MECHANISMS IN THE INDUCTION OF ALLERGIC CONTACT DERMATITIS TO NICKEL

A thesis submitted for the degree of Doctor of Philosophy of the University of Aston in Birmingham

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August, 1982

The University of Aston in Birmingham

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Ph.D Thesis 1982

SUMMARY

Nickel is frequently reported as an agent causing allergic contact dermatitis in humans and was selected as an example for the investigation into the molecular mechanisms of the sensitivity. Nickel, as a hapten, may not constitute the complete antigen. The protein or cellular conjugates of nickel formed in the skin may represent the antigenic complexes, but the number and nature are unknown.

In this submission, allergic contact dermatitis to nickel is reviewed; data on the immunological processes from various human and animal-model systems relevant to this problem are appraised and experimental investigations into the antigenic complexes of nickel are described.

The albino guinea-pig was selected as a suitable animal model and a test system was developed to induce and elicit allergic contact dermatitis to nickel in this species. Under the exposure conditions of this system, an incidence of the sensitivity of 70-100% in a test population was experimentally induced. This consistently high incidence was not achieved by the use of standard published animal methods.

Time-coursed *in vivo* exposure studies, employing $^{63}NiCl_2$ as a radiotracer, were performed to qualitatively and quantitatively examine the absorption and accumulation of nickel in the skin.

The results indicated that soluble nickel salts are poorly absorbed across the skin. Maximum plasma levels were seen after 12 hours of exposure, although these represented only a very small percentage of the applied nickel salts (0.06%). Absorbed nickel was excreted in the urine.

Microautoradiography of *in vivo* exposed skin indicated that transappendageal passage may be the main route of entry into the body. Accumulation of nickel was apparent in the highly keratinized areas of the skin, and labelling of basal and supra-basal epidermal cells was observed. Dermal accumulation was not seen.

Fractionation of the *in vivo* exposed epidermis by zonal ultracentrifugation and gel filtration revealed four main areas of nickel localization. It was found associated with a microsomal fraction, with albumin, with an epidermal protein fraction (molecular weight 8,700) and with low molecular weight (<5,000) residues.

The antigenicity of each isolated nickel-containing fraction was assessed by *in vitro* lymphocyte transformation. The primary antigens in this system were the epidermal protein fraction (molecular weight 8,700) and the microsomal fraction. The low molecular weight residues possessed some very weak antigenic properties.

Allergic contact dermatitis appears to be a complex sensitivity reaction to more than one antigen in the skin. The recognition of antigen by the immune system is protein-carrier dependant and the processing of haptenic or antigenic information by microsomal constituents may be an important function in the induction phase of the dermatitis.

Key Words:- allergic contact dermatitis, nickel dermatitis, delayed contact hypersensitivity, hapten absorption, antigen formation.

CONTENTS

Page

SECTION IV (cont'd)

LIST OF TABLES

LIST OF TABLES

LIST OF TABLES

LIST OF FIGURES

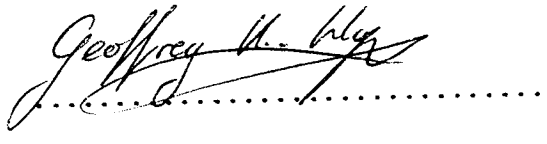
LIST OF FIGURES

DECLARATION

The research presented in this thesis was undertaken in the laboratories of the Huntingdon Research Centre.

Statistical analysis of the lymphocyte transformation assays was undertaken in collaboration with Dr. D.S. Chanter and Mr. A. McAllister of the Statistics Unit, Huntingdon Research Centre. No other aspect of the work was performed in collaboration. The thesis has not been submitted for other awards.

Aspects of the absorption and accumulation of nickel in guinea-pigs were published in Nickel Toxicology, Academic Press, 1980. Other aspects are intended for publication.



Geoffrey K. Lloyd, B.Sc.

ACKNOWLEDGEMENTS

It is with pleasure that I record my sincere gratitude to the friends and colleagues who have contributed in various ways to this project.

To Dr. Len Levy (University of Aston) and Mr. Ron Davies (HRC) for their guidance, enthusiasm and encouragement.

To Mr. Micheal Brown and Mr. Adrian Ball for their expert technical assistance and animal husbandry.

To Mr. Adrian Jackson for introducing me to the field of radioactivity.

To Mr. Greg Dash for expert assistance in the enzyme analyses.

To Mrs. Pat Uphill and Mrs. Julia Rose for the cell culture phases.

To Dr. Dennis Chanter and Mr. Arthur McAllister for the statistics.

To Mrs. Doreen Wright and Mrs. Marie Gardiner for their excellent skill and care in preparation of the manuscript.

To my wife Gill and daughters Joanna and Hannah for their support, encouragement, patience and for being there.

SECTION I - GENERAL INTRODUCTION

Contact Dermatitis - The Problem

In the assessment of human safety, the steadily increasing number of substances to which we are exposed, is of constant concern. The adverse effects which many substances exert on the body's prime defence organ, the skin, can be a serious medical problem (Shupack, 1977). In the past, soaps, cosmetics and pesticides have been recognised as potential sources of skin disorders. More recently a multitude of occupational and environmental factors have been recognised and are contributing to the vast list of skin contactants.

The exact prevalence of skin disorders resulting from contact with external agents has not been established with any certainty. It often accounts for 10% or more of the people attending dermatology clinics (Fregert 1974). In 1968, industrial dermatitis (defined as non-infective dermatitis of external origin) accounted for 470,000 days of incapacity in 15,200 spells of absence (3 or more days of medically certified sickness absence) for a population at risk over 14 million in the U.K. (Newhouse 1972). These figures outweighed and contrasted those for all other diseases prescribed under the National Insurance Act of 1965; 43 other diseases accounted for 190,000 days of incapacity in 7,200 spells of absence. Wilkinson (1980) considered that these figures understated the problem, since many employees continue working with some degree of dermatitis without losing work.

In the U.S.A. skin disorders associated with environmental agents are considered to be amongst the greatest public health problems. Occupational skin disorders in the U.S.A. in 1975 represented 46% of all occupational diseases (Katz, et al, 1979; Johnson et al, 1979).

The majority of these skin disorders were forms of contact dermatitis (Katz, et al, 1979).

Recognition of the increasing potential for various materials to contact the skin has led to the application of methods (including animal tests and human epidemiology) in an attempt to predict, or identify, the hazards. Clearly the predictive tools or use of these tools are inadequate since the list of materials causing contact dermatitis is steadily lengthening and new case reports enter the dermatological literature with every publication.

The skin's vital function is one of protection, which is achieved through several properties. Contact dermatitis may be defined as an inflammation caused by exposure to external agents and can be considered to be one such defence mechanism. The inflammation may be non-specific and produced in response to tissue injury or destruction, or an immunological response to foreign bodies (e.g. bacteria) or contacting chemicals.

Contact dermatitis is therefore a range of conditions, which may appear clinically similar, but may be divided into two basic classes; those that involve the immune system and those that do not.

Primary irritant contact dermatitis is a cutaneous inflammation produced in response to injury after contact with an agent. Almost all substances can act as skin irritants provided the concentration and duration of exposure are sufficient. The response can occur in most individuals, at the point of contact. The agent acts directly through a mechanism which damages the skin, and the immune system is not involved.

Allergic contact dermatitis may be defined as a cutaneous inflammatory response produced via a specific immunological reaction to a foreign substance (allergen). The term allergic usually refers to a specifically acquired increased reactivity which damages the host.

In contrast to irritant agents, allergens need not produce cutaneous damage to elicit a response and repeated contact is usually required before the allergy is induced. It is the inflammatory response which is adverse to the host and not necessarily the primary toxic properties of the contactant.

In early reports, allergic contact dermatitis was estimated to account for only 15 - 20% of occupational contact dermatitis (Adams 1969). More recently, however, the incidence has been estimated at 51 - 65% and varies with the degree and type of industrialization (Wilkinson et al, 1980; Fregert, 1975). This apparent increase in the frequency of allergic reactions compared to irritant contact dermatitis perhaps reflects the increased skill and attention paid to allergic manifestations and the very different and difficult medical problem of correct diagnosis and prevention. Failure to establish the aetiology is a common cause for the recurrence and persistence of occupational dermatitis (Adams, 1969)

Contact allergy, once acquired is easily maintained and readily triggered by very low exposures to the allergen. Exacerbation of the dermatitis can occur at the place of renewed contact and in severe cases can encompass large areas of the skin.

Simple precautions and personal hygiene, which minimise dermal contact, are usually effective in the prevention of occupational or non-occupational hazards from irritant materials. However, the low exposures which can maintain an allergy are not easily controlled by ordinary protective measures. Changes of occupation, or the learning of a new trade are often necessary for people with contact allergy but for some the contactant may be unknown or of non-occupational origin, or the person unable to qualify for a new job. In these cases the condition can be

prolonged and result in long periods of unemployment, repeated hospitalization and permanent disablement.

For the prevention of allergic contact dermatitis and prediction of potential which any contactant may have to induce the disorder, a detailed knowledge of the cause and effect is required.

Several contact dermatitis research groups have been established with the aim of providing information on the frequency of allergic contact dermatitis to many old and new materials. The International Contact Dermatitis Research Group and the North American Contact Dermatitis Group have been especially active in this regard.

The information generated by such international projects shows that allergic contact dermatitis is repeatedly diagnosed to certain substances throughout the world. These agents are usually simple compounds of low molecular weight (frequently under 500) (Adams, 1969) but represent a wide range of chemical types such as metals and their salts, monomeric compounds used in the production of plastics and rubbers, para-substituted aromatic amines, medications and germicides and metabolic products of plants. This information is helpful but shows no clear pattern as to why these materials should induce allergic contact dermatitis and therefore limits prediction of hazard.

It is the overall aim of this project to study the biological mechanisms of the induction of allergic contact dermatitis, in the search for a better understanding of the dermatological problem by investigation of one allergen in detail.

The metals, as a group, are frequently reported within the "top ten" agents responsible for allergic contact dermatitis and constitute a major proportion of the overall incidence. Of the metals, nickel and its salts represent a major hazard. A leading nickel manufacturer has been reported as saying "Nickel is with you and does things for you from the time you get up in the morning until you go to sleep at night". (International Nickel Company Inc.). This statement serves to demonstrate the ubiquitous nature of nickel in the environment and the considerable potential exposure to the general public.

Thus, nickel is selected, as an example of a contact allergen, for further investigation.

Incidence of Allergic Contact Dermatitis to Nickel

It has been known for many years that repeated skin contact with nickel and/or nickel salts can result in dermatitis. One of the first reports of "nickel itch" (Herxheimer, 1912) described the disorder following occupational exposure in nickel miners and smelters.

Since this early publication, many investigators have reported cases of allergic contact dermatitis to nickel. These reports range from epidemiological surveys to single case histories describing a new clinical manifestation or exposure factor. The incidence of sensitivity to nickel in the general population is unknown. The answer to this problem can only be obtained by testing whole population groups which are formed in respect of the wide spectrum of geographical, climatic, social, occupational, health and economic factors. The practical difficulties of testing such a population probably inhibit such investigations.

Only recently has this problem been approached. In three studies, the incidence of nickel sensitivity has been surveyed in "normal" volunteers who may, or may not, have been attending dermatology clinics for therapy. In Turku, Finland (Peltonen, 1979) positive patch testing to nickel was found in 4.5% of the population examined, in 8% of the females and 0.8% of the males. A history of dermatitis following metal contact was reported for the majority of the nickel sensitive persons and was clinically manifest at the time of testing in 36% of these subjects.

A similar study in Denmark, using veterinary student volunteers (Kieffer, 1979) resulted in incidences of 5.5% in the population examined, in 9.8% of the females and 2.8% of the males. The clinical history of dermatitis following metal contact was also taken into account, but it was not concluded to be of reliable predictive value.

Prystowsky (1979) studied 1,158 "normal" volunteers and 127 patients attending dermatology clinics for evaluation of contact dermatitis. The incidences of positive patch test reactions in the "normal" group were 5.8% of the total, 9% of the females and 0.9% of the males. In the patients already attending clinics, 11% of the total showed positive patch test reactions, thus indicating that data from the clinics was not applicable for extrapolation to the general population.

However, with the exception of these reports, epidemiological surveys by dermatologists from 1937 to the present day have been limited to patients attending dermatology clinics for treatment of skin disorders. The results of several surveys are presented as in Table 1. All surveys are based on the data obtained from patch testing (the elicitation of dermal reactions under an occlusive dressing) which like many laboratory examinations is subject to sources of error and variation of technique between different investigators (Hjorth, 1977). These data, although not strictly comparable are the only available major source of information indicating the prevalence of nickel sensitivity.

TABLE 1

Epidemiological Surveys of Allergic Contact Dermatitis to Nickel

Location	Year	No. Dermatitis cases Examined	% Incidence NI Allergy			Reference	Remarks
			Total	♂	♀		
New York, U.S.A.	1937	-	12.3	-	-	Baer et al (1973)	Only relative percentages given
London, G.B.	1953	1028	12.7			Calnan (1957)	Almost all cases were female
	1954	891	22.2				
	1955	885	20.3				
	1956	931	15.7				
Denmark	1960	-	4.6	-	-	Marcusson (1962)	0.6% occupational 4% non-occupational
New York, U.S.A.	1961	-	11.2	-	-	Baer et al (1973)	Only relative percentages given
Holland	1962-67	3151	4-9	1.3-3	2.7-6	Malten & Spruit (1969)	
Australia	1964-72	1000	4.7	-	-	Burry (1980)	
Scandinavia	1968	5558	5.9	-	-	Magnusson et al (1968)	
South Sweden	1969	712	7.9	0	7.9	Agrup (1969)	
Europe	1969	4825	6.7	1.8	10.2	Fregert et al (1969)	Survey of nine clinics
New York, U.S.A.	1968-70	-	13.1	-	-	Baer et al (1973)	Only relative percentages given
Poland	1970	1205	4.9	2.1	2.8	Rudzki & Kleniewska (1970)	
North America	1970-72	1200	11	-	-	Rudner (1977)	
	1972-74	3000	13				
	1974-75	1900	13.3				
	1975-76	2000	12.4				
Malmö, Sweden	1971	-	8.9	-	-	Christensen & Möller	Incidence ratio female to male 13/1
	1972	-	8.5	-	-		
	1973	-	10	-	-		
Geneva, Switzerland	1975	1000	12.2	3.5	8.7	Brun (1975)	
London, G.B.	1967-76	16571	9.1	2.3	15.3	Cronin (1980)	
Scotland	1970-77	1312	16	2	14	Husain (1977)	
Australia	1972-78	1000	8.6	-	-	Burry (1980)	
Denmark	1973-77	3225	6.4	1.3	5.1	Hammershøy (1980)	
Barcelona, Spain	1973-77	4600	14.9	-	-	Romaguera & Grimalt (1980)	20.9% occupational 68.2% non-occupational
Spain	1977	2806	18.8	-	-	Camarasa (1979)	All patients had contact dermatitis 19.2% of Ni +ve were housewives
Salvador, Brazil	1978	536	7.1	1.3	5.8	Moriearty et al (1978)	
Finland	1978	980*	4.5	0.8	8.0	Peltonen (1979)	
California, U.S.A.	1978	1158*	5.8	0.9	9	Prystowsky (1979)	
		127	11.0				
Denmark	1979	415*	5.5	2.8	9.8	Kieffer (1979)	

* Subjects examined were not attending dermatology clinics

Baer and co-workers (1973) reported a comparison study of the incidence of allergic contact dermatitis amongst patients attending a dermatology clinic in New York, USA, during 1937, 1961-62 and 1968-70. The incidence of nickel dermatitis over these years remained fairly constant at approximately 11-13% of the one patient-population. No further information relating to sex - distribution or occupational distribution was supplied.

