DITHIOCARBAMATE-MEDIATED UPTAKE OF NICKEL(II) BY CELLS *IN VITRO*

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BY

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ABSTRACT

Dithiocarbamate-mediated nickel(II) uptake was studied in five cell lines: 1) cultured human B-lymphoblasts, 2) rabbit alveolar macrophages, 3) human peripheral lymphocytes, 4) human erythrocytes and 5) human polymorphonuclear leukocytes. Two different incubation protocols were employed: concurrent incubation of nickel(II) with the ligand, and sequential incubation of ligand followed by nickel(II) incubation.

The effects of various experimental parameters such as ligand concentration, cell number and available nickel(II) concentration on nickel(II) uptake were examined for most of the above cell types. During concurrent incubations, the effect of ligand concentration on nickel(II) was maximum at 10^{-6} M sodium diethyldithiocarbamate (DDC) or ammonium pyrrolidinedithiocarbamate (APDC) for all cell types (except polymorphonuclear leukocytes). Enhanced uptake was evident at higher concentrations ($\geq 10^{-3}$ M DDC or APDC) for sequential incubations. By contrast, ammonium dithiocarbamate (AD) had no enhancing effect on nickel(II) uptake in either protocol (tested only for human peripheral lymphocytes and human erythrocytes). Distribution studies indicated that enhanced cytosolic uptake of nickel(II) occurred for 10^{-7} - 10^{-5} M DDC. The observed effects on nickel(II) uptake of ligand concentration, Ni^{2+} concentration and cell number were interpreted on the basis of an 'Equilibrium Model' with several possible pathways. The nickel(II) uptake data were consistent

with the possibility that ligand uptake precedes metal-ion uptake. The latter process may well involve the protonated form of the dithiocarbamate as an ionophore.

As a means of enhancing cell-associated nickel(II), peripheral lymphocytes from nickel-sensitized and non-sensitized individuals were pretreated with APDC in veronal buffer. The transforming ability of these cells were then studied by use of a lymphocyte transformation (proliferation) test. This approach was not successful in enhancing the response to nickel(II) because of the inherent toxicities of the veronal buffer and APDC.

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LIST OF ABBREVIATIONS

AD	Ammonium Dithiocarbamate
APDC	Ammonium Pyrrolidinedithiocarbamate
cpm	Counts per minute
DD	Doubly Distilled
DDC	Diethyldithiocarbamate
DDI	Distilled Doubly Deionized Water
DDW	Distilled Deionized Water
DPP	Differential Pulse Polarography
EAAS	Electrothermal Atomic Absorption Spectrometry
EDTA	Ethylenediaminetetraacetic acid (disodium salt)
HEPES	N-2-Hydroxyethylpiperazine-N'-2-ethanesulfonic acid
HSA	Human Serum Albumin
LTT	Lymphocyte Transformation Test
$[Ni^{2+}]_t$	Total Concentration of Nickel(II)
PBS	Phosphate Buffered Saline
PHA	Phytohaemagglutinin
PIPES	Piperazine-N,N'-bis[2-ethane-sulfonic acid]
PMNs	Polymorphonuclear Leukocytes
RAMs	Rabbit Alveolar Macrophages
RBCs	Red Blood Cells

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1. INTRODUCTION

1.1. Toxic Effects of Nickel and its Compounds.

Nickel as a pure metal or alloy has many important industrial and nonindustrial uses: in electronic components, steel alloys, stainless steel, jewelry and many other items. Exposure and direct handling of nickel metal or alloys, or of nickel compounds, have caused various toxic effects in man and in experimental animals (Sunderman, 1977; Cecutti and Nieboer, 1981).

In experimental animals, toxic effects of nickel and nickel compounds have been studied extensively. Exposure to binary nickel compounds (e.g., Ni_3S_2) by inhalation induced respiratory tract irritations and lesions and malignant tumours (Sunderman, 1981). By contrast, inhalation of soluble nickel salts (e.g., $NiCl_2$) has not produced tumours but has suppressed immunological response of the respiratory system (Gardner, 1980; Casarett-Bruce *et al*, 1981). When nickel or nickel compounds were administered orally or by injection in rodents, levels or activities of enzymes (especially those related to iron metabolism) and metabolites were modified or suppressed (Sunderman, 1977). Pathological lesions of major organs were also evident (Cecutti and Nieboer, 1981). Tumour development was found following injection or implantation of relatively insoluble nickel compounds (Sunderman, 1981, 1984a). *In vitro* studies have shown that solid nickel compounds or water soluble salts lyse cells (Waters *et al*, 1975), impair phagocytic activity *in vitro* and damage murine

macrophage membranes (Rae, 1975). Cell culture studies have also demonstrated the genotoxicity of both types of nickel compounds (Sunderman, 1981, 1984b; Leonard et al, 1981).

Toxic effects of nickel and nickel compounds in man can be subdivided into two main categories: 1) occupationally related (carcinogenesis, nickel carbonyl poisoning and respiratory hypersensitivity reactions) and 2) nonoccupationally related (contact dermatitis). The cancer-related studies, nickel carbonyl poisoning and occupational asthma case studies will only be reviewed briefly, whereas the main focus will be on contact dermatitis.

1.1.1. Carcinogenesis.

Nickel has been shown to increase risks of various types of cancers, of which nasal and lung cancers are the most prevalent. The incidence of these two cancers (commonly associated with nickel refining processes such as roasting, smelting and perhaps electrolysis) has been related most consistently to exposure to insoluble salts (Ni_3S_2 and/or Ni oxides) (Cecutti and Nieboer, 1981; Sunderman, 1984c). The mechanism of nickel carcinogenesis is unknown. However, the carcinogenic potential of the different compounds seems to correlate with the degree to which the lung can remove the material. This depends in part, upon the solubility of the compounds: the more soluble, the more quickly removed. Water-insoluble compounds (e.g., Ni_3S_2, Ni powder and NiO) have all been demonstrated to be carcinogenic, whereas water-soluble salts (e.g., $NiCl_2$, $NiSO_4$) have no carcinogenic

properties (Leonard et al, 1981).

Crystallinity of the nickel compounds also appears to be predisposing to carcinogenicity (Nieboer et al, 1985). At the molecular level, metal-ion induced somatic mutations are postulated. These may involve B- to Z-DNA conformational changes, DNA damage (e.g., strand breaks and cross-links), non-isomorphous replacement of endogenous metal ions necessary for replication and repair (e.g., Mg^{2+} and Zn^{2+}), and modifications of chromatin structure (Sunderman, 1984a; Nieboer et al, 1985).

1.1.2. Nickel Carbonyl Poisoning.

Nickel carbonyl is taken up by respiratory tissues and can cause pulmonary edema. It is a highly volatile liquid which is lipid soluble and has the chemical formula $Ni(CO)_4$. Numerous occupational exposures have been documented and fatalities are known (Nieboer et al, 1984d). Clinically, symptoms resemble pneumonia. Antabuse and DDC have been used to reduce the body burden of nickel in individuals who have been exposed to it during accidental gassing (Sunderman, 1977; Nieboer et al, 1984d).

1.1.3. Occupational Asthma.

Very few cases of nickel-related occupational asthma have been reported for workers exposed industrially to nickel compounds (see Dolovich et al, 1984 for summary). An antibody with nickel specificity has been characterized in the serum of a patient (Dolovich et al, 1984; Nieboer et al, 1984a). The antibody recognizes nickel(II) bound to the natural

copper(II)-binding site of human serum albumin (HSA). Cu^{2+} bound at this site is not recognized.

1.2. Nickel Contact Dermatitis.

1.2.1. Prevalence.

Unlike occupational asthma, contact dermatitis is more commonly found in the general population. Numerous non-occupational sources of nickel such as jewelry, coins, clothing fasteners and others have all been shown to be associated with nickel dermatitis (Menné and Hjorth, 1982; Fischer et al, 1984a and b; Nieboer et al, 1984d; van Ketel, 1985). Dermatitis from dermajet intradermal injections has also been reported (Lachapelle and Tennstedt, 1982; Lachapelle et al, 1982).

Nickel sensitivity is found in approximately 5% of the general population, with a tenfold higher incidence in women than in men (Menné and Hjorth, 1982; Sun, 1980; Nieboer et al, 1984d; Kieffer, 1979). It is one of the most common skin allergens in the general population (e.g., Cavelier et al, 1985; Dooms-Goossens et al, 1980; Kalimo and Lammintausta, 1984; Sun, 1980). The greater incidence in women (9% compared to 1% for men) is probably due to a greater exposure to nickel-containing items such as jewelry, cutlery, and other household items (Nieboer et al, 1984d).

1.2.2. Clinical Aspects.

Nickel dermatitis is characterized by erythema, edema and eczema (Menné and Hjorth, 1982). Primary lesions usually occur at sites on which direct contact with the metal has

taken place. Papulo-vesicular eruptions are common, and the usual area of lesions are the web of the fingers. Secondary eruptions distant from the metal contact sites are found in 75% of patients (Menné and Hjorth, 1982).

1.2.3. Recognition Phases of Contact Dermatitis.

The following summary concerning the various phases of contact dermatitis is based on the review of this topic by Adams (1983). Nickel contact dermatitis is a delayed reaction (delayed hypersensitivity). It flares up 48 h after exposure to a secondary challenge with nickel. The development of allergic contact dermatitis occurs in four different phases: refractory, induction, elicitation and persistence. In the refractory period, an individual is exposed to the allergen but remains unaffected. It is hypothesized that the hapten penetrates the skin, conjugates with an epidermal protein and during the induction phase the complex interacts with and is carried by T-lymphocytes to regional lymph nodes via dermal lymphatics. In the lymph node, the T-lymphocytes become immunoblasts which divide into memory and effector cells. Memory cells retain the information of previous contact with the antigen and upon recontact, these cells proliferate and produce a new population of sensitized small lymphocytes. Effector cells circulate in peripheral blood. During elicitation, which involves secondary challenge of the antigen, the hapten again penetrates the dermis, combines with a protein and is recognized by the effector cells which comprise only < 10% of the total lymphocyte population at the

contact site. Upon contact, effector cells release chemotactic substances that cause the inflammatory skin reaction by inducing vascular permeability, attraction of mononuclear cells, retention and proliferation of cells within the area. Often, during both the induction and elicitation phases, macrophages or Langerhans' cells may be involved in processing and presentation of the antigen to the T-cells. Persistence involves continued presence of the specific effector cells capable of recognizing the hapten with the ability to produce inflammation.

1.2.4. Penetration of Nickel into the Skin.

The refractory, induction and the elicitation phases require penetration of the hapten into the skin; hence, binding of nickel to the skin is important. Spruit et al (1965) have shown that externally applied ionic Ni^{2+} bound reversibly to the dermis of human cadaverous skin. Further, the nickel permeated the dermis and was shown to penetrate deeper via the sweat duct and hair follicles (Spruit et al, 1965; Kalimo et al, 1985). Rapid accumulation of nickel within 1 h was demonstrated for keratinized areas (stratum corneum) and hair shafts of albino guinea pigs (Lloyd, 1980) and for the upper keratin layer of human skin (Kalimo et al, 1985).

1.2.5. Diagnostic Tests.

Nickel contact dermatitis can be diagnosed by three conventional methods employed for other contact allergens: 1) patch test, 2) leukocyte migration test and 3) lymphocyte

transformation test. The most commonly used clinical method is the skin patch test, which involves application of a 2.5% solution of $NiSO_4$ in petrolatum on an A-1 patch to an area of skin (Spruit *et al*, 1980). The classic positive patch test reaction is characterized by erythema, edema and small closely set vesicles. The patch test is based upon the immune response after secondary challenge of nickel. The main advantage is that the test is reliable most of the times and rapid, however false positive and false negative results have been reported (Fischer and Rystedt, 1985; Kieffer, 1979; Svejgaard *et al*, 1978). Patients may also become sensitized as a result of the patch test.

The *in vitro* leukocyte migration test (LMT) has the advantage of being rapid (72h) and specific for delayed hypersensitivity. Only 1% of the lymphocytes tested need to be sensitized to indicate a positive LMT result. Risks of sensitization of the patient do not exist and hence testing of the patient can be done at all clinical stages. LMT is based on the release of a soluble mediator (macrophage inhibiting factor) from sensitized lymphocytes on contact with the specific antigen. It measures the extent of inhibition of the migration of macrophages at openings of capillary tubes. Some investigators have found a discrimination in results between non-allergic controls and patients (e.g., in culture medium RPMI-1640 with $NiSO_4$; Jordan and Dvorak, 1976), while others (e.g., Thulin, 1976) report an overlap in results between patients and controls. Thulin found that in TC medium 199,

Ni^{2+} bound to bovine albumin, human epidermal protein or human serum albumin scored positively, but not $NiCl_2$ alone. Albumin has also been shown to be a carrier protein for Cr^{3+} in the LMT of chromium-sensitized leukocytes (Thulin and Zachariae, 1972). A more recent study again showed no difference in LMT results for nickel sensitive patients and controls (Nordlind and Sandberg, 1983).

The *in vitro* lymphocyte transformation test (LTT) can detect nickel sensitivity by culturing peripheral lymphocytes with the appropriate nickel compound. This results in transformation of lymphocytes into lymphoblasts, with subsequent proliferation. As with LMT, there are inconsistencies in LTT findings.

Discrimination between controls and patients has been reported by most researchers when employing $NiSO_4$ as the allergen (Silvennoinen-Kassinen, 1980; Forman and Alexander, 1972; Gimenez-Camarasa *et al*, 1975; Nordlind, 1984) and also with $NiCl_2$ (Millikan *et al*, 1973). Equal transformation abilities have been demonstrated for $NiSO_4$ and nickel(II) acetate (Hutchinson *et al*, 1972) illustrating that Ni^{2+} ion is the active component. For some patients, lymphocyte transformation could only be demonstrated for high concentrations of $NiSO_4$, whereas for others low concentrations sufficed. Hence, there appears to be no consistent nickel-dose dependency (Gimenez-Camarasa *et al*, 1975; Svejgaard *et al*, 1978). Nonspecific weak mitogenic effects of nickel(II) acetate and $NiSO_4$ have been observed, and this

might explain the lack of discrimination between patients and controls (Pappas et al, 1970; Svejgaard et al, 1978).

A lymphokine (also called a blastogenic factor which induces the transformation of lymphocytes into lymphoblasts) has been identified in supernatants of nickel-sensitized lymphocyte cultures. This substance when incubated with control cells was able to induce transformation (Macleod et al, 1982).

1.2.6. Cells Involved in Nickel Contact Dermatitis.

1.2.6.1. T-Cells and Macrophages.

Silvennoinen-Kassinen (1980) has shown that of the three major cell types involved in immune responses (T-lymphocytes, B-lymphocytes and macrophages), T-lymphocytes and macrophages play a critical and interdependent role in in vitro transformation. In two studies of human skin biopsies following test applications of $NiSO_4$ and by use of monoclonal antibody markers, the majority of the cells in the cellular infiltrate were identified as helper/inducer T-lymphocytes (Kanerva et al, 1984; Scheynius et al, 1984).

1.2.6.2. Langerhans' Cells.

Langerhans' cells (LC) have been implicated in contact hypersensitivity. They have been found in close apposition with mononuclear cells at sites of positive allergic-contact reactions due to mercuric chloride (Silberberg et al, 1976). LCs are characterized by receptors commonly found on B-lymphocytes and macrophages (F_c and C_{3b}). They also have surface antigens (Ia and DR) which may be involved in their

antigen presenting function in a genetically restricted manner. LCs resemble monocytes in that they process the antigen before presenting it to the lymphocyte (Silberberg et al, 1976).

Evidence that Langerhans' cells play an important role in nickel contact dermatitis may be summarized as follows.

1) LCs can take up metal-ion allergens including nickel(II) (Braathen, 1980).
2) LCs have been shown to be present at patch test sites by skin biopsies of nickel sensitive patients (Sjöborg et al, 1984).
3) Human epidermal cell suspensions from nickel-sensitive individuals consist of 3-5% LCs (Braathen, 1980).
4) In vitro proliferation of T-lymphocytes, in response to $NiSO_4$, was enhanced when epidermal cells were added to the culture medium. The response was higher than that found for T-lymphocytes + macrophages + $NiSO_4$, or for T-lymphocytes + $NiSO_4$. Inhibition occured by pretreating the epidermal cells with anti-DR antiserum (Braathen, 1980).
5) Skin exposed to grenz-rays (low energy X-rays) suppressed nickel contact dermatitis at the irradiated site. Employing monoclonal antibodies, a decrease in LCs was demonstrated in treated skin versus untreated skin (Lindelöf et al, 1985).

It has been suggested that LCs may transport the nickel antigen to the dermis and lymph nodes, where they are

postulated to participate in the T-cell mediated response (Braathen, 1980).

1.2.7. Genetic Predisposition for Nickel Contact Dermatitis.

Allergy to a single antigen or multiple antigens may be related to the presence of a human leukocyte antigen (HLA) (Silvennoinen-Kassinen and Tiilikainen, 1980). However, most studies appear to show that there is no genetic predisposition for nickel contact dermatitis. Nickel allergy was not HLA-A, -B, -C or -D_w1-4 associated (Menné and Holm, 1983; Silvennoinen-Kassinen and Tiilikainen, 1980; Liden et al, 1981). Nevertheless in a recent clinical survey of nickel contact dermatitis patients, evidence is presented that, compared to a control group of nonallergic women, patients exhibited somewhat higher serum IgE levels (Belda et al, 1985). The nickel-sensitized individuals also had an increased frequency of other allergic symptoms (namely, reactions from adhesive tape, mosquito bites and hives). The latter was termed contact sensitivity syndrome. These observations suggest some hereditary control.

1.3. The Effect of Dietary Nickel(II) and Chelation Therapy on Nickel Contact Dermatitis.

In addition to topical application of drugs, two major approaches to the treatment of nickel contact dermatitis have been employed: 1) low nickel diet, and 2) chelation therapy with sodium diethyldithiocarbamate (DDC) or disulfiram (tetraethylthiuramdisulfide [TETD], Antabuse).

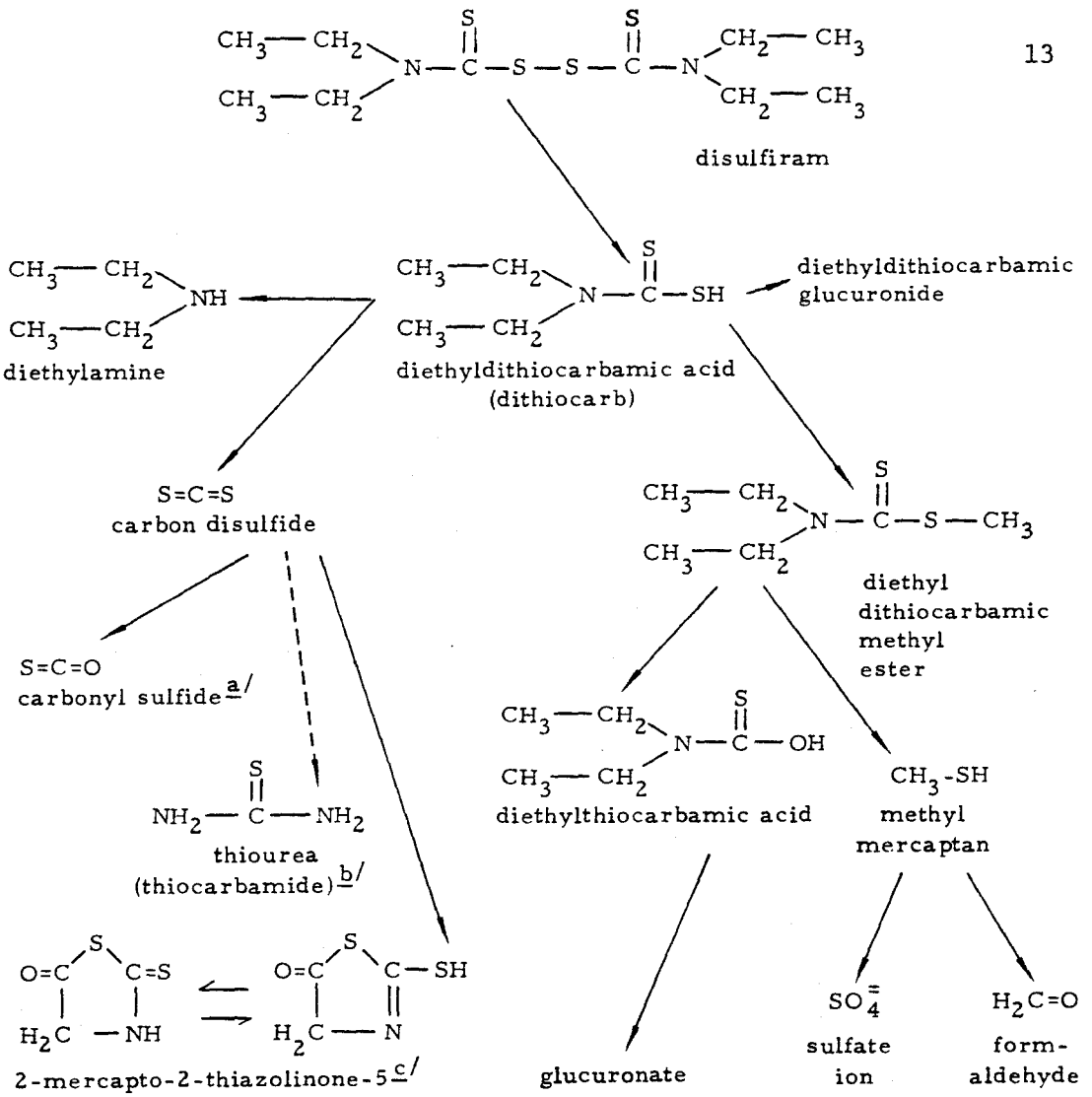
Diets containing low levels of nickel improved or reduced

the flares of dermatitis (Veien et al, 1985). Dietary supplements comparable or exceeding the daily nickel intake (approximately 500 µg/day) aggravated both primary and secondary eruptions (e.g., Fisher, 1978; Christensen et al, 1981). These observations imply that the body burden of nickel has impact on the clinical course of the nickel dermatitis in sensitized individuals. Interestingly, only 1-10% of ingested nickel is absorbed from the gastrointestinal tract. Major excretory pathways of absorbed nickel include urine and sweating (Cecutti and Nieboer, 1981).

Disulfiram (also called Antabuse) has been employed in the treatment of alcoholism, as an antidote to nickel carbonyl poisoning and in the treatment of nickel contact dermatitis. After disulfiram is absorbed into the body, it is metabolized to diethyldithiocarbamate, a well known chelating agent for Ni^{2+}. Other decomposition products or metabolites of disulfiram include carbon disulfide (CS_2), carbonyl sulfide (SCO), thiourea and methyl mercaptan (Figure 1A)(Hayes, 1982).

Considerable evidence exists that DDC, and especially Antabuse, induces nickel(II) uresis. Case studies and case series have revealed that these therapeutic agents clinically improve the dermatitis flares (Spruit et al, 1978; Christensen, 1982; Menné et al, 1980). Hence, lowering the body burden of nickel(II) appears to reduce the number and severity of the flares. This approach, however, is not generally recommended perhaps because of some fundamental toxicological concerns.

A



B

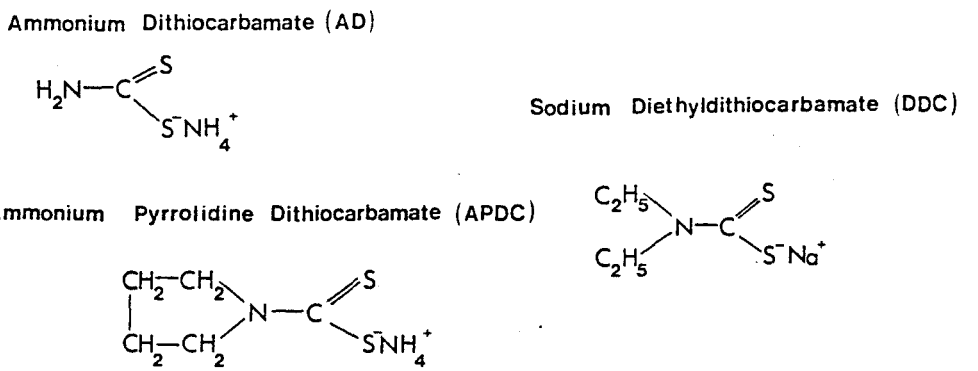


Figure 1. A: The metabolism of tetraethylthiuramdisulphide (Antabuse; also known as disulfiram) to diethyldithiocarbamate and other products. Reproduced from Hayes, 1982. B: Structural formulae of AD, DDC and APDC.

It is evident from radiotracer and whole body autoradiography studies that DDC promotes the retention and the accumulation of nickel in lipid rich organs (e.g. in adipose tissue) of mice (Oskarsson and Tjälve, 1980; Tjälve et al, 1984). Even though DDC treatment lowers the nickel burden of the target tissue (the lung) in mice exposed to nickel carbonyl (either by inhalation or injection), this redistribution to lipid tissues is of concern (Tjälve et al, 1984). Unlike DDC, D-penicillamine which forms polar complexes with nickel(II) was shown to decrease the nickel-63 radioactivity in all tissues (Oskarsson and Tjälve, 1980). As for nickel, DDC-induced redistribution of cadmium (Gale et al, 1982), copper and zinc (Aaseth et al, 1979) and thallium (Vyth et al, 1983) have been reported in DDC-treated mice. Redistribution of endogeneous metals (e.g. Zn, Cu, Mn, Fe) might be expected to have serious metabolic consequences if they occurred. Further, retention and deposition of toxic metals in critical organs is potentially disastrous. For example, DDC appears to worsen Tl-related neurological symptoms in man (Vyth et al, 1983), and DDC-enhanced uptake of nickel by rat liver and kidney promoted excess heme oxygenase activity (Sunderman et al, 1983).

DDC has also been shown to have immunomodulating effects. In vivo it can enhance immune responses in both man (Renoux et al, 1983) and guinea pigs (Neveu et al, 1982), whereas in vitro it inhibits mitogen-induced proliferative responses (Corke, 1984). As a means of promoting cell-mediated immune

responses in cancer patients, DDC has been suggested as a possible drug (Renoux et al, 1983).

1.4. Nickel(II) in Body Fluids.

Concentrations of nickel(II) in body fluids, such as serum and urine, reflect ambient exposure. In unexposed individuals, $\leq$ 2 µg/L of nickel is normal in the serum and in urine (Sunderman et al, 1984; Nieboer et al, 1984d). In a recent ultracentrifugation study, 76% of serum nickel was shown to be associated with the high-molecular-weight fraction (relative molecular mass $\geq$ 50,000), and 24% to low-molecular-weight molecules (Sanford and Nieboer, unpublished results). The latter constitutes the fraction of plasma nickel available for glomerular filtration in the kidney. Based upon the findings of Lucassen and Sarkar (1979), the low-molecular-weight nickel(II)-binding component is most probably L-histidine, and the high-molecular-weight one likely human serum albumin (HSA). The primary and natural Cu^{2+}/Ni^{2+} binding site of HSA is characterized by a square planar chelate ring formed by the N-terminus alpha amino nitrogen, the first two peptide nitrogens and the 3-nitrogen of the imidazole ring of residue 3 (Glennon and Sarkar, 1982; Sarkar, 1984; Dolovich et al, 1984) (see Figure 2). In man, about 65% of kidney-filtered nickel appears to be reabsorbed by the renal tubular system (Sanford and Nieboer, unpublished results).

1.5. Objectives of the Thesis.

The main objective of this thesis is to study

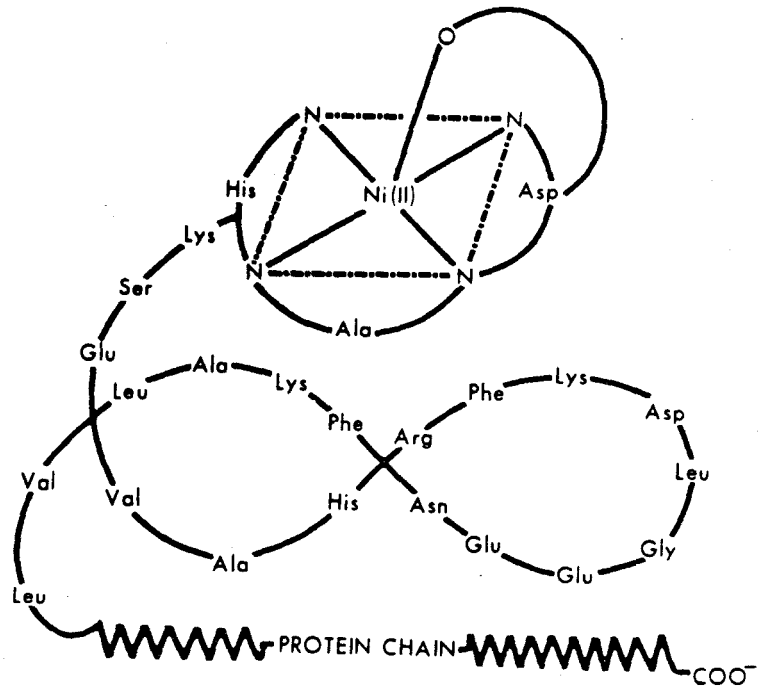


Figure 2. Proposed nickel(II) binding site in human serum albumin. The figure is reproduced with permission from Sarkar, 1981.

dithiocarbamate-mediated nickel(II) uptake by various cell types, namely: rabbit alveolar macrophages, B-lymphoblasts of human origin, human peripheral lymphocytes, human polymorphonuclear leukocytes and human erythrocytes. Among these are the cells that constitute the major cell types in blood. Human peripheral lymphocytes are of special interest because, as already discussed, they are involved in the immunological mechanism of nickel-induced contact dermatitis. B-lymphoblasts (a cultured cell line derived from B-lymphocytes) are selected to supplement the work on peripheral lymphocytes and rabbit alveolar macrophages (RAMs) provide a convenient source of phagocytes. The dithiocarbamate ligands selected for study are sodium diethyldithiocarbamate (DDC), ammonium pyrrolidinedithiocarbamate (APDC) and ammonium dithiocarbamate (AD). Previous nickel(II) uptake studies focused on the concurrent incubation of Ni^{2+} and DDC (Stafford, 1984), whereas the current work also examines a sequential incubation protocol in which cells are first incubated with ligand and then with the metal ion. Effects on nickel(II) uptake of Ni^{2+} concentration and cell number are assessed, as well as the intracellular distribution of cell-associated nickel. Due to the limited solubility of nickel(II) dithiocarbamate complexes, the effect on uptake experiments of adsorptive losses of these complexes will also be investigated.

Previous work has established that DDC enhances the amount of cell-associated nickel(II) (Stafford, 1984). Thus,

it might be pertinent to assess the effect of ligand-enhanced nickel(II) uptake on transformation (proliferation) of peripheral lymphocytes obtained from both nickel-sensitized and nonsensitized individuals.

2. MATERIALS AND METHODS

2.1. Materials.

2.1.1. Chemical Reagents.

In Table 1, details are provided for the routine chemical reagents used.

2.1.2. Buffers.

Veronal buffer (pH=7.4). The stock solution of veronal buffer consisted of the following components in DDW: 0.015 M barbital, 0.0045 M sodium barbital and 0.77 M NaCl. For all experiments, the stock buffer was diluted fourfold with DDW giving final concentrations of 0.0038 M barbital, 0.0011 M sodium barbital and 0.19 M NaCl. Throughout the thesis, veronal buffer refers to the diluted stock unless specified otherwise.

