in cultured human cells *in vitro*, positive results were obtained for inhibition of protein-DNA binding activities, inhibition of p53 binding to DNA and for induction of gene expression, induction of DNA strand breakage and sister chromatid exchange. Chromosomal aberrations were not observed in cultured human cells (IARC, 1991). [The Working Group noted the low concentrations employed.] Cobalt(II) chloride induced aneuploidy in cultured human lymphocytes.

In vivo, cobalt(II) chloride administered by intraperitoneal injection induced aneuploidy (pseudodiploidy and hyperploidy) in bone marrow and testes of Syrian hamsters, micronuclei in bone marrow in male BALB/c mice, and enhanced the micronuclei frequencies induced by the three other mutagens tested.

A gene expression mechanism is involved in several tissue and cellular responses induced by soluble cobalt (generally cobalt chloride) mimicking the pathophysiological response to hypoxia, a response which involves various genes including those coding for erythropoiesis and for growth factors for angiogenesis (Gleadle *et al*., 1995; Steinbrech *et al*., 2000; Beyersmann, 2002). Up-regulation of erythropoietin gene expression was observed *in vivo* after a single intraperitoneal injection of cobalt chloride (60 mg/kg bw) into rats (Göpfert *et al*., 1995) and might be of relevance in explaining the polyglobulia noted in humans treated with high doses of cobalt (Curtis *et al.*, 1976). In Chinese hamster ovary cells, cobalt also up-regulated the expression of haeme oxygenase-1, a potent antioxidant and anti-inflammatory mediator which helps to maintain cellular homeostasis in response to stress and injury (Gong *et al*., 2001).

In studies designed to explore the molecular mechanisms of gene response to hypoxia, cobalt (12 and 60 mg/kg bw as cobalt chloride) was found to up-regulate the expression of the *PDGF-B* gene in lungs and kidneys of male Sprague-Dawley rats (Bucher *et al*., 1996). Since PDGF is an important growth factor which modulates cell proliferation and the expression of several proto-oncogenes mainly in mesenchymal cells, this effect of cobalt might explain how it may exert fibrogenic and/or carcinogenic properties, but this remains to be documented.

(*d*) *Other cobalt compounds*

Few results are available with other cobalt(II) salts.

Molecular analysis of lung neoplasms of $B6C3F_1$ mice exposed to cobalt sulfate heptahydrate showed the presence of K-*ras* mutations with a much higher frequency (55%) of G > T transversion at codon 12 than in controls (0%). This provides suggestive evidence that cobalt sulfate heptahydrate may indirectly damage DNA by oxidative stress (National Toxicology Program, 1998).

Cobalt sulfate has been shown to induce chromosomal aberrations and aneuploidy in plant cells, chemical changes in bases in purified calf thymus DNA and in isolated human chromatin in the presence of hydrogen peroxide, and cytoskeletal perturbation of micro-

tubules and microfilaments and p53 protein in mouse fibroblasts treated *in vitro*. Cell transformation of Syrian hamster embryo cells has been induced by cobalt sulfate *in vitro*.

A number of mammalian genes (metallothionein MT-IIA, heat-shock proteins hsp70, c-fos) are transcriptionally regulated by a *cis*-acting DNA element located in their upstream regions. This DNA element responds to various heavy metals, including cobalt, to stimulate the expression of these genes (Murata *et al.*, 1999). *MT-IIA* and *hps70* but not *c-fos* RNA transcripts were increased in HeLa S_3 cells exposed to high concentrations of cobalt sulfate (> 10 μM). Metal response element (MRE)-DNA binding activity was not inhibited by cobalt sulfate in Hela cells *in vitro* while the results for heat shock element (HSE)-DNA binding activity were inconclusive. It is unknown whether MT-IIA and hps70 induction plays a role in the pathophysiological processes involved in cobalt carcinogenesis.

Cobalt(II) acetate was found to induce cell transformation *in vitro*. Cobalt(II) acetate and cobalt(II) molybdenum(VI) oxide ($CoMoO_4$) enhanced viral transformation in Syrian hamster embryo cells. Cobalt(II) acetate was shown to induce DNA base damage in female and male Fischer 344/NCr rats. Cobalt sulfide particles were found to induce DNA strand breaks and alkali-labile sites in Chinese hamster ovary cells. Data on the induction of gene mutations in Chinese hamster cells by cobalt sulfide particles are conflicting. Cobalt sulfide was shown to induce morphological transformation in Syrian hamster embryo cells; the crystalline form of cobalt sulfide being more active than the amorphous form.

Cobalt(III) nitrate induced gene mutations in *Pisum abyssinicum* chlorophyll. Eight of 15 cobalt(III) complexes with aromatic ligants were found to be positive in a DNA repair assay and four among the eight were also mutagenic to *Salmonella typhimurium*. Cobalt(III) complexes with desferal-induced scission of double-stranded DNA, and a cobalt(III) Schiff-base complex induced inhibition of zinc-finger transcription factors.

4.5 Mechanistic considerations

It had been assumed that, as for other metals, the biological activity of cobalt-metal particles, including their genotoxic effects, were mediated by the ionic form of cobalt and could be revealed by testing soluble compounds. However, Lison *et al.* (1995) demonstrated *in vitro* that cobalt metal, and not its ionic (II) species, was thermodynamically able to reduce oxygen in ROS independently of the Fenton reaction. During this process, soluble cobalt ions are produced which have several major cellular targets for induction of genotoxic effects and may, in turn, take part in a Fenton reaction in the presence of hydrogen peroxide. Moreover, since metallic cobalt forms particles which can be inhaled, assessment of genetic effects should also take into consideration: (i) that the primary production of ROS is related to the specific surface properties of the particles or the presence of transition metals, together with other parameters such as particle size, shape and uptake; and (ii) that excessive and persistent formation of ROS by inflammatory cells can lead to secondary toxicity. Since the mechanisms leading to the genotoxic effects of metallic cobalt are complex, assessment of its mutagenic effects should not be restricted

to the genetic effects of metallic cobalt alone but should be complemented by those of cobalt in association with carbides, and of cobalt salts.