From many of the other published surveys the incidence of nickel sensitivity in females is strikingly higher than in males.

Surveys performed at St. John's Hospital, London, during 1953-56 (Calnan, 1956 and 1957) showed that 46 to 53% of the patients examined had a form of allergic contact dermatitis. Of these subjects 27-48% (12-25% of total cases) were diagnosed to be sensitive to nickel sulphate. Almost all these cases were female and metal suspender belt clasps were considered to be the major cause.

Similarly, in a study in South Sweden (Agrup, 1969), 712 persons (250 men, 462 women) attending a dermatology clinic were examined for allergic contact dermatitis using a series of test substances, including nickel sulphate. Positive reactions were elicited in 56 women but in none of the males.

Nickel is considered to be the commonest sensitizer in women (Cronin, 1971).

Although these surveys are not strictly comparable, it may be concluded that allergic contact dermatitis to nickel is a disorder that has been recognised for many years, but that still remains a medical problem in everyday life. The surveys indicate that the incidence in the general population is around 5% and in patients attending dermatology clinics is on average 11%.

Nickel sensitivity appears more frequently in females than males. This may be attributable to a greater potential exposure of environmental origin (see below).

Sources of Skin Contact with Nickel

(a) Occupational exposure

The occupations in which the workers can be potentially exposed to nickel and its salts are listed in Table 2.

Technicological improvements and advances in industrial medicine and hygiene have aided considerably in controlling the exposure to nickel in many industries, to the extent that allergic contact dermatitis to nickel is seen relatively infrequently today as an "occupational disease", (National Research Council 1975, Marcusson, 1960) and nickel is not considered to be a major occupational allergen in males or females (Wilkinson, et al, 1980).

Early reports by Bulmer and Mackenzie (1926) and by Dubois (1931) described the preventative measures undertaken to reduce the high incidence of nickel dermatitis seen in industry at that time.

Bulmer and Mackenzie concluded from their survey in a nickel refinery that the development of the disease was closely related

TABLE 2

Occupations with Potential Exposure to Nickel

Artists
Battery manufacturers
Car Manufacturers and workers
Cashiers and shop assistants
Cemented-carbide manufacturers
Ceramic manufacturers and workers
Dentists and dental technicians
Disinfectant manufacturers
Duplicator machine workers
Dyers
Electronics workers
Electroplaters
Enamellers
Hairdressers
Ink makers
Insecticides manufacturers
Jewellers
Nickel-alloy manufacturers
Nickel catalyst manufacturers
Nickel miners, refiners, smelters
Paint manufacturers
Pen manufacturers
Rubber workers
Spark-plug manufacturers
Stainless-steel manufacturers and workers
Textile dyers and mordanters
Welders

(Adams 1969, Fisher 1967, NIOSH 1977)

to working in hot environments where finely divided nickel dust was present. Workers exposed to the heat of the furnaces or to the humidity and heat of the electrolysis areas were most often affected. In 1923, 43 cases of nickel dermatitis resulted in a total of 4,016 hours lost working time. The preventive medical programme implemented drastically reduced these figures to 22 cases causing only 72 lost hours.

Dubois reported on a survey in a Swiss nickel plating factory. In the 2-year period, 1928-1929, 370 workers were employed in the plating room but only 20 had been able to continue work without interruption due to dermatitis. The plating solution contained $NiSO_4$ maintained at a temperature of 85°C and produced a thick cloud reducing visibility to less than one metre.

In comparison, no cases of dermatitis were observed in plating rooms where cold solutions were used.

More recently, Marcussen (1960) reviewed the published case reports of occupational nickel dermatitis in Denmark between 1930 to 1960. The author noted that the incidence of the disease had largely ameliorated in major industries. However, an increasing incidence was apparent in minor occupations where, for example, women may be employed as salesgirls, cashiers and hairdressers. The results of this survey indicated that 86.5% of the cases were of non-occupational origin, 4% were due to nickel plating processes and 9.5% were related to other occupations.

In Barcelona, Spain, a survey of nickel sensitive patients attending dermatology clinics during 1973-77 (Romaguera and Grimalt, 1980) classified 20.9% to occupational exposure, of which 88.8% were female. Non-occupational exposure was reported to account for 68.2% of the nickel sensitive patients.

A survey of occupational dermatoses (Fregert, 1975) indicated that nickel was the most common sensitizer in women particularly amongst cleaning staff.

Thus, nickel dermatitis still remains an occupational problem in some industries, particularly electroforming, electroplating and electrolysis processes and especially when dermal exposure is high. (National Research Council, 1975, Wall and Calnan, 1980).

(b) Non-occupational exposure

Non-occupational exposure to nickel is potentially a greater problem because the general population can be affected. The use of nickel and nickel-containing commodities increases by approximately 10% per year (National Research Council, 1975) and the number and type of commodities that a person can contact are considerable.

The control of this exposure from the private use of nickel-containing products would be impractical and the education of the general population to full awareness and appreciation of the hazards would be a difficult task.

A list of some of the non-occupational sources of nickel is presented in Table 3. This list is by no means exhaustive but serves to demonstrate the formidable potential exposure to any one person.

Malten and Spruit (1969) reviewed the relative importance of various sources of non-occupational exposure to nickel. Nickel-containing commodities and nickel-containing detergents were concluded to be the principal sources.

The low concentrations of nickel in commercial detergent powders (2-9 ppm in the Netherlands, Malten and Spruit, 1969, 10 ppm in England, Wells, 1956 and 0.4-0.7 ppm in Austria, Ebner, et al, 1978) were not considered to be sufficient to produce the dermatitis. In the Netherlands, ethylenediaminetetraacetic acid (EDTA) was added to the available detergents to chelate nickel, but the incidence of the disorder did not decline during a 3-year period after the addition of EDTA (Malten and Spruit, 1969).

Although the nickel content of detergents was not considered sufficient to produce the dermatitis, the possibility that these low levels could maintain the condition was, however, not excluded.

In women there are three main sources of "continuous" contact with nickel: jewellery, clothing fasteners and appliances and stainless steel kitchens. The prevalence of reports of allergic contact dermatitis due to costume jewellery and clothing fasteners indicates the importance of these commodities for skin contact with nickel (Rostenberg and Perkins, 1951; Calnan, 1956; Fregert and Rorsman, 1966; Fisher, 1967; Watt, et al, 1968; Fregert, 1974;

TABLE 3

Commodities containing Nickel

Cigarette lighters
Clothing fasteners (press studs, zips, buckles etc)
Coinage
Dental materials and instruments
Detergents
Fungicides
Hair pins and curlers
Hairdyes
Handbag and purse locks and handles
Insecticides
Jewellery (earrings, rings, spectacle frames, necklace clasps etc)
Keys
Lipstick holders
Metallic eyelets of shoes
Nickel pigments (paints)
Pens
Pen knives
Powder compacts
Prostheses and other medical appliances
Scissors
Stainless steel kitchens and kitchen utensils
Taps (Ni plated under chromium plating)
Thimbles
Tobacco smoke
Watches, watch straps and buckles

(Fisher 1967, Fregert 1974, National Research Council 1975)

Grimalt and Romaguera, 1978; Yoshikawa, et al, 1978; Moriearty, 1978; Peltonen, 1979; Cronin, 1980).

However, the relative importance of skin contact with nickel from stainless steel kitchens and utensils has not been reviewed. There are no reported studies to demonstrate whether nickel is released by the action of sweat or sweat in combination with detergents or whether enough nickel is released to provoke reactions if the contact is short but frequent.

The potential for the release of nickel from stainless steel and other alloys is an important consideration in the use of protheses and other medical appliances. Nickel containing alloys have been implanted in man in the form of a wide variety of therapeutic devices and prostheses, including stainless steel and nickel wires as suture materials, stainless steel infusion cannulae, heart-valve replacements, total joint replacements and dental castings and filling materials. Allergic dermatitis and metal implant rejection has been attributed to nickel and the resultant sensitivity published in several reports (Stoddart, 1960; Tinckler, 1972; Barranco and Soloman 1972; Pegum, 1974; Elves, et al, 1975; Mayor, et al, 1980).

Samitz and Katz (1975) demonstrated that nickel is released from commonly implanted metal alloys by the action of sweat, blood and physiological saline solution. These authors considered that the action of body fluids, and corrosion and fatigue products of alloys could release nickel into the local tissue sufficient to provoke the reported actions.

The Clinical Pattern of Allergic Contact Dermatitis to Nickel

The early case reports of allergic contact dermatitis to nickel in nickel miners, smelters, refiners and platers described the disease as "nickel itch" (Herxheimer, 1912). The dermatitis began as an itching of the web of the fingers spreading to the fingers themselves, wrists and forearms.

More recent reports describe the dermatitis as a papular or papulovesicular eruption with a tendency for lichenification in chronicity (Fisher, 1967; Calnan, 1956; Wilson, 1956; Cronin, 1972; Epstein, 1956; Christensen and Möller, 1975).

In an analysis of 400 cases of nickel dermatitis, Calnan (1956) classified patterns of the eruptions into three groups:

primary eruptions : involving areas in direct contact with nickel

secondary eruptions : involving areas where the dermatitis spreads but with no obvious nickel contact (75% of Calnan's cases)

associated eruptions : areas of dermatitis that appear to have no direct relation to the primary eruption or to nickel sensitivity, e.g. seborrhoeic, atopic

The primary eruption is to be anticipated, in that any area of the skin may become affected if it is in direct contact with nickel for a sufficiently long period. Primary eruptions are most frequently

reported on the earlobes, forearms, back and central abdomen and may be resultant from the wearing of ear-rings, costume jewellery including watchstraps, brassiere fasteners and jean stud fasteners respectively (Wilson, 1956; Calnan, 1956; Epstein, 1956; Wahlberg and Skog, 1971; Brandrup and Larsen, 1979; Cronin, 1980).

The secondary eruptions were a common observation in nickel sensitive women when the primary contact was with suspender fasteners (Calnan, 1950). The eruption was not clearly associated with the primary contact and was often seen as a bilateral spread affecting eyelids, sides of the neck and elbow flexures. The secondary eruptions were also reported to occur before the primary eruption or flare without exacerbation of a previous primary site (Calnan, 1956; Wilson, 1956).

The secondary eruptions were peculiar to nickel allergic dermatitis and according to Calnan (1956) were not observed in cases resultant from occupational exposure. There appears to be no adequate explanation for this phenomenon although Fisher (1967) has suggested that close observation and careful recordings of the history of the dermatitis may reveal that nickel did contact the secondary areas and that "wandering" of nickel objects over the skin may explain the appearance of the reactions over widespread areas.

In the cases reported by Cronin (1980) during 1967-76, the incidence of suspender dermatitis had ameliorated and secondary eruptions were seen in only 2% of patients.

Secondary eruptions of the hands are frequent observations in cases of contact allergy to nickel where the primary eruption is elsewhere on the body. The incidence of hand dermatitis amongst a population of nickel sensitive patients has been reported to be between 16-52% (Calnan, 1956; Wilson, 1956; Cronin, 1972; Christensen and Möller, 1975).

The hand dermatitis was clinically characterized by Christensen and Möller (1975) as a pompholyx, i.e. a recurring itching eruption with deep seated vesicles, with some or no erythema and localised on the palms, volar aspects and sides of the fingers.

Another clinical feature common to nickel dermatitis, but seen infrequently with other contact allergens is the persistence, in some cases, of the eruptions after removal of the subjects from evident sources of exposure. Hand dermatitis appeared to be continuous in 50% of cases reported by Fregert (1975) in a 10 year follow-up survey. Christensen and Möller (1975) calculated a mean duration of 7.9 years for the hand dermatitis in their patients.

The factors responsible for the observed chronicity are unknown but the following may be contributory to the clinical picture:-

1. Nickel fixation within the skin.

2. Subtle re-exposure to nickel from inadequately identified sources.

3. Dietary ingestion of nickel. Experimental oral administration of nickel at levels similar to the expected normal daily intake has been demonstrated to exacerbate dermal reactions

in nickel sensitive persons (Christensen and Möller, 1975; Mennè and Thorboe, 1976; Cronin, et al, 1980).

4. Atopy-nickel sensitivity relationship. Atopy is a familial condition in which there is the frequent occurrence of allergic manifestations (asthma, hay fever, dermatitis). The condition is believed to be inherited and Immunoglobulin E mediated. Epstein (1956) and Watt and Baumann (1968) observed concomitant atopy and nickel sensitivity in their dermatological surveys. Several investigations refute this relationship and have seen no significant connection (Wilson, 1956; Caron, 1964; Calnan, 1956 and Fisher, 1967). Raised Ig E levels have not been demonstrated in nickel sensitive individuals (Wahlberg and Skog, 1971).