Phosphate buffered saline (pH 7.2-7.4). To prepare 1 L of phosphate-buffered saline, the following materials were dissolved in DDW (molarity is given in parenthesis): 8 g NaCl (137 mM), 0.20 g KCl (2.7 mM), 0.12 g KH_2PO_4 (0.88 mM) and 0.91 g Na_2HPO_4 (6.4 mM). The final pH of the solution was in the range 7.2 to 7.4.

Potassium phosphate buffer (pH=6.3). To 250 ml of DDW, 17 g KH_2PO_4 (0.5 M) and 21.8 g K_2HPO_4 (0.5 M) were added.

2.1.3. Culture Media.

Alpha-MEM. The α-MEM culture medium used for HSV B-lymphoblasts was purchased from Gibco, Grand Isle, N.Y.. It was supplemented with the following components (% v/v): 1% penicillin(85 U ml^{-1})-streptomycin(100 μg ml^{-1}), 1%

Table 1. Chemical Reagents

Chemical Reagent	Source	Grade	Comment
Diethyldithiocarbamate (DDC)	BDH, Toronto, Ontario	AnalaR	stock solutions were daily prepared in veronal buffer.
Ammonium pyrrolidinedithiocarbamate (APDC)	BDH	Spectrosol	stock solutions were daily prepared in veronal buffer.
Ammonium Dithiocarbamate(AD)			prepared according to the method of Mathes(1950). (Obtained from A. Stafford, McMaster University).
Trypan Blue ,0.4%	Gibco, Burlington, Ontario		purchased as a solution.
Bromothymol Blue	BDH		0.1 g dissolved in 250 ml of $4x10^{-5}$ N NaOH.
Bacto-Phytohaemagglutinin(PHA).	BDH		5 ml sterile DDW was used to rehydrate the PHA.
Na_2HPO_4	BDH	ACS Assured	
KCl	BDH	ACS Assured	
K_2HPO_4	BDH	ACS Assured	
KH_2PO_4	BDH	Assured	
$NiSO_4.6H_2O$	BDH	Analytical	
Tris(hydroxymethyl)aminomethane	Fisher, Don Mills, Ontario	ACS certified	
NH_4Cl	J.T.Baker Phillipsburg, N.J.	Analyzed reagent	
Disodium ethylenediaminetetraacetic acid ($Na_2EDTA.2H_2O$)	J.T.Baker	Analyzed reagent	

Table 1. Continued

Chemical Reagent	Source	Grade	Comment
$NaHCO_3$	Fisher	ACS certified	
NaCl	BDH	Analytical reagent	
Barbital IV (Sodium diethyl-barbiturate)	Fisher	Purified	
Barbital	Fisher	Purified	
NH_4OH	Merck, Toronto, Ontario	Ultrapure	13.4 M solution.
HNO_3	J.T.Baker	Ultrex	60% (w/v) solution.
4-Methylpentan-2-one (MIBK)	BDH	Laboratory Reagent	

L-glutamine (2 mM), 1% HEPES buffer (8 mM), 1% sodium bicarbonate (1.5 mM) and 15% heat inactivated fetal bovine serum (FBS). All of these components, except the serum, were purchased from Gibco. The FBS was purchased from Flow Laboratories, Missisauga, Ontario.

RPMI. The RPMI culture medium, used for Crowe B-lymphoblasts, was supplemented with the same components as described above. In addition, 0.05 mM 2-mercaptoethanol (BDH) was included. When this medium was used for maintaining peripheral lymphocytes, 2-mercaptoethanol was omitted and the FBS was replaced by 10% heat inactivated human AB serum (obtained from Dr. Singal, McMaster University).

2.1.4. Reagents Employed in Radioisotope Laboratory Techniques.

The radiolabelled reagents and the constituents of counting scintillation liquids are described in Table 2.

2.1.5. Miscellaneous Solutions.

Ficoll-Hypaque was obtained from Dr. Singal (McMaster University). It was prepared in the following manner: 75.5 g of Ficoll (Pharmacia, Sweden) was added to 7 L of DDW. To 440 ml of Ficoll, 19x50-ml bottles of sodium hypaque (Winthrop, Aurora, Ontario) were added. The density of the final solution was 1.075 g L^{-1}.

Lytic Solutions

Tris/Veronal/NH_4Cl lytic solution (osmolarity=323 mOSM), consisted of 1 part of 2.06 g Tris (amine form) per 100 ml of veronal buffer and 9 parts of 0.83 g of NH_4Cl per 100 ml of

Table 2. Radiolabelled Reagents and Scintillation Liquids

Reagent	Source	Comment
Aqueous Counting Scintillation fluid (ACS fluid)	Amersham, Oakville, Ontario	
Toluene	Fisher, Don Mills, Ontario	Toluene/PPO/POPOP was used as scintillation fluid.
2-5-Diphenyloxazole, PPO	BDH, Toronto, Ontario	Scintillation Grade 5 g L^{-1} toluene.
1,4-Di-2-(5-phenyl-oxazolyl)-benzene, POPOP	BDH	Scintillator Laboratory Reagent 0.1 g L^{-1} toluene.
$^{63}NiCl_2$ (in 0.1 ml of 0.5 M HCl)	New England Nuclear Corporation (NEN), Lachine, Quebec	This solution was diluted to 5 ml with DDW giving a total $[Ni^{2+}]$ = 6.5×10^{-4} M and an activity of 10.31 mCi mg^{-1}. ~$65\text{-}70 \times 10^5$ cpm 10 μl^{-1}.
[^{3}H-methyl] thymidine	NEN, Quebec	6.7 Ci $mmol^{-1}$; total of 5 mCi.

DDW. Final concentrations were: 0.017 M Tris and 0.14 M NH_4Cl. The final pH was adjusted to 7.4 with 6 N HCl.

$Na_2EDTA/NaHCO_3/NH_4Cl$ lytic solution contained 0.155 M NH_4Cl, 0.01 M $NaHCO_3$ and 0.1 mM EDTA in DDW. Its final pH was adjusted to 7.4 with 1 N NaOH or 1 N HCl.

2.2. Analytical Techniques.

2.2.1. Liquid Scintillation Analysis.

A Beckman model LS 5801 beta counter was used for most of the radioactive analyses of $^{63}NiCl_2$. Sample aliquots (0.9-1.0 ml) were counted for 1 minute in 10 ml of ACS fluid (xylene based). Vials were always left overnight prior to counting to avoid chemiluminescence (Kasprzak and Sunderman, 1979). For the $^{63}Ni^{2+}$ measurements a window setting of 0 to 500 was shown to be suitable.

Quench Corrections. No quenching was observed for veronal buffer or for any of the cell types except the RBCs. Because of the red colour of the hemolysate from RBCs, calibration curves were established to correct for colour quenching. This was achieved by centrifugation (1500 rpm for 10 min) of RBC cell suspensions in veronal buffer, containing $1x10^7$ cells ml^{-1} to $40x10^7$ cells ml^{-1}. The supernatants were decanted and the cells from each tube were transferred in 3 aliquots of 0.3 ml DDW or veronal buffer into a liquid scintillation vial containing 100 µl $^{63}NiCl_2$. Controls containing only 100 µl of $^{63}NiCl_2$ and veronal buffer were also counted.

2.2.2. Methods of Nickel Determination.

Two methods of

ultratrace nickel analysis were employed, namely electrothermal atomic absorption spectrometry (EAAS) and differential pulse polarography (DPP). A detailed description of the DPP method, which was used to determine the nickel content in $^{63}NiCl_2$ stock solutions, is described by Flora and Nieboer (1980). Nickel standards and nickel-free buffers were provided by P. I. Stetsko (McMaster University).

Details of the EAAS measurements are provided by Brown et al (1981) and constitute routine procedures. The material in this section focuses on sample preparation preceding the analytical measurements, although a general outline of the procedure is provided. Since nickel uptake by cells was at ultratrace levels only, extraneous sources of nickel contamination from laboratory wares was removed by acid washing employing standard preventive protocols (Nieboer and Jusys, 1983).

Prior to analysis, cells were lysed by adding 400 µl DDI followed by freeze(liquid N_2)-thawing. The freeze-thaw cycle was repeated two or three more times and cell lysis was checked using a light microscope. To each tube 1.6 ml of DDI was added followed by 100 µl of concentrated 60% HNO_3 (Ultrex) to precipitate protein. Mixing was continued for 1 min and tubes were stoppered and wrapped in Parafilm. Following incubation at 70°C (water bath) for 10 min, the tubes were vortexed for 10 s and centrifuged at 2000 rpm for 30 min. Approximately 1.5 ml of protein-free supernatant was transferred into labelled glass centrifuge tubes and the

nickel was extracted as described below.

Standard solutions (20, 40, 60 and 80 µg L^{-1}) of nickel were prepared by serial dilutions of a 1 µg ml^{-1} stock solution, prepared in 1% HCl(DD). To the blanks (consisting of 2 ml of DDI) and standards (containing 2 ml of prepared standard), 100 µl of 60% HNO_3 was added. Subsequently, the acidified blanks, standards and samples (see above) were adjusted to pH 7. This neutralization step consisted of adding 3 drops of bromothymol blue, 38 µl potassium phosphate buffer, followed by concentrated ammonium hydroxide (Ultrex) dropwise until the colour changed from yellow to blue. After the addition of 375 µl of 2% APDC (freed of any nickel by preincubation with MIBK), the tubes were vortexed and left to stand at room temperature for 5 min. Subsequently, 1.5 ml of MIBK was added to each tube and the mixture was vortexed for 35 s. Each MIBK layer was then transferred to labelled EAAS sample cups. The nickel content of samples and standards were determined on a Perkin-Elmer model 703 atomic absorption spectrometer equipped with an automatic sampler (Perkin-Elmer model AS-1).

2.2.3. Cell Harvester.

This instrument (Titertek Cell Harvester or Skaatron model Mash II) was used to isolate [^{3}H-methyl]thymidine-labelled DNA of lysed lymphocytes. Prior to harvesting the cells, Linbro microtitre plates were frozen and thawed to ensure complete lysis. Lysed cells were then washed out of the wells by aspiration and by the addition of DDW two or

three times. DNA is adsorbed onto fibreglass filter papers. DNA-impregnated filters were dried in a 60°C oven for 2 h, and were transferred to scintillation vials containing Toluene/PPO/POPOP for radioactive measurements.

2.3. Cell Maintenance and Preparation.

2.3.1. HSV B-Lymphoblasts.

The human HSV B-Lymphoblast cell line was donated by B. Zimmerman of Sick Children's Hospital, Toronto. Cells were cultured in Corning flasks (model No. 25100) containing supplemented α-MEM medium and were maintained in an incubator at 37°C with 5% CO_2 tension and near 100% humidity.

In preparation for experimental work, the cells were transferred from the flasks to 50-ml Falcon tubes (model 2095) and were pelleted at 1500 rpm for 10 min. The supernatant was decanted and the cell pellet was resuspended in 30 ml of veronal buffer. Subsequently the cells were centrifuged at 1500 rpm for 10 min. After decantation, the cells were repelleted twice more and the cell number was determined by using a haemacytometer and a light microscope; cell viability was assessed by the trypan blue exclusion method (Johnstone and Thorpe, 1982). The cell concentration was subsequently adjusted to 2×10^7 cells ml^{-1}.

2.3.2. Crowe B-Lymphoblasts.

The human Crowe B-lymphoblast cell line employed was also donated by B. Zimmerman. These cells were cultured in Corning polystyrene flasks (model 25110) containing supplemented RPMI culture medium and were maintained and prepared for

experimentation in the same manner as described above for the HSV lymphoblasts.

2.3.3. Human Mononuclear Leukocytes (Lymphocyte Fraction).

On the day of experimentation, blood was removed from healthy donors by venipuncture (green-capped vacutainer tubes with heparin as anticoagulant). The cell separation technique described below was adapted from Boyum (1968b). Blood (10 ml) was first diluted with 30 ml of PBS (50-ml Falcon tube) and then was layered on top of 10 ml of Ficoll-Hypaque. Centrifugation at 1500 rpm for 10 min followed (see Figure 3 for schematic of the density gradient-dependent separation of human blood cells). Using a Pasteur pipette, the mononuclear (lymphocyte) layer above the Ficoll-Hypaque interface was transferred to another tube. This lymphocyte fraction was diluted with 20 ml of PBS and was then pelleted at 1500 rpm for 10 min. The pellet was resuspended in 30 ml of veronal buffer and was again centrifuged (1500 rpm for 10 min). To remove red blood cells, the cell pellet was treated with 300 µl of DDW for 30 s. Veronal buffer (about 20 ml) was immediately added and the cells were again pelleted at 1500 rpm for 10 min. Another wash with 20 ml of veronal buffer followed. Lymphocyte cell number and viability were determined as before, and the cell concentration was adjusted to 2×10^7 cells ml^{-1}.

2.3.4. Separation of Human Monocytes (Adherent Cell Population) from Peripheral Lymphocytes.

The following protocol is based on the method of Pennline

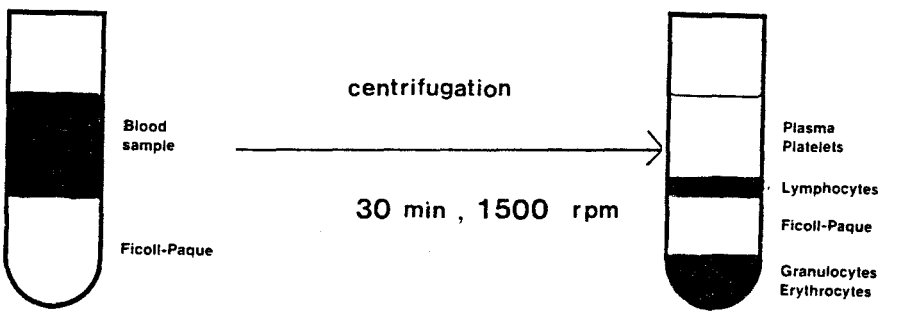


Figure 3. Separation of human blood cells by Ficoll-Hypaque (also called Ficoll-Paque) density-gradient. Reproduced from Ficoll-Paque brochure by Pharmacia Fine Chemicals, 1975.

(1981). After the removal of the mononuclear cell ring from a Ficoll-Hypaque gradient, the cells were washed two times in 20-ml aliquots of RPMI (no AB serum) for 10 min (centrifugation at 1200 rpm). The cell number and viability were determined and cells were plated at a cell concentration $2x10^6$ cells ml^{-1} in RPMI in Falcon (model No. 3003) petri dishes (8 ml per petri dish). Following an incubation for 1 h at 37°C, supernatants containing nonadherent cells were decanted into two 50-ml Falcon tubes. The plates were washed three times each with 3 ml of RPMI. These washes were pooled with previously collected supernatants and centrifuged at 1500 rpm for 10 min with 20 ml RPMI. The cell pellets were pooled and washed for 10 min (centrifugation at 1500 rpm in 20 ml veronal buffer). Cell number and viability were assessed.

2.3.5. Human Red Blood Cells.

Experiments with human erythrocytes were usually performed on the same day as the lymphocyte experiments. Subsequent to the removal of the lymphocyte ring (see Section 2.3.3), the plasma and the Ficoll-Hypaque layer were aspirated (see Figure 3). The packed RBCs were then diluted with 30 ml of veronal buffer and centrifuged at 1500 rpm for 10 min. They were washed twice more with 20-ml aliquots of veronal buffer. The cell number was assessed and the cell concentration was adjusted to $2x10^8$ cells ml^{-1}.

2.3.6. Human Polymorphonuclear Leukocytes.

The procedure described in this section is based on the isolation method of Boyum (1968a). Heparinized blood was

removed from healthy volunteers by venipuncture (green-capped vacutainer tubes). The blood (approximately 10 ml) was spun at 1500 rpm for 10 min. The top plasma layer was removed using a Pasteur pipette till 1/4" was left above the buffy coat layer. The mixed cell suspension (PMNs, mononuclear leukocytes and RBCs) was diluted in a 50-ml Falcon tube with 30 ml of PBS and then was layered over 10 ml of Ficoll-Hypaque. The mixture was subsequently centrifuged at 1500 rpm for 30 min and the mononuclear (lymphocyte) cell ring was carefully removed using a Pasteur pipette; the top plasma layer was also removed. To 5 ml of PMNs and RBCs suspension in a 50-ml Falcon tube, 45 ml of lytic solution ($NH_4Cl/Na_2EDTA/NaHCO_3$) was added. The tube was shaken manually for 5 min until the crimson red colour turned to a deep-purple. After centrifugation at 1500 rpm for 10 min, the supernatant containing the RBC lysate was decanted. If the PMN pellet was still red, then the lytic step was repeated with 10 ml of lytic solution. After lysis, the PMNs were washed twice with 20 ml of veronal buffer (centrifugation at 1500 rpm for 10 min). The cell number and cell viability were determined and the cell suspension was adjusted to a cell concentration of $2x10^7$ cells ml^{-1}.

2.3.7. Rabbit Alveolar Macrophages.

For the isolation of macrophages from rabbit lungs only plasticware was employed, since macrophages have a tendency to stick to glass surfaces. The following procedure was adopted from M^{c}Gee and Myrvik (1981). Using 40 ml of veronal buffer

per lavage, rabbit alveolar macrophages were removed by three pulmonary washes. In each case, 40 ml of veronal buffer was injected into the rabbit lungs, and the trachea was clamped. After a gentle massage of the lungs, the clamp was removed and the wash was decanted into a 50 ml Falcon tube. The cells of the three washes were pelleted at 1500 rpm for 10 min and were pooled after decantation of the supernatants. The pooled cells were resuspended in 30 ml of veronal buffer and centrifuged at 1500 rpm for 10 min. Red blood cells were removed by suspending the cells for 5 min at 37°C in 5 ml of lytic solution (Tris/veronal/NH_4Cl). Immediately after the incubation, 25 ml of veronal buffer was added and the suspension was centrifuged at 1500 rpm for 10 min. The supernatant containing the RBC lysate was decanted and the lysis step was repeated if required. A 20-ml veronal-buffer wash followed (centrifugation at 1500 rpm for 10 min). The macrophages were resuspended in veronal buffer and the cell number and viability were determined. The cell concentration was adjusted to $2x10^7$ cells ml^{-1}.

2.4. Incubation Protocols.

2.4.1. Concurrent Incubation Protocol.

For experiments in which the ligand was concurrently incubated with Ni^{2+}, 100 µl of $^{63}Ni^{2+}$ at the appropriate concentration(s) was first added to the incubation tubes. The rest of the components were added in the following order: 700 or 800 µl of veronal buffer, 100 µl of ligand, followed by 100 µl of cells at appropriate concentration(s). After incubation

at 37°C and near 100% humidity, 3 ml of veronal buffer was added and the cells were pelleted at 1500 rpm for 10 min. The cells were washed twice more in 3 ml of veronal buffer (centrifugation at 1500 rpm for 10 min). They were subsequently transferred to scintillation counting vials using three aliquots of 300 µl DDW (or veronal buffer). Radioactivity was measured in the same manner as described in Section 2.2.1.

In some experiments, a transfer step was incorporated in the above protocol. Immediately following the second wash step, the cell pellet was transferred in 1.0 ml of veronal buffer to a new tube. The original incubation tube was washed with 2 ml of veronal buffer, which was then decanted into the new tube. Subsequently, cells were centrifuged at 1500 rpm for 10 min and cell pellets were transferred to scintillation vials with the aid of three 0.3-ml aliquots of DDW.

2.4.2. Sequential Incubation Protocol.

The following protocol was used for experiments in which cells were first preincubated with the ligand and then incubated with the metal ion. Ligand (900 µl) was first added to the incubation tubes followed by 100 µl of cell suspension. After incubation at 37°C and near 100% humidity, the cells were pelleted at 1500 rpm for 10 min and the incubation medium was decanted. The cells were washed twice in 2 ml of veronal buffer (centrifugation at 1500 rpm for 10 min), and were subsequently resuspended in 1.0 ml of $^{63}NiCl_2$ at the appropriate concentration. To samples destined for EAAS

analysis, 1.0 ml of cold Ni^{2+} (as $NiSO_4$) was employed instead. After incubation at 37°C and near 100% humidity, 3 ml of veronal buffer was added and the cells were pelleted at 1500 rpm for 10 min. Washing with 3 ml of veronal buffer was repeated twice more. The washed cell pellet was transferred to scintillation vials by three aliquots of 300 µl of DDW (or veronal buffer) or was prepared for EAAS analysis (see Section 2.2.2).

2.4.3. Assessment of Nonspecific Losses of Nickel-63 and Its Mass Balance.

For experiments described in Section 2.4.1 and 2.4.2, the nickel-63 content was determined in the following fractions: the washes, the cell pellet and losses to the incubation tube wall. The latter was achieved by vortexing the washed incubation tubes with scintillation cocktail fluid (usually 10 ml). Losses in 'no cell' controls (absence of cells) were also assessed.

2.5. Nickel(II) Uptake Studies.

The following experiments were performed in polystyrene (model 2057) or polypropylene (model 2059) 15-ml Falcon tubes. The incubation medium was veronal buffer.

2.5.1. Dependence of Uptake on Time.

The effects of various incubation times were studied for HSV B-lymphoblasts. For the concurrent metal ion/ligand incubation experiments, incubation times of 0 to 240 min were employed. A range of 0 to 120 min was selected for the experiments with sequential ligand and metal ion incubation

steps. These experiments were done in the presence and in the absence of ligand (10^{-6} M ligand for concurrent incubation and 10^{-5}-10^{-3} M ligand for preincubation experiments) with $2x10^6$ cells/100 µl/incubation tube. A 2 h incubation was used for further concurrent incubation experiments, and for sequential incubations a 1 h ligand incubation followed by 1 h metal ion incubation time were employed.

2.5.2. Dependence of Uptake on Nickel(II) Concentration.

Nickel(II)-dose response uptake curves (concurrent incubations) were established in the absence and in the presence of ligand (10^{-6} M) and involved the following cell types ($2x10^6$ cells/100 µl/incubation tube): Crowe B-lymphoblasts, human peripheral lymphocytes, human PMNs and RAMs. For human RBCs, a cell concentration of $2x10^7$ cells/100 µl/incubation tube was used. The final concentrations of $^{63}NiCl_2$ in the incubation medium ranged from $6.5x10^{-10}$ to $6.5x10^{-7}$ M (expressed as total amount of nickel).

2.5.3. Dependence of Uptake on Cell Number.

The effect of cell number on $^{63}Ni^{2+}$ uptake with or without DDC (concurrent incubation protocol) was studied for Crowe B-lymphoblasts, human peripheral lymphocytes, human RBCs and RAMs. Cell numbers in the range $1x10^5$ to $5x10^6$ were introduced into the incubation tube in 100 µl of buffer for lymphoblasts, lymphocytes and RAMs, whereas a more extensive range ($1x10^6$ to $4x10^7$ cells in 100 µl/incubation tube) was used for human RBCs.

2.5.4. Nickel(II) Distribution Studies.

Following uptake studies using concurrent incubation (see Section 2.4.1), the isolated cell pellet was lysed by the addition of 300 µl of DDW, vortexed, frozen (liquid N_2) and then thawed. The freeze-thaw cycle was repeated three more times, and the extent of lysis was checked by light microscopy. The lysate fraction was decanted after centrifugation at 12,300 g for 60 min at 4°C. Subsequently, the membrane pellets were washed twice with 300 µl DDW (centrifugation at 12,300 g for 30 min at 4°C). The decanted lysate and subsequent washings were pooled for scintillation counting. The washed membrane pellet was transferred to a scintillation vial with three aliquots of 300 µl of DDW. Uptake by whole cells (control) was also determined (see Section 2.4) for reference purposes. The above fractionation procedure is based upon the method reported by Ong and Lee, 1980b.

2.5.5. Loss of Cell-Associated Nickel(II) to Culture Media.

Following the preincubation experiment under sterile conditions described in Section 2.4.2, 1.0 ml of complete α-MEM culture medium supplemented with 15% FCS was added to each tube containing the lymphoblast cell pellet. For peripheral lymphocytes, 1.0 ml of RPMI or α-MEM supplemented with 10% human AB serum was employed. Cells were incubated at 37°C near 100% humidity for periods of 0.5 to 72 h. Subsequently, cells were isolated by centrifugation (1500 rpm for 10 min) and the pellet was washed twice with 1.0-2.0 ml

veronal buffer. For scintillation counting, cell pellets were transferred with three 0.3-ml aliquots of veronal buffer. Samples with cold Ni^{2+}, (as $NiSO_4$; Section 2.4.2) were incubated similarly. For these samples, viabilities were determined and the nickel content was assessed by EAAS analysis (Section 2.2.2). The nickel content of the cells prior to incubation in α-MEM was also determined.

2.6. Lymphocyte Transformation.

2.6.1. Lymphocyte Transformation Test.

This procedure is based upon the study by Al-Tawil et al (1981). For this test, lymphocytes from patch-tested nickel sensitive and non-sensitive individuals were cultured. Sterile culture techniques were used for all steps of the procedure. All of the solutions were either autoclaved or filtered through a 0.22 μm Nalgene filter to destroy or remove possible bacterial contamination.

The lymphocyte fraction, removed from the Ficoll-Hypaque gradient (see Section 2.3.3), was washed twice in 20 ml of RPMI (no serum) at 1300 rpm for 10 min. The cell viability was determined and cell concentration was adjusted to $1x10^6$ cells ml^{-1} in RPMI supplemented with 20% human AB serum. The antigen ($NiSO_4$) and PHA solutions were prepared in the RPMI culture medium to the following final concentrations: PHA, 5-100 μg ml^{-1} ; and $NiSO_4$, 50 μg ml^{-1} ($1.9x10^{-4}$ M), 25 μg ml^{-1} ($9.5x10^{-5}$ M), 12.5 μg ml^{-1} ($4.75x10^{-5}$ M) or 6.25 μg ml^{-1} ($2.4x10^{-5}$ M). $NiSO_4$ solutions were prepared in RPMI by serial dilutions of a stock 1% (w/v) solution.

Incubation was carried out in 96-multiwell Linbro round-bottomed plates at 37°C, 5% CO_2 and near 100% humidity. After adding 100 µl of cell suspension to the wells, 100 µl of RPMI, or 100 µl of $NiSO_4$ or 100 µl of PHA were pipetted in and the plates were subsequently shaken for 5 min on an orbital action shaker. Cells were then cultured in quadruplicate for a maximum of 4 d with PHA, for 8 d with RPMI and for 8 d with $NiSO_4$. Twenty four h prior to the day of harvesting, sets of 4 replicate samples were pulsed with 25 µl of 1 µCi [^{3}H-methyl]thymidine. Cells were then harvested and the radioactivity was assessed by scintillation counting (see Section 2.2.3).

The optimal dose of PHA required for transformation was determined by varying the PHA concentrations (range 5 to 100 µg ml^{-1}) employing specimens from three nickel non-sensitive control subjects.

2.6.2. Transformation of APDC-Preincubated Cells.

Sterile techniques were employed for all steps. Cells from both nickel sensitive and nickel non-sensitive individuals were tested. Lymphocytes were isolated from heparinized blood (diluted in PBS) by Ficoll-Hypaque gradient (Section 2.3.3). The mononuclear cell ring was isolated and twice washed in 20 ml PBS at 1500 rpm for 10 min. After determining cell number and viability, a fraction of the cells were incubated for 30 min in 10^{-3} M APDC or in veronal buffer: $2x10^6$ cells ml^{-1} of incubation medium per incubation tube. Following the incubation at 37°C, 2 ml of veronal buffer was

added to each tube and cells were pipetted up and down with a Pasteur pipette. Cells were then pooled into a 50-ml Falcon tube (transferred with veronal buffer). Cells were pelleted at 1500 rpm for 10 min, the supernatant was decanted and cells were resuspended in 10 ml RPMI (with no AB serum). Cell number and viability were redetermined, and cells were resuspended in RPMI supplemented with human AB serum for the lymphocyte transformation test (see Section 2.6.1).

3. RESULTS

3.1. Specific Activity of Nickel-63.

The total (labelled and unlabelled) nickel concentration in the $^{63}NiCl_2$ stock solution was found to be 6.5 $\pm$ 0.4 x10^{-4}M, giving a corrected specific activity of 0.61 mCi μmol^{-1} (see Table 3).

3.2. Reporting of Data.

Unless otherwise specified, the experimental data reported in tables and figures are given as the mean of three replicates $\pm$ one standard deviation. In figures, standard deviations smaller than the data point symbols are not indicated.

In subsequent sections of this thesis a number of specific quantities are calculated from the data. These are defined in Equations 1 to 3. The assessment of standard deviations in these mathematical operations have been carried out according to the rules of propagation of errors (e.g. Skoog and West, 1976).

$$\text{Relative Uptake}(\%) = \frac{\text{Radioactivity(ligand)} - \text{Radioactivity(no ligand)}}{\text{Radioactivity (no ligand)}} \times 100 \quad (1)$$

$$\text{Relative Distribution in the Cell Compartment (\%)} = \frac{\text{Radioactivity(cell compartment)}}{\text{Radioactivity(whole cell)}} \times 100 \quad (2)$$

$$\text{Stimulation Index} = \frac{\text{Radioactivity (stimulant)}}{\text{Radioactivity (no stimulant)}} \quad (3)$$

Table 3. Specific Activity of $^{63}Ni^{2+}$ Stock Solution

Experiment No. or Value	$[Ni^{2+}]_t$ (M)[a,b]	Specific Activity (mCi μmol^{-1})[c]
1	6.93×10^{-4}	0.57
2	6.22×10^{-4}	0.63
3	6.31×10^{-4}	0.62
Mean Value	$6.5 \pm 0.4 \times 10^{-4}$	$0.61 \pm .04$
Manufacturer's value	5.8×10^{-4}	0.69

[a]Concentration of nickel was assessed by DPP.

[b]The total nickel concentration of an older stock solution was found by DPP to be $[Ni^{2+}]_t = 7.0 \times 10^{-4}$ M, with a specific activity of 0.57 mCi μmol^{-1}. This solution was used in a few of the experiments reported.

[c]The specific activity was calculated from the given radioactivity (2 mCi) and $[Ni^{2+}]_t$ or from the given specific activity (11.42 mCi mg^{-1}).

The Relative Uptake represents the ligand-induced increment of nickel uptake expressed relative to what is bound to control cells. The Relative Distribution corresponds to the percentage of nickel present in each cellular compartment, relative to nickel associated with the whole cells. The Stimulation Index compares the amount of cell proliferation in the presence and absence of a mitogen (e.g., PHA) or of an antigen ($NiSO_4$).

3.3. Mass Balance and Nonspecific Losses of Nickel-63.

The background counts observed in 'no cell' control experiments are summarized in Tables 4 to 6. It is evident that for constant $[Ni^{2+}]_t$ ($6.5x10^{-8}$ or $6.5x10^{-9}$ M) background counts increase significantly at certain concentrations of ligand. This occurs at ligand concentrations $\geq 10^{-5}$ M for concurrent incubation (Table 4) and at 10^{-2} M for sequential incubation (Table 5). Similarly at constant ligand concentration (10^{-6} M DDC), background counts increase significantly at $[Ni^{2+}]_t \geq 6.5x10^{-8}$ M (Table 6). It is interesting to note that when a transfer step is incorporated in the concurrent protocol, these high background counts are no longer evident (Table 4).