The results of genotoxicity assays with cobalt salts demonstrate clearly their mutagenic potential. Recent experimental studies have contributed to better delineate the molecular mechanisms involved in the genotoxic (and carcinogenic potential) of cobalt ions. These mechanisms may conceivably apply both to soluble cobalt compounds — for example, cobalt chloride or sulfate — and also to cobalt-metal or hard-metal particles which are readily solubilized in biological media. *In vivo*, however, the bioavailability of cobalt(II) is relatively limited because these cations precipitate in the presence of physiological concentrations of phosphates ($Co_3(PO_4)_2$); K_s: 2.5×10^{-35} at 25 °C) and bind to proteins such as albumin.

In vitro in mammalian cells, two mechanisms seem to apply :

(1) a direct effect of cobalt(II) ions causing damage to DNA through a Fenton-like mechanism;
(2) an indirect effect of cobalt(II) ions through inhibition of repair of DNA damage caused by endogenous events or induced by other agents.

In vitro, cobalt(II) has been shown to inhibit the excision of UV-induced pyrimidine dimers from DNA in a dose-dependent fashion. Inhibition of repair by cobalt(II) resulted in the accumulation of long-lived DNA strand breaks suggesting a block in the gap-filling stage (DNA polymerization) of repair. Ability to inhibit repair was not correlated with cytotoxicity. It has been shown that repair of X-ray-induced DNA damage is not sensitive to cobalt. All inhibitory metals inhibited closure of single-strand DNA breaks (Snyder *et al.*, 1989).

In vitro, ionic cobalt(II) was shown to inhibit nucleotide excision repair processes after ultraviolet (UV) irradiation as measured by the alkaline unwinding method. A concentration as low as 50 μM cobalt chloride inhibited the incision as well as the polymerization step of the DNA repair process in human fibroblasts treated with UV light. As the repair of DNA damage is an essential homeostatic mechanism, its inhibition may account for a mutagenic or carcinogenic effect of cobalt(II) ions. Concentrations less than 1 mM cobalt chloride did not affect the activity of bacterial fpg but significantly reduced the DNA binding activity of the mammalian damage recognition protein XPA. Competition with essential magnesium ions and binding to zinc finger domains in repair proteins have been identified as potential modes of indirect genotoxic activity of cobalt(II) ions. It has also been reported that the DNA binding activity of the p53 protein, which is a zinc-dependent mechanism, can be modulated by cobalt(II) ions (Kasten *et al.*, 1997; Palecek *et al.*, 1999; Asmuss *et al.*, 2000).

This indirect mutagenic effect of cobalt on repair enzymes is not restricted to cobalt salts but has been shown to apply also to in-vitro exposure to metallic cobalt. De Boeck *et al.* (1998) examined the effects of cobalt-metal particles using the alkaline Comet assay on methyl methanesulfonate (MMS)-treated isolated human lymphocytes. MMS induced DNA strand breaks and alkali-labile sites in the lymphocytes in a dose-dependent manner. Post-incubation of MMS-treated cells for 2 h, in the absence of cobalt, resulted in signi-

ficantly less DNA damage, implying that repair took place. Post-treatment with cobalt particles at a non-genotoxic dose for 2 h, after treatment with 5.5 µg/mL MMS, resulted in higher damage values compared with post-incubation values. These results may reflect inhibition by the cobalt particles of the ongoing repair of MMS-induced DNA lesions, which had presumably reached the polymerization step. Simultaneous exposure of lymphocytes to 5.5 µg/mL MMS and 1.2 µg/mL cobalt for 2 h resulted in higher damage values, conceivably representing an interference of cobalt particles at the incision of methylated bases, allowing more alkali-labile apurinic sites to be expressed, which, in the absence of cobalt, would be repaired. The authors concluded that metallic cobalt could cause persistence of MMS-induced DNA lesions by interference during their repair.

Since the previous IARC evaluation of cobalt in 1991, additional information has been obtained on the genotoxicity of the various cobalt species.

Cobalt(II) ions have been shown to substitute for zinc in the zinc-finger domain of some important proteins, such as those controlling cell cycling and/or DNA repair processes in animal and human cells.

Cobalt-metal particles produce mutagenic effects *in vitro* by two different mechanisms:

- directly through the production of ROS resulting in DNA damage, and
- indirectly by releasing Co(II) ions which inhibit DNA repair processes.

Moreover, when cobalt-metal particles are mixed with metallic carbide particles (mainly tungsten carbide), they form a unique chemical entity which:

- produces higher amounts of ROS than cobalt alone *in vitro*,
- has a stronger mutagenic activity than cobalt alone *in vitro* in human cells, and
- is mutagenic in rat lung cells in *vivo*.

A physicochemical mechanism to explain this increased toxicity has been proposed.

In humans, a specific fibrosing alveolitis (so-called hard-metal disease) occurs in workers exposed to dusts containing metallic cobalt such as hard metal or cemented microdiamonds. Fibrosing alveolitis may be a risk factor for lung cancer in humans.