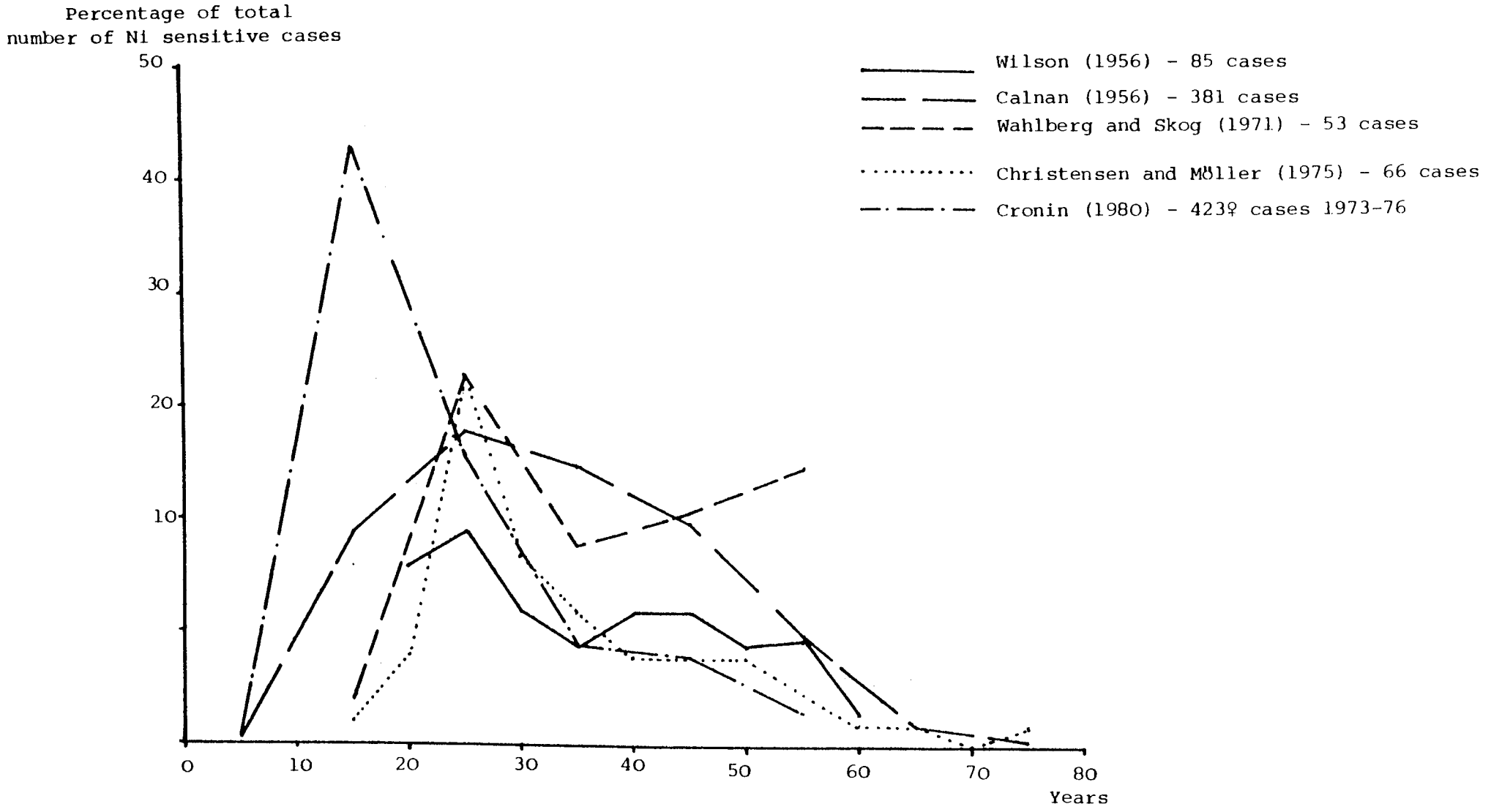
Age of Initial Diagnosis and Duration of Nickel Dermatitis

The ages of initial onset of nickel dermatitis as reported by Calnan (1956), Wilson (1956), Wahlberg and Skog (1971), Christensen and Möller (1975) and Cronin (1980) are presented in Figure 1. From these data it is apparent that susceptibility is highest amongst the 10-30 year age group and declines markedly in the 30-40 year age group. This is particularly apparent in women but not men (Cronin, 1980).

The greater potential occupational and non-occupational exposure (particularly costume jewellery) to people 10-30 years of age, may be a possible explanation for the higher frequency of diagnosed cases. Lack of awareness of exposure and failure to appreciate the consequences may be contributory.

FIGURE 1

Age of initial diagnosis of nickel sensitivity



The observed frequency of the dermatitis may also be influenced by a greater cosmetic awareness of skin blemishes by people in this younger age group who are perhaps more likely to attend dermatology clinics.

Calnan (1956) suggested that, regardless of the age of onset, the dermatitis is difficult to heal and the sensitivity, once acquired is persistent. In 15% of Calnan's cases the sensitivity was persistent for at least 5 years and still present in approximately 1% of cases after 40 years.

In a retrospective survey of 64 patients, Wilson (1956) reported 50% of cases suffered from intermittent eruptions of the dermatitis irrespective of whether contact with nickel containing metal had been avoided or not. Patients were examined from 6 months to 5 years after first diagnosis. On patch testing with $NiSO_4$ all cases examined showed positive reactions to challenge.

Fregert (1975) reported a 10 year retrospective survey of female patients with occupational hand dermatitis resultant from nickel allergy. Approximately 50% of cases had almost continuous dermatitis and 50% showed periodic symptoms. None of the cases was completely healed.

In 1973 TeLintum and Nater reported on the persistence of positive patch test reactions in nickel in a group of 57 persons previously diagnosed as sensitive to nickel. The interval before repeat patch testing was 2-15 years. Only 18 persons ($\sim$ 32%) were negative.

Although the published data on the duration of nickel allergy in humans is somewhat scanty, it would appear that nickel sensitivity, once acquired, is seldom lost irrespective of whether or not the initial dermatitis clears up. Complete avoidance of contact with nickel containing metals may be impossible considering the ubiquitous nature of nickel in the environment and subtle re-exposure may be responsible for the persistence of the allergy. However, the potential to respond to an allergen is not known to reduce with age, although preventative measures (personal hygiene, and an awareness of the cause) can reduce the incidence of exacerbation.

SECTION II - IMMUNOLOGICAL ASPECTS OF ALLERGIC CONTACT DERMATITIS

General Concepts

The present concept of some forms of contact dermatitis as allergic or immune diseases was initiated by Jadassohn in 1985. As a dermatologist he recognised that certain skin reactions could not be simply explained by the toxic properties of the contacting chemicals, but were due to a specific increased sensitivity acquired by the patients to the substances after repeated contact.

On the basis of experimental studies in the guinea-pig, Landsteiner and Jacobs (1935) postulated a theory, which has since become a landmark, concerning the relationship of immunogenicity to chemical structure. According to this concept, low molecular weight substances, capable of inducing the hypersensitivity, were unable to produce the reactions alone. Their allergenicity was only produced on conjugation with carrier proteins. The term hapten was introduced to define the incomplete allergen.

A second fundamental concept by Landsteiner and Chase (1942) concerned the existence of two basic types of immune reactions. The authors succeeded in transferring contact sensitivity in guinea-pigs from hypersensitive donors to normal recipients using viable lymphocytes from the donors. The hypersensitivity was not transferred when serum instead of lymphocytes was used. Immune reactions were divided into antibody-mediated (immediate type, serum transferable) and cell-mediated (delayed type, lymphocyte transferable) hypersensitivities.

More recently it has been shown (Cooper, 1972; Cooper and Ada, 1972) that although lymphocytes are morphologically indistinguishable they can be functionally divided into two sub-groups: the bursa dependent cells (B-cells) which are precursors of antibody-producing plasma cells and the thymus dependent cells (T-cells) which are responsible for cell-mediated immune responses and play a decisive role in contact sensitivity.

The types of tissue damaging immune responses were classified into four categories (Coombs and Gell, 1975) of which, types I to III are considered to be antibody-mediated, and type IV cell-mediated. This classification is as follows:-

Type I reaction (anaphylactic, antibody-dependent).
Initiated by antigen reacting with tissue cells (basophils and mast cells) passively sensitized by antibody produced by B-cells, leading to the release of pharmacologically active substances (histamine).

Type II reaction (cytotoxic).
Initiated by antibody reacting with either an antigenic component of a cell or an antigen which has become intimately associated with a cell. Damage to the cell may then occur via the activation of complement.

Type III reaction (damage by antigen-antibody complexes). Initiated when precipitating antibody (usually IgM) reacts in intercellular spaces with antigen, forming

(a) microprecipitates in and around small vessels causing damage to cells secondarily;

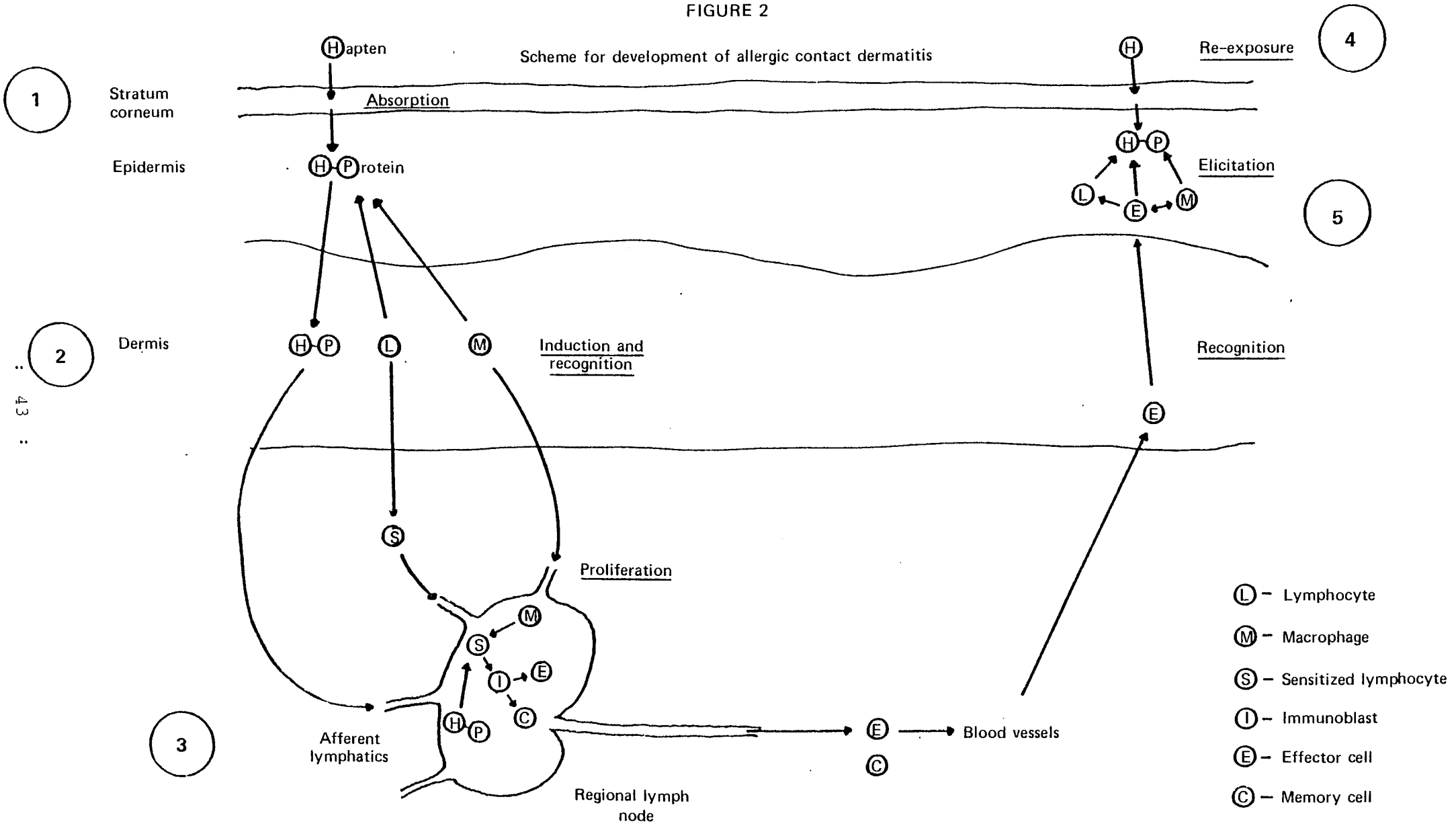
(b) precipitates in cellular membranes and interfering with the function of the membranes;

(c) soluble antigen-antibody complexes in the blood, which may be deposited in vessel walls or basement membranes, causing local inflammation and/or complement activation.

Type IV reaction (delayed, tuberculin-type, cell-mediated). Initiated by the reaction of sensitized lymphocytes (T-cells) responding specifically to antigen by the release of soluble mediators (lymphokines). Free antibody is not involved. The synonym, delayed hypersensitivity, is used to denote that the reaction peaks within 24-48 hours, in contrast to types I to III reactions which usually peak within a few minutes to 12 hours.

Allergic contact dermatitis is a type IV response. The general concept of the mechanisms are presented diagrammatically in Figure 2. This scheme is divided into several chronological events:

FIGURE 2


Scheme for development of allergic contact dermatitis
Hapten
Absorption
Stratum corneum
Epidermis
Protein
Dermis
Induction and recognition
Proliferation
Afferent lymphatics
Regional lymph node
Blood vessels
Re-exposure
Elicitation
Recognition
1
2
3
4
5
L – Lymphocyte
M – Macrophage
S – Sensitized lymphocyte
I – Immunoblast
E – Effector cell
C – Memory cell

1. Absorption and conjugation of the hapten.

2. Recognition of the allergen and induction of sensitivity.

3. The proliferative phase.

4. Re-exposure to hapten.

5. Recognition and elicitation of immune response.

The time from initial contact to elicitation of the response can be 5-7 days (Polak, 1977) assuming the allergen is recognised on first contact. In practice induction may span several days to several years.

In order to maintain some clarity, current knowledge and concepts of the mechanisms of each event will be discussed generally. Facts specifically pertinent to nickel contact dermatitis and requiring some detailed discussion will be presented in later sections.

Absorption and Conjugation of the Hapten

From the early work of Landsteiner and Jacob it is apparent that for compounds to induce contact sensitivity they must penetrate the skin and be sufficiently chemically reactive to form stable conjugates with skin proteins or cells.

The skin's barrier to external chemical insult is the highly keratinized stratum corneum, which is generally considered to be the rate-limiting step in absorption of compounds applied to the skin. However, consideration must be given to skin appendages (hair follicles and gland ducts) and to areas of damaged skin where

the stratum corneum is broken. Contact sensitizers must, therefore, be able to penetrate the skin by at least one of these routes.

Once past the stratum corneum, the potential for the hapten to produce contact sensitivity is to some extent dependant on its ability to form a complete antigen by conjugation with skin proteins or cells. It seems a reasonable assumption that if the hapten is sufficiently reactive to conjugate with one protein it will also react with several other skin or serum proteins or amino-acids (Polak, 1974).

It can be anticipated that the various antigens formed in situ will consist of both high and low molecular weight conjugates. The latter molecules may be amino-acid conjugates capable of further penetration through the skin and passage through the lymphatic system to the regional lymph node. Berrens (1965) proposed that these small conjugates act as intermediate messengers detailing the haptenic information to the reticuloendothelial system and are responsible for induction of sensitivity.