From Figure 4 it is seen that there is a loss of nickel-63 in concurrent incubation experiments (with transfer) at ligand concentrations $> 10^{-7}$ M DDC. This is also found in concurrent incubations that exclude the transfer step (data not shown). It is evident from Figure 4 that this phenomenon occurs whether cells were present or not in the experiment. Washing of buffer-washed incubation tubes with scintillation counting fluid indicated

Table 4. Background Counts for "No Cell" Experiments (Concurrent Incubation)[a]

$[Ni^{2+}]_t$ (M)	LIGAND	NO LIGAND	Ligand Concentration (M) 10^{-8}	10^{-7}	10^{-6}	10^{-5}	10^{-4}	10^{-3}
$6.5x10^{-8}$	DDC	30±10	ND	27±9	30±10	600±180	300±70	210±80
$6.5x10^{-9}$	DDC	16±3	29±9	24±2	17±3	110±20	81±7	84±2
$6.5x10^{-8}$	DDC[b]	13±4	ND	21±1	22±3	30±2	26±8	26±1
$6.5x10^{-8}$	APDC	22±4	27±1	30±6	26±4	500±90	450±120	200±100
$6.5x10^{-9}$	APDC	16±3	26±4	28±4	25±3	100±20	43±5	55±5
$6.5x10^{-8}$	APDC[b]	21±1	ND	29±4	24±6	25±1	23±1	22±1

[a] cpm ± standard deviation (n=3); ND, not determined.

[b] Experiments included a transfer step to a new set of incubation tubes.

Table 5. Background Levels for "No Cell" Experiments (Sequential Incubation)[a]

$[Ni^{2+}]_t$ (M)	LIGAND	NO LIGAND	Ligand Concentration (M) 10^{-7}	10^{-6}	10^{-5}	10^{-4}	10^{-3}	10^{-2}
6.5×10^{-8}	DDC	28±8	32±5	28±9	34±4	25±10	29±7	590±260
6.5×10^{-8}	APDC	30±7	32±7	28±5	26±5	31±10	35±6	250±50

[a] cpm ± standard deviation (n=3).

Table 6. Background Levels for "No Cell" Experiments for Nickel Dose Response (Concurrent Incubation Without Transfer)[a]

	Nickel(II) Concentration (M)			
Condition	6.5×10^{-10}	6.5×10^{-9}	6.5×10^{-8}	6.5×10^{-7}
Control (No DDC)	28±2	19±1	30±2	47±10
10^{-6}M DDC	27±4	69±8	430±50	960±30

[a] cpm ± standard deviation (n=3).

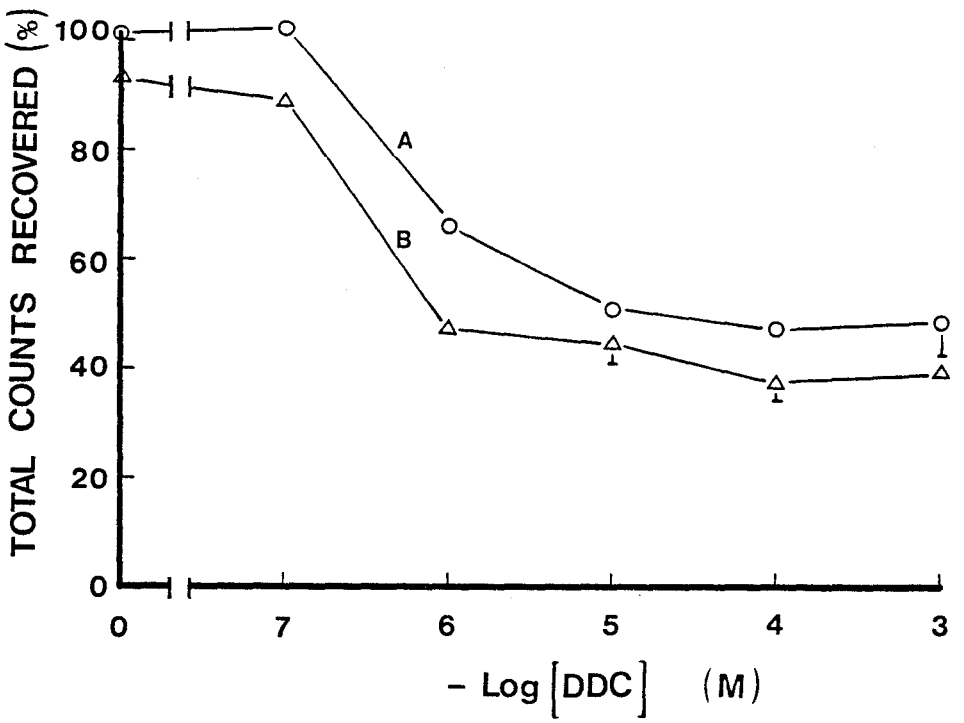


Figure 4. Effect of DDC concentration on the percentage of total counts of nickel-63 recovered (concurrent incubation with transfer). Curve A: "no cell" control experiment; Curve B: in the presence of peripheral lymphocytes (1.4×10^6 cells). $[Ni^{2+}]_t = 6.5 \times 10^{-8}$ M. Absence of ligand is denoted by zero on the concentration scale.

significant adsorption of the label onto tube walls (data not reported). In sequential incubation experiments, a higher recovery of total counts was found at all concentrations of DDC and APDC in the absence or presence of cells (Figure 5).

As shown in Figure 6, three wash steps were sufficient to remove excess unbound nickel-63 from cell suspensions. The majority of the counts were removed in the first wash; <15% in the second wash; and <10% in the third.

3.4. Human B-Lymphoblast Studies.

3.4.1. General Comments.

Unless specified otherwise, Crowe B-lymphoblast cells were employed. Prior to experimentation, cell viability was 85-90%. During the experiment an additional cell mortality of <15% occurred. The data describing nickel dose and cell concentration effects on nickel(II) uptake were corrected for 'no cell' background levels. In other types of experiments, the 'no cell' backgrounds were negligible (relatively speaking).

3.4.2. Effect of Ligand Concentration on Nickel(II) Uptake by B-Lymphoblasts.

Data displayed in Figures 7,8 and 9 exhibit a maximum in nickel(II) uptake at a DDC or APDC concentration of 10^{-6} M. A comparison of the data for APDC and DDC (Figure 7) reveals that the maximum relative uptake for APDC is ~3-fold higher than that for DDC. At the lower Ni^{2+} concentration examined (i.e., 6.5×10^{-9} M) no substantial difference was observed between the relative uptake for the two ligands (Figure 8). From the data in Figure 9, it is evident that the introduction of a transfer step has not altered

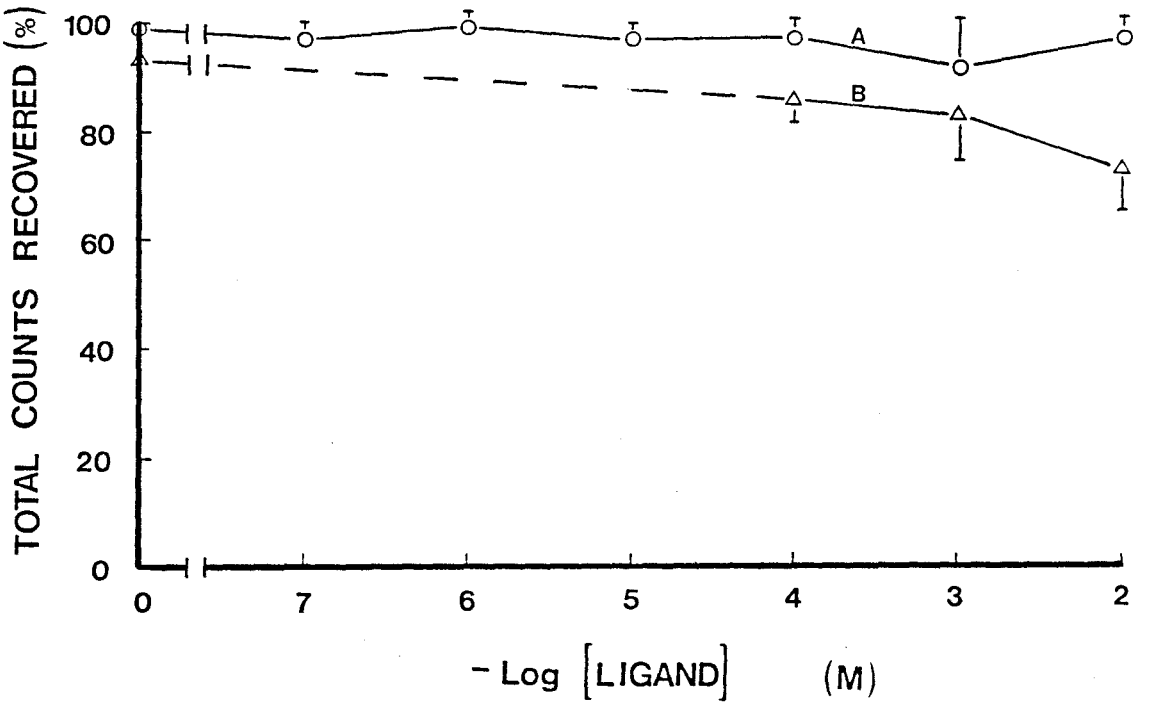


Figure 5. Effect of ligand concentration on the percentage of total counts of nickel-63 recovered (sequential incubation). Curve A: "no cell" control experiment. Curve B: in the presence of HSV B-lymphoblasts (1.9×10^6 cells). $[Ni^{2+}]_t = 7 \times 10^{-8}$ M. Data points represent the average of the mean values for both DDC and APDC ($\pm$ standard deviation), where n=6 for Curve A and n>9 for Curve B. Absence of ligand is denoted by zero on the concentration scale.

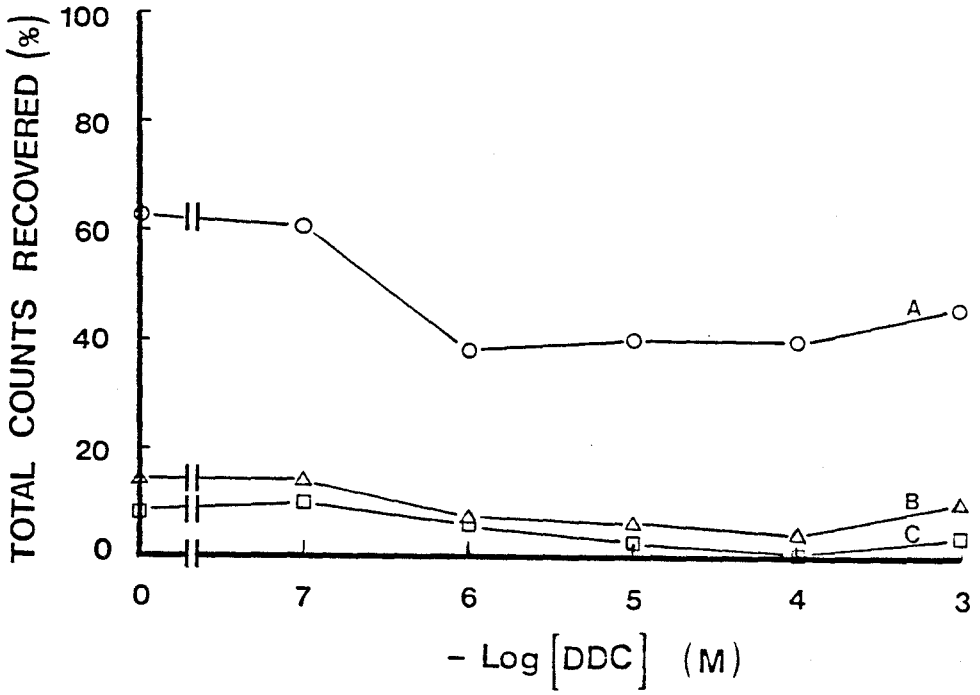


Figure 6. Effectiveness of washing procedure in the removal of unbound nickel-63 from RAMs (concurrent incubation with transfer). Curve A: % of counts recovered in the first supernatant; B: % of counts recovered in the second supernatant; and C: % of counts recovered in the third supernatant. $[Ni^{2+}]_t = 6.5 \times 10^{-8}$ M. Absence of ligand is denoted by zero on the concentration scale.

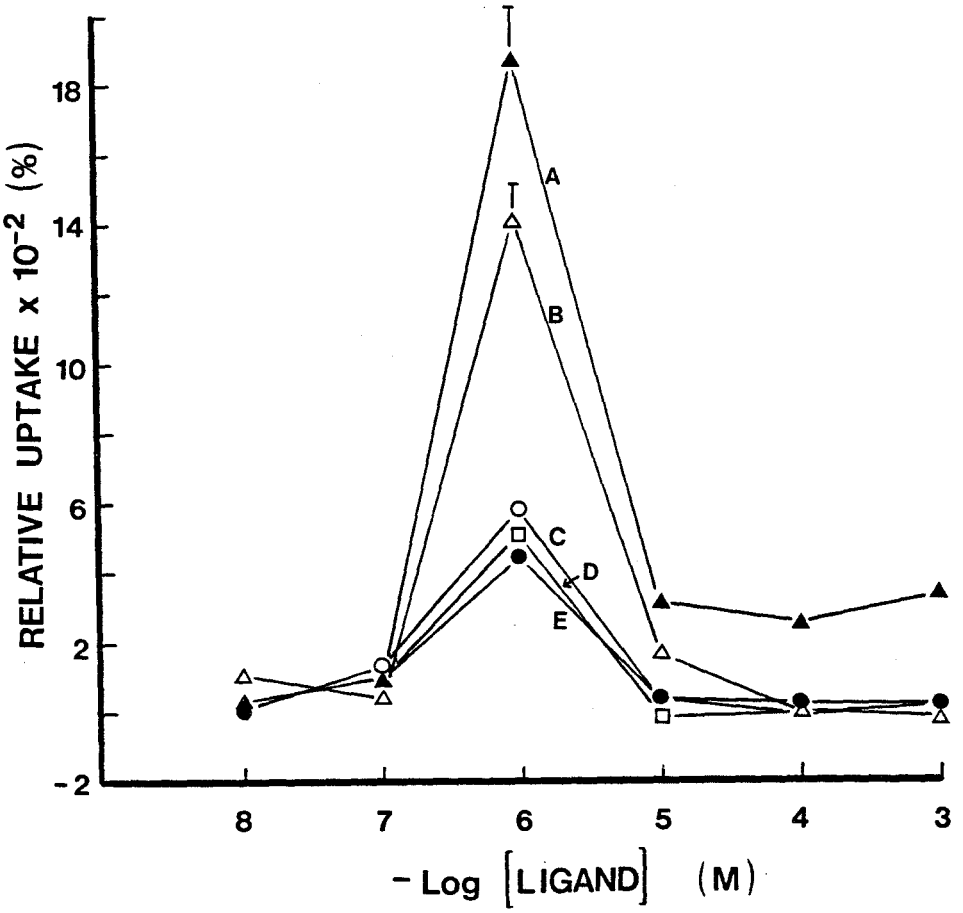


Figure 7. Effect of ligand concentration on nickel(II) uptake by B-lymphoblasts (concurrent incubation). Curves A (2.4×10^6 cells) and B (2×10^6 cells) correspond to APDC, and C (1×10^6 cells), D (2×10^6 cells) and E (1.4×10^6 cells) refer to DDC. $[Ni^{2+}]_t = 6.5 \times 10^{-8}$ M.

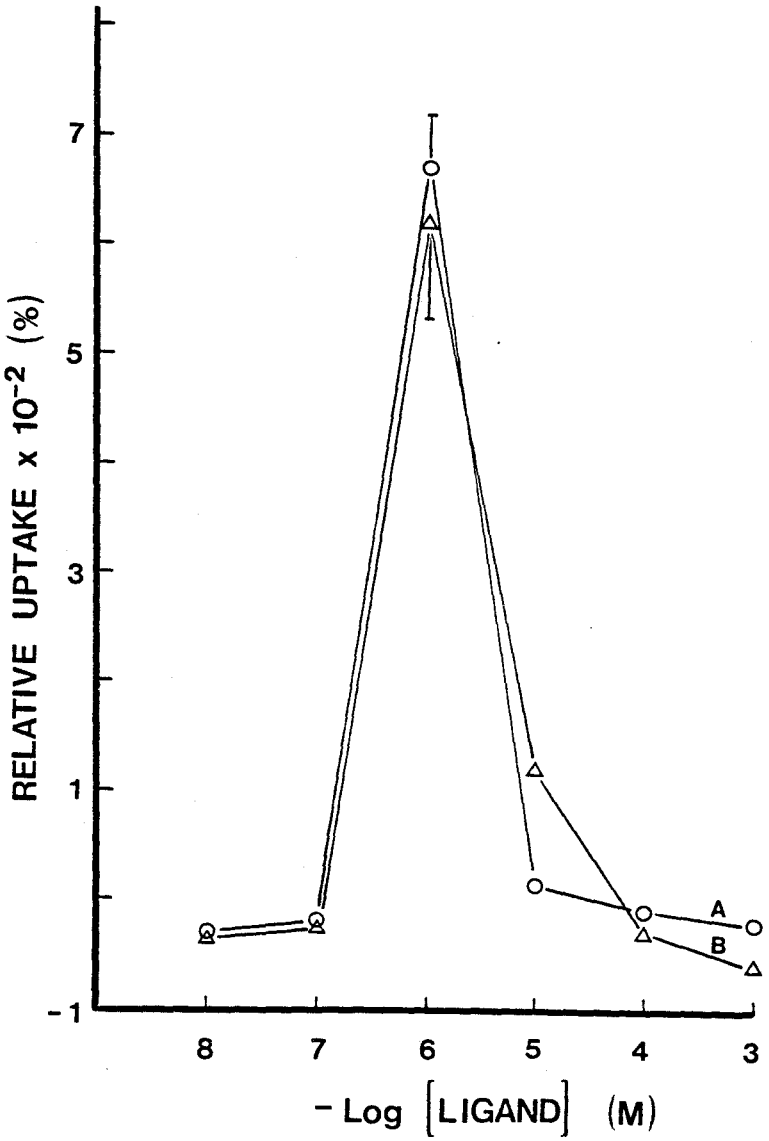


Figure 8. Effect of ligand concentration on nickel(II) uptake by B-lymphoblasts (concurrent incubation). Curve A ($2x10^6$ cells) corresponds to DDC and B ($2x10^6$ cells) refers to APDC. $[Ni^{2+}]_t=6.5x10^{-9}$ M.

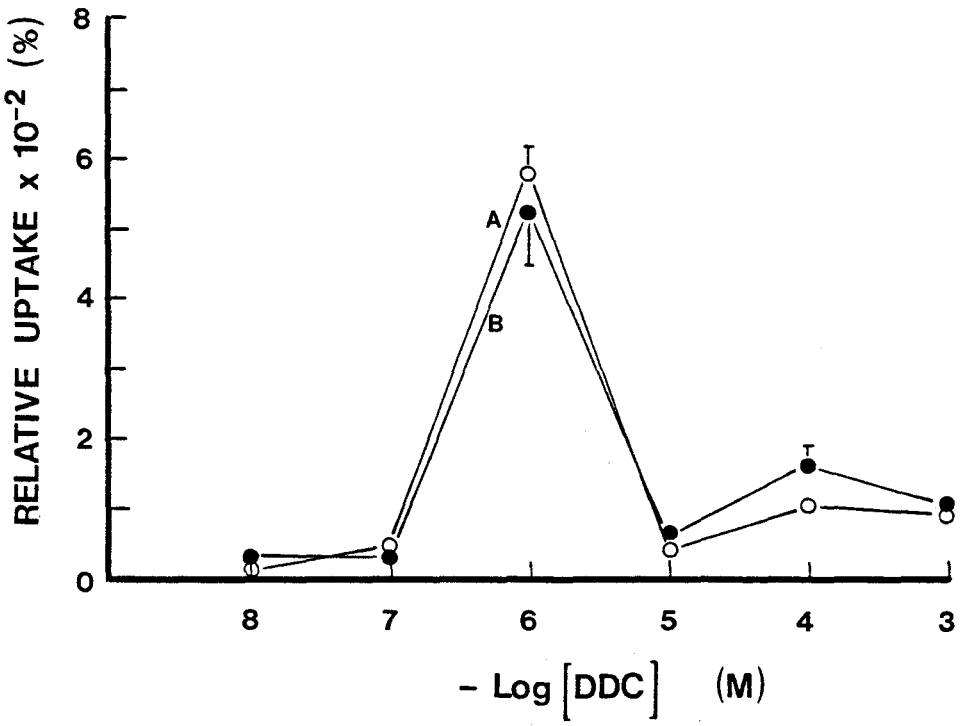


Figure 9. Effect of DDC concentration on nickel(II) uptake by B-lymphoblasts (concurrent incubation). The experiment corresponding to Curve A (3.2×10^6 cells) does not include transfer step while B (3.2×10^6 cells) does. $[Ni^{2+}]_t = 6.5 \times 10^{-8}$ M.

the trend in relative uptake.

For the sequential-incubation protocol, a maximum in relative uptake was observed at 10^{-3} M APDC, while that for DDC appears to approach saturation at concentrations $\geq 10^{-3}$ M (Figure 10). The relative uptake at 10^{-3} M APDC in sequential experiments is similar to that found at 10^{-6} M APDC in concurrent incubations. For DDC, nickel uptake is more enhanced in the sequential protocol (c.f., Figures 9 and 10).

3.4.3. Effect of Incubation Times on Nickel(II) Uptake.

For incubation periods of 60-240 min, very little time dependence is seen in the absence of DDC (Figure 11). By contrast, in the presence of 10^{-6} M DDC there is a time dependence $\leq$ 60 min.

For each incubation step of the sequential protocol, a time dependence of nickel uptake is evident at 10^{-3} M DDC (Figure 12). Incubation times $\geq$ 1 h are shown to be the most suitable time for the ligand-preincubation step. Incubation times $\geq$ 30 min appear suitable for nickel(II) uptake. For the experimental time interval, the actual uptake (observed counts) were not significantly different in the absence and presence of 10^{-5}M DDC (data not shown). Thus at this concentration of DDC, the relative uptake shows no strong dependence on time.

3.4.4. Effect of Nickel(II) Concentration on Nickel(II) Uptake.

The response to nickel dose depicted in Figure 13 reveals that the relative uptake remains approximately constant for $[Ni^{2+}]_t$ in the range 6.5×10^{-10} M to 6.5×10^{-7} M ($-\log [Ni^{2+}]_t = 9.2$ to 6.2). By contrast, the actual uptake increased with increasing nickel(II) concentration; both in the absence and presence of 10^{-6} M DDC.

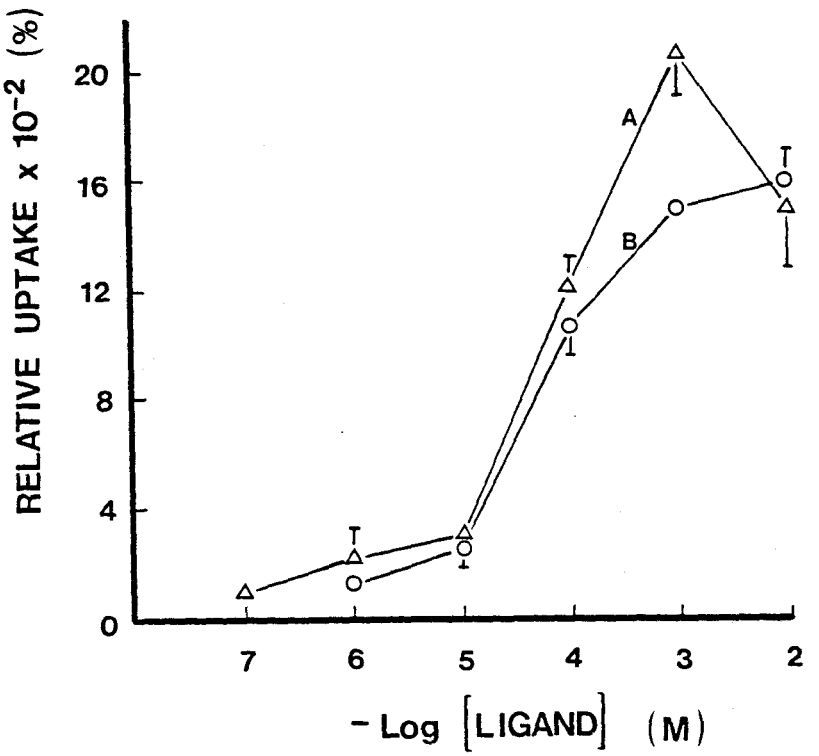


Figure 10. Effect of ligand concentration on nickel(II) uptake by B-lymphoblasts (sequential incubation). Curve A (3.2×10^6 cells) corresponds to APDC and B (3.2×10^6 cells) refers to DDC. $[Ni^{2+}]_t = 6.5 \times 10^{-8}$ M.

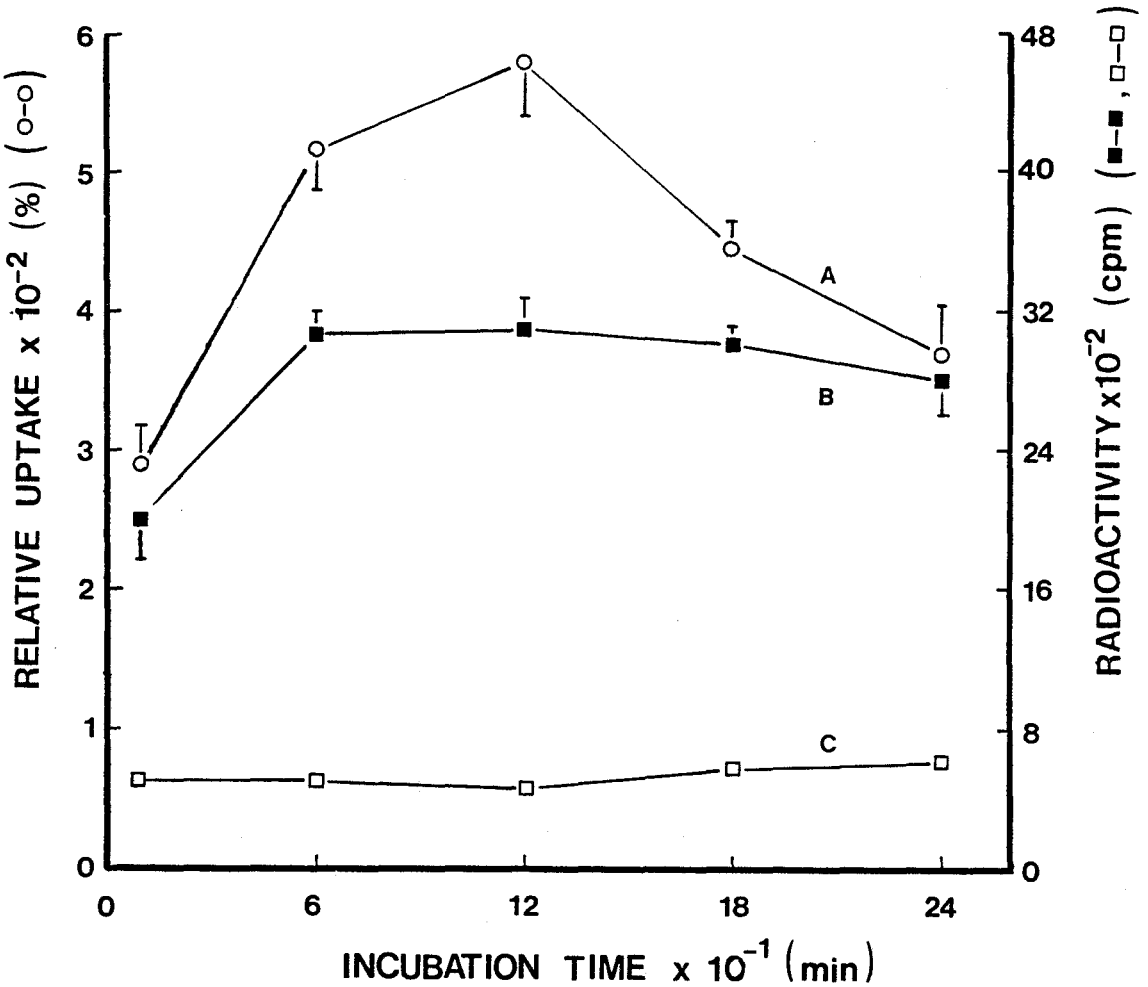


Figure 11. Effect of incubation time on nickel(II) uptake by B-lymphoblasts (concurrent incubation). Curve A gives the relative uptake. Curves B (at 10^{-6} M DDC) and C (no DDC) refer to the observed nickel(II) uptake expressed in cpm. $[Ni^{2+}]_t = 7.0 \times 10^{-9}$ M; 2×10^6 HSV B-lymphoblasts.

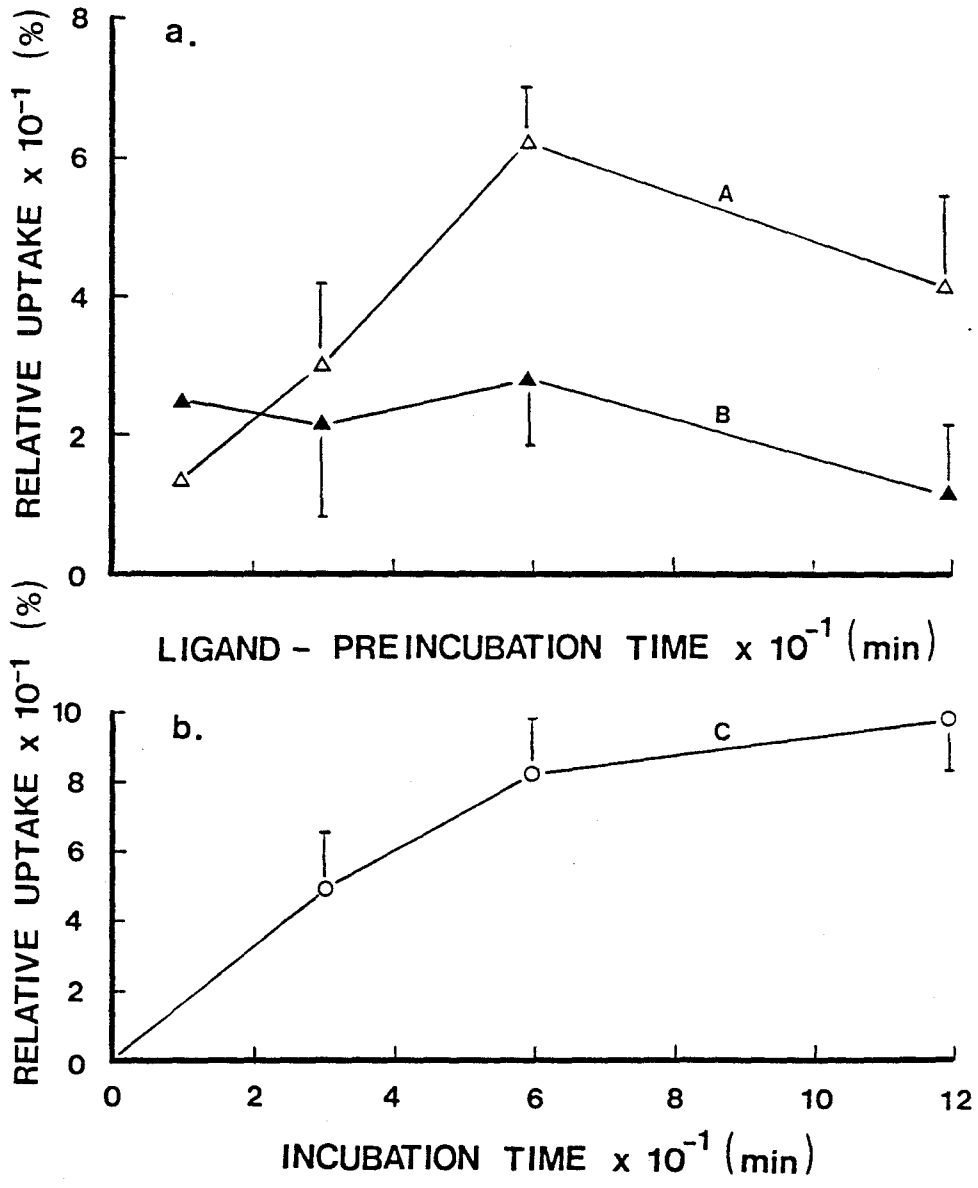


Figure 12. Effect of incubation time on nickel(II) uptake by B-lymphoblasts (sequential incubation).

a. Effect of ligand-preincubation time on nickel(II) uptake (the subsequent nickel incubation time was for 1 h). Curve A ($1x10^6$ cells) corresponds to 10^{-3} M DDC and B refers to 10^{-5} M DDC ($1x10^6$ cells).

b. Effect of nickel(II) incubation time on nickel(II) uptake at 10^{-4} M DDC ($2x10^6$ cells) for a ligand-preincubation time of 1 h. For both a and b, HSV B-lymphoblasts were employed; $[Ni^{2+}]_t = 7x10^{-8}$ M.