The large molecular weight (M.Wt.) conjugates are more likely to be "fixed" within the skin, where they are "recognised" by the sensitized lymphocytes. These fixed macromolecules may explain the chronicity of the eruptions seen in nickel dermatitis.

Rostenberg (1965) postulated that the conjugated hapten projects from the surface of the protein molecule, thus changing its spatial configuration so that the immune system recognises the conjugate as foreign, but not disrupting its structure to such an extent that the protein is denatured or destroyed.

Denaturation may explain why strong irritants which can vigorously alter protein structure such as acids, alkalis or phenols etc. are not frequently reported as being allergic contact sensitizers.

In studies using azobenzene arsonate (ABA) as a hapten (Leskowitz, 1962 and 1963), guinea-pigs sensitized to ABA-guinea-pig albumin would crossreact with an azobenzene-albumin conjugate. When the animals were then desensitized with the latter conjugate, this cross-reactivity was lost, but reactivity to ABA-albumin remained. These results suggested that a considerable contribution to the contact sensitivity was due to specific recognition of the hapten.

When the carrier protein was oxidised to its open chain form, reactivity and cross-reactivity with a different hapten on the same non-oxidised protein carrier were unaffected. This suggests that the tertiary structure of the carrier protein does not play an important role in the specificity of the reaction, but the primary structure, including the hapten, is an important factor.

The relevance of skin derived protein conjugates has been demonstrated by several researchers using 2,4 dinitrochlorobenzene (DNCB) or picryl chloride as haptens (Parker and Turk, 1970a; Nishioka, et al 1971; Nishioka and Amos, 1973; Milner, 1974; Miyagawa, et al, 1977; Miyagawa, et al, 1978). In these reports, conjugates of skin sub-cellular organelles and soluble proteins have been demonstrated to elicit sensitivity responses.

However, the ability to elicit contact sensitivity does not appear to be restricted to hapten conjugates with carriers of skin origin. Red blood cell stromata (Landsteiner and Chase, 1941),

serum proteins (Salvin and Smith, 1961), some amino acids (Eisen and Belman, 1955; Jansen, et al, 1964; Frey, et al, 1969; Samitz, et al, 1975) and intact lymphocytes (Geczy and Baumgarten, 1970) have been demonstrated as efficient carriers.

Thus, the diversity of hapten-carrier conjugates, which are able to elicit reactions, may be considered to illustrate the complexity of the underlying mechanisms. Contact sensitivity may represent a complex reaction to several conjugates formed in situ and of the same allergenicity (Polak, 1980). However, the exact number and structure of conjugates formed in vivo in any allergic contact dermatitis are unknown.

Recognition of the Allergen by Lymphoid Cells and the Induction of Sensitivity

The complete antigen must remain in the skin at the area of exposure for a defined minimal length of time after exposure. In guinea-pigs the excision of the skin at the site of antigen exposure, within 8 hours of contact, completely inhibits the induction of sensitivity. Excision of the skin site up to 16 hours after exposure results in a reduction of the number of animals showing the sensitivity and excision 24 hours after exposure has no effect on the incidence of sensitivity (Frey and Wenk, 1957; Macher and Chase, 1969; Turk and Stone, 1963). These workers considered that the majority of this minimal contact time is necessary for antigen recognition by lymphocytes.

There are two possible mechanisms by which lymphocytes could be sensitized during the induction phase of the allergy:-

1. The lymphocytes are sensitized at the site of exposure (i.e. the skin). Antigen inexperienced T-cells bearing membrane receptors for antigens in the skin, circulate in the lymph in low numbers and encounter the antigen at the site of hapten application. This encounter results in a "switching on" of the cells (peripheral sensitization) (Medawar, 1958).

2. Antigen inexperienced T-cells or macrophages contact the antigen at the site of hapten application. The antigen or antigenic information is transported via the lymphatic vessel to regional lymph nodes where the information is transferred from the macrophages to T-cells. The induction of sensitivity takes place in the lymph node (central sensitization) (Parker and Turk, 1970b).

Polak and Macher (1974) successfully sensitized guinea-pig lymphocytes cultured in vitro with DNCB in a protein-containing medium. This demonstration serves to indicate that sensitivity can be induced outside the lymph node and supports the concept of peripheral sensitization.

However, the necessity for the regional lymphatic system in the development of contact sensitivity was demonstrated by the elegant investigation of Frey and Wenk (1957). Skin islands of guinea-pig flank were prepared in such a manner as to leave intact only one of the possible pathways of transport from the skin (i.e. either blood vessels,

lymph vessels or nerves of the skin syncytium). Contact sensitivity to DNCB was induced via the skin island only when the lymph vessels were intact. This work was later verified using different techniques by Epstein, et al (1963).

From the reports of several authors (Dumonde, 1967; Unanue and Feldman, 1971; Waldron, et al, 1973; Thomas, et al, 1977; Rosenstreich and Rosenthal, 1973; Rosenthal and Shevach, 1973), it would appear that the macrophage has an important role in the induction phase of contact sensitivity. The antigen-induced in vitro T-lymphocyte transformation, which is considered to be the in vitro correlate of in vivo delayed hypersensitivity, has been shown to be macrophage dependant. Lymphocyte populations from sensitized guinea-pigs lost their ability to transform to immunoblasts in response to antigen, when the preparations are depleted of macrophages (Rosenstreich and Rosenthal, 1973). This transformation ability was restored by the addition of macrophages only and not by the addition of lymphocytes, fibroblasts or granulocytes.

Thomas, et al, (1977) proposed that the sequence of events leading to the activation of T-lymphocytes, consists of the uptake and processing of antigen or hapten by the macrophages, formation of immunogenic complexes, and T-lymphocyte binding to antigen-modified macrophages. It was suggested that lymphocytes do not recognize hapten-carrier conjugates per se, but only the macrophage processed antigen. Furthermore, the question arises as to whether macrophages stimulate T-lymphocytes by just presenting haptenic determinants, or processed antigen or another form of antigenic information, such as a specific soluble stimulation factor (Polak, 1978).

An alternative hypothesis has been proposed from studies of the role of the major histocompatibility complex (MHC) in contact sensitivity. Initially, the research of Rosenthal and Shevach (Rosenthal and Shevach, 1973; Shevach and Rosenthal, 1973; Shevach, 1976) indicated that the compatibility of the Ia membrane molecule (the product of the I gene region of the MHC) in the guinea-pig was a prerequisite for the stimulation of T-lymphocytes by antigen-modified macrophages (i.e. syngeneic macrophages but not allogeneic, act as stimulator cells to T-cells). However, Thomas, et al, (1977) demonstrated that allogeneic antigen-modified macrophages could stimulate T-cells, providing the dual stimulation by the mixed lymphocyte reaction (the recognition of foreign cells in culture) could be inhibited. This was achieved using hybrid strains of guinea-pig or by specific chemical manipulation of the cells. When allogeneic antigen-modified macrophages were used to both activate and stimulate manipulated T-cells (primary and secondary responses), then the secondary response only occurred with allogeneic macrophages and not syngeneic.

It was postulated from these experiments that the 'true' antigen may be a three-way conjugate of the hapten, carrier protein and the Ia region. The Ia region need not be strain specific and thus not a prerequisite for the induction of sensitivity, but it is probably the 'point' of stimulation during the T-cell/macrophage interactions.

On the basis of this role of the Ia region it seems reasonable to assume that other cells which bear Ia markers could also act as stimulator cells. It is from this assumption that the possible role of the Langerhans' cell has developed.

The Langerhans' cell was first described by Paul Langerhans (1898) as a dendritic epidermal cell, but it has received little attention until recently. The cells have been identified by means of both light microscopy (Juhlin and Shelley, 1977) and electron microscopy (Birbeck, et al, 1961). It is interesting to note that most of the agents used to 'stain' Langerhans' cells for light microscopy (e.g. cobalt, gold, nickel, mercury or p-phenylenediamine) are also recognised as haptens in allergic contact dermatitis (Juhlin and Shelley, 1977; Shelley and Juhlin, 1976).

Using these techniques Langerhans' cells can be demonstrated predominately in a basal and supra-basal position in the epidermis of several species including guinea-pig and man.

Several reports have now demonstrated that Langerhans' cells also bear immunological markers similar to other cells of the reticuloendothelial system, notably F_c receptors (binding the F_c portion of IgG), C_3 receptors (binding the C_3 component of complement) and Ia antigens (Stingl, et al, 1977; Rowden, 1977; Rowden, et al, 1977; Klareskog, et al, 1977; Stingl, et al, 1978a; Rowden, 1980).

The apposition and assumed interaction of Langerhans' cells with T-cells has been demonstrated by electron microscopy of epidermis in allergic contact dermatitis responses, but not in irritant reactions. This is considered to be analogous to the macrophage/T-cell interaction in sensitivity responses (Silberberg, 1971; Silberberg, 1972; Silberberg, et al, 1973; Silberberg, et al, 1974; Silberberg, et al, 1976).

Using in vitro cell culture techniques and inbred guinea-pig strains, Stingl, et al, (1978b), were able to demonstrate that syngeneic, but not allogeneic, antigen-pulsed Langerhans' cell-enriched epidermal cell suspensions were capable of eliciting antigen-sensitized T-cell transformation. This capacity was equal in magnitude to that seen with syngeneic antigen-pulsed peritoneal macrophages. These results indicated that Langerhans' cells may have an antigen presenting function analogous to macrophages. However, it is important to note that the methods of preparing the Langerhans' cell-enriched epidermal cell suspensions were based on rosetting with red blood cells through the F_c receptors. This technique would also isolate any other F_c receptor bearing cells including macrophages and these may be responsible for the T-cell stimulation in vitro.

The mounting experimental data on the function and characteristics of the Langerhans' cell (reviewed by Wolff, 1972; Silberberg-Sinakin, et al, 1978) is gradually directing attention

towards the role of a peripheral outpost of the immune response to haptens. It has been hypothesized that, haptens penetrating the stratum corneum conjugate with soluble proteins and are trapped by epidermal macrophages and Langerhans' cells via the Ia regions of the membranes (Silberberg-Sinakin et al, 1978). Antigenic information is then presented to opposing and circulating antigen-inexperienced T-cells. This hypothesis in relation to the early concepts of Landsteiner and co-workers is presented diagrammatically as Figure 3.

It is apparent that the T-cells can be stimulated by the allergen via several mechanisms, all of which are largely undefined (see Figure 4).

1. presentation of haptenic or antigenic information by macrophages;

2. direct stimulation of T-cells by hapten-carrier conjugates;

3. presentation of haptenic or antigenic information by Langerhans' cells.

These mechanisms need not be mutally exclusive and the conjugate of hapten with skin protein may still be the first step (Polak, 1978). However, it appears that antigens are recognised as foreign in the skin. The development of a population of sensitized lymphocytes capable of response

FIGURE 3

The possible role of Langerhans' cell and macrophage in allergic contact dermatitis

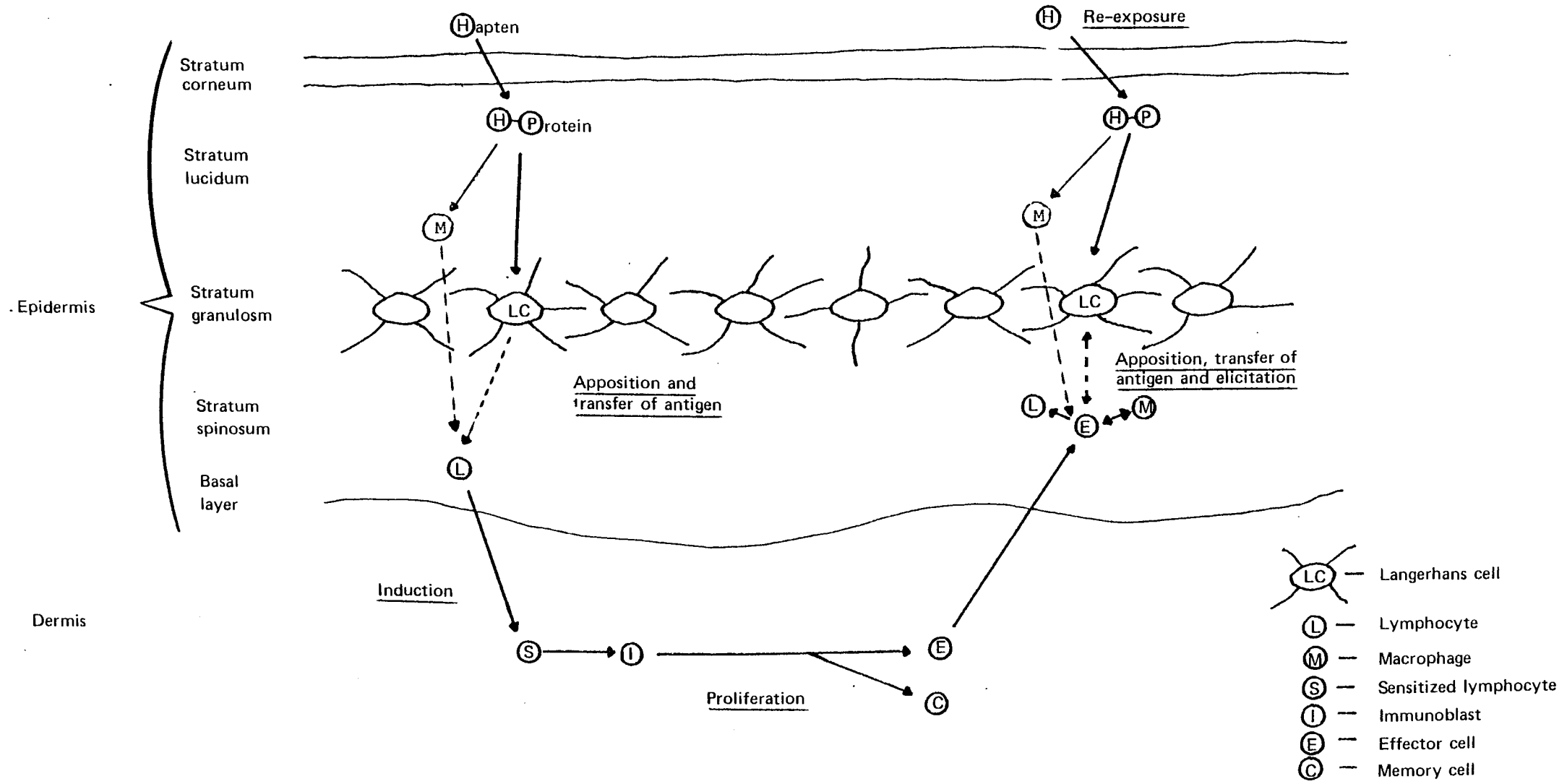
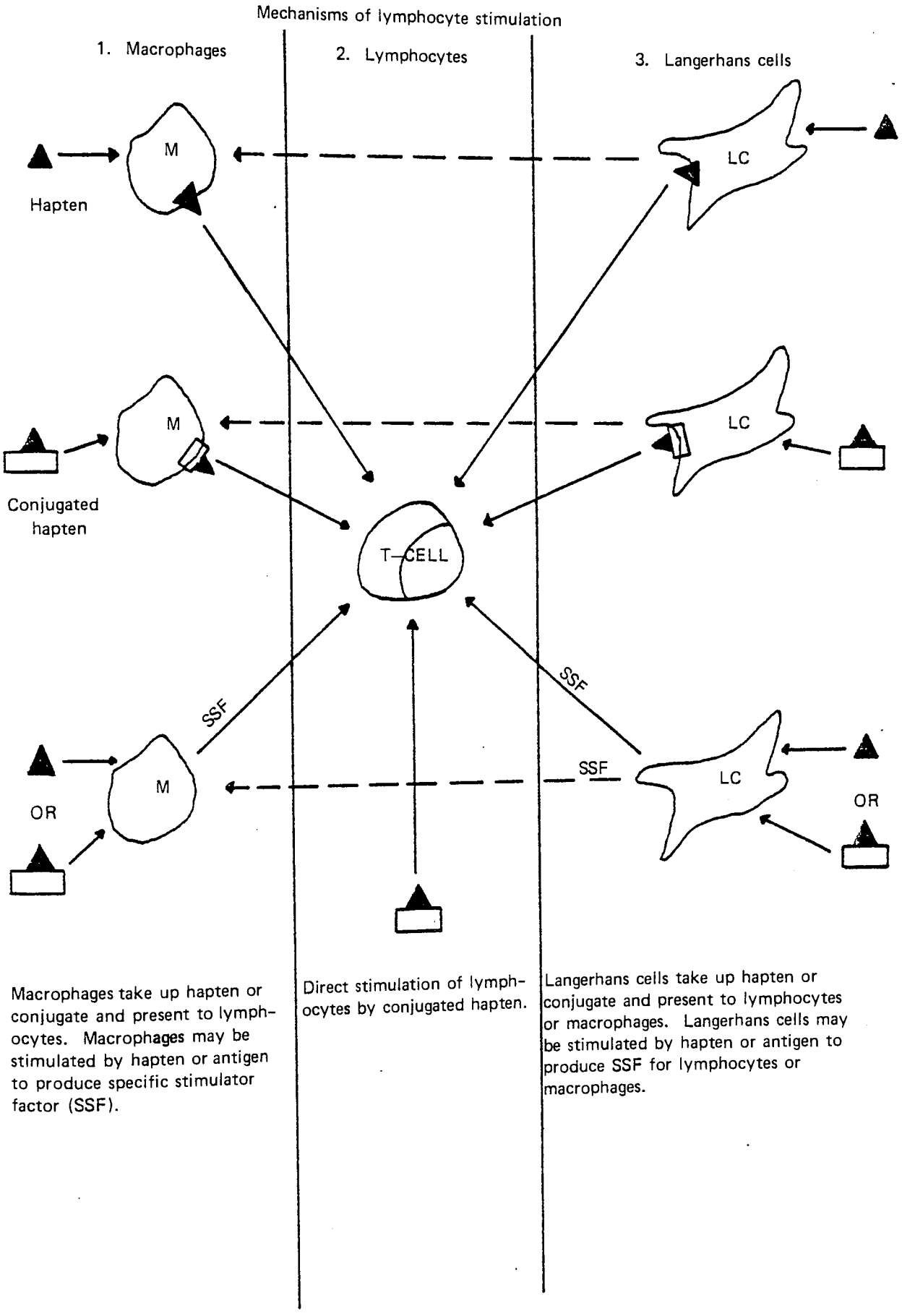


FIGURE 4



(adapted from Polak, 1978)

against the antigen (proliferative phase) occurs in the regional lymph node.

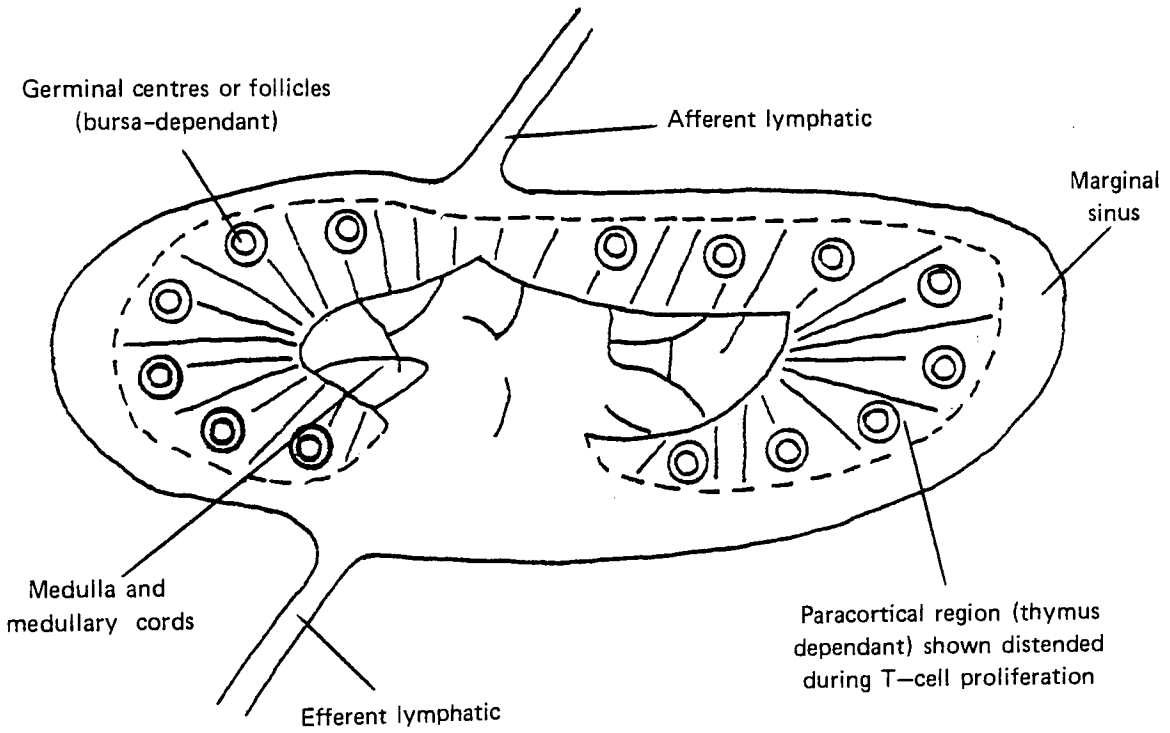
Proliferative Phase

T-cells, specifically triggered during the previously described events of induction, drain from the skin via the lymphatics into the regional lymph node. These cells accumulate in the paracortical area (thymus dependent) where proliferation takes place (Turk and Oort, 1970).

In normal animals the population of lymphocytes in the paracortical region is small and those cells present, are considered to be passing through. Within a few hours after the application of hapten to the skin, the triggered T-cells draining from the skin site accumulate in the paracortical region, which becomes extended and compresses the medulla (Emerson and Thirsh, 1971). The appearance of an active regional lymph node is presented as Figure 5.

FIGURE 5

Diagrammatic representation of an immunologically active regional lymph node



The importance of the regional lymph node in the proliferative phase of the development of contact sensitivity was further demonstrated in the experiments of Frey and Wenk (1957). The regional lymph nodes of guinea-pigs were excised at various times after the skin application of hapten (DNCB). Excision at times up to 48 hours, inhibited the development of the sensitivity. Excision at 3 or 5 days after hapten application inhibited the development in 80 or 50% of animals respectively and a later excision had little influence on the incidence of the sensitivity.

These findings indicate that the proliferative phase of the development of contact sensitivity takes 5-6 days. This time period, necessary for the complete production of a population of sensitized lymphocytes, has since been confirmed by several authors (Turk and Stone, 1963; Bloom and Chase, 1967; Turk and Polak, 1967).

During the proliferative phase, the triggered T-cells are transformed within the lymph node into immunoblasts (large pyroninophilic cells with high protein synthesis) (Oort and Turk, 1965) which themselves give rise to a population of small T-cells (Turk and Oort, 1967). These progeny form a morphologically homogeneous population leaving the lymph nodes via the efferent ducts. Although morphologically homogeneous, the existence of two subpopulations of sensitized T-cells in the cell-mediated immune response has been demonstrated by several authors (Bloom, et al, 1964; Bloom, et al, 1972; Rocklin, et al, 1970; Kano and Bloom, 1973; Polak, 1976).

One of these populations of T-cells, usually termed memory cells, recirculates between the thymus dependent areas of the lymph nodes,

the spleen and the lymphatic vessels, and stores information of the previous contact with the hapten. These T-cells are similar to antigen-inexperienced immunocompetent cells. Upon a new exposure to the same antigen they are able to proliferate and differentiate into a new progeny of sensitized T-cells via the immunoblastic stage. The memory cells do not themselves participate in the elicitation phase.

However, if in this first response, the dermal exposure to hapten is sufficiently prolonged for circulating memory cells to contact the antigen, then they can be triggered at this stage. The resultant response by triggered memory cells may be termed a "booster effect" (Davies, et al, 1968).

The second population of sensitized lymphocytes is the effector T-cells. These cells possess the ability to react to a new exposure to the same antigen by release of soluble agents (lymphokines) but are incapable of further proliferation and differentiation. These cells participate directly in the elicitation phase. The lymphokines are capable of inducing inflammatory skin reactions at the site of hapten application.

In the skin island experiments of Frey and Wenk (1957) previously mentioned, the authors demonstrated that contact sensitivity could only be induced when the lymphatic connection was maintained from the skin island. They also demonstrated that once the sensitivity was induced dermal reactions could be elicited without intact lymphatics but with unimpaired blood vessels.

Thus the afferent arc of the immune response is dependent on the lymphatic system and the efferent arc on the blood vessels.

Elicitation Phase

The elicitation phase or secondary response occurs on re-exposure of the skin to the sensitizer. The hapten penetrates the skin and conjugates with skin proteins as previously described under Absorption and Conjugation of the Hapten. It is reasonable to assume that the same set of antigenic conjugates are formed.

The specific effector T-cells, produced as described above and circulating in the blood and skin milieu recognise the antigenic information and are stimulated by this contact to release lymphokines. The result is an inflammatory reaction at the site of exposure which under experimental circumstances peaks at 24-48 hours after re-exposure and ameliorates (under experimental conditions) over the next few days.

The following events at the site of hapten application may be observed in the course of development of the skin reaction:-

1. attraction of mononuclear cells;

2. increased vascular permeability;

3. retention and proliferation of the cells in the infiltrate;

4. damage to tissue.

The histological appearance of the dermal reactions has been studied by numerous authors. Polak and Mom (1949) described the microscopic events following patch testing in humans sensitized to DNCB or p-phenylenediamine. At 12 hours post-challenge marked primary vesiculation and the beginning of spongiosis were observed in the epidermis, and inflammation was evident in the dermis. At 24 to 48 hours post-challenge there was a spread of the spongiosis, intercellular bridges were broken and the inflammation in the dermis was at a peak.

This general picture was supported by Charpy, et al (1954) and Meischer (1961). Charpy also observed histological changes in the dermis within the first hour, and Meischer reported epidermal infiltration by lymphoid cells at 6 hours post-challenge.

At the start of the dermal reactions the inflammatory cells are arranged perivascularly. These cells are essentially small lymphocytes. After 24 hours basophils appear in the epidermis in small numbers, but increase as the reactions proceed. A small proportion of eosinophils may also be observed after 3 days (Dvorak, et al, 1974). The inflammatory cell infiltrate is essentially of a mixed nature but consists predominantly of lymphocytes with some macrophages.

Silberberg (1971) used transmission electron microscopy techniques to study the inflammatory response. Monocytes resembling lymphocytes were observed in close apposition to epidermal Langerhans cells within 4 hours of patch testing with mercuric chloride.

It may be reasonable to expect that the main population of monocytic cells in the inflammatory response (i.e. lymphocytes) are predominantly those produced as a result of the induction exposure to the hapten. However, work by Najarian and Feldman (1961 and 1963) by Turk (1962) and by Turk and Oort (1963) demonstrated that this assumption is incorrect. By transferring ^{3}H-thymidine labelled T-cells from hypersensitive donors to normal recipients (passive transfer of sensitivity) it was shown that the majority of infiltrating cells of the elicitation phase were not the ^{3}H-labelled sensitized lymphocytes but non-sensitized mononuclear cells. The number of labelled cells (sensitized T-cells) formed only 10% of the infiltrating population. Similar work by Turk and Polak (1967) suggested an even lower figure of 0.01% and studies by Bloom, et al (1970) concluded 0.1 to 1.6% of infiltrating cells were specifically sensitized.

It seems improbable, therefore, that this small population of sensitized T-cells would be able to activate the large total number of lymphoid cells participating in the inflammatory response by direct cell to cell contact alone. Soluble agents released by the effector cells were considered to be the mediators, which were termed lymphokines (Dumonde, et al, 1969).

Soluble Mediators of the Inflammatory Response

Lymphokines were first defined as cell-free soluble factors that are produced by sensitized lymphocytes upon stimulation with a specific antigen but that show no immune specificity and are therefore different from antibodies (Dumonde, et al, 1969).

The secretion of lymphokines commences within hours following antigenic stimulation, continues for several days afterwards and is independent of DNA synthesis and cell proliferation (Bloom, et al, 1974). The cellular source of the majority of lymphokine activities is still unknown. Although sensitized T-cells have been shown to produce some lymphokines, it is not clear that these cells are the only source and production by other cells (B-cells, macrophages) must be considered as secondary sources.

Similarly the lymphokines have only been classified in terms of their biological activities and none of the factors have been definitively chemically identified or purified. The lymphokines frequently described and most documented are presented in Table 4, together with their in vitro activities. It should be considered that the different factors are classified by their biological activities and may merely reflect the different assay systems. These factors may, in fact, be one molecular entity or conversely the activities attributed to any one factor may be due to more than one type of molecule.