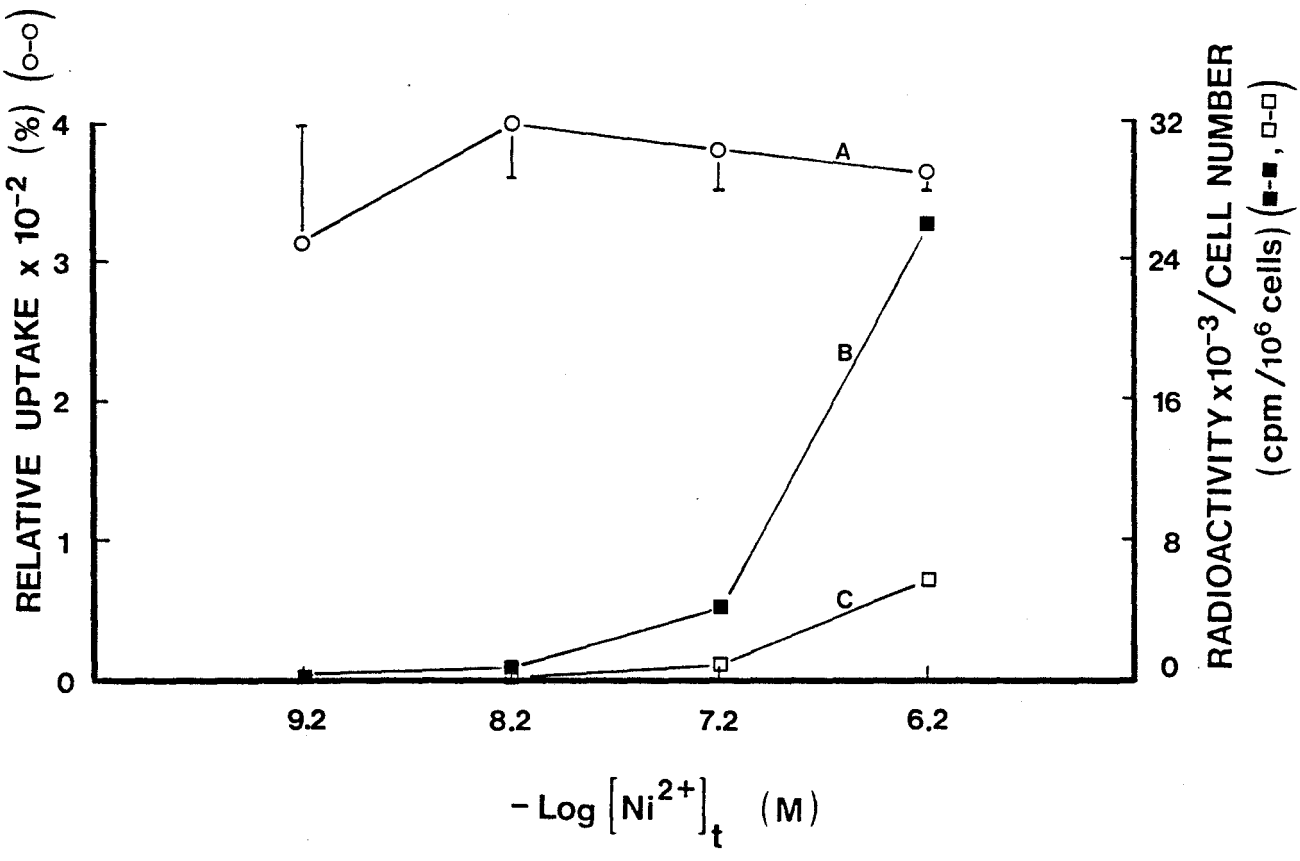


Figure 13. Effect of nickel(II) concentration on nickel(II) uptake by B-lymphoblasts (concurrent incubation). Curve A depicts relative uptake at 10^{-6} M DDC; in Curves B (at 10^{-6} M DDC) and C (no DDC) the observed nickel-63 counts per 10^6 cells are plotted. The experimental cell number was $2x10^6$ for all three curves.

3.4.5. Effect of Cell Concentration on Nickel(II) Uptake.

An increase in cell number clearly decreases the actual and relative uptake of nickel(II) (see Figure 14). At cell number $\geq 2 \times 10^6$, the actual and relative nickel(II) accumulations remain approximately constant.

3.4.6. Effect of Ligand Concentration on Relative Uptake of Nickel(II) by Cellular Compartments.

A maximum in relative uptake at 10^{-6} M DDC is exhibited (Figure 15) for whole cells and for the two cell compartments examined (cell lysate and pellet). At 10^{-6} M DDC, the highest relative uptake is indicated for the cell lysate.

3.4.7. Retention of Nickel(II) Following Incubations in α-MEM.

From the data summarized in Table 7, it is evident that during post-incubations in α-MEM supplemented with FCS, a higher percentage of nickel is retained by those cells which have been preincubated with 10^{-3} M APDC. After 24 h in the α-MEM medium, the loss in cell viability is greatest for cells preincubated with 10^{-2} M APDC.

3.5. Rabbit Alveolar Macrophage Studies.

3.5.1. General Comments.

Prior to and after experiments, the viabilities of RAMs were checked. Freshly isolated cells had viabilities of ~80% and by the end of the experiment, cell mortality had increased by about 25%. Trends and magnitudes of nickel(II) uptake showed no apparent dependence on cell viability. Data for nickel-dose response and cell concentration effects were corrected for 'no cell' background levels. In other types of experiments, the 'no cell' backgrounds

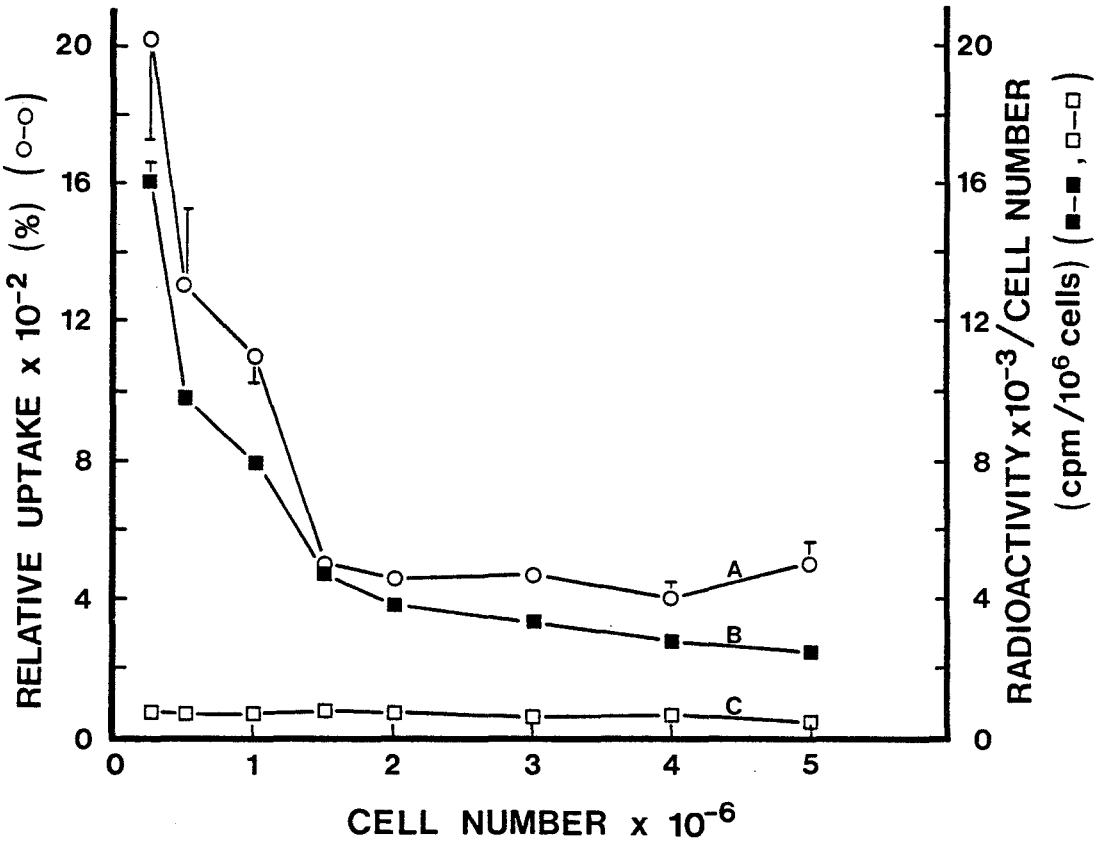


Figure 14. Effect of cell concentration on nickel(II) uptake by B-lymphoblasts (concurrent incubation). Curve A corresponds to relative uptake at 10^{-6} M DDC; Curves B (at 10^{-6} M DDC) and C (no DDC) give the observed nickel(II) uptake per 10^6 cells. Although not shown in the figure, the total amount of cell-associated nickel-63 label in samples increased with cell number (i.e., the product of the quantities plotted on the abscissa and the ordinate at the right increased with cell number). $[Ni^{2+}]_t = 6.5 \times 10^{-8}$ M.

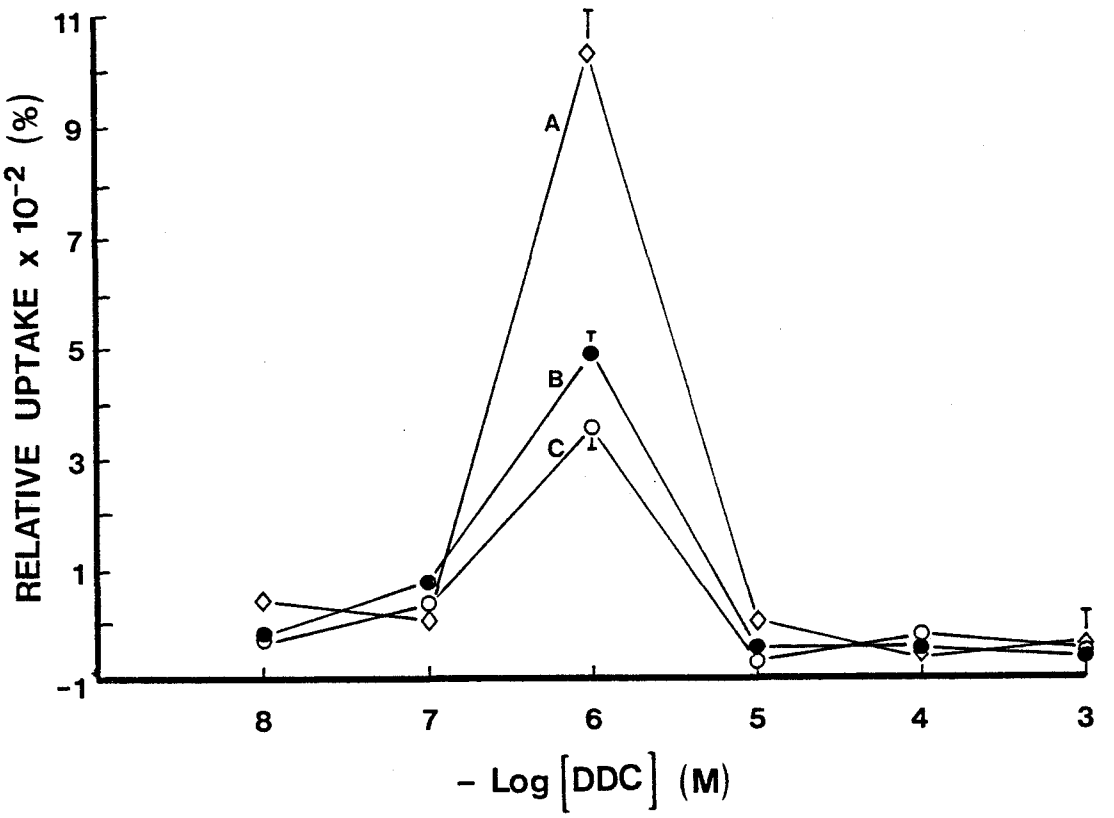


Figure 15. Effect of DDC concentration on nickel(II) uptake by cellular compartments of B-lymphoblasts (concurrent incubation with transfer). Curve A corresponds to cell lysate and B and C refer to the whole cell and pellet, respectively. $[Ni^{2+}]_t = 6.5 \times 10^{-8}$ M; 1.8×10^6 cells.

Table 7. Percentage of Nickel(II) Retained by B-Lymphoblasts Incubated in α-MEM[a]

Time in MEM + FCS, t (h)	APDC Concentration[b,c,d] None	10^{-3} M	10^{-2} M
0	100 (80)	100 (72)	100
0.25	16±4	50±28	74±36
0.5	15±9 (83)	63±23 (83)	74±39 (69)
2	10±3 (81)	43±7 (82)	56±28 (64)
24	7±1 (69)	27±16 (51)	8±1 (15)

[a] Nickel uptake involved the sequential protocol in veronal buffer (Section 2.5.5); $[Ni^{2+}]_t$ in the nickel uptake step was 6.5×10^{-8} M.

[b] Percentages are calculated relative to the amount of Ni^{2+} associated prior to the α-MEM/FCS incubation step.

[c] Values in parentheses denote cell viability (in %) at time t.

[d] Mean ± standard deviation (n=3).

were negligible.

3.5.2. Effect of Ligand Concentration on Nickel(II) Uptake.

The data in Figures 16 and 17 illustrate the dependence of nickel(II) uptake on ligand concentration for both DDC and APDC. Both responses exhibit a maximum at 10^{-6}M. At higher concentrations of ligand $\geq 10^{-5}$ M, inhibition of nickel(II) uptake is revealed. Insertion of a transfer step into the concurrent incubation protocol did not affect the trends of nickel(II) uptake (Figure 17).

Trends in the APDC-concentration dependence in sequential incubation experiments were similar to those observed for B-lymphoblasts (c.f., Figures 10 and 18). A maximum in relative uptake was seen at 10^{-4} M DDC and 10^{-3} M APDC. Maximum uptake values were similar to those found in concurrent incubations (c.f., Figures 16-18).

3.5.3. Effect of Nickel(II) Concentration on Nickel(II) Uptake.

Relative and actual accumulations (Figure 19) increase sharply for $[Ni^{2+}]_t > 6.5x10^{-9}$ M ($-\log [Ni^{2+}]_t=8.2$). The enhancement effect of DDC becomes more pronounced at $[Ni^{2+}]_t \geq 6.5x10^{-8}$ M ($-\log [Ni^{2+}]_t=7.2$).

3.5.4. Effect of Cell Concentration on Nickel(II) Uptake.

In the presence of DDC, an increase in cell number appears to decrease the actual amount of nickel(II) taken up by RAMs (Figure 20). Not much dependence is seen in the absence of DDC. With an increase in cell number, the trend in relative uptake seems to decrease although their differences are not statistically different. It is clear by comparing the data in Figure 14 for

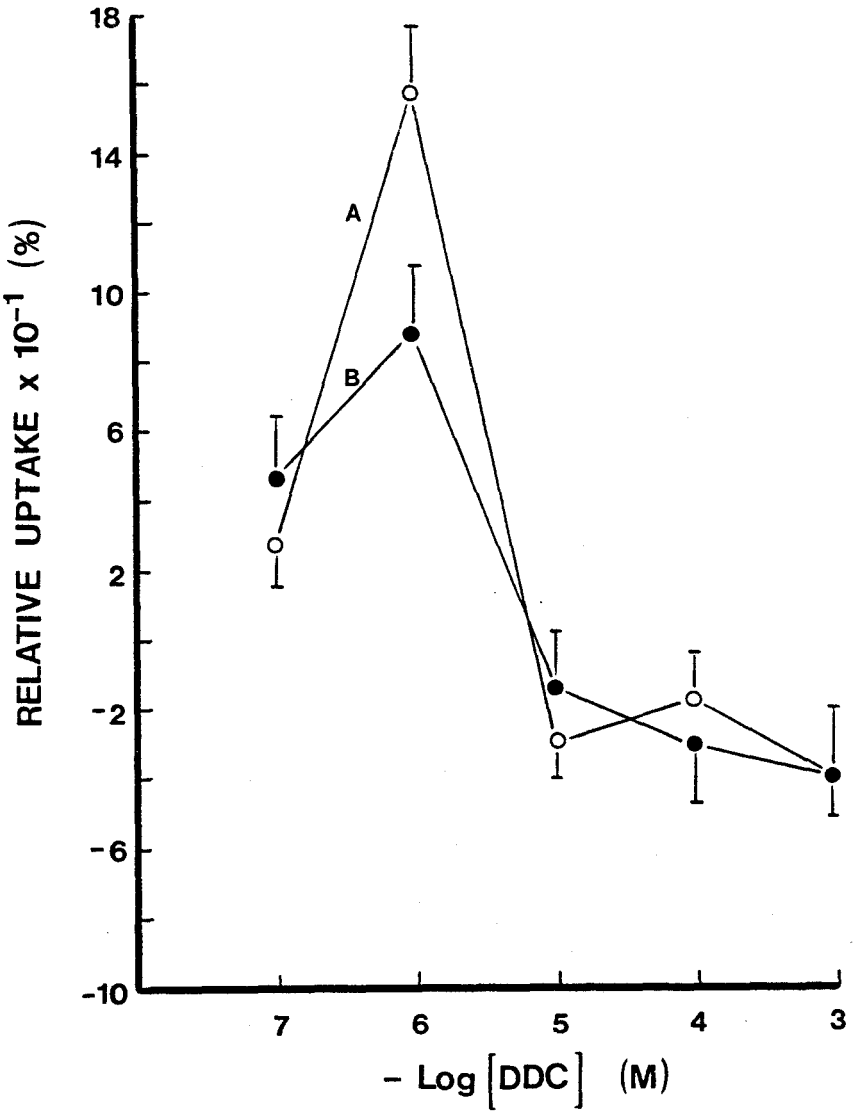


Figure 16. Effect of DDC concentration on nickel(II) uptake by RAMs (concurrent incubation). For both Curve A (1.7×10^6 cells) and B (1.5×10^6 cells), $[Ni^{2+}]_t = 6.5 \times 10^{-8}$ M.

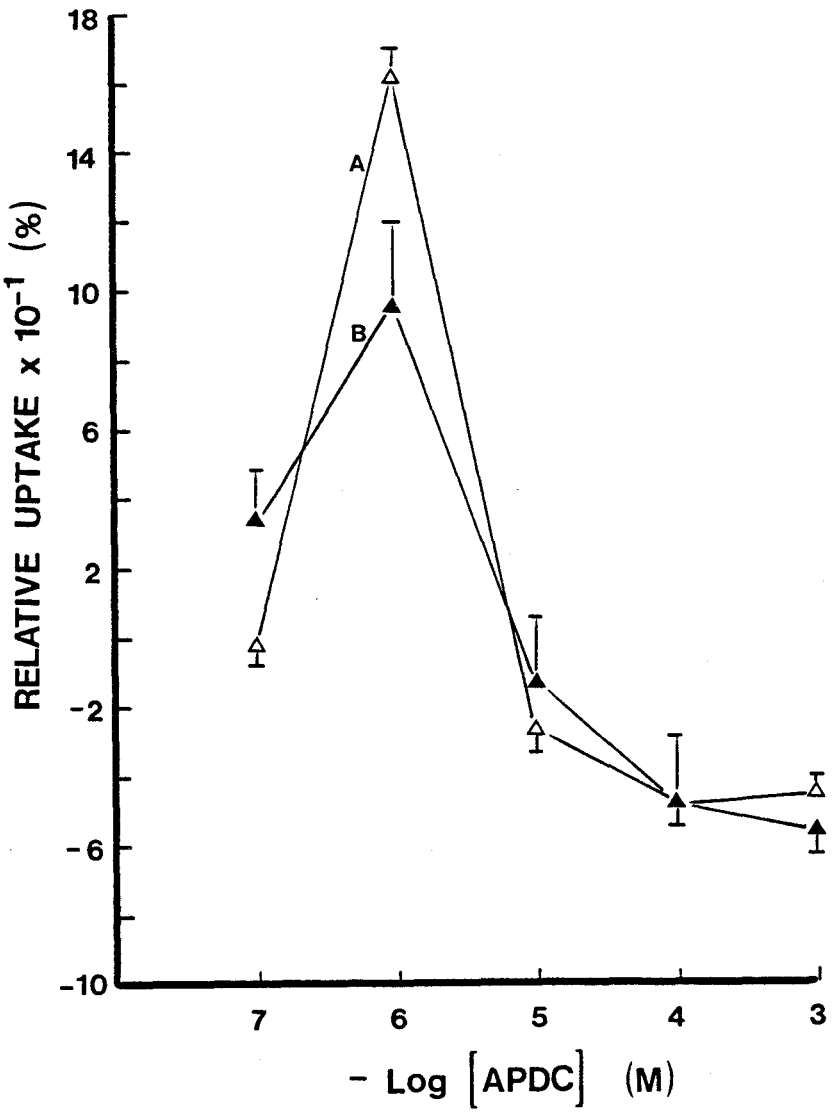


Figure 17. Effect of APDC concentration on nickel(II) uptake by RAMs (concurrent incubation). Curve A (1.8×10^6 cells) corresponds to an experiment without transfer and B (1.5×10^6 cells) includes a transfer step. $[Ni^{2+}]_t = 6.5 \times 10^{-8}$ M.

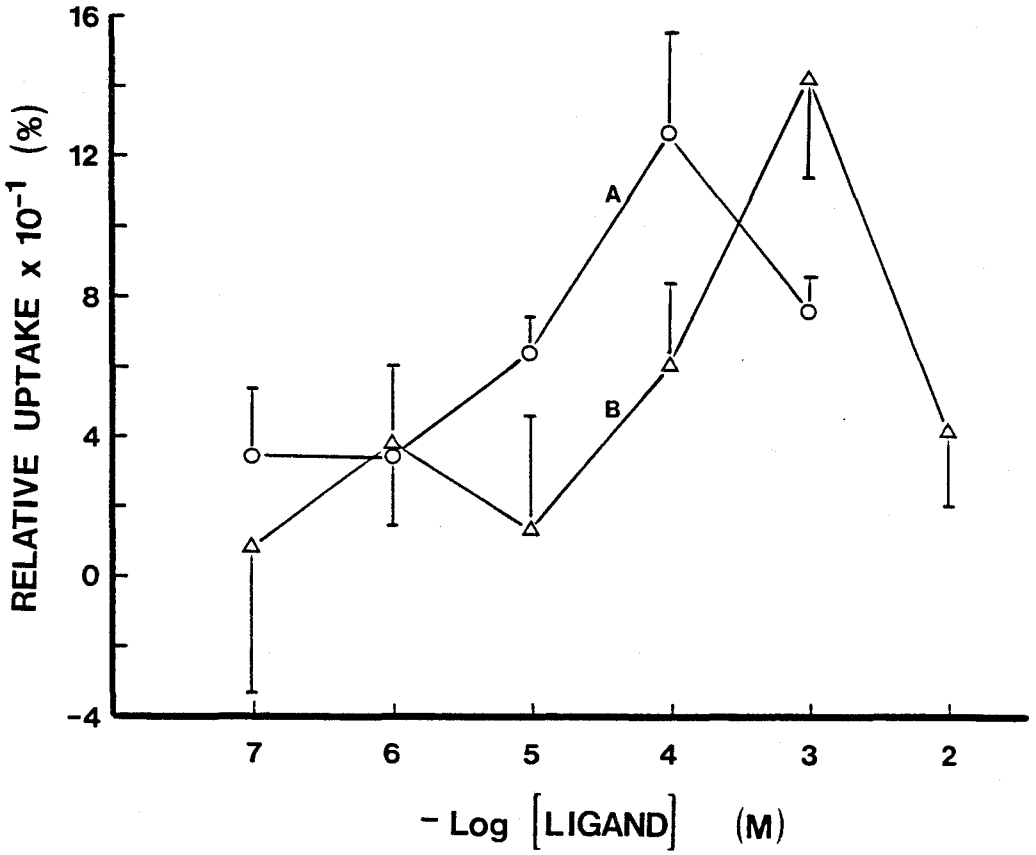


Figure 18. Effect of ligand concentration on nickel(II) uptake by RAMs (sequential incubation). Curve A corresponds to DDC and B to APDC. $[Ni^{2+}]_t = 6.5 \times 10^{-8}$ M; 1.8×10^6 cells.

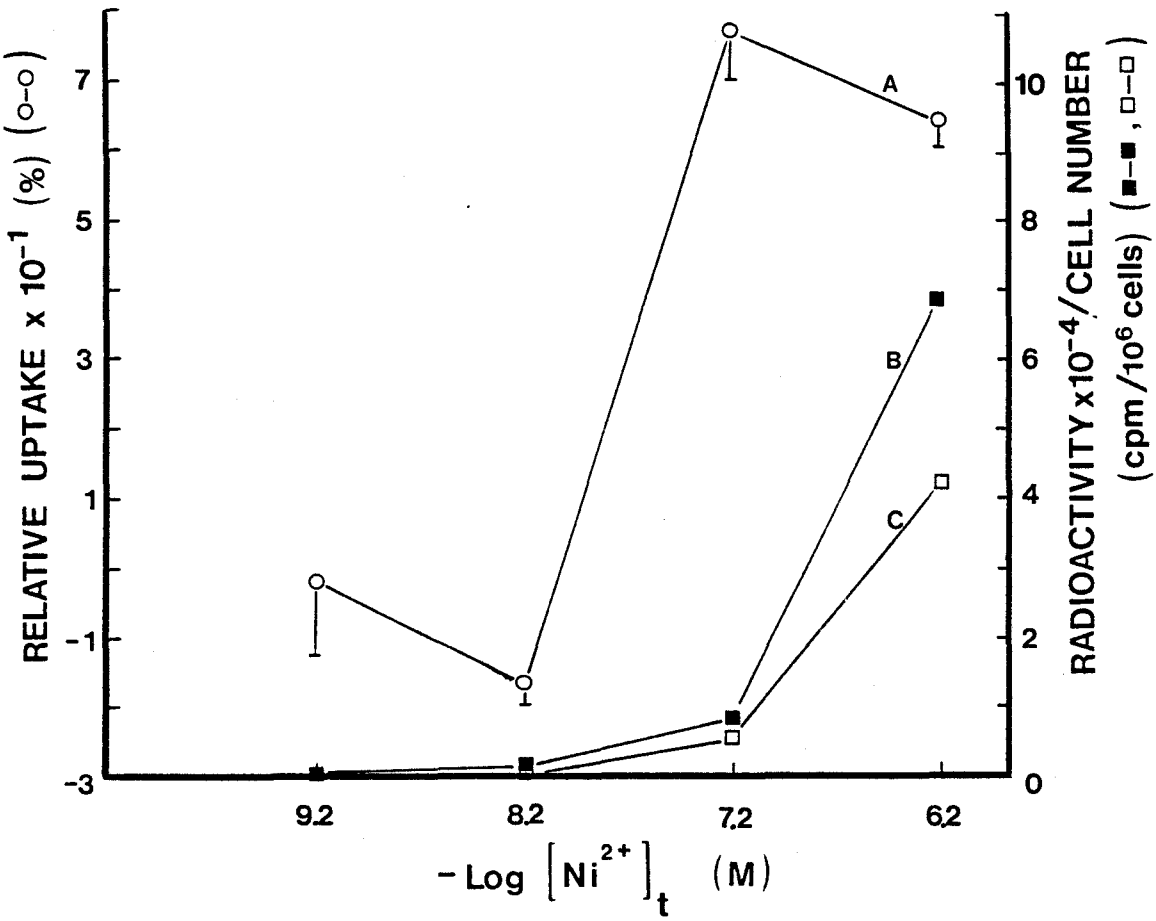


Figure 19. Effect of nickel concentration on nickel(II) uptake by RAMs (concurrent incubation). Curve A corresponds to relative uptake while Curves B (10^6 M DDC) and C (no DDC) show the observed uptake per 10^6 cells. Cell number=1.6×10^6.

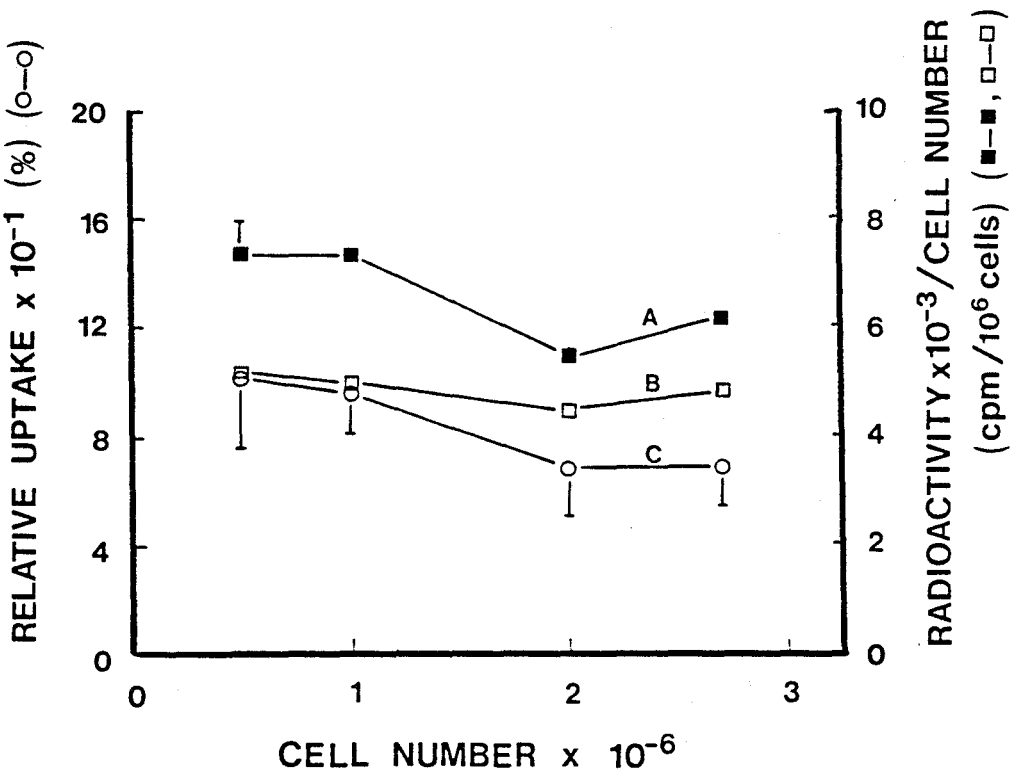


Figure 20. Effect of cell concentration on nickel(II) uptake by RAMs (concurrent incubation). Curves A (at 10^{-6} M DDC) and B (no DDC) correspond to actual accumulations while C denotes the relative uptake (10^{-6} M DDC). $[Ni^{2+}]_t = 6.5 \times 10^{-8}$ M.