TABLE 4

Lymphokines - Products of Activated Lymphocytes

Function and Name	Synonym	In Vitro Activity	Comment
1. Affecting Macrophages			
migration inhibition factor	MIF	inhibits migration of normal macrophages agglutinates macrophages in suspension increases adherence and surface area of macrophages	released by effector T-cells M.Wt. ~ 45,000*
macrophage activating factor	MAF	increases glucose oxidation and phagocytosis	has not been separated from MIF
chemotactic factor	CHF or CFM	macrophage "attractant" - causes macrophages to migrate along gradient through micropore filter	has not been separated electrophoretically from MIF M.Wt. 45,000/12,000*
2. Affecting Lymphocytes			
mitogenic factor or lymphocyte transformation factor	MF or LTF	induces blast cell formation and ^{3}H-thymidine uptake by normal lymphocytes	M.Wt. 20,000/45,000*
chemotatic factor	CFL	nonsensitized lymphocyte "attractant"	possibly distinct from CHF
3. Affecting other cells			
lymphotoxin	LT	cytotoxic to target cells-resulting in cell lysis and general tissue damage	action may be direct or via macrophages M.Wt. 45,000*
4. Other factors			
skin reactive factor	SKF	causes increased vascular permeability in skin site	possibly an assay phenomenon due to several factors

(Bloom et al 1974 and Valdimarsson 1976)

* as released by guinea pigs cells

Aims and Objectives

In the preceeding section a review of the immunological processes of allergic contact dermatitis has been presented. This may be briefly summarized as follows:

Allergic contact dermatitis is a delayed-type, cell-mediated immune response comprising several different chronological events. Initially sensitizing materials (haptens) must be absorbed into the skin and conjugate with skin proteins or cells to form a complete antigen or set of antigens. The antigen may be taken up by macrophages or Langerhans' cells and a stimulus presented to circulating T-lymphocytes. T-cells may also be stimulated directly by the antigens. Specifically triggered T-cells undergo proliferation in the paracortical areas of regional lymph nodes to form populations of at least effector cells and memory cells. On re-exposure of the hapten, the same processes of absorption and conjugation may occur. Circulating effector T-cells recognise antigenic information and are stimulated to release lymphokines. The result is an inflammatory reaction at the site of exposure.

The review attempts to define the basic concepts and to highlight the mechanisms for which little data is available or inconclusive. This, by no means, describes all the immunological processes and mechanisms involved, since a complete account is outside the scope of the project, which is to examine mechanisms of induction. However, it is apparent that the areas worthy of further investigation and elucidation are those of hapten absorption, conjugation and recognition. There is particularly little knowledge of the nature of hapten conjugation and of which conjugates act as antigens.

Nickel is ubiquitous in the environment and presents considerable potential exposure to the general public. Allergic contact dermatitis to nickel has been recognised for many years but still remains a medical problem in everyday life. Nickel, a prime example of a hapten in allergic contact dermatitis, is therefore chosen for investigation of hapten absorption, conjugation and antigenicity.

The proposed experimental protocol is as follows:-

ASPECT	MODEL/TECHNIQUES USED	OBJECTIVES
induction of sensitivity	*in vivo* animal models	to select an animal model for further studies, to select and develop methods to induce and demonstrate Ni sensitivity
absorption and accumulation	radiotracer techniques including microautoradiography	to qualitatively and quantitatively assess hapten absorption and accumulation
conjugation	zonal centrifugation and gel filtration chromatography	to quantitatively assess and isolate hapten conjugates
antigenicity	*in vitro* lymphocyte transformation	to identify which conjugates represent antigenic stimuli

SECTION III - EXPERIMENTAL SENSITIZATION WITH NICKEL

OBJECTIVES

The objective of this section of the project is to select an animal model suitable for application to the investigation of the molecular mechanisms of allergic contact dermatitis to nickel and to select and develop methods to induce and demonstrate the hypersensitivity in the selected species.

INTRODUCTION

Selection of Species

The criteria required of any one animal species in the investigation of human allergic contact dermatitis are:-

1. ability for the hypersensitivity to be experimentally induced and elicited;
2. of similar immunology to humans;
3. of similar skin structure and reactivity to humans;
4. ability for the dermatitis to be clinically manifest;
5. inexpensive, routinely available and easy to house.

The guinea-pig would appear to be the species of choice according to the above criteria. The ability of the guinea-pig to develop hypersensitivity reactions under experimental conditions is well documented. In fact, many of the concepts of allergic

contact dermatitis were developed using the guinea-pig as a model.

The rabbit rarely shows contact sensitivity and rats and mice can be sensitized only with some difficulty (Davies, 1970).

The ability of the guinea-pig to respond to contact allergens is not very different from the human but because of cutaneous structural differences the dermatitis is not as easily demonstrated (Magnusson and Kligman, 1970). It is believed that this limitation can be overcome by experimental manoeuvres designed to intensify and potentiate the sensitivity.

Experimental Induction of Hypersensitivity to Nickel in Man

The data available of the experimental sensitization of man are very limited. The reason for this absence may be that it is considered ethically unacceptable to purposefully induce a disease state in the human. The majority of reports of human testing are primarily concerned with the diagnosis of the condition and therefore bear little relevance to the investigation of the underlying mechanisms of induction.

The first reports of Burckhardt (1935) and Haxthausen (1936) suggested that allergic contact sensitization to nickel can be induced easily in humans using a 25% $NiSO_4$ solution. However, these results are not generally acceptable, since a 10% $NiSO_4$ solution capable of producing primary irritation was used for the elicitation (challenge application), their subjects were not pre-screened for

prior sensitivity and the authors did not use repeated tests at lower concentrations to rule out spurious responses.

Vandenberg and Epstein (1963) used a "triple-freeze" technique to induce sensitivity in American male prisoner "volunteers". This technique consisted of repeated freezing of an area of skin, application of 25% $NiCl_2$ and occlusion for 48 hours. Their results indicated that 9% of subjects were sensitive following the first period of induction, which increased to 26% following a second induction period after 4 months. This hypersensitivity appeared to persist, since patch testing 6 months later still produced strong reactions, although the subjects did not appear to be clinically reactive to their normal nickel containing apparel (watch straps, identity badges, etc).

Vandenberg and Epstein concluded that experimental sensitization to nickel was possible, but under carefully controlled conditions. Nickel is a weak allergen but prolonged exposure raises the frequency of sensitization.

Magnusson and Kligman (1970) reported comparative sensitization rates to nickel using the Landsteiner-Draize (Draize, et al, 1944) and Human Maximization (Kligman, 1966) techniques. Positive reactions could not be demonstrated by the former method but by the latter method, using much higher induction rates and concentrations, positive reactions were achieved in 48% of human volunteers.

Experimental Induction of Hypersensitivity to Nickel in Guinea-Pigs

The experimental data on the induction of the hypersensitivity in guinea-pigs are slightly greater than those in humans but are more conflicting.

Data supporting the induction of allergic nickel dermatitis in guinea-pigs have been published by some investigators (Stewart and Cromia, 1934; Nilzen and Wikström, 1955; Magnusson and Kligman, 1970; and Wahlberg, 1976) but their results have not been confirmed by other workers (Hunziker, 1960; Samitz and Pomerantz, 1958).

Nilzen and Wikström (1955) reported a method of sensitizing guinea-pigs to nickel sulphate by repeated topical painting of an aqueous mixture of 4% nickel sulphate and 1% of a surfactant (sodium lauryl sulphate). The authors demonstrated that sensitivity was induced with this mixture, but were unable to demonstrate induction by nickel sulphate alone.

Using the same technique Samitz and Pomerantz (1958) produced dermal reactions considered to be irritant in nature. No sensitivity to nickel sulphate was demonstrable.

The guinea-pig maximization test, developed by Magnusson and Kligman and consisting of intradermal injection and topical application of the allergen was used by the investigators to induce sensitivity to nickel sulphate. Positive results were reported in 11 of 20 animals tested (55%) (Magnusson and Kligman, 1970). The Landsteiner-Draize test was also used in comparison but demonstrable sensitivity could not be induced in guinea-pigs by this method.

The results of the guinea-pig Maximization test were later confirmed by Wahlberg (1976) who reported successful induction of sensitivity by this method and by a method consisting of intramuscular injection, intradermal injection and topical painting. This latter method was developed by Polak and Turk (1968).

In a study comparing two test methods (Maurer, et al, 1979), successful induction of nickel sensitivity in the guinea-pig was reported in 100% of the test animals by the Optimization technique (i.d. injection only) and in 35% of animals by the Maximization technique.

More recently, the abilities of three test methods to detect contact allergens, were compared using 19 known human sensitizers including nickel (Goodwin, et al, 1981). Positive responses consistent with sensitivity to nickel were seen with the Maximization test and a newly developed method, termed the single injection adjuvant test. However, the incidences were low with both methods (10-20% of test groups). No positive reactions were observed with the modified Draize method.

The above limited data appear to be weighed in favour of the ability of the guinea-pig to be sensitized to nickel. However, induction appears to be difficult and a technique for the consistent induction and demonstration of the sensitivity may require further development.

Selection of Test Methods

There are many techniques and variations of techniques currently available for the detection and identification of contact allergens, which are employed in many research establishments. The basic test methods may be divided into three groups according to the route of administration.

1. topical application methods - Büehler test (Büehler, 1964) and open epicutaneous test (Klecak, et al, 1977);

2. intradermal injection methods - Draize test (Draize, 1959), Freunds complete adjuvant test (Klecak, et al, 1977) and the Optimization test (Maurer, et al, 1978);

3. combined intradermal injection and topical application methods - Split adjuvant method (Maguire and Chase, 1967) and Guinea-pig maximization test (Magnusson and Kligman, 1970).

Of these test methods only the guinea-pig Maximization technique and Optimization tests have shown with any certainty that allergic contact dermatitis to nickel can be induced (Magnusson and Kligman, 1970; Wahlberg, 1976; Maurer, et al, 1979).

In test method comparison studies using strong allergens such as DNCB or chlormethylimidazoline hydrochloride (CMI) the maximization test, split adjuvant method and optimization test have been shown to produce a greater frequency of sensitization than topical methods

(Fahr, et al, 1976 and Maurer, et al, 1978). The topical application test methods do, however, appear useful in the detection and identification of strong allergens only, but since nickel may be considered to be a weak allergen, these topical techniques are not considered to be applicable.

Two techniques were selected for further investigation, the Maximization test as an example of combined intradermal and topical administration and the Optimization test as an example of solely intradermal administration. Neither of these tests are considered to be entirely relevant since the routes of induction do not mimic human exposure, particularly that of the Optimization test. However, the objective is to demonstrate that the guinea-pig can be sensitized to nickel (i.e. it has the immunological capability to recognise nickel as a hapten/allergen) and is, therefore, a suitable animal model for use in further investigations.

Since the studies reported in this thesis were undertaken, two additional techniques for the identification of contact allergens have been published (Goodwin, et al, 1981; Sato, et al, 1981). The authors of each report considered the newly developed techniques to be as sensitive in detecting contact allergens as the Maximization technique. Thus, both techniques are worthy of future examination but are not considered to divert the objectives of the experiments performed and reported in this part of the thesis.

MATERIALS AND METHODS

Nickel Containing Test Compound

Nickel sulphate heptahydrate ($NiSO_4.7H_2O$) was obtained from Fisons Scientific Apparatus, Loughborough, Leicestershire, England. The analytical grade preparation was used which was stated to be 99% pure on minimal assay. Other metal impurities were as follows:

Cobalt	(Co)	<0.0005%
Copper	(Cu)	<0.001 %
Iron	(Fe)	<0.002 %
Lead	(Pb)	<0.001 %
Zinc	(Zn)	<0.0025%

Aqueous w/v solutions of $NiSO_4$ were prepared freshly at the time of dosing.

Animals

The guinea-pigs used in this study were random-bred albino animals of the Dunkin Hartley strain. Animals of both sexes at approximately 8 to 10 weeks of age and weighing 300 to 400 g were obtained from LAC accredited commercial breeding stations.

All animals were housed in metal cages with wire mesh floors and had free access to water and a standard commercial pelleted guinea-pig diet enriched with Vitamin C (Spratts' guinea-pig diet No. 21067). Autoclaved hay or fresh cabbage were supplied once weekly as dietary supplement.

Animal room temperature was maintained at approximately 21°C and artificial lighting was controlled on a 12 hour light/dark cycle.

All animals were examined prior to testing and only guinea-pigs of good health and condition were used.

Preliminary Test Methods

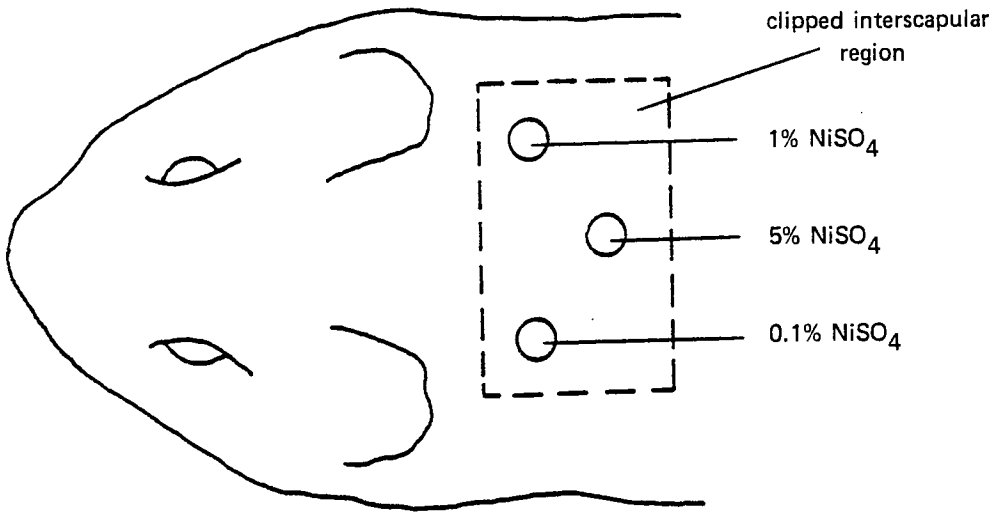
The clinical appearance of primary irritant dermatitis and allergic contact dermatitis can be very similar in humans and particularly in guinea-pigs. In order to diagnose allergic reactions it is, therefore, of prime importance to define those dosages which cause irritant reactions. For example, if the dermatitis is elicited in the skin of a hypersensitive subject using a concentration of allergen which is known to be non-irritant, then it is reasonable to assume that the reactions are immunologically based and not of a direct toxic nature.

For this reason preliminary investigations were undertaken in an attempt to define the concentrations of aqueous nickel sulphate which can elicit irritant reactions following intradermal injection and topical application to guinea-pigs.