B-lymphoblasts and that for RAMs (Figure 20) that the DDC enhancement effect is considerably lower for RAMs.

3.5.5. Effect of Ligand Concentration on Nickel(II) Uptake by Cellular Compartments of RAMs.

As for B-lymphoblasts, the degree of enhanced nickel(II) uptake is greatest for the lysate compartment (Figure 21). Maximum relative uptakes for the whole cell and the two compartments are again seen at 10^{-6} M DDC. A comparison of the whole cell data in Figure 21 (curve B) with that plotted in Figure 16 indicates that the insertion of a transfer step in the concurrent incubation protocol did not affect the relative uptake.

3.6. Human Erythrocyte Studies.

3.6.1. General Comments.

The quench curves illustrated in Figure 22 were used to correct for quenching due to haemoglobin in the scintillation counting of nickel-63 for whole cells and cell lysate. In the following studies, all uptake data reported have been corrected for quenching. Although relative uptake data was not affected by quenching, quench-corrections were nevertheless made in most instances. Counting efficiency was dependent on RBC number and was independent of DDC concentration.

3.6.2. Effect of Ligand Concentration on Nickel(II) Uptake.

From Figures 23 and 24, it is evident that the maximum uptake is again found at 10^{-6} M for APDC and DDC. By comparison of the effect of these two ligands at 10^{-6} M, APDC is seen to enhance nickel uptake to a greater extent than DDC. Similar trends were observed at $[Ni^{2+}]_t = 6.5 \times 10^{-9}$ M (data not shown). Nickel uptake

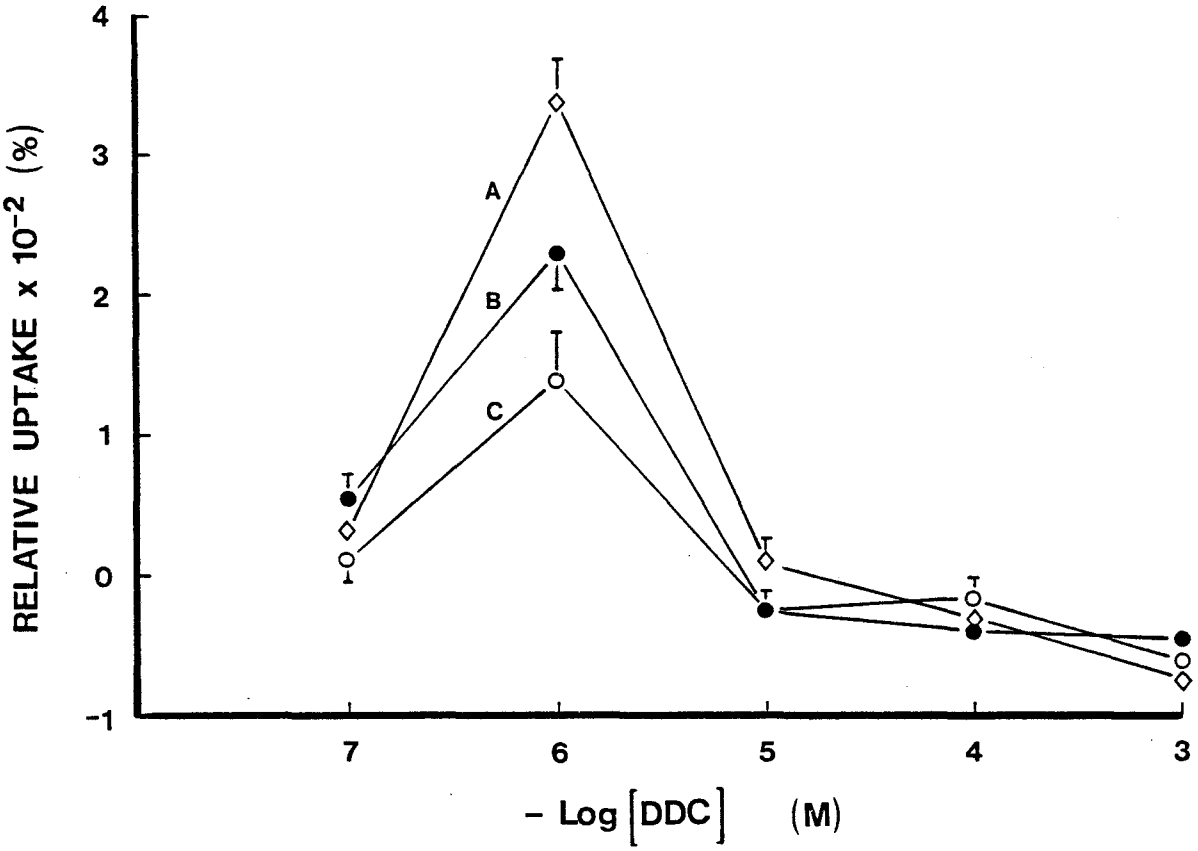


Figure 21. Effect of DDC concentration on nickel(II) uptake by cellular compartments of RAMs (concurrent incubation with transfer). Curve A corresponds to cell lysate and B and C refer to the whole cell and pellet, respectively. $[Ni^{2+}]_t = 6.5 \times 10^{-8}$ M; 1.5×10^6 cells.

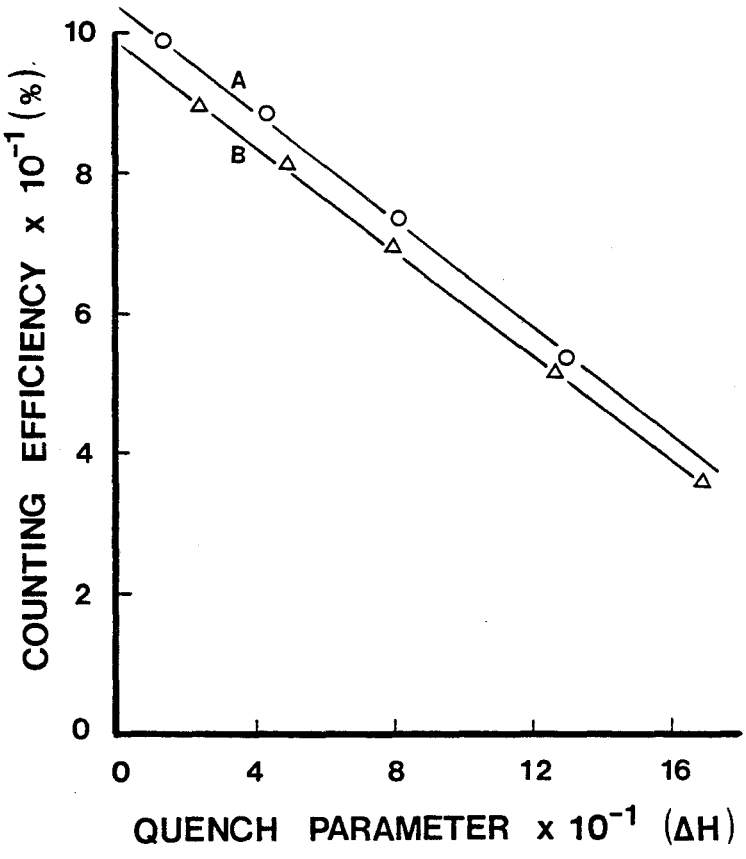


Figure 22. Nickel-63 quench curves for whole RBCs and RBC lysate. ΔH represents the difference between the pulse height number (H) of $^{63}Ni^{2+}$ alone and that in the presence of haemoglobin. A: RBC lysate, B: whole RBCs; $[Ni^{2+}]_t = 6.5 \times 10^{-8}$ M.

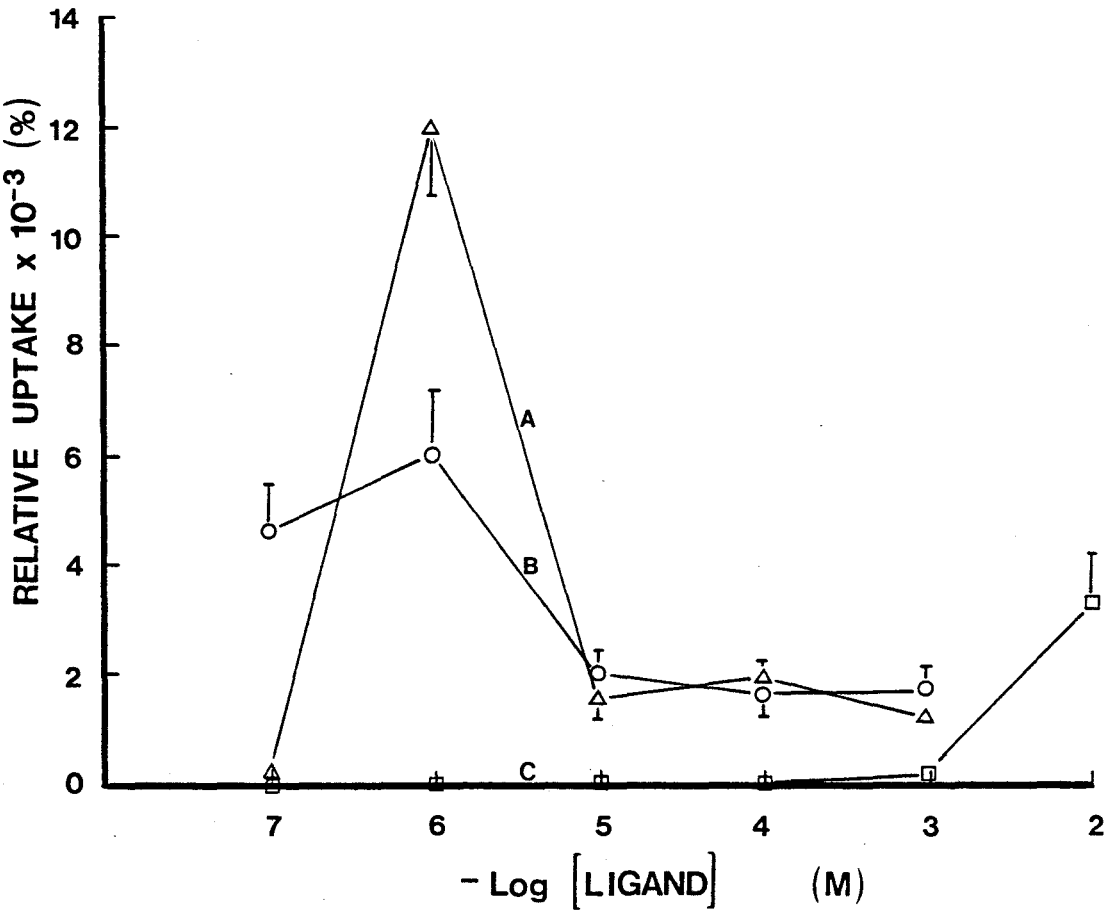


Figure 23. Effect of ligand concentration on nickel(II) uptake by RBCs (concurrent incubation). Curve A corresponds to APDC, while B and C refer to DDC and AD, respectively. $[Ni^{2+}]_t = 6.5 \times 10^{-8}$ M; 20×10^6 cells, donor AS.

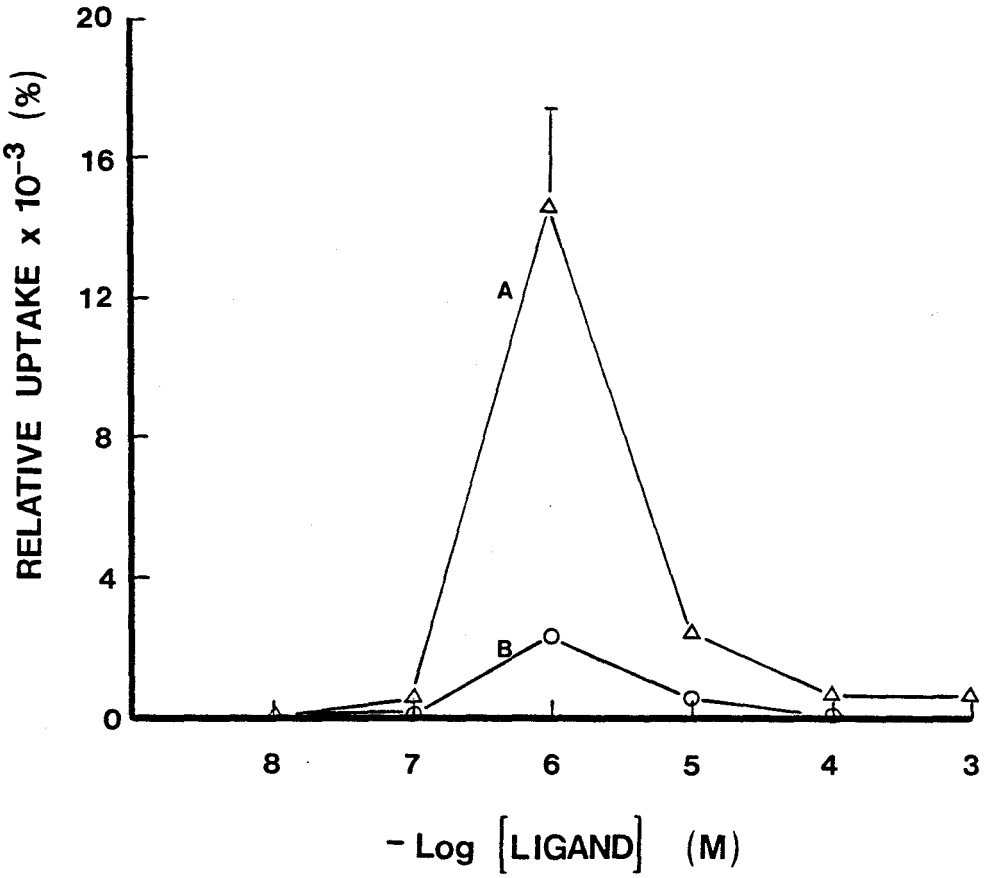


Figure 24. Effect of ligand concentration on nickel(II) uptake by RBCs (concurrent incubation). Curve A corresponds to APDC and B refers to DDC. $[Ni^{2+}]_t$=6.5x10^{-8} M; 20x10^6 cells, donor RT.

was not affected by AD at concentrations $\leq 10^{-3}$ M.

In sequential incubation experiments, APDC is again more effective (Figures 25 and 26). DDC-induced uptake peaks at 10^{-3} M, while that for APDC reaches saturation at $\geq 10^{-3}$ M. The data in Figure 25 clearly illustrate that cells from different donors have different affinities for nickel(II) (c.f., curves C and E which correspond to the same number of cells). For APDC, the relative uptake is ~2 fold greater for concurrent incubation compared to the sequential protocol (c.f., curves A in Figure 23 and curve E in Figure 25; also see Figure 26). As for concurrent incubations, AD has relatively little effect on nickel uptake (Figure 26).

3.6.3. Effect of Nickel(II) Concentration on Nickel(II) Uptake.

As in other cell types, it is evident that an increase in nickel concentration increases both the relative and actual accumulation of nickel (Figure 27). Although not apparent from the figure, uptake does increase with $[Ni^{2+}]_t$ in the absence of ligand.

3.6.4. Effect of Cell Concentration on Nickel(II) Uptake.

From the data in Figure 28, it is seen that an increase in cell number decreases the actual nickel(II) uptake per 10^6 cells in the presence of 10^{-6} M DDC more significantly than the relative uptake. In the absence of DDC the actual uptake per 10^6 cells also is reduced with increasing cell number. At cell number $\geq 4 \times 10^6$, the relative uptake remains approximately constant (see Figure 14).

3.7. Human Leukocyte Studies.

3.7.1. Polymorphonuclear Leukocytes.

In the absence of ligand, PMNs take up nickel in similar amounts to lymphocytes (Figure 29). As shown in Figure 30, the

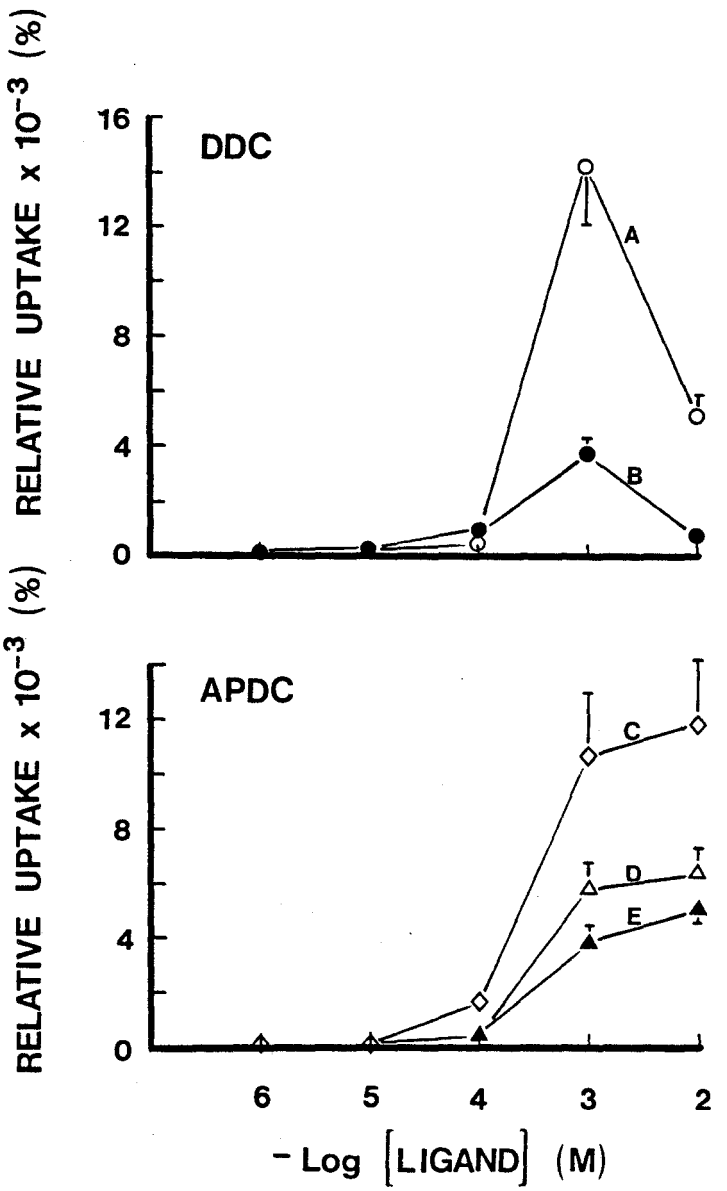


Figure 25. Effect of ligand concentration on nickel(II) uptake by RBCs (sequential incubation). Curves A ($10x10^6$ cells, donor SM) and B ($35x10^6$ cells, donor SE) correspond to DDC. Curves C ($20x10^6$ cells, donor DD), D ($35x10^6$ cells, donor SE) and E ($20x10^6$ cells, donor AS) refer to APDC. $[Ni^{2+}]_t=7x10^{-8}$ M for Curve A; $[Ni^{2+}]_t=6.5x10^{-8}$ M for Curves B, C, D and E.

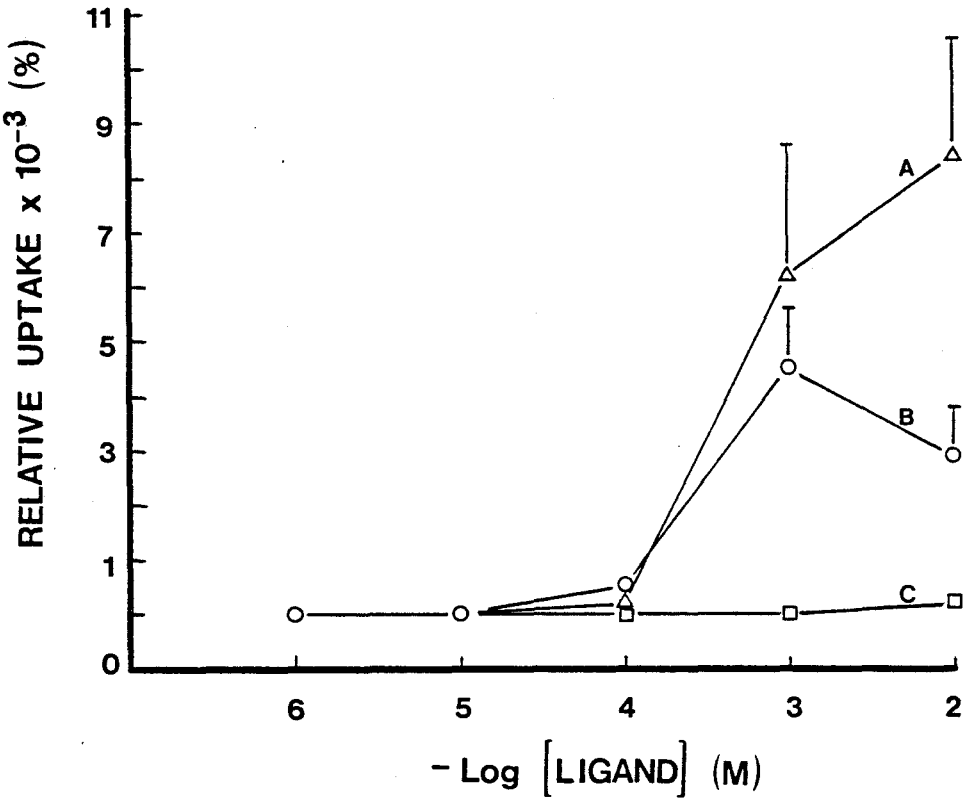


Figure 26. Effect of ligand concentration on nickel(II) uptake by RBCs (sequential incubation). Curve A corresponds to APDC; B and C refer to DDC and AD, respectively. $[Ni^{2+}]_t = 7 \times 10^{-8}$M; 10×10^6 cells, donor FR.

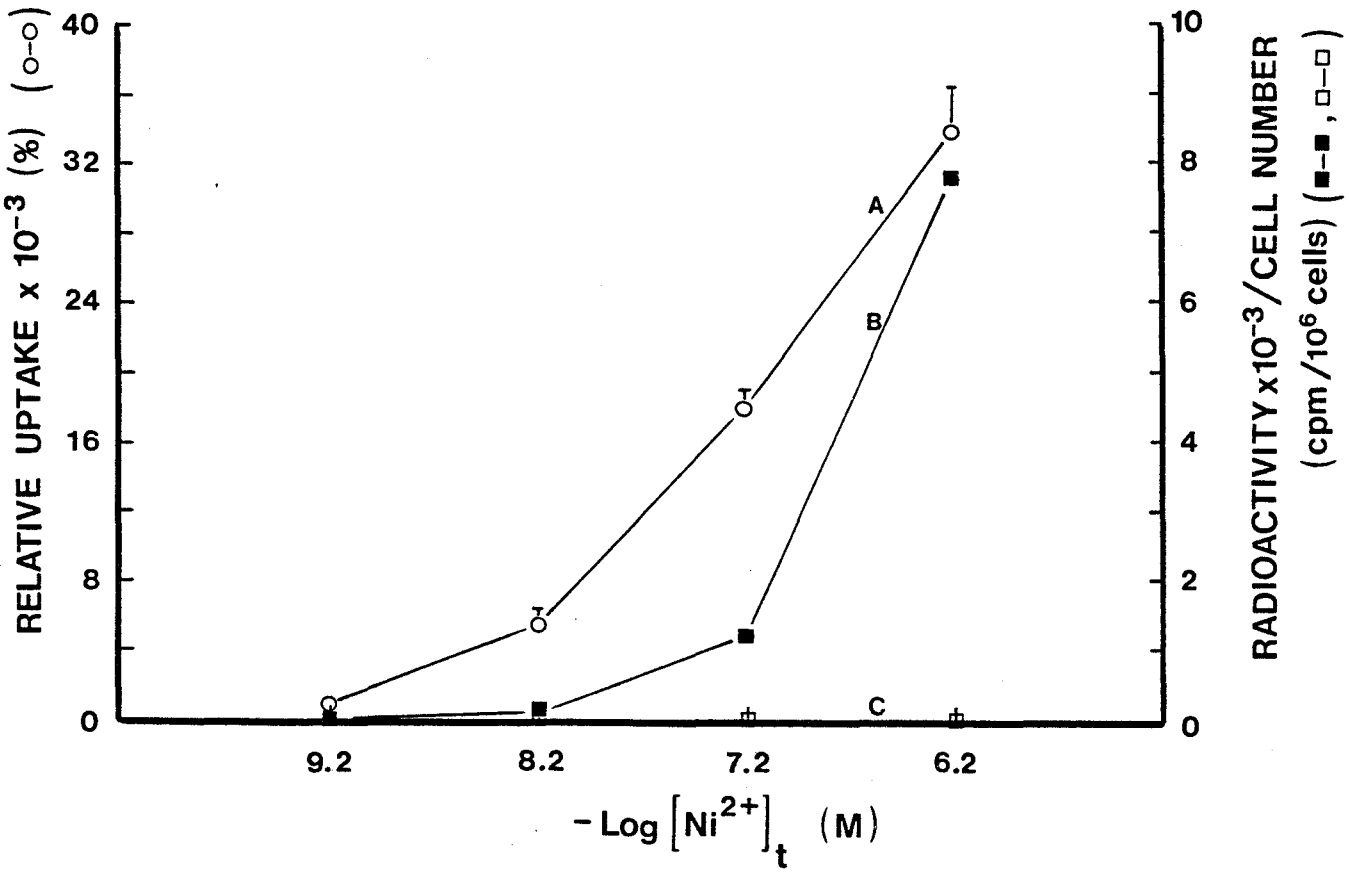


Figure 27. Effect of nickel concentration on nickel(II) uptake by RBCs (concurrent incubation). Curve A corresponds to the relative uptake at 10^{-6} M DDC; while B (at 10^{-6} M DDC) and C (no DDC) refer to actual uptake per 10^6 RBCs. Cell number=20x10^6, donor KC.

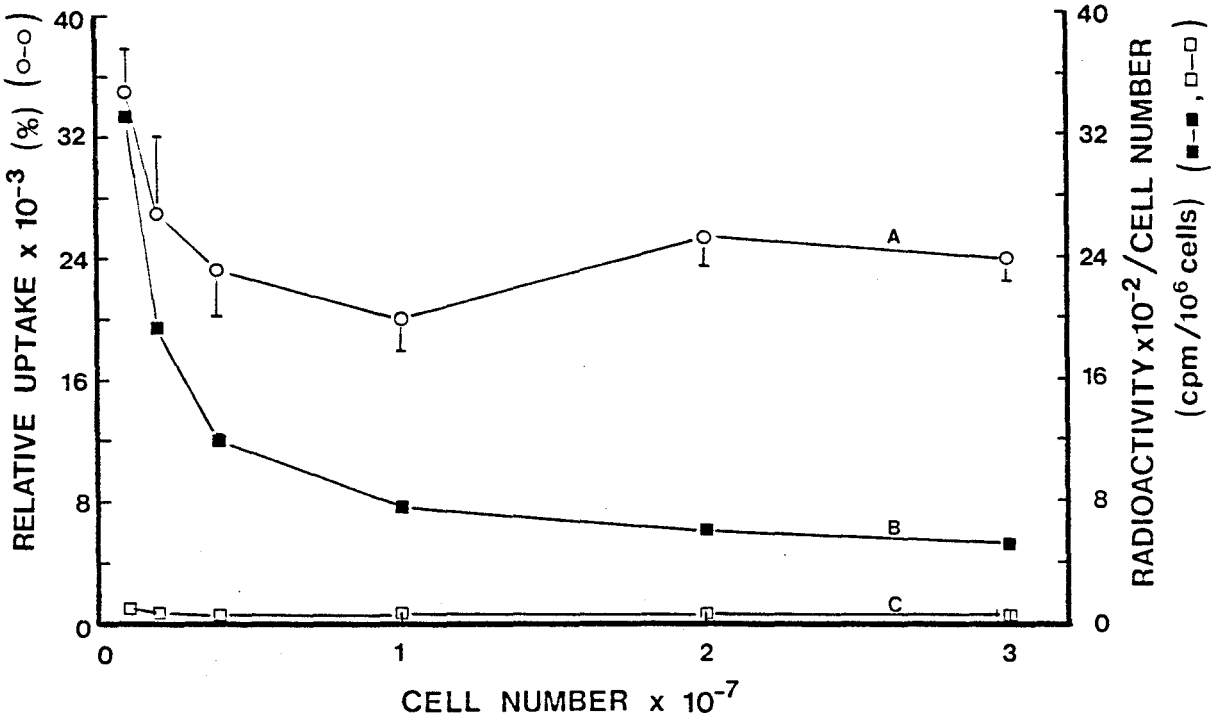


Figure 28. Effect of cell concentration on nickel(II) uptake by RBCs (concurrent incubation). Curve A corresponds to the relative uptake at 10^{-6} M DDC; while B (at 10^{-6} M DDC) and C (no DDC) give the actual uptake per 10^{6} cells. $[Ni^{2+}]_t = 6.5 \times 10^{-8}$ M; donor SE.

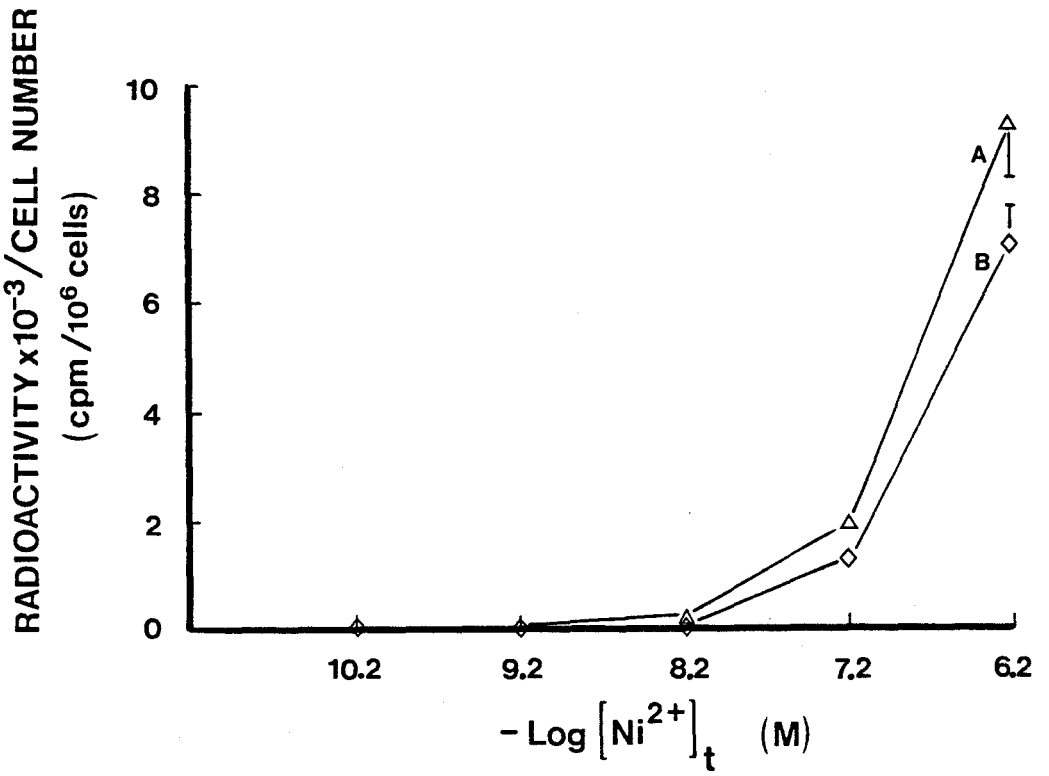


Figure 29. Effect of nickel concentration on nickel(II) uptake by PMNs and lymphocytes of donor PS (1 h incubation). Curve A (1.2×10^6 cells) is for lymphocytes and B (2×10^6 cells) for PMNs.