A total of 10 healthy guinea-pigs each received intradermal injections of three aqueous concentrations of $NiSO_4$ (5%, 1% and 0.1% w/v) in the interscapular region which was clipped free of hair. The dosage volume was 0.1 ml/site. The position of the injection sites is presented in Figure 6.

FIGURE 6

Preliminary tests – position of intradermal injections



24 hours after the injections, each site was examined for signs of irritation. Reactions were assessed in terms of erythema (0-4 scale), oedema (0-4 scale) and diameter. Any necrosis or ulceration was also noted. The scoring system of erythema and oedema was as follows:-

Erythema formation:

No erythema	0
Slight erythema (barely perceptible redness)	1
Well-defined erythema (area of redness easily discernable)	2
Moderate erythema (intense redness)	3
Severe erythema (beet redness)	4

Oedema formation:

No oedema	0
Slight oedema	1
Well-defined oedema (area well-defined by definite raising)	2
Moderate oedema (raised approximately 1 millimetre)	3
Severe oedema (raised more than 1 millimetre and extending beyond the area of exposure)	4

A second series of 10 guinea-pigs each received topical applications of four concentrations of nickel sulphate (10%, 5%, 1% and 0.1% w/v) on the flank regions which were clipped free of hair. Each concentration was applied to a 2 x 2 cm square piece of surgical lint until saturated (volume $\backsim$ 0.4 ml/site) which was then placed on a section of occlusive

adhesive tape (Blenderm, 3M Company, Minnesota, USA) and which was, in turn, placed on a length of adhesive bandage (Elastoplast, Smith and Nephew Co. Ltd., Hull, England). The whole bandage was tightly wrapped around the trunk of one animal in such a manner that two sites were applied to each clipped flank. The position of the topical sites are presented in Figure 7.

The dressings were removed after an exposure period of 24 hours and the test sites were examined for signs of irritation at 1 hour and 24 hours after removal. Any reactions were assessed in terms of erythema (0-4 scale) and oedema (0-4 scale) as shown above.

The concentrations which produced minimal dermal irritation by intradermal injection and by topical application were selected for the induction phases of the main studies. The concentrations which did not produce irritation by topical application were selected for the elicitation (challenge) phases.

Main Study Procedures

1. Optimization Test (Maurer, et al, 1978)

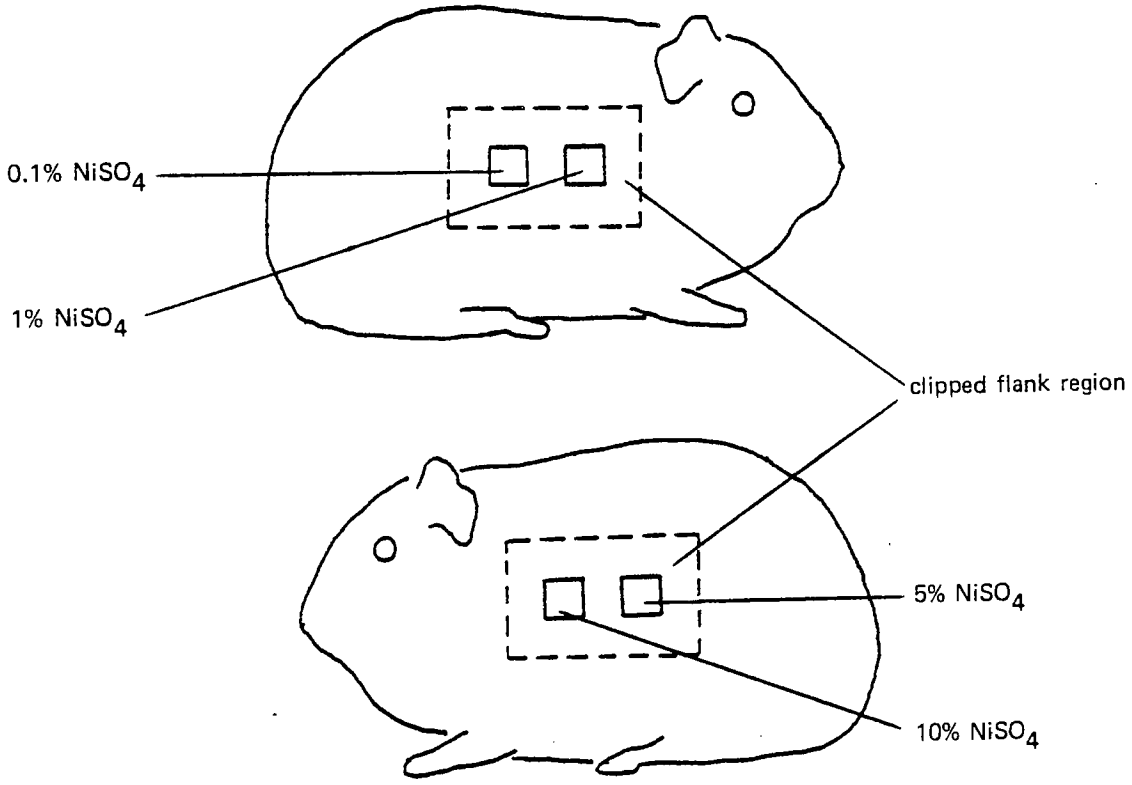
The flanks, mid-dorsal and interscapular regions of a group of guinea-pigs were clipped free of hair prior to injection.

Induction:

A 0.1% w/v aqueous solution of nickel sulphate was injected intradermally into the flank and mid-dorsal regions of each animal on day 1. The injections were repeated in the mid-dorsal region

FIGURE 7

Preliminary tests – position of topical applications



only on days 3 and 5. The dosage volume was 0.1 ml per injection site.

On days 8, 10, 12, 15, 17 and 19 each animal received an intradermal injection in the interscapular region of 0.1% nickel sulphate in 50% Freunds complete adjuvant containing killed mycobacteria (Difco, Michigan, USA). The dosage volume was 0.1 ml/ site again.

Challenge:

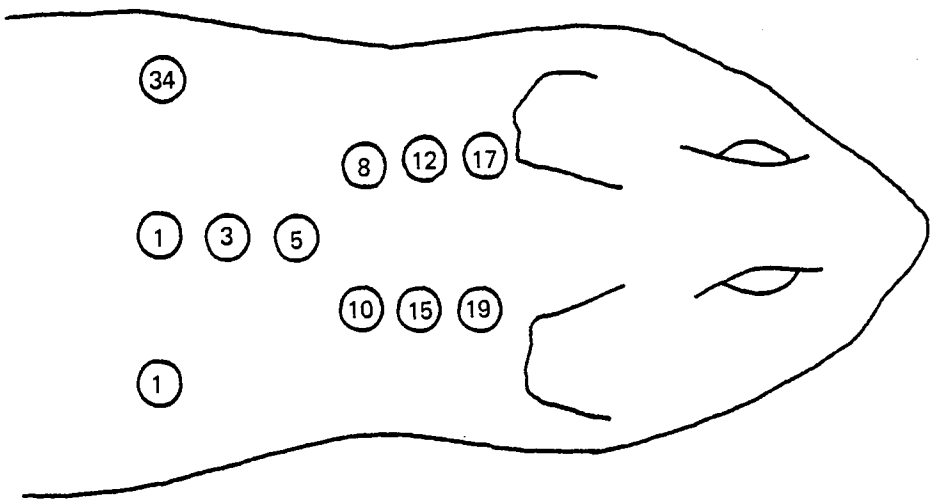
Fourteen days after the final induction injection 0.1 ml of 0.1% aqueous nickel sulphate was injected intradermally into the previously unused flank. This single injection constituted the challenge exposure.

The sequence and positions of intradermal injections are presented in Figure 8.

Twenty-four hours after each induction injection on days 1, 3 and 5 and after the challenge injection, any dermal reactions were recorded in terms of diameter (mm) and skin-fold thickness (mm). The value "reaction volume", as described by Maurer, was obtained by the multiplication of the diameter and thickness for each animal. The mean "reaction volume" over the first three induction days assessed for each animal is considered to represent the skin irritation "threshold" for that guinea-pig. Any challenge reaction greater than the threshold is considered to be indicative of an allergic reaction and the animal termed positive.

FIGURE 8

Optimization test – sequence and position of intradermal injections



DAY

1 – 0.1% aq. $NiSO_4$

3 and 5 – 0.1% aq. $NiSO_4$

8, 10, 12, 15, 17 and 19 – 0.1% $NiSO_4$ in Freunds complete adjuvant (50%)

34 – Challenge injection 0.1% aq. $NiSO_4$

Where the results of the intradermal challenge were negative or inconclusive, a second challenge was performed 14 days later, which consisted of topical application only. Approximately 0.2 ml of 5% aqueous nickel sulphate was applied to a patch of filter paper (Whatman 3M, 2 x 2 cms) which was adhered to a bandage of occlusive tape and Elastoplast as previously described in the Preliminary Test Methods. The patch was applied to an unused area of the clipped flank and the bandage wrapped around the trunk of the animal for a period of 24 hours. After a further 24 hours from removal of the patch (i.e. 48 hours from application) dermal reactions at the site of application were assessed in terms of erythema and oedema according to the scales described under Preliminary Test Methods.

According to Maurer, the presence of any discernible reaction should be considered to be an indication of an allergic response and the animal termed positive.

2. Maximization Test (Magnusson and Kligman, 1970)

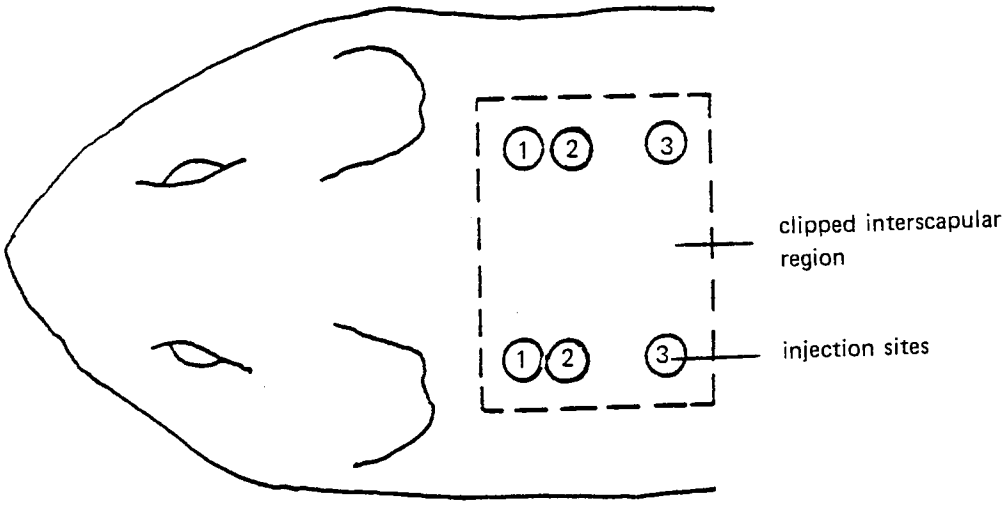
The induction procedures consisted of both intradermal injection and topical application as follows:

Induction:

Three pairs of intradermal injections (0.05 ml/site) were made within the clipped interscapular region of each guinea-pig (Figure 9).

FIGURE 9

Maximization test – position of intradermal induction injections



Injections:

1. Freunds complete adjuvant/water (50 : 50)
2. 0.1% aq. $NiSO_4$
3. Freunds complete adjuvant/0.2% aq. $NiSO_4$ (50 : 50)

(a) 2 injections of Freunds complete adjuvant/ water emulsion (50:50);

(b) 2 injections of 0.1% w/v aqueous nickel sulphate;

(c) 2 injections of Freunds complete adjuvant/0.2% aqueous nickel sulphate emulsion (50:50). The final concentration of nickel sulphate was, therefore, 0.1% w/v.

One week after the intradermal injections (day 8), a topical induction procedure was performed. Approximately 0.4 ml of 10% aqueous nickel sulphate was applied to a patch of filter paper (Whatman, 3M, 3 x 6 cms). The patch, adhered to occlusive bandage backed with Elastoplast was applied over the interscapular region and the bandage wrapped around the trunk of the animal. The exposure period was 48 hours, after which time the bandage was removed.

Challenge:

Fourteen days after the topical induction application (day 22), the guinea-pigs were challenged on the previously clipped but untreated skin of the flank. Approximately 0.2 ml of 5% aqueous nickel sulphate was applied to a patch of filter paper (Whatman, 3M, 2 x 2 cms). The patch was applied to the skin of the flank by means of a bandage similar to that described above. The bandage was removed after 24 hours and dermal reactions assessed in terms of erythema and oedema at 1, 24, 48 and 72 hours after removal of the dressings (25, 48, 72 and 96

hours after application of nickel sulphate). Reactions were assessed according to the numerical scales described under Preliminary Test Methods.

Dermal reactions of a time course characteristic of sensitivity (increasing to a peak at 48-72 hours after application) and greater than those expected from preliminary irritancy tests, were considered to be indicative of an allergic response.

Where the results of the challenge procedure were inconclusive a second challenge was performed one week after the first using the alternate unused flank.

A proportion of the animals were treated during the induction phase with the vehicles without nickel sulphate but were challenged as the test animals. These guinea-pigs served as control animals to ensure that the treatment procedure alone did not produce a non-specific dermal sensitivity.

3. Modified Maximization Test

The results obtained by the above two methods will be described in detail in later sections. However, it is necessary to state at this point that conclusive and reproducible positive sensitivity could not be induced by the standard methods. Evidence of sensitivity was produced from the Maximization test but results of the Optimization test were generally inconclusive. The former method was selected for further development, in an attempt to reproducibly induce a reasonably high frequency of sensitivity in any one series of guinea-pigs.

It was considered that the irreproducibility may be due to the weak allergenicity of nickel, previously indicated from human experimentation by Vandenberg and Epstein (1963). The following modifications were made to the Maximization test in order to increase the exposure of guinea-pigs to nickel without producing increased local or systemic toxicity.

1. The volume of intradermal injection was increased from 0.05 ml to 0.1 ml/site, thus doubling the intradermal exposure.

2. A second series of intradermal injections was performed one week after the first standard series, thus doubling the intradermal exposure again.

3. In the standard test method filter paper was used for all topical applications. It was noted that considerable amounts of the colour of nickel sulphate solutions were retained in the patches, possibly indicating that sufficient allergen was not available to the skin site. Filter paper was replaced by surgical lint, which is of a more open matrix, in an attempt to make the nickel sulphate more available to the skin site. The subsequent increase in exposure of guinea-pigs was not assessed but the applied volume was doubled.