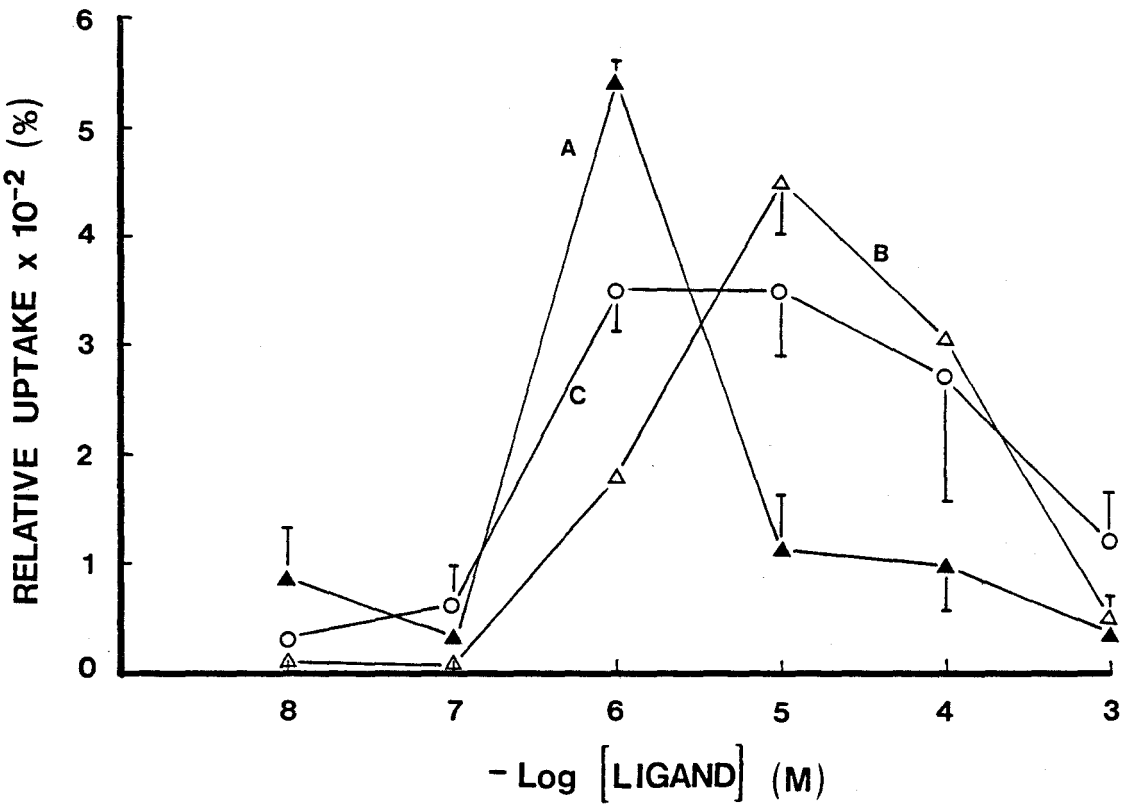


Figure 30. The effect of ligand concentration on nickel(II) uptake by PMNs (concurrent incubation). Curves A (1.5×10^6 cells, donor SE) and B (1.2×10^6 cells, donor FR) refer to APDC and C (1.2×10^6 cells, donor FR) corresponds to DDC. $[Ni^{2+}]_t = 6.5 \times 10^{-8}$M.

ligand concentration for maximum nickel uptake appears to depend upon the donor (c.f., curves A and B) and the ligand (c.f., curves B and C). Optimum ligand concentration is in the 10^{-6}-10^{-5} M range.

3.7.2. Human Peripheral Lymphocytes.

3.7.2.1. General Comments.

Immediately after isolation, lymphocyte viability was at least 75-90%. During short-term incubations, cell mortality did not increase by more than 15%. Data reported for nickel-dose response and cell-concentration dependence have been corrected for 'no cell' background levels. Comparable corrections for other experiments were not required.

3.7.2.2. Effect of Ligand Concentration on the Nickel(II) Uptake.

From Figures 31-33 it is evident that the relative nickel uptake is maximum at ligand concentrations of 10^{-6} M in concurrent experiments. DDC and APDC appear to be equally effective in enhancing nickel(II) uptake (c.f., curves A and B, Figure 31). Although there is no significant difference in the trends with ligand concentration of the relative uptake, lowering the available nickel concentration to 7×10^{-9} M from 7×10^{-8} M Ni^{2+} reduces the relative uptake (Figure 32). Removal of monocytes from the lymphocytes did not alter the general nickel uptake pattern (Figure 33).

Nickel uptake by APDC-preincubated peripheral lymphocytes shows a strong dependence on ligand concentration. Saturation appears to be approached at [APDC] $\geq 10^{-3}$ M (Figure 34). The data

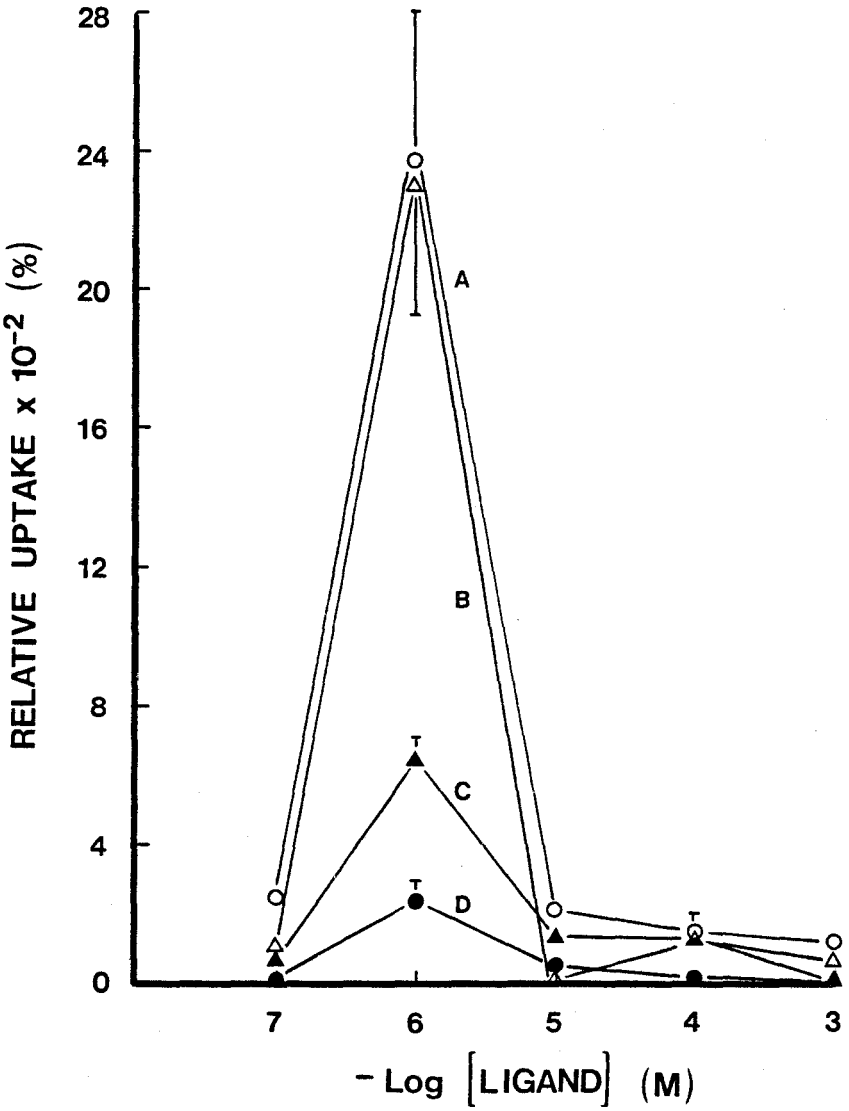


Figure 31. Effect of ligand concentration on nickel(II) uptake by peripheral lymphocytes (concurrent incubation). Curves A ($2x10^6$ cells, donor FR) and D ($2x10^6$ cells, donor AS) correspond to DDC. Curves B ($2x10^6$ cells, donor FR) and C ($2x10^6$ cells, SE) refer to APDC. $[Ni^{2+}]_t$=6.5 or $7x10^{-8}$ M.

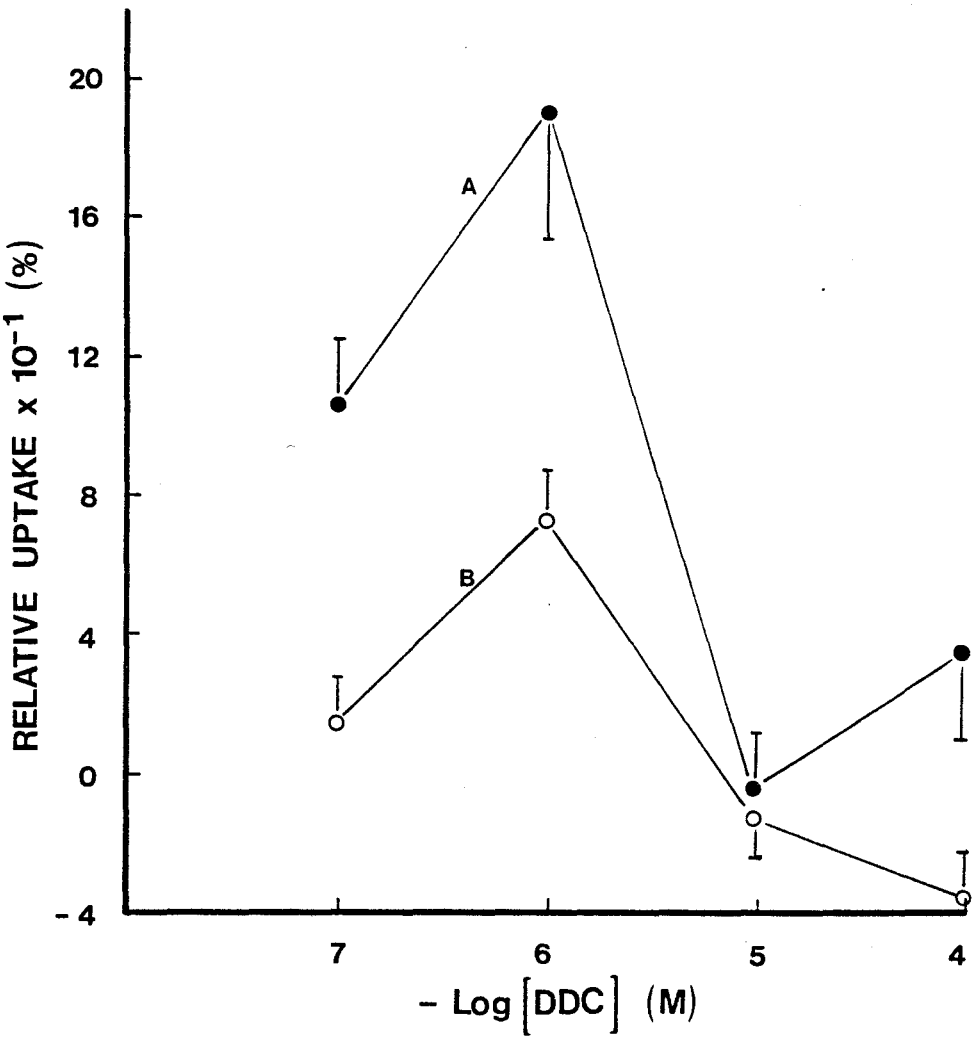


Figure 32. Effect of DDC and nickel concentrations on nickel(II) uptake by peripheral lymphocytes (concurrent incubation). Curve A corresponds to $[Ni^{2+}]_t = 7 \times 10^{-8}$ M and B to $[Ni^{2+}]_t = 7 \times 10^{-9}$ M; 1×10^6 cells, donor RM. Data points correspond to the mean of two replicates and the error bars denote the average deviation.

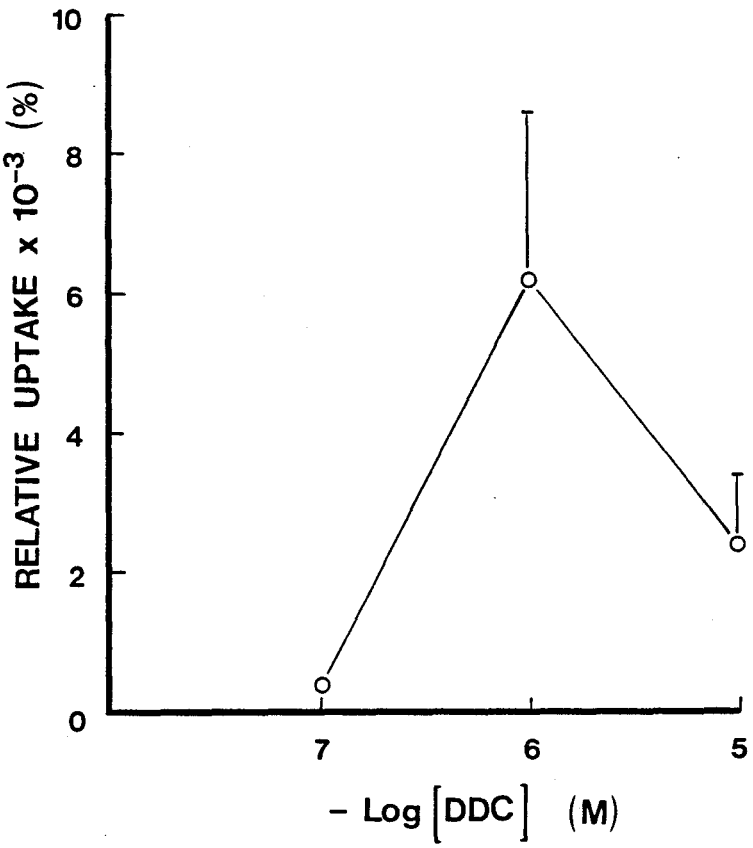


Figure 33. Effect of DDC concentration on nickel(II) uptake by lymphocytes from which monocytes have been separated (concurrent incubation); 0.6×10^6 cells (donor KC). $[Ni^{2+}]_t = 6.5 \times 10^{-8}$ M.

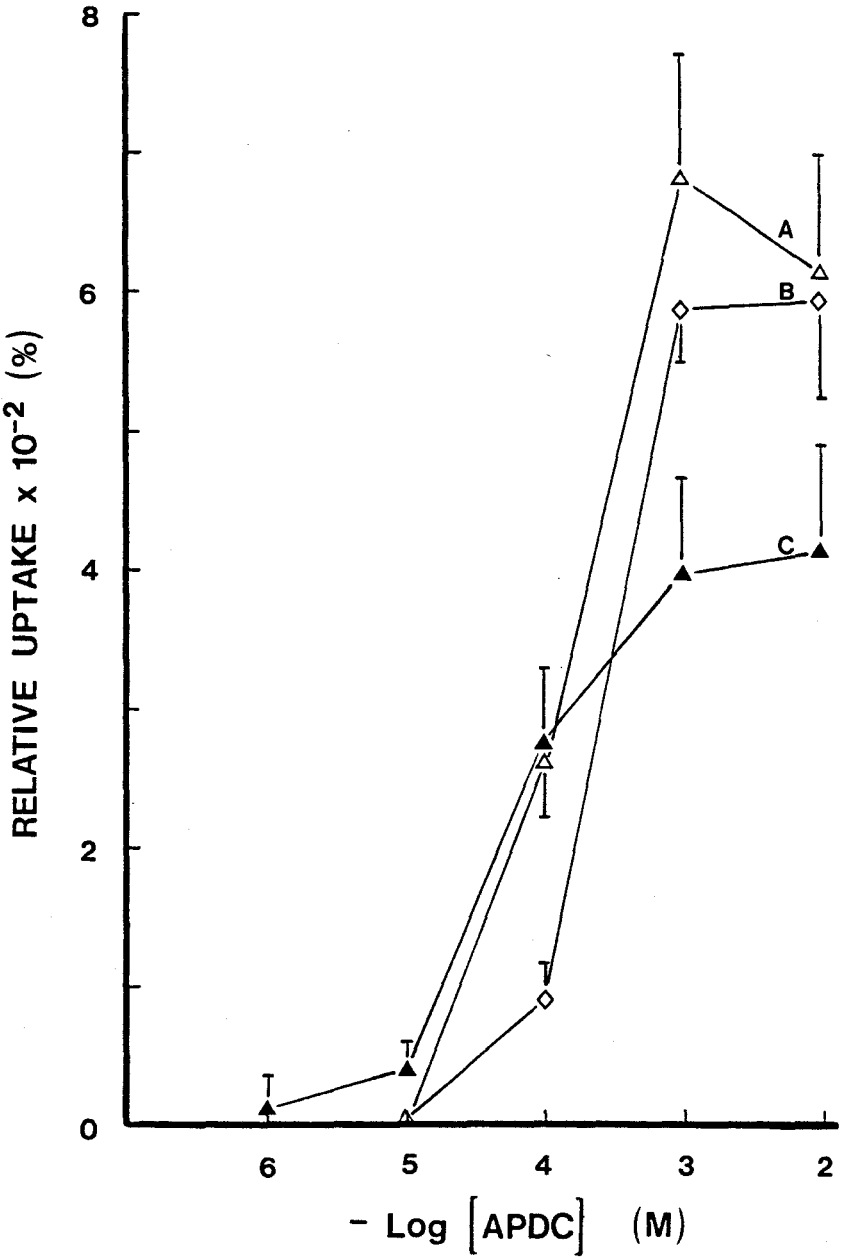


Figure 34. Effect of APDC concentration on nickel(II) uptake by peripheral lymphocytes (sequential incubation). Curves A (1.25×10^6 cells, donor AS) and C (2×10^6 cells, donor SM) are for individuals not sensitized to nickel, while Curve B (1.25×10^6 cells, donor LS) is for a nickel-sensitive individual. $[Ni^{2+}]_t = 6.5$ or 7×10^{-8} M.

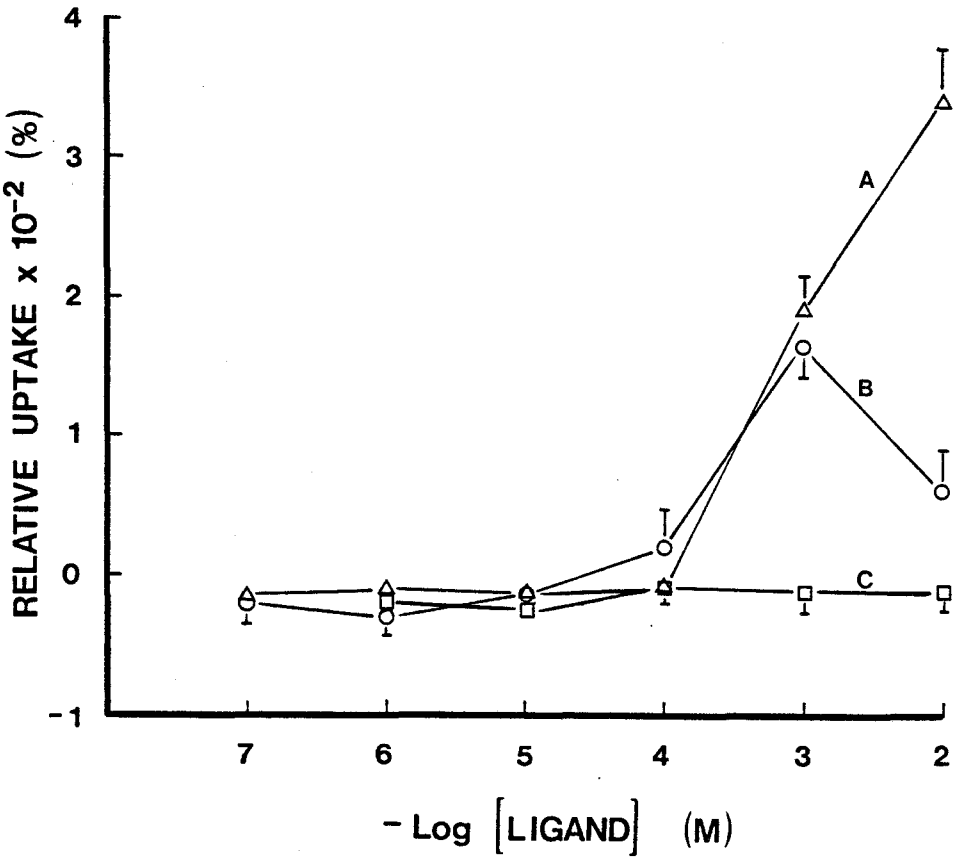


Figure 35. Effect of ligand concentration on nickel(II) uptake by peripheral lymphocytes (sequential incubation). Curves A (APDC), B (DDC) and C (AD) all correspond to 2×10^6 cells (donor FR); $[Ni^{2+}]_t = 7 \times 10^{-8}$M.

summarized in Figure 35 illustrate that in sequential experiments the nickel uptake depends on the nature of the dithiocarbamate ligand: APDC is somewhat more effective than DDC, while AD has little effect, as with RBCs.

3.7.2.3. Effect of Nickel(II) Concentration on Nickel(II) Uptake.

It is clear from Figure 36 (curve B) that in the presence of 10^{-6} M DDC, the nickel uptake increases sharply at $[Ni^{2+}]_t \geq 6.5 \times 10^{-8}$ M ($\log [Ni^{2+}]_t \leq 7.2$). The relative uptake (curve A) increases gradually with increasing nickel concentration. This implies that in the absence of ligand, nickel uptake also increases sharply at $[Ni^{2+}]_t \geq 6.5 \times 10^{-8}$ M (curve C).

3.7.2.4. Effect of Cell Concentration on Nickel(II) Uptake.

The dependence of cell associated nickel on cell number is shown in Figure 37, and is similar to that observed in RBCs, lymphoblasts and RAMs. Thus as the number of cells increases, the relative uptake and the actual nickel uptake per 10^6 cells diminish.

3.7.2.5. Relative Uptake by Cell Compartments.

The relative uptake of nickel(II) is greatest for the cell lysate compartment (Figure 38). All compartments exhibit a maximum in relative uptake at 10^{-6} M DDC. By comparing curve A in Figure 31 with curve B in Figure 38 it is clear that the incorporation of a transfer step into the concurrent incubation protocol has not altered the trends of nickel(II) uptake.

3.7.2.6. Cellular Retention of Nickel(II) in Complete Physiological Medium.

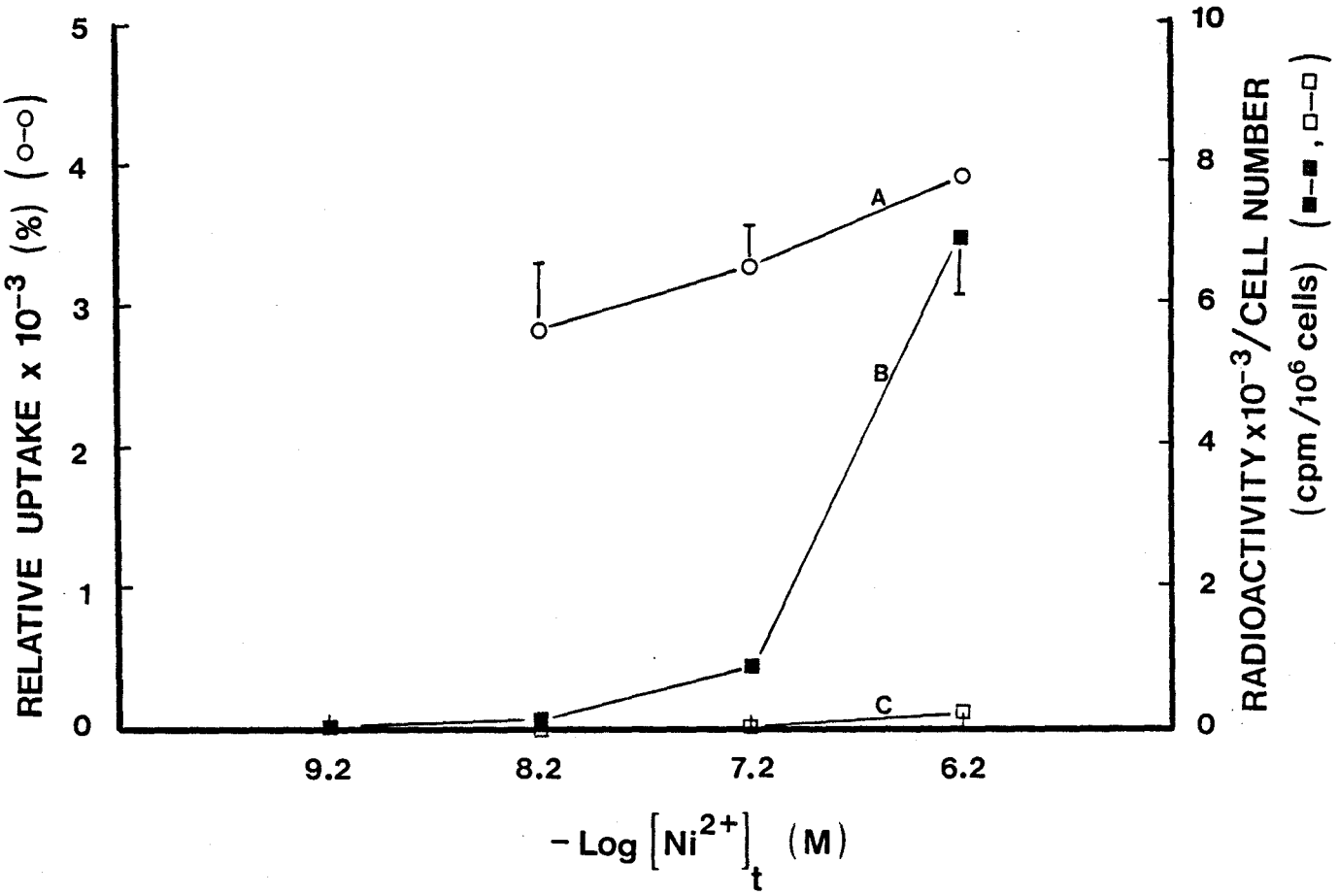


Figure 36. Effect of nickel concentration on nickel(II) uptake by peripheral lymphocytes (concurrent incubation). Curve A represents the relative uptake (10^{-6} M DDC), while Curves B (10^{-6} M DDC) and C (no DDC) refer to the actual nickel uptake. Experiments were conducted with $2x10^{6}$ cells, donor SE.

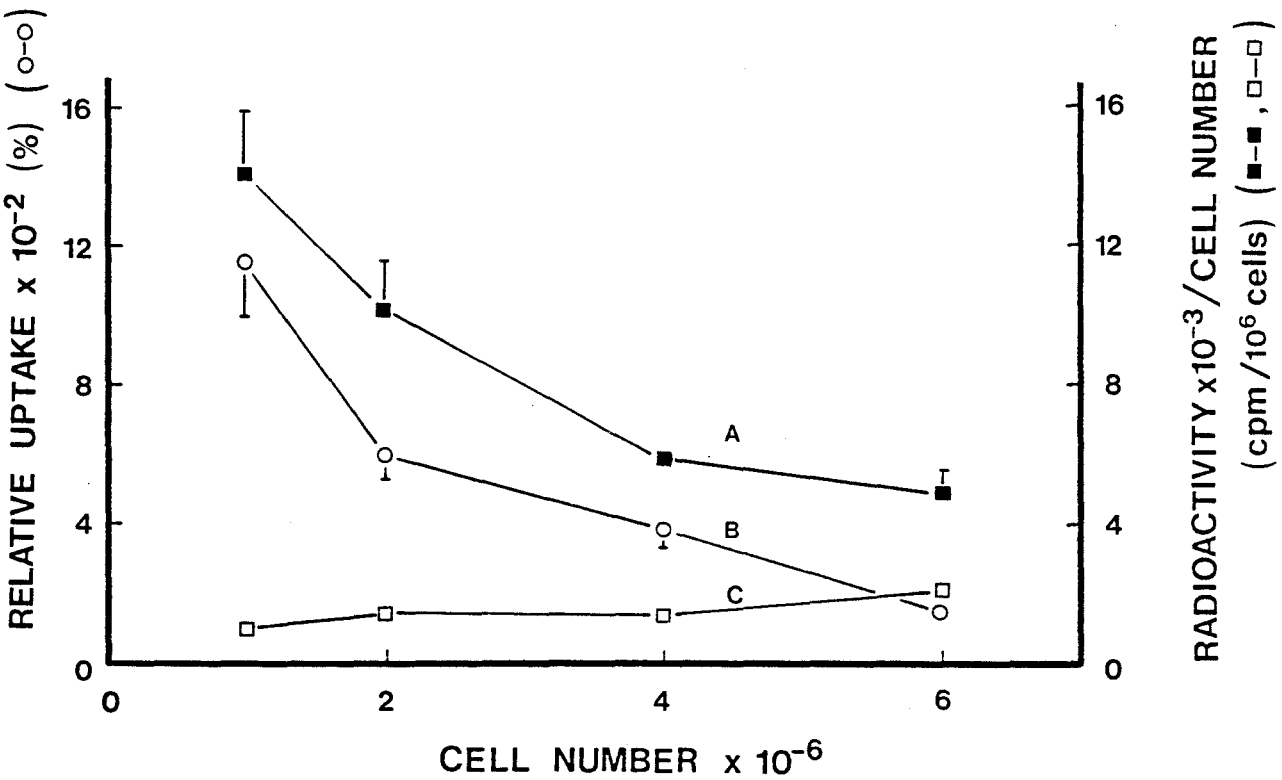


Figure 37. Effect of cell number on nickel(II) uptake by peripheral lymphocytes (concurrent incubation). Curve B describes relative uptake at 10^{-6} M DDC, and A (10^{-6} M DDC) and C (no DDC) the actual nickel uptake. $[Ni^{2+}]_t$=6.5x10^{-8} M; cells were from donor RT.

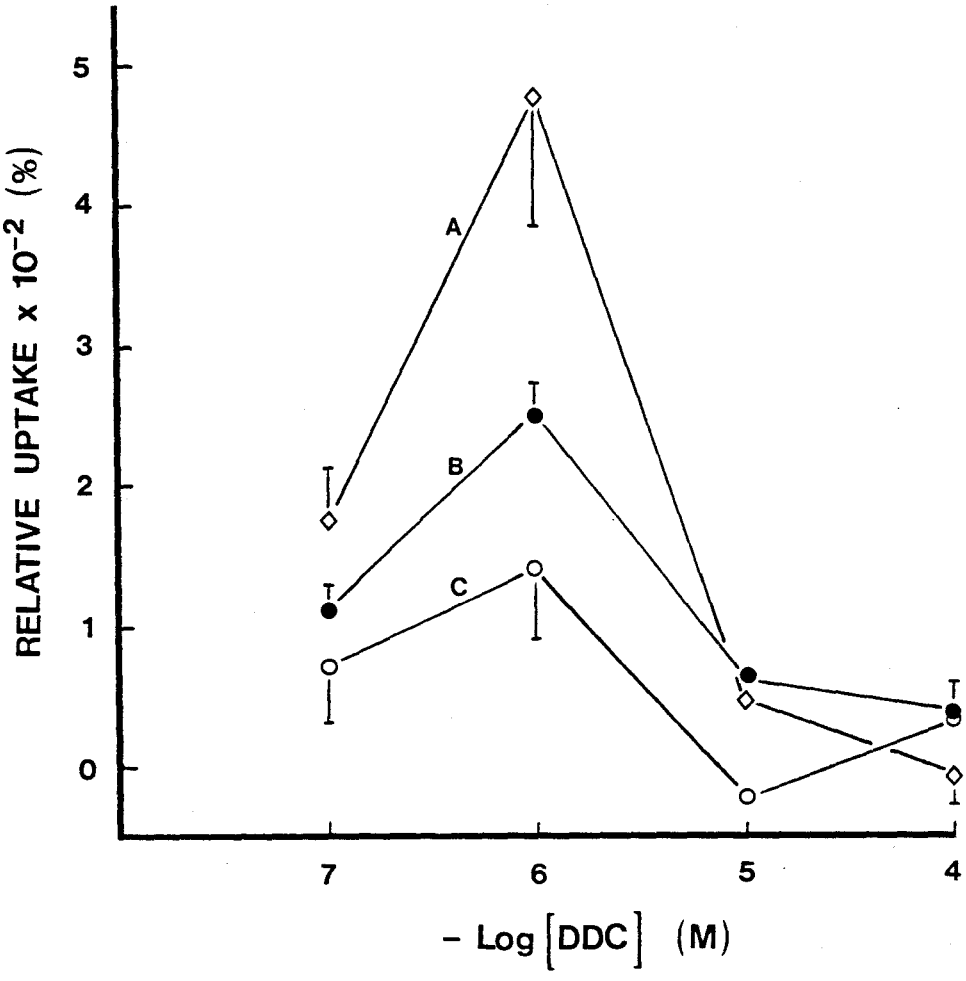


Figure 38. Effect of DDC concentration on the relative uptake of nickel-63 by cellular compartments of peripheral lymphocytes (concurrent incubation with transfer). Curve A corresponds to cell lysate, B to the whole cell and C to the cell pellet. $[Ni^{2+}]_t$=6.5x10^{-8} M; cell number=1.4x10^6 cells, donor FR.

Table 8. Percentage of Nickel(II) Retained by Peripheral Lymphocytes in α-MEM Medium[a,b,c]

Time, t (h)	Subject	Absence of APDC %Retained	Absence of APDC Viability	APDC (10^{-4} M) %Retained	APDC (10^{-4} M) Viability	APDC (10^{-3} M) %Retained	APDC (10^{-3} M) Viability
0	RM	100	87	100	84	100	91
	AG	100		100		100	
	AS	100	88	100		100	74
	FR[d]	100	86±4	100		100	89±2
1	RM	11±2	95	90±40	95	80±20	87
	AS		80				73
18	AG	12±3	94	15	0	22±6	
24	AS		91				54
	FR[d]	6±1	96±2			26±4	96±1
48	FR[d]	7±1	98±1			22±4	93±1
72	AS[e]		0				0
	FR[d]	5±1	96±3			19±3	86±7

[a]Nickel uptake involved the sequential protocol. Both the ligand preincubation and nickel uptake steps were done in veronal buffer. $[Ni^{2+}]_t$ in the nickel uptake step was 6.5×10^{-8} M, except for donor AS for which $[Ni^{2+}]_t = 1 \times 10^{-4}$ M. Subsequent to nickel uptake, cells were incubated in α-MEM medium supplemented with 10% human AB serum.

[b]Percentages are calculated relative to the amount of Ni^{2+} associated prior to α-MEM incubation step. Viabilities were assessed at time, t.

[c]Mean ± standard deviation (n=3).

[d]FR cells were preincubated with APDC for 15 min instead of 1h. RPMI medium was employed in the retention study.

[e]Poor viabilities were also observed in the controls (no nickel).

Data summarized in Table 8 reveal that the retention of nickel by cells suspended in complete physiological medium is higher for APDC-preincubated cells. This observation was confirmed by EAAS when cells were preincubated in 10^{-2} M APDC, then with 10^{-4} M Ni^{2+}, followed by a 1 h incubation in culture medium. For most of the experiments reported in Table 8, the ligand-preincubation time was 1 h. When this time period was reduced to 15 min the viability assessed at the end of incubation in the physiological complete medium improved for APDC-preincubated cells (see columns 4 and 8 of Table 8).

3.7.2.7. Lymphocyte Transformation Studies.

From the plots in Figures 39 and 40 it is clear that the time dependence of both PHA-induced mitogenic effect and the antigenic effect of $NiSO_4$ in lymphocyte cultures are distinctly different. The mitogenic effect of PHA was found to be dependent upon the dose. A dose-response study showed that 10 μg ml^{-1} elicited a maximum response. The plots of stimulation index *versus* incubation time depicted in Figure 39 clearly show that 50 μg ml^{-1} was less effective that 10 μg ml^{-1}. PHA concentrations below 10 μg ml^{-1} also showed a reduced response.

A comparison of responses A and B with C and D in Figure 40 indicates that a nickel-sensitive individual exhibited larger stimulation indices (especially for cells cultured with $NiSO_4$ $\geq$ 6.5 days). This discrimination often occurs for a range of $NiSO_4$ concentrations used in the incubation medium (Figure 41). The data reported in Figure 41 also illustrates that the magnitude of nickel(II) stimulation depends on its concentration and on the

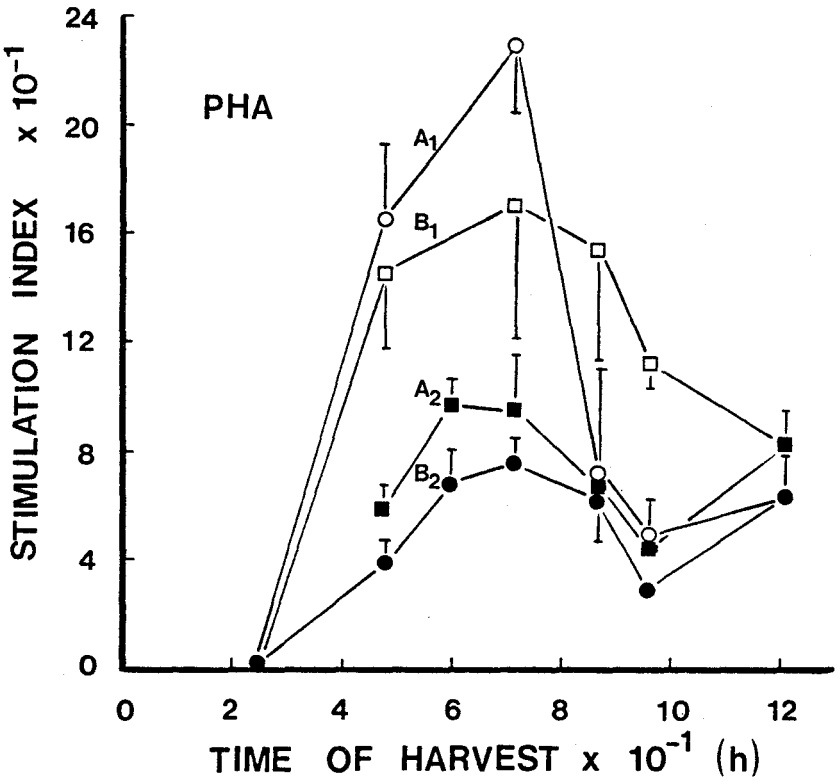


Figure 39. Time dependence of PHA-induced lymphocyte transformation. Curve A corresponds to donor RM and B, donor PS. The subscript 1 denotes a PHA concentration of 10 µg/ml and the subscript 2 indicates 50 µg/ml. Cell number: $1x10^5$ cells/well.

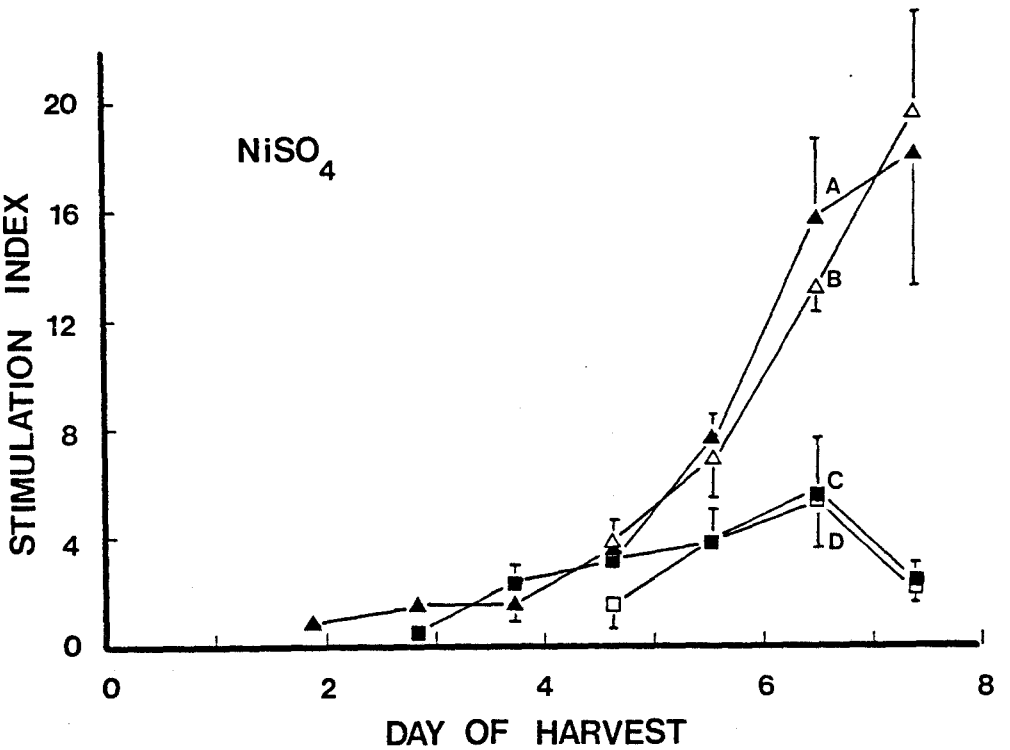


Figure 40. Dependence on incubation time and antigen concentration of the nickel(II)-induced antigenic response in the lymphocyte transformation test. Curves A (25 µg $NiSO_4$ ml^{-1}; $9.5x10^{-5}$ M) and B (12.5 µg $NiSO_4$ ml^{-1}; $4.3x10^{-5}$ M) are for a nickel sensitive donor LS while Curves C ($9.5x10^{-5}$ M $NiSO_4$) and D ($4.3x10^{-5}$ M $NiSO_4$) are for a non-sensitized individual (donor AS). Data points correspond to the mean of three or four replicates $\pm$ one standard deviation.

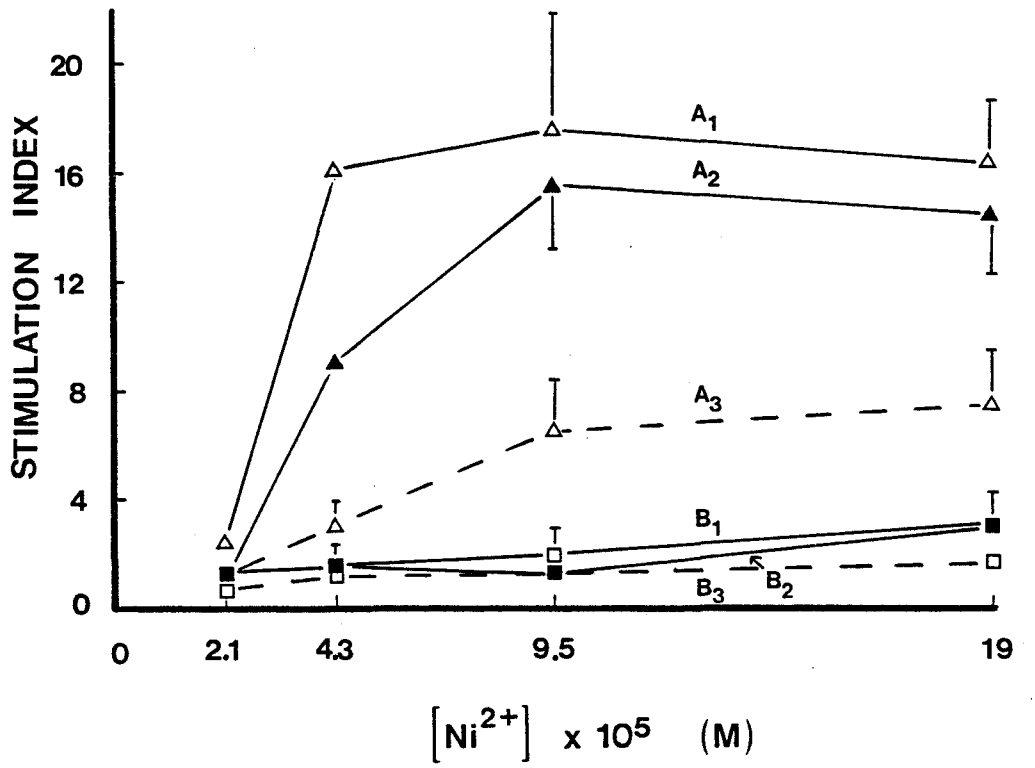


Figure 41. Effect of nickel concentration and culture duration on nickel-induced transformation of lymphocytes. Curves A_1 (Day 8), A_2 (Day 7) and A_3 (Day 6) are for a nickel sensitive individual (BP) while Curves B_1 (Day 7), B_2 (Day 8) and B_3 (Day 6) correspond to a non-sensitized person (SE). Data points correspond to the mean of three or four replicates $\pm$ one standard deviation.

Table 9. The Effect of Pretreatment on Peripheral Lymphocyte Proliferation as Measured by ^{3}H-Thymidine Uptake

Subject	Day	Condition[b]	Pretreatment[a] None	Veronal	10^{-3} M APDC
AS	3	Control	8±2	4±1	2±1
		PHA	620±40	1.1±0.2	1.2±0.1
LS[d]	3	Control	7±1	3.4±0.5	3±1
		PHA	470±50	3.9±0.8	3±1
AS	7	Control	9±2	2.4±0.7	1.4±0.5
		$NiSO_4$[c]	36±4	4±1	1.20±0.03
LS	7	Control	4.3±0.5	3.1±0.5	2.2±0.9
		$NiSO_4$	28±4	4±1	------

[a] [^{3}H]-thymidine uptake is indicated as $x10^{2}$ cpm. Mean ± standard deviation (n=4).

[b] Control condition excludes antigen or mitogen. Concentration of PHA and $NiSO_4$ were 10 μg/ml and $4.8x10^{-5}$ M, respectively.

[c] Decrease in counts for veronal and APDC preincubated cells were also found at other concentrations of nickel(II) ($<19x10^{-5}$ M) on days 4-8.

[d] LS, nickel sensitive donor.

incubation duration (also see Figure 40).

An examination of the data compiled in Table 9 shows that in comparison to the control cells (untreated), veronal or APDC pretreated cells had significantly lower ^{3}H-thymidine uptake. As shown already in Figures 39 and 40, the extent of stimulation of cells is much higher through a PHA (mitogen) mediated transformation relative to $NiSO_4$-induced transformation (see column 4).

3.8. Relative Cellular Uptake of Nickel(II).

3.8.1. Cellular Distribution.

An examination of the data in Table 10 shows that, relative to the whole cell, comparable amounts of nickel-63 accumulated in the cell lysate and pellet for both B-lymphoblasts and peripheral lymphocytes. By contrast, more nickel-63 was associated with the pellet compared to the cell lysate for RAMs while the reverse was true for human RBCs. In the presence of 10^{-6} M DDC, a larger amount of nickel content in the lysate was evident in all cases except for RBCs.

3.8.2. Relative Cellular Affinities for Nickel(II).

In the absence of ligand in concurrent incubations, nickel accumulates in cells in the following sequence:
RBCs < B-lymphoblasts $\leq$ human peripheral blood leukocytes < RAMs (see Tables 11 and 12). For all cell types studied, 10^{-6} M DDC or 10^{-6} M APDC enhanced nickel(II) accumulation. In most instances, the enhancement observed for APDC was larger than for DDC.

A comparison of the amount of nickel accumulated by peripheral blood leukocytes of donor FR with those of donors AS and SE

Table 10. Summary of Relative Distributions of Nickel(II) in Cell Compartments for Various Cell Types (Concurrent Incubation with Transfer)[a,b]

Cell Type (Subject)	No Ligand		10^{-6}M DDC	
	Pellet	Lysate	Pellet	Lysate
B-lymphoblasts	44±2	29±2	33±3	56±4
	47±7	38±5	28±3	46±7
RAMs	62±11	23±2	45±5	31±1
	70±4	26±3	52±8	38±3
Lymphocytes (FR)	40±13	43±8	27±1	71±6
RBCs	31±10	74±21	14±3	61±6

[a] Percentage of nickel(II) associated with whole cells; mean ± standard deviation (n=3).

[b] $[Ni^{2+}]_t$ was 6.5×10^{-8}M.

Table 11. Levels of Nickel(II) (pmoles/10^6 cells) Accumulated by Lymphoblasts and Alveolar Macrophages (Concurrent Incubation)[a,b,c]

Cell Type	No Ligand	10^{-6}M DDC	10^{-6}M APDC
B-lymphoblasts	0.5±0.1	6±1	11±2
RAMs	9.5±0.5	16±3	28.1±0.8

[a]The total nickel available in the incubation medium was 65 pmoles; cellular nickel was calculated from:
(nickel available) (counts of cell pellet)/(counts added to incubation medium).

[b]Average of means ± standard deviation ($n \geq 6$).

[c]The uptake values were adjusted to that for 10^6 cells employing experimental plots of uptake versus cell number.

Table 12. Levels of Nickel(II) (pmoles/10^6 cells) Accumulated by Different Blood Cells of the Same Subject (Concurrent Incubation)[a,b,c,d]

Subject	Cell Type	Cell Number ($x10^6$)	No Ligand	10^{-6}M DDC	10^{-6}M APDC
FR	L	1	0.4±0.2	10.8±0.9	21±1
FR	P	1.2	0.73±0.04	3.3±0.2	4.1±0.3[e]
AS	L	1	2.1±0.5	9±2	-------
AS	R	1	0.09±0.01	5.2±0.7	9.3±0.6
SE	L	1	1.6±0.3	9.3±0.5	16±1
SE	P	1.5	1.5±0.4	12±1	10±1
SE[f]	R	1	0.03±0.01	2.66±0.08	-------

[a]The total nickel available in the incubation medium was 65 pmoles; cellular nickel was calculated from:
(nickel available)(counts of cell pellet)/(counts added to incubation medium).

[b]Mean ± standard deviation (n=3).

[c]For lymphocytes and RBCs, the uptake was adjusted to that for 10^6 cells employing experimental plots of uptake *versus* cell number.

[d]L, lymphocyte; P, PMN; R, RBC.

[e]At 10^{-5}M APDC.

[f]With transfer step.

suggests that some interindividual variability occurs (see Table 12). This same conclusion may be drawn from the difference in nickel uptake by RBCs from donors AS and SE. For RBCs and peripheral lymphocytes (donor FR) the highest nickel levels were observed in the presence of 10^{-6} M APDC.

For sequential incubation experiments, it was not possible to express the experimental results per 10^6 cells because plots of uptake versus cell number were not established. Consequently, relative affinities of the various cell types for nickel could not be assessed. However, it is still clear from the data in Table 14 that RBCs take up less nickel(II) than lymphocytes since even when the RBC number is 16 fold greater than that of peripheral lymphocytes, nickel uptake is still smaller (see data for donor AS).

Although the data in Tables 13 and 14 correspond to different cell numbers, it is clear that APDC is again more effective than DDC in enhancing uptake. The lymphocyte and RBC data in Table 14 illustrate that the actual nickel uptake peaks at 10^{-3} M DDC, while for APDC there is no similar decrease at 10^{-2} M. It is also evident from the last entry in Table 14 that increasing the nickel concentration by 1400-fold increases the nickel accumulation by at least a 100-fold, both in the presence and absence of ligand. Finally, it may be concluded that nickel-sensitized cells (donor LS) take up similar amounts of nickel as do control cells (nonsensitized donor AS).

Table 13. Levels of Nickel(II)(pmoles) Accumulated by Lymphoblasts and Alveolar Macrophages (Sequential Incubation)[a,b]

Cell Type	Cell Number ($x10^6$)	No Ligand	10^{-3}M DDC	10^{-2}M DDC	10^{-3}M APDC	10^{-2}M APDC
B-lymphoblasts	2	0.82±0.07	10.4±0.9	9.2±0.7	-----	-----
	3.2	1.1±0.7	-----	-----	18±2	18.2±0.6
RAMs	1.8	11±2	19±2	(25±1)[c]	27±1	15±1

[a]The total nickel available in the incubation medium was 65 pmoles; cellular nickel was calculated from:
(nickel available)(counts of cell pellet)/(counts added to incubation medium).

[b]Mean ± standard deviation (n=3).

[c] For 10^{-4}M DDC.

Table 14. Levels of Nickel(II)(pmoles) Accumulated by Different Blood Cells of the Same Subject (Sequential Incubation)[a,b]

Subject	Cell Type	Cell Number ($x10^6$)	No Ligand	10^{-3}M DDC	10^{-2}M DDC	10^{-3}M APDC	10^{-2}M APDC
FR[c]	L	2	3.8±0.9	13±2	8±2	26±6	21±4
FR[d]	R	10	0.39±0.09	18±2	11±2	24±7	33±3
AS	L	1.25	3.1±0.4	----	----	24±1	22±1
AS	R	20	0.14±0.01	20±1	6.6±0.4	----	----
AS	R	20	0.54±0.05	----	----	21±2	27.7±0.4
SE	R	35	0.7±0.1	27±1	5.5±0.4	40±10	46±2
LS[e]	L	1.25	3.0±0.2	----	----	20.8±0.9	21±2
WT[f,g]	L	2	410±90	----	----	----	3500±300

[a]The total nickel available in the incubation medium was 65 or 70 pmoles; mean ± standard deviation; cellular nickel was calculated from: (nickel available)(counts of cell pellet)/(counts added to incubation medium).

[b]L, lymphocyte; R, RBC.

[c]Average of mean ± standard deviation (n=3).

[d]Counts are not corrected for quenching.

[e]Nickel sensitive individual.

[f]The total nickel available in the incubation medium was 100,000 pmoles; cellular nickel was assessed by EAAS (Section 2.2.2).

[g]Mean ± average deviation (n=2).

4. DISCUSSION

4.1. Consequences of the Insolubility of Nickel(II) Dithiocarbamate Complexes.

The nonspecific losses of nickel-63 to the surface of the incubation tubes in the presence of ligand can be rationalized on the basis of the relatively low solubility of nickel(II) dithiocarbamate complexes. It appears that the resultant pools account for the increase in background counts at ligand concentrations greater than 10^{-6} M (Tables 4 to 6). Since no losses were observed when the ligand concentration was $\leq 10^{-6}$ M, with a corresponding $[Ni^{2+}]_t \leq 6.5 \times 10^{-9}$ M, the solubility product (K_{sp}) for the $Ni(DTC)_2$ complexes may be estimated. Thus,

$$Ni^{2+}(aq) + 2DTC^{-}(aq) \rightleftharpoons Ni(DTC)_2(solid) \qquad (4)$$

$$\begin{aligned} K_{sp} &= [Ni^{2+}][DTC^{-}]^2 \\ &= (6.5 \times 10^{-9})(1 \times 10^{-6})^2 \\ &= 6.5 \times 10^{-21} \end{aligned} \qquad (5)$$

with DTC representing either DDC or APDC and square brackets denoting concentration in moles L^{-1}. This K_{sp} value is in good agreement with that of 10^{-23} reported for $Ni(DDC)_2$ in 0.1M KNO_3 at 25°C (Hulanicki, 1967). A comparison of these two values suggests that $Ni(DDC)_2$ is slightly more soluble at physiological pH in veronal buffer.

Precipitation problems were avoided by the incorporation

of a transfer step or by switching to the sequential protocol (Tables 4 and 5). Although there was potential for interference in nickel uptake studies in concurrent incubations without transfer, the similarity of results of such experiments with those including a transfer step suggest that this precipitation phenomenon did not have a serious effect. Nevertheless, one suspects that it may have reduced reproducibility. An effect (recovery exceeding 100% relative to whole cells) could be demonstrated in distribution experiments, presumably because the manipulations of the samples were more elaborate. All experiments of this type were therefore conducted employing the concurrent protocol with a transfer step.

A pertinent question is whether at concentrations of ligand > 10^{-6} M the nickel complex was available in the solution phase. Control experiments summarized in Figure 6 provide evidence that at least 50% of nickel-63 remained in the aqueous phase. The potential interference of the precipitation phenomena was not recognized previously (e.g. Nieboer et al, 1984c; Stafford, 1984).

4.2. Equilibrium Model of Metal-Ion Uptake.

To facilitate the discussion, known metal-ion uptake mechanisms are summarized in Figure 42. Under steady-state conditions, all uptake schemes shown in this figure are regulated by equilibrium parameters including compartment pH, ligand and metal concentrations, association constants, solubility in different phases and partition coefficients

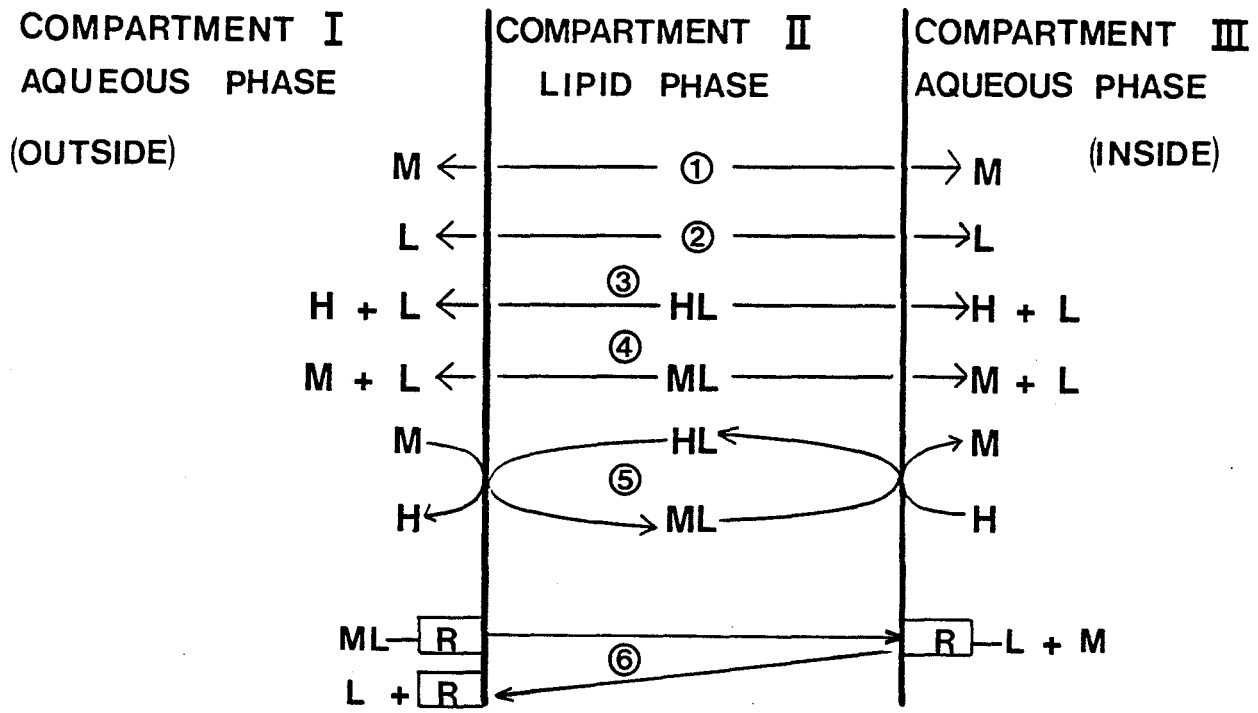


Figure 42. Equilibrium model for the transport of metal-ion species across biological membranes. Processes 1-6 denote various modes of transporting metal ions across biological membranes: 1, cation diffusion, ion exchange or ion channel; 2, ligand diffusion or exchange; 3, partitioning via neutral, lipid-soluble protonated ligand; 4, partitioning via neutral, lipid-soluble metal complex; 5, exchange by extraction; and 6, receptor-mediated transfer. Symbols: M, metal ion; L, ligand; HL is a neutral protonated ligand molecule; ML is a neutral metal-ligand complex; and R denotes a receptor. Charges on M, L, HL and ML and metal-ligand stoichiometry are omitted for convenience.

(Williams, 1981). At rest, the inside of cells is negatively charged with respect to the outside. Consequently, cations can diffuse inward (Pathway 1 in Figure 42) unless, of course, it moves against a concentration gradient. In the latter case an energy source is required (e.g., Na^+/K^+ ATPase driven pump). Often cation influx is balanced by the antiport of another cation to maintain charge balance inside the cell. Ion selective channels may also provide a mode of entry. For example, the channel-forming antibiotic gramicidin for monovalent ions (Pressman, 1976) and Ca^{2+} channels (Naschshen, 1984; Tsien, 1983).

Even though the mentioned pathways exist, membrane permeability to cations is generally low. It is well known that membranes are permeable to weak acids and bases, and this is employed for intracellular pH measurements (Nieboer et al, 1984b; Nuccitelli and Deamer, 1982). Neutral ligands are usually weak bases and therefore are transported readily across membranes (Pathway 3). When L is an anion, uptake by diffusion and exchange is low. Many energy dependent anion pumps are known; such as those for phosphate, sulphate and chloride. As already indicated, weak acids penetrate membranes and this is illustrated by Pathway 3 (e.g. CH_3COOH, Finean et al, 1978; 8-hydroxyquinoline across bacterial membranes, Albert et al, 1953). If in addition, HL is lipid soluble it may accumulate in all three compartments. This has been demonstrated in phospholipid vesicles with such ligands as 8-hydroxyquinoline, dithizone and DDC (Epand et al, 1985).

By analogy to Pathway 3, the transport of neutral metal complexes is also possible (Pathway 4), especially if ML is lipophilic (e.g., Zn complex of 8-hydroxyquinoline, Epand et al, 1985; and $In(Oxine)_3$ by leukocytes, Clay et al, 1983). It is also known that negatively charged complexes do not cross the membrane readily (e.g. $[Ni(EDTA)]^{2-}$, Nieboer et al, 1984c; the Zn^{2+} complex of 8-hydroxyquinoline-5-sulfonic acid and $[Zn(XAN)_2]^{2-}$ with XAN=xanthurenate, Epand et al, 1985). Since neutral metal complexes are often polar, the lack of charge does not assure permeability (e.g. $[Ni(His)_2]^{o}$, His=L-histidine; Nieboer et al, 1984c; May and Williams, 1977).

Another possibility is exemplified by the carrier protein metallothionein, which transports Zn^{2+}, Cd^{2+} and Cu^{2+}/Cu^{+} across cell membranes by pinocytosis (Williams, 1984; Cain and Holt, 1983). This mechanism is likely limited to macromolecular ligands. A modification of this macromolecular pathway is known for transferrin (Pathway 6). In this case, the macromolecule binds to a receptor on the outside of the cell, next the ferritransferrin/receptor complex is internalized, the iron is then released intracellularly, and the apotransferrin/receptor complex is recycled to the outside of the plasma membrane (Dautry-Varsat et al, 1983).

Pathway 5 is essentially the combination of 3 and 4, and might be expected when both HL and ML are lipophilic. Although this mechanism has not been established in biological systems, it forms the basis of extracting metal complexes into organic solvents (Laitinen and Harris, 1975).

For the present study, the compartments in the equilibrium model of Figure 42 may be designated as follows: OUTSIDE (Compartment I), corresponds to the incubation medium; INSIDE (Compartment III), to the cell cytosol; and the membrane (Compartment II) now represents the cell pellet. At the centrifugation speeds employed, the cell pellet includes the plasma membrane, nuclei, mitochondria, lysosomes and cell debris. The supernatant fraction is equated to the cytosol (Finean et al, 1978).

Equilibrium data available for the interaction between DDC, APDC and nickel(II), as well as the protonation data for the ligands, are compiled in Table 15. These parameters will be useful in the discussion of the experimental data.

4.3. Nickel(II) Uptake by Cells (in Absence of Ligand).

4.3.1. Equilibrium Considerations.

To interpret the results it is helpful to consider the cell as a ligand ($\bar{L}$) without differentiation of internal and external binding sites. It is possible then to express the basic interaction of a metal ion, M, with $\bar{L}$ as in Reaction (6).

$$M + \bar{L} \rightleftharpoons \overline{ML} \tag{6}$$

$$\bar{K} = \frac{(\overline{ML})}{(M)\,(\bar{L})} \tag{7}$$

$$\frac{(\overline{ML})}{(\bar{L})} = \bar{K}(M) \tag{8}$$

The bar over $\bar{L}$, $\overline{ML}$ and $\bar{K}$ signifies that these parameters

Table 15. Equilibrium Parameters for Dithiocarbamates

Parameter	DDC	APDC	Condition and/or Comment	Reference
pK_a^a (aq)	3.6	3.1	20-24°C, μ = 0.1 M	Stary and Freiser, 1978
$p\bar{K}_a$ $(CHCl_3)^b$	7.0	6.1	24-25°C, μ = 0.1 M	Stary and Freiser, 1978
log β_2^c	12.9	13.2	22-25°C, μ = 0.01 M	Scharfe et al., 1973
$t_{\frac{1}{2}}$ (pH = 7.0)	8.3 h	(>8.3 h)		Everson and Parker, 1974
$t_{\frac{1}{2}}$ (pH = 1)	7.5 s	30 min		Scharfe et al., 1973
pH ($t_{\frac{1}{2}}$ = 10 h)	6.84	3.98	20°C	Hulanicki, 1967
$P^d_{CHCl_3/H_2O}$	2.3×10^3	1.1×10^3	20-24°C, μ = 0.1 M	Stary and Freiser, 1978
log K_{sp}	-23.1	--	25°, 0.1 M KNO_3	Hulanicki, 1967

[a] $HDTC\ (aq) \rightleftharpoons H^+\ (aq) + DTC^-\ (aq)$

[b] $\overline{HDTC}\ (org) \rightleftharpoons H^+\ (aq) + DTC^-\ (aq)$

[c] The formation constant (β_2) is for the following reaction: $Ni^{2+}\ (aq) + 2DTC^-\ (aq) \rightleftharpoons Ni(DTC)_2(aq)$

[d] Partition Coefficient = $[\overline{HDTC}]_{CHCl_3} / [HDTC]_{H_2O}$

involve the cell as a ligand; parentheses denote concentrations in appropriate units. The cellular binding sites include intracellular and membrane-bound proteins as well as polynucleotides and low molecular weight molecules such as free amino acids. Consequently, the equilibrium constant, $\bar{K}$, is viewed as an average, composite constant for the various (n) types of available binding sites, i.e. $\bar{K} = \sqrt[n]{\bar{K}_1\ \bar{K}_2\ \bar{K}_3 \ldots\ldots \bar{K}_n}$. If C_M denotes the total amount of metal added then

$$(M) = C_M - (\overline{ML}) \tag{9}$$

Since less than 4% of the total nickel added becomes associated with all but one of the cell lines employed ($\leq$ 10% for RAMs), as a first approximation $(\overline{ML})$ may be considered to be small relative to C_M. Substitution of (9) into (8) yields (10) and (11).

$$\frac{(\overline{ML})}{(\bar{L})} = \bar{K}[C_M - (\overline{ML})] \tag{10}$$

$$\frac{(\overline{ML})}{(\bar{L})} = \bar{K}C_M \qquad \text{for } (\overline{ML}) << C_M \tag{11}$$

If $\bar{C}_L$ represents the total ligand concentration added (i.e. the number of cells x total concentration of binding sites per cell), $(\overline{ML}) << \bar{C}_L$ when working well below saturation conditions. This is the case in the present study since very low nickel-63 concentrations were employed. Thus equation (7) and (11) may be rewritten as in (12) and (13).

$$\bar{K} = \frac{(\overline{ML})}{\bar{C}_L C_M} \tag{12}$$

$$\frac{(\overline{ML})}{\bar{C}_L} = \bar{K} C_M \tag{13}$$

4.3.2. Discussion of Uptake Data.

It is evident for all cell types that when the total amount of nickel added increases, the cell-associated nickel increases also. This is to be expected since according to the equilibrium expression (13) $(\overline{ML})$ is proportional to C_M at constant $\bar{C}_L$. ($\bar{K}$ may well be invariant, and might decrease somewhat if additional and weaker binding sites become available at high C_M.)

By contrast to the uniform dependence of uptake on C_M, variation of cell number at constant C_M was dependent on cell type. For macrophages little dependence was indicated, for B-lymphoblasts and RBCs the uptake decreased somewhat with increasing cell number, while for peripheral lymphocytes there was a trend to a higher nickel uptake. In terms of Equation(13), at constant $\bar{K}$ and C_M the uptake per 10^6 cells (i.e., $(\overline{ML})/\bar{C}_L$) should remain approximately constant, as observed for macrophages. An increase in this relative uptake parameter suggests that the value of $\bar{K}$ increases, while a decrease implies that either the assumption $C_M >> (\overline{ML})$ is no longer valid or that $\bar{K}$ decreases. Co-operativity (intercellular binding) might be expected to increase $\bar{K}$, while aggregation (clumping) might block extracellular sites

(effectively lowering $\bar{K}$). Divalent cations, like Ca^{2+}, are known to promote co-agglutination of RBCs (Wallach, 1972).

In the absence of added ligand and for the same C_M value, the following uptake sequence was observed: RBCs < B-lymphoblasts $\leq$ human peripheral blood leukocytes < RAMs. Thus under conditions far removed from saturation, either $\bar{K}$ and/or the total concentration of binding sites per cell increase along this series.

It is interesting that Barton et al (1980) assigned 13% of the nickel in human whole blood to RBCs and 63% to the buffy coat (which contains the peripheral lymphocytes) and the remainder 24% to the plasma fraction. Furthermore, since the nickel content of whole blood is higher than that in serum or plasma (Sunderman et al, 1984), the current study supports the observation by Barton et al, 1980 that the peripheral leukocyte fraction accounts for this.

Interestingly, nickel-63 interaction with lymphocytes has previously been demonstrated by autoradiography (Hutchinson et al, 1975).

4.4. Effect of Dithiocarbamates on Nickel(II) Uptake.

4.4.1. Equilibrium Considerations.

In the presence of ligand, Reaction (14) and Equilibrium Expressions (15) and (16) may be assumed to apply by analogy to Equations (6), (7) and (13).

$$M + \bar{L}' \rightleftharpoons \overline{ML}' \qquad (14)$$

$$\bar{K}' = \frac{(\overline{ML}')}{(M)(\bar{L}')} \qquad (15)$$

$$\frac{(\overline{ML}')}{\bar{C}_L'} = \bar{K}'C_M \qquad \text{for } (\overline{ML}') << C_M, \bar{C}_L \qquad (16)$$

$\bar{L}'$ (and $\bar{C}_L'$) now includes not only the concentration of the cells' intracellular and cell-membrane binding sites, but also that of any dithiocarbamate taken up.

4.4.2. Discussion of Uptake Data.

4.4.2.1. Concurrent Incubation Protocol Studies.

For the various cells, the patterns of nickel uptake as a function of APDC and DDC concentration is remarkably similar. In concurrent incubations, trends exhibited a sharp rise in nickel uptake between 10^{-7} M and 10^{-6} M ligand and a sharp decrease between 10^{-6} M and 10^{-5} M. At ligand concentrations > 10^{-5} M and < 10^{-7} M nickel uptake approximated background levels (absence of ligand). The only deviation from these trends occurred for PMNs, where maximum uptake occurred between 10^{-6} M and 10^{-5} M, and for RAMs where at ligand concentrations between 10^{-5} M - 10^{-3} M negative uptake (removal) was observed.

The occurrence of the maximum at 10^{-6} M coincides with the ligand concentration at which complex formation is complete. This may be deduced from the equilibrium expression for β_2 and the data compiled in Table 15. The concentration of the fully-formed complex at various ligand concentrations can be estimated from these parameters.

$$\frac{[M(DTC)_2]}{[M]} = \beta_2[DTC]^2 = 10^{13}[DTC]^2 \qquad (17)$$

Since very little dithiocarbamate (DTC) is used up in complex formation because of the low concentration of nickel(II) employed, C_{DTC} approximates the equilibrium concentration of DTC (i.e., C_{DTC}= [DTC]). Thus, assuming that the reaction is complete when $[M(DTC)_2]/[M]=100$, it follows from Equation (17) that this occurs when $C_{DTC}^2=10^{-11}$ with $C_{DTC}=3\times10^{-6}$ M.

The first inclination is to associate the sharp drop in uptake at concentrations > 10^{-6} M to the precipitation phenomenon. However, approximately 50% of the nickel-63 was still available in the aqueous phase during incubation (see Figure 6). This interpretation may thus be discounted. Since anionic complexes of DTC for nickel(II), (e.g., $[Ni(DTC)_3]^-$), are not known, the formation of such species can also be rejected. The most likely explanation is that at high external ligand concentrations DDC and APDC, like other ligands (e.g., L-His, EDTA, HSA etc., Nieboer *et al*, 1984c; Stafford, 1984), remove nickel(II) from cells. In terms of the equilibrium model depicted in Figure 42, Compartment I thus seems to be favoured by virtue of the presence of high concentrations of added ligand.

4.4.2.2. Sequential Incubation Protocol Studies.

The nickel uptake curves observed using the sequential protocol were considerably different from those observed in the concurrent incubations. A much higher ligand concentration was required to achieve significant uptake. In the case of DDC,

uptake increased from 10^{-5} to 10^{-3} M decreasing at 10^{-2} M. Exceptions occurred for RAMs in which case the maximum was at 10^{-4} M (Figure 18) and for B-lymphoblasts where uptake approached saturation at 10^{-3} M (Figure 10). By contrast, uptake patterns for APDC levelled off at ligand concentration $\geq 10^{-3}$ M. Again, deviation from this pattern was observed for RAMs and B-lymphoblasts for which a maximum was observed at 10^{-3} M. It may be concluded from the positive relative uptakes of nickel(II), that DDC and APDC can be accumulated by all cell types studied. Since the relative uptakes of nickel(II) are approximately of the same order of magnitude for the two incubation protocols, it is tempting to conclude that the same principles govern nickel uptake. Thus it seems plausible that the ligand is taken up first (Pathway 2 and/or 3), followed by the metal ion (Pathway 1 and/or 5 and/or 4). From this point of view, ligand-preloaded cells at concentrations of 10^{-4} to 10^{-2} M appear to generate concentrations in Compartment I comparable to 10^{-7} to 10^{-5} M in concurrent incubations. In the latter case, the relative uptake decreased at ligand concentrations $> 10^{-6}$ M for all cell types for both APDC and DDC (PMNs excluded). As indicated, reduced uptake at 10^{-2} M relative to 10^{-3} M ligand occurred frequently in the sequential protocol for DDC, but infrequently for APDC. This suggests that the extent of ligand release from ligand-preloaded cells and its accumulation in Compartment I may be cell-dependent. This might well be related to cell characteristics such as lipid content which would influence the partitioning of ligand

and metal between Compartments I, II and III (Figure 42).

4.4.2.3. Relative Effectiveness of Dithiocarbamates.

Compared to APDC and DDC, AD did not enhance nickel uptake significantly for the cell types that were studied (peripheral lymphocytes and RBCs). From the chemical structural formulae, it is clear that APDC and DDC are more lipophilic than AD (Figure 1B). It is interesting that in studies with synthetic lipid vesicles, only lipophilic chelating agents forming neutral complexes rendered zinc lipid soluble; polar ligands like EDTA did not (Epand et al, 1985). Thus the same kind of principles seem to govern nickel(II) uptake in synthetic lipid vesicles and in whole intact living cells.

The reason for the observation that APDC generally promoted uptake more effectively than DDC is not immediately apparent. The thermodynamic data compiled in Table 15 are not of much help. The similarity of log β_2 and pK_a values predict that APDC and DDC would be equally effective while $p\bar{K}_a$ and partition coefficients (P_{CHCl_3/H_2O}) imply that DDC might be better than APDC. Neither do differences in the decomposition of the ligands during the experimental procedure provide an explanation, since DDC the less stable of the two ligands (Figure 1 and Table 15) has a half-life of 8 h at pH=7.

The accumulation of ligand by the cell during the ligand preincubation step (sequential protocol) confirms the existence of Pathway 2 and/or 3. Since the $p\bar{K}_a$ for DDC and APDC are between 6 and 7 (see Table 15 and Figure 42), Pathway 3 is highly probable at physiological pH values. Consequently,

Pathway 5 for nickel(II) uptake should also be possible. Unfortunately, no evidence is available in the present study to assess the relative importance of Pathways 1 and 5, nor of 4.

4.4.2.4. Nickel(II) and Cell Concentration Effects (Concurrent Incubation).

Regardless of the nickel concentration used (in the range 10^{-10} to 10^{-7} M), the presence of ligand (at 10^{-6} M) enhanced actual nickel(II) uptake. Positive relative uptakes were also evident for all cell types at $[Ni^{2+}]_t > 6.5 \times 10^{-9}$ M ($-\log [Ni^{2+}]_t = 8.2$). Hence, as in the absence of ligand, the nickel taken up (expressed as the amount per 10^6 cells) increased with C_M and the rationalization used in the absence of ligand again applies, namely ($\overline{ML}'$) increases with C_M at constant $\overline{C}'_L$ (see Section 4.3.2 and compare Equations 13 and 16).

In the presence of 10^{-6} M DDC, cell concentration effects were less dependent on cell type. For peripheral lymphocytes, B-lymphoblasts and RBCs, the nickel uptake (per 10^6 cells) pattern decreased rapidly and then leveled off, whereas for RAMs a more gradual decrease was apparent. Since at least 19% of nickel(II) was taken up in the experiments at the higher cell concentrations, these decreasing patterns of nickel uptake suggest that the assumption $C_M >> (\overline{ML}')$ is no longer valid. There seems to be no obvious reason to interpret the decrease with $\overline{C}_L$ as resulting from a systematic decrease in $\overline{K}'$ (e.g., blocking of external sites by aggregation).

4.4.2.5. Relative Cellular Affinities for Nickel(II) (Concurrent Incubation).

By comparing nickel uptake in the presence of 10^{-6} M DDC for the different cells, an order identical to the sequence found for nickel uptake without ligand (Section 4.3.2.) is revealed: RBCs < B-lymphoblasts $\leq$ peripheral leukocytes < RAMs (Tables 11 and 12). It seems likely that the different abilities for nickel(II) accumulation are determined by cell-dependent characteristics (e.g., cell size; lipid and protein composition of the plasma membrane, cytosol and organelles; the nature and number of membrane and intracellular proteins and of nuclear non-protein metal-binding components such as polynucleotides). It is noteworthy that the larger and nucleated cells take up more nickel(II) than RBCs.

4.4.3. Cellular Compartmentalization of Nickel(II) (Concurrent Incubation with Transfer).

4.4.3.1. Relative Distribution of Nickel(II) in Absence and Presence of Ligand.

It is evident that cellular compartmentalization of nickel(II) in the presence and in the absence of 10^{-6} M DDC is dependent upon cell-type (see Table 10). The distribution percentages do not quite add up to 100%. A deficit in the pellet fraction due to its incomplete removal from the incubation tube (after the final centrifugation step prior to transfer to the the scintillation vial) is the most likely explanation (see Section 2.5.4 for protocol). Without ligand, both B-lymphoblasts and peripheral lymphocytes exhibit approximately equal distributions of nickel(II) between the pellet and cytosol. The bulk of the nickel-63 was bound in the

cytosol of RBCs (74%). This finding is similar to that for the distribution of lead-203, for which 85% was present in the cytosol (Ong and Lee, 1980b). As found for ^{203}Pb, it is possible that haemoglobin is the major binding component for nickel(II) in the RBC cytosol (Ong and Lee, 1980b). It was shown that of membrane associated ^{45}Ca and ^{203}Pb more than 88% was associated to protein and less than 10% was associated with the lipid fraction (Ong and Lee, 1980a). By contrast to the RBCs, more nickel(II) was found in the pellet than in the cytosol of RAMs. By comparison of these distribution data (in the absence of added ligand) to those of Stafford (1984), there is good agreement for RAMs and RBCs. Whereas an equal distribution between the two compartments was found for B-lymphoblasts, Stafford (1984) reported slightly more in the pellet (60%) than in the lysate (40%). It is most likely that the origin of the B-lymphoblast explains these differences: Crowe cells were used in this study whereas HSV cells were employed by Stafford (1984).

The presence of ligand was shown to increase the lysate-associated nickel(II) for all cell types, except RBCs for which there was little change. However, a noticeable decrease in pellet-associated nickel(II) was evident for the latter. For RBCs, unexplained losses of label relative to the whole cell were evident for both the pellet and cytosol fractions. In the presence of ligand, Stafford (1984) found that nickel(II) uptake by the pellet was enhanced for RAMs and B-lymphoblasts at 10^{-6} M DDC. By contrast, the presence of

ligand enhanced cytosolic uptake in the present study (see Section 4.4.3.2. below). Since Stafford employed the concurrent protocol without transfer, this discrepancy most likely reflects transfer of label from the adsorbed pool in the incubation tube to whole cells and the pellet fraction.

4.4.3.2. Relationship of Relative Uptake and Relative Distribution.

The relative compartmentalization of nickel(II) in the pellet and the cytosol can be related to the corresponding relative uptakes. This is easily demonstrated by the following considerations based on Equation (2) (Section 3.2).

$$C_p = X_p C_{cell} \qquad (18)$$

$$C_c = X_c C_{cell} \qquad (19)$$

$$C'_p = X'_p C'_{cell} \qquad (20)$$

$$C'_c = X'_c C'_{cell} \qquad (21)$$

In these expressions, X_p is the fraction of nickel bound to the pellet, X_c that bound to the cytosol, and X'_p and X'_c as the corresponding fractions in the presence of ligand; C denotes the concentration of nickel(II) and subscripts 'c', 'p' and 'cell' refer to the cytosol, pellet and whole cell, respectively. Expressions that include the superscript prime(') correspond to the fractional distribution in the presence of ligand. Relative uptake (R) can be related to the

relative distribution by substituting Equations 18-21 into Equation (1) (Section 3.2).

$$R_{cell} = \frac{C'_{cell}}{C_{cell}} - 1 \qquad (22)$$

$$R_p = \left(\frac{X'_p}{X_p}\right)\left(\frac{C'_{cell}}{C_{cell}}\right) - 1 \qquad (23)$$

$$R_c = \left(\frac{X'_c}{X_c}\right)\left(\frac{C'_{cell}}{C_{cell}}\right) - 1 \qquad (24)$$

The basic difference between the relative uptake and the fractional distribution is that the relative uptake of each compartment in the presence of ligand is compared with the amount of nickel taken up by the same compartment in the absence of ligand, whereas the fractional distribution is expressed relative to the amount of nickel(II) associated with the whole cell (see Equations 1 and 2, Section 3.2). From Figures 15, 21 and 38 it may be seen that at 10^{-7} to 10^{-5} M DDC, the ratios of relative uptakes R_p/R_{cell} is < 1.0 and R_c/R_{cell} is > 1.0. An inspection of Equations 22 to 24 indicates that if R_p/R_{cell} is < 1.0, then X'_p/X_p is also < 1.0; similarly if R_c/R_{cell} is > 1.0, then X'_c/X_c > 1.0. An examination of the data in Table 10 confirms that X'_p/X_p < 1.0 and X'_c/X_c > 1.0. Hence, as already concluded in the previous section, the presence of ligand enhanced cytosolic uptake of nickel(II) by B-lymphoblasts, RAMs and peripheral lymphocytes.

4.4.4. Loss of Nickel(II) from Cells in Complete Physiological Medium.

Generally, it is clear from the studies conducted in veronal buffer that nickel(II) can be transported across the plasma membrane of cells in the presence (likely Pathways 1 and 5, although 4 may not be discounted, Figure 42) and absence of DDC (Pathway 1, Figure 42). Previous studies have also shown nickel association to Chinese Hamster Ovary cells in simple salts/glucose medium (Abbracchio et al, 1982). These simple media are characterized by high free nickel(II) concentration (Abbracchio et al, 1982). However, in vivo it is unlikely that nickel(II) exists in free ionic form because of the presence of amino acids (e.g., L-histidine) and proteins (e.g., albumin) that can bind to nickel(II) (Lucassen and Sarkar, 1979). Complete physiological medium consists of serum proteins (e.g., albumin) and essential amino acids (e.g., L-histidine), some of which have been shown to remove cell-associated nickel(II) in simple media (Stafford, 1984; Nieboer et al, 1984c; Abbracchio et al, 1982). Therefore it is not surprising that nickel is removed from nickel-pretreated cells when incubated in complete medium (see Tables 7 and 8). A similar observation was reported by Veien et al (1980), who were able to remove more than 90% of nickel-63 from peripheral lymphocytes by 3 washes with RPMI-1640 culture medium.

APDC-pretreatment of B-lymphoblasts and peripheral lymphocytes helped to retain cell-associated nickel(II) in complete medium. After 1 h for lymphocytes or 15 min for

lymphoblasts, less that 50% of nickel is removed by the components in the medium, whereas in the absence of ligand in excess of 80% of nickel(II) is released. From the point of view of viability, it is important to point out that a ligand pretreatment time as short as 15 min promoted the retention of nickel(II) (see data for donor FR in Table 8.).

4.4.5. Lymphocyte Transformation.

PHA, a well known T-cell specific mitogen that stimulates transformation of lymphocytes, is used as a control in most lymphocyte transformation tests. PHA-stimulated transformation is almost ten fold greater than that found by $NiSO_4$ (Figures 39 to 41). Although nickel(II) accumulates at similar levels for both nickel-sensitized and non-sensitized lymphocytes (compare uptake values for LS and AS in Table 14), it is interesting that transformation only occurs for the sensitized individual. Equal binding of nickel(II) to both sensitized and nonsensitized cells concurs with data reported by Hutchinson et al (1975) and Veien et al (1980). This is not surprising since the process of transformation is likely receptor-driven, and such sites would be expected to comprise a small proportion of the membrane surface proteins that bind to nickel(II) (see below).

Although a few studies suggest that $NiSO_4$ is a weak mitogen that stimulates lymphocytes from non-sensitized individuals to divide (Pappas et al, 1970; Svejgaard et al, 1978), the current data provides no such evidence. Specific transformation of nickel sensitized cells, which is

demonstrated in Figures 40 and 41, is supported by many other studies (Al-Tawil et al, 1981; Forman and Alexander, 1972). Even though nickel-induced transformation is well documented, the mechanism remains unclear. Two possible molecular mechanisms have been postulated: 1) Ni^{2+} may directly bind to cell surface receptors to induce transformation (Hutchinson et al, 1975); or 2) a nickel(II)-protein complex (possibly with serum albumin) may bind to a cell surface receptor (Thulin, 1976). In the latter case, Ni^{2+} acts as a hapten. In a humoral response to Ni^{2+} (occupational asthma), the Ni^{2+}-HSA complex was the antigen for an antibody with nickel(II) specificity (Dolovich et al, 1984).

Pretreatment of peripheral lymphocytes with APDC in veronal buffer was employed as a means of promoting nickel(II) uptake. It was reasoned that this nickel might become receptor associated and thus affect transformation. Since relatively high concentrations of nickel(II) ($2.4\text{-}9.5 \times 10^{-5}$ M) were required for the transformation test, the sequential protocol was used to avoid any precipitation in culture media (which was evident during concurrent incubation; data not reported).

From the data summarized in Table 9, it is evident that veronal buffer inhibited cell proliferation even under control conditions (data for culture medium with serum and no added nickel(II) salts or PHA). Since barbitals (the active ingredient of veronal buffer is diethyl barbiturate) are known to be lipophilic, they may affect membrane fluidity (Ho and Harris, 1981). It is therefore possible that this buffer

modified membrane fluidity and in this manner affected cell proliferation and perhaps reduced viabilities as well (see footnote e to Table 8 and see Table 9). This is somewhat surprising since veronal buffer is sometimes used for cell-related experiments (e.g., McGee and Myrvik, 1981; Harington et al, 1971). In addition, although not obvious from the data in Table 9, there is evidence from other experiments that PHA-induced cell proliferation was inhibited by APDC itself (data not shown).

In vitro inhibitory effects of DDC have been reported in studies of PHA activation of human peripheral lymphocytes (Neveu et al, 1980). In fact immunological effects of DDC have been extensively studied because of the possibility that it may resemble the in vitro and in vivo immunoenhancing potential of levamisole, a sulfhydryl drug used in rheumatoid arthritis (Corke, 1984). Preincubation of lymphocytes with DDC resulted in the inhibition of PHA-induced stimulation in vitro (Corke, 1984). By contrast, in vivo DDC displayed an immunoenhancing effect (see Section 1.3.) (Neveu et al, 1982).

4.5 Concluding Remarks.

From the current study, it is clear that adsorption of metal-ligand complexes can interfere with experimental procedures. This adsorption problem with nickel(II) dithiocarbamates has not been previously recognized, although it is well known that precipitation occurs in unbuffered solutions for complexes between DDC and metal ions(e.g., Ni^{2+}, Cd^{2+}, Co^{2+}, Zn^{2+}) (Gale et al, 1981). Thus, when working with

ligands that form adsorptive metal complexes, it is important to establish appropriate 'no cell' controls and to assess metal mass balance to enable one to estimate the extent for potential interference. The adsorbed metal complex represents a pool of nickel-63 on the tube wall. Systematic and adventitious contributions from this pool were found to occur, especially in fractional distribution studies. This problem was circumvented in two ways: 1) by incorporating a transfer step in the concurrent incubation protocol; and 2) by adopting the sequential procedure.

Ligand-enhanced uptake of nickel(II) was demonstrated in both experimental protocols. Although the exact mechanism by which DDC or APDC enhances uptake is unknown, it might be possible to distinguish between the various postulated pathways (Figure 42) on the basis of proton-gradient build up. On uptake in lipid vesicles, DDC-induced pH gradients between the outside and the inside have been reported, and were measured by the use of the fluorescent probe quinine (Epand et al, 1985). According to Pathway 3, ligand uptake would be accompanied by a concomitant uptake of H^+. Alternatively, a decrease in internal pH could also result if uptake were coupled to OH^- efflux (e.g., by Pathway 2). Accumulation of a large concentration of protons such that physiological pH is no longer maintained intracellularly can lead to cell death. Not only can cell viability be lowered from such a pH imbalance, but also from the toxic effects of dithiocarbamate degradation products (e.g., CS_2 in Figure 1A; Hayes 1982; Everson and

Parker, 1974). By analogy, uptake of ML by Pathway 5 would result in an increase of internal pH and this could be assessed by standard procedures (Nuccitelli and Deamer, 1982). Under acidic conditions (pH $\leq$ 6), decomposition of DDC would occur (Everson and Parker, 1974). For APDC, decomposition does not occur until pH < 5.

Even though intracellular ligand concentration appears to be important in enhancing nickel(II) uptake (see Section 4.4.2.2), the uptake process may well involve ionophoretic abilities of the dithiocarbamates (i.e., Pathway 5, Figure 42). An interesting study on the ability of DDC to act as an ionophore for artificial phospholipid vesicles (unilamellar) has recently been completed (Epand, Stafford and Nieboer, unpublished results). In PIPES buffer (pH=7.0), the concentration of DDC required for maximal removal of vesicular-entrapped nickel(II) in the presence of an external concentration of 10^{-3} M EDTA was 10^{-6} M, which is the same concentration for maximal enhanced nickel(II) uptake found in the present work (concurrent incubation). EDTA itself was not very effective in removing the nickel. By contrast, for RAMs, B-lymphoblasts and human RBCs, EDTA removed accumulated nickel-63 label, while DDC was much less effective or promoted its continued association with the cells.

As already mentioned, EDTA is a polar ligand which forms a water soluble complex with Ni^{2+} ($[NiEDTA]^{2-}$), while that with DDC has low water solubility and is soluble in organic solvents. Thus, it appears that the lipophilicity of DDC and

of the resultant nickel(II) complexes enhances Ni^{2+} uptake. This conclusion explains results in which DDC-treated mice (administered 10 min before or after nickel-63 injection) exhibited a larger total radioactivity of nickel-63 after 4 h than controls not treated with the ligand (Oskarsson and Tjälve, 1980; Tjälve et al, 1984). High levels were especially found in lipophilic tissue (e.g., adipose tissue). Similarly, enhanced uptake of $^{201}Tl^{+}$ by brain in mice has been reported (concurrent administration of metal and DDC; Vyth et al, 1983) and of Cd^{2+} by rat brain (pretreatment with DDC; Cantilena et al, 1982) when compared to the injection of metal alone. Consequently, concurrent administration of ligand and metal or ligand-pretreatments result in enhanced metal uptake both in vivo and in vitro (current study) experiments.

Fractional distribution studies in the presence of ligand promoted larger deposition of Ni^{2+} in the cell lysate. It would be interesting to determine if the redistribution of nickel(II) from the kidneys and the lungs to the liver, brain and RBCs in DDC-treated mice (Oskarsson and Tjälve, 1980) is accompanied by a similar cytosolic increase.

Antabuse or DDC have a number of clinical applications. Of course, the use of Antabuse in the treatment of chronic alcoholism is well known (Eneanya et al, 1981). Both chelating agents have been employed in the treatment of nickel carbonyl poisoning and in alleviating nickel contact dermatitis (Nieboer et al, 1984d; see also Section 1.3.). Serious side effects occur in the use of Antabuse, although the possibility of

alterations in metal redistribution of essential metals (i.e., Fe, Cu and Zn) have not been considered. Since DDC is a metabolite of Antabuse (see Figure 1A), similar problems might occur in its clinical application. It may be concluded from the work reported here, and that reviewed in the previous paragraphs, that metal redistribution might indeed be expected. Tissue enhancement of metals may well have toxic consequences. For example, Sunderman et al (1983) have demonstrated that enhanced nickel uptake due to DDC promoted heme oxygenase activity in rat liver and kidney. Further, DDC therapy of thallium intoxication in man worsened thallium-related neurological symptoms (Vyth et al, 1983).

It may be concluded that lipophilic chelating agents promote the cellular association of Ni^{2+}. It was hoped to improve the lymphocyte transformation test by enhancing the amount of cell-associated Ni^{2+} by pretreating the peripheral lymphocytes with APDC. This approach failed mainly because of the toxicity of the veronal buffer. Furthermore APDC, like DDC, appeared to decrease cell proliferation on its own. Evidence was presented that APDC indeed increased peripheral lymphocyte nickel levels in complete physiological medium. Therefore, in principle, ligand pretreatment appears to be a useful experimental tool. Future studies with less toxic buffers and lipophilic ligands are warranted.

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