The final test procedure was briefly as follows:-

Day 1 - Three pairs of intradermal injections into the clipped interscapular region (0.1 ml/site).

Day 8 - Repeat intradermal injections (0.1 ml/site) (Figure 10).

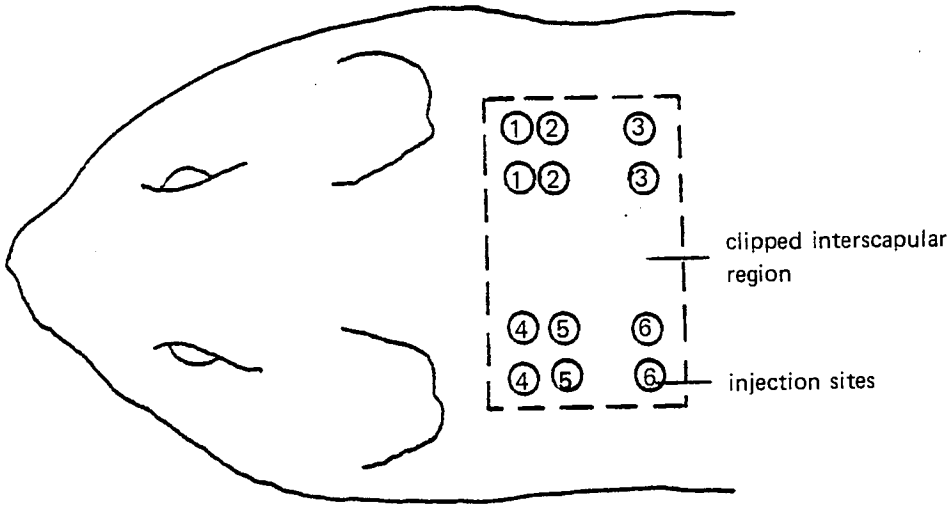
Day 15 - Topical applications of approximately 0.8 ml of 10% aqueous nickel sulphate on a surgical lint patch (3 x 6 cms) over the interscapular region. Occlusion for 48 hours.

Day 29 - Topical challenge application of approximately 0.4 ml of 5% aqueous nickel sulphate on a surgical lint patch (2 x 2 cms) on the unused flank. Occlusion for 24 hours.

The method and timings of assessment of dermal reactions and evaluation of results were as previously described in the standard Maximization test.

FIGURE 10

Modified maximization test – position of intradermal induction injections



Injections:

1 and 4	Freunds complete adjuvant/water (50 : 50)
2 and 5	0.1% aq. $NiSO_4$
3 and 6	Freunds complete adjuvant/0.2% aq. $NiSO_4$ (50 : 50)

RESULTS

Preliminary Tests

The intradermal and topical irritancy of several concentrations of aqueous nickel sulphate were assessed in a series of 20 guinea-pigs. The individual responses are presented in Tables 5 and 6.

The intradermal injection of 5% aqueous nickel sulphate produced moderate to severe reactions at the site of injection. Necrosis and/or ulceration was noted in 5 animals. The mean diameter of the response was 12 mm. Injection of a 1% solution produced slight to well-defined reactions of 6 mm mean diameter.

Only slight erythema was observed at the sites of injection of a 0.1% solution in 3/10 animals. No response was noted in the remaining seven animals receiving intradermal injections.

In the preliminary tests for irritancy by topical application reactions were assessed 1 hour and 24 hours following a period of 24 hours occlusion. Well-defined dermal reactions were generally observed at the 1 hour reading following application of a 10% solution of nickel sulphate. These reactions decreased to slight to well-defined at the 24 hours reading.

Slight dermal reactions were observed in only 2 animals (Nos. 14 and 17) at the 1 hour reading following exposure to a 5% solution. Slight erythema continued in guinea-pig No. 17 to the 24 hour reading.

TABLE 5

Preliminary Tests - Irritancy of Nickel Sulphate by Intradermal Injection

Animal No.	Reaction	Concentration			Comment
		5%	1%	0.1%	
1	E O D	4 3 10	2 1 5	0 0 0	
2	E O D	3 3 12	1 1 8	0 0 0	
3	E O D	4 4 15	2 2 10	1 0 2	Necrosis at 5%
4	E O D	2 3 9	1 1 4	0 0 0	
5	E O D	4 3 15	1 1 5	0 0 0	Slight ulceration at 5%
6	E O D	4 4 15	2 1 6	0 0 0	Necrosis at 5%
7	E O D	4 4 12	2 1 6	1 0 3	Necrosis and slight ulceration at 5%
8	E O D	3 3 10	0 0 0	1 0 3	
9	E O D	4 3 10	1 1 8	0 0 0	
10	E O D	4 4 13	1 0 5	0 0 0	Slight necrosis at 5%
Mean Response	E O D	3.6 3.4 12	1.3 0.9 6	0.3 0 1	

E - erythema
O - oedema
D - diameter (mm)

TABLE 6

Preliminary Tests - Irritancy of Nickel Sulphate by Topical Application and Occlusion

Animal No.	Reaction	Time of observation							
		1 hour				24 hours			
		CONCENTRATION				CONCENTRATION			
		10%	5%	1%	0.1%	10%	5%	1%	0.1%
11	E	2	0	0	0	2	0	0	0
	O	2	0	0	0	1	0	0	0
12	E	2	0	0	0	1	0	0	0
	O	2	0	0	0	1	0	0	0
13	E	1	0	0	0	1	0	0	0
	O	2	0	0	0	0	0	0	0
14	E	1	1	0	0	0	0	0	0
	O	1	0	0	0	0	0	0	0
15	E	2	0	0	0	1	0	0	0
	O	1	0	0	0	0	0	0	0
16	E	2	0	0	0	1	0	0	0
	O	2	0	0	0	1	0	0	0
17	E	3	1	0	0	2	1	0	0
	O	2	1	0	0	0	0	0	0
18	E	2	0	0	0	0	0	0	0
	O	1	0	0	0	0	0	0	0
19	E	2	0	0	0	1	0	0	0
	O	2	0	0	0	0	0	0	0
20	E	1	0	0	0	0	0	0	0
	O	1	0	0	0	0	0	0	0
Mean Response	E	1.8	0.2	0	0	0.9	0.1	0	0
	O	1.6	0.1	0	0	0.3	0	0	0

E - erythema
O - oedema

There were no dermal reactions at the 1% or 0.1% levels.

From these results it is apparent that 10% aqueous nickel sulphate is irritant by topical application and occlusion. Concentrations of 5% and 1% are irritant by intradermal injection.

The following concentrations were therefore selected for use in the main studies:-

1. for intradermal injection - 0.1% aqueous $NiSO_4$

2. for topical induction - 10%

3. for topical challenge - 5% and below

10% aqueous nickel sulphate was selected for topical induction in the Maximization test, since the production of some irritation is believed to potentiate the rate of sensitization (Magnusson and Kligman, 1970). The increased cellular infiltration at the site of irritation, allowing greater contact of lymphocytes with the allergen, may explain this potentiation.

Optimization Test

The results of this main study are presented in Table 7.

Using the criteria for assessing positive or negative reactions as described by Maurer, et al (1978), it would appear that evidence of sensitivity was produced in four of the ten animals at the first challenge stage (intradermal injection challenge).

TABLE 7

Optimization Test - Dermal Scores for Individual Guinea Pigs

Animal No.	Induction Day				First Challenge (intradermal)		Second Challenge (topical)			Overall result
	Reaction	1	3	5	24 Hrs. AD	Result	Reaction	48 Hrs. AD	Result	
1	D	3	2	5	5	+	E	0	-	±
	T	1	1	2	2		O	0		
	RV	3	2	10	10					
	TV	-	-	5						
2	D	2	2	3	0	—	E	2	+	±
	T	0.5	1	1	0.5		O	1		
	RV	1	2	3	0					
	TV	-	-	2						
3	D	1	2	1	2	+	E	2	+	+
	T	1	2	2	2		O	1		
	RV	1	4	2	4					
	TV	-	-	2.3						
4	D	2	2	1	3	+	E	1	+	+
	T	1	1	1	2		O	1		
	RV	2	2	1	6					
	TV	-	-	1.7						
5	D	3	4	3	5	-	E	0	-	-
	T	3	3	3	2		O	0		
	RV	9	12	9	10					
	TV	-	-	10						
6	D	2	2	2	2	-	E	0	-	-
	T	1	1	1	1		O	0		
	RV	2	2	2	2					
	TV	-	-	2						
7	D	0	0	1	3	+	E	0	-	±
	T	0.5	0.5	1	2		O	0		
	RV	0	0	1	6					
	TV	-	-	0.3						
8	D	3	5	4	3	-	E	0	-	-
	T	2	3	3	2		O	0		
	RV	6	15	12	6					
	TV	-	-	11						
9	D	0	0	0	0	-	E	0	-	-
	T	0.5	0.5	0.5	0.5		O	0		
	RV	0	0	0	0					
	TV	-	-	0						
10	D	3	3	3	3	-	E	0	-	-
	T	2	1	2	1		O	0		
	RV	6	3	6	3					
	TV	-	-	5						

D - diameter of reaction (mm)
T - thickness of skin fold at reaction site (mm)
RV - "reaction volume"
TV - 'threshold volume'

E - erythema
O - oedema
+ - positive
- - negative
± - inconclusive

At the second challenge stage, positive reactions were again seen in two of the above animals and in one other guinea-pig not previously showing positive intradermal reactions. No dermal responses were observed at the second challenge in the remaining two animals positive at intradermal challenge.

The overall results appeared to indicate, therefore, that 2/10 animals (20%) were sensitized. This sensitivity was demonstrable by both intradermal and topical challenge. In a further three animals positive dermal reactions were demonstrable by only one method of administration. The evidence for sensitivity to nickel sulphate in the latter animals was, therefore, inconclusive. The remaining five animals did not show positive dermal reaction at either challenge.

These results do not agree with those of Maurer, et al (1979) where positive reactions were reported in the total test group.

Maximization Test

The results of this technique in the first series of guinea-pigs are presented in Table 8.

Dermal reactions of a time course characteristic of sensitivity (increasing to a peak at 48-72 hours after application) and greater than those expected from preliminary irritancy tests (Table 6) were considered to be indicative of an allergic response.

Using these criteria, positive reactions were observed in 7 of the first series of 15 animals (46.6%). In these animals dermal scores of 0 (no reaction) to 2 (well-defined reaction) were observed at the 25 hour reading (1 hour after removal of bandage). Dermal

TABLE 8

Maximization Test - Dermal Reactions at First Challenge (Day 22) of Individual Guinea Pigs

Treatment	Animal No.	Reaction	Reactions after Challenge Application at				Results (+ve or -ve)
			25 Hrs.	48 Hrs.	72 Hrs.	96 Hrs.	
$NiSO_4$ (5%)	1	E	1	2	2	0	+
		O	0	1	1	1	
	2	E	1	2	2	1	+
		O	0	1	2	1	
	3	E	1	2	2	1	+
		O	1	2	3	2	
	4	E	0	2	2	1	+
		O	0	1	2	1	
	5	E	2	2	2	1	±
		O	1	2	1	1	
	6	E	1[F]	1[F]	1[F]	0	-
		O	1	1	1	0	
	7	E	0	0	0	0	-
		O	0	0	0	0	
	8	E	2	2	2	2	±
		O	1	1	1	1	
	11	E	0	2	2	1	+
		O	0	2	2	1	
	12	E	0	0	0	0	-
		O	0	0	0	0	
	13	E	0	0	2	1	+
		O	0	0	1	0	
	14	E	0	2[F]	2[F]	1[F]	-
		O	0	2	2	1	
	15	E	0	1[F]	1[F]	0	-
		O	0	0	0	0	
	16	E	0	2[F]	1[F]	0	-
		O	0	0	0	0	
	17	E	0	0	0	0	-
		O	0	0	0	0	
Control	9	E	0	0	0	0	-
		O	0	0	0	0	
	10	E	0	0	0	0	-
		O	0	0	0	0	
	18	E	0	0	0	0	-
		O	0	0	0	0	
	19	E	0	0	1[F]	0	-
		O	0	0	0	0	
	20	E	0	0	0	0	-
		O	0	0	0	0	

E - erythema
O - oedema
F - focal reaction only

+ - positive
- - negative
± - inconclusive

reactions generally increased to well-defined to moderate at the 48 and 72 hour readings and ameliorated slightly at 96 hours.

In one animal (No. 8) well-defined erythema with slight oedema were observed at the 25 hour reading and persisted throughout the observation period. Since these reactions were greater than expected from irritancy tests but were not of the characteristic time course, their nature was uncertain.

In four other animals (Nos. 6, 12, 13 and 14) focal dermal reactions were observed. These reactions involved only a small area of the test patch site and could not be considered truely indicative of sensitivity.

No dermal reactions were observed in the control animals.

These results were considered to agree with those of Magnusson and Kligman (1970). Using the same technique these authors demonstrated sensitivity to nickel sulphate in 55% of guinea-pigs tested.

In order to confirm these results the animals (including the controls) were rechallenged under the same conditions one week after the first challenge (day 28). The results are presented in Table 9.

Two animals removed their bandages during the 24 hours exposure period and were deleted from further interpretations.

Dermal reactions indicative of sensitivity to nickel sulphate were observed in five of the thirteen remaining test guinea-pigs (38%). However, the reactions of only three of these five animals (Nos. 1, 3 and 11) could be correlated with positive reactions at first challenge. Animals Nos. 4 and 16 had previously shown only inconclusive

TABLE 9

Maximization Test - Dermal Reaction at Second Challenge (Day 29) of Individual Guinea Pigs

Treatment	Animal No.	Reaction	Reactions after Challenge Application at				Results (+ve or -ve)
			25 Hrs.	48 Hrs.	72 Hrs.	96 Hrs.	
$NiSO_4$ (5%)	1	E O	1 0	3 3	2 3	2 2	+
	2	E O	0 0	0 0	0 0	0 0	-
	3	E O	0 0	3 2	2 2	1 1	+
	4	E O	0 0	0 0	0 0	0 0	-
	5	E O	Animal removed bandage				
	6	E O	Animal removed bandage				
	7	E O	0 0	0 0	0 0	0 0	-
	8	E O	0 0	0 0	0 0	0 0	-
	11	E O	0 0	3 2	3 2	2 2	+
	12	E O	0 0	0 0	0 1	0 0	-
	13	E O	0 0	0 0	0 0	0 0	-
	14	E O	0 0	2 2	2 2	1 1	+
	15	E O	0 0	0 0	0 0	0 0	-
	16	E O	0 0	1 1	1 1	0 0	+
	17	E O	0 0	0 0	0 0	0 0	-
Control	9	E O	0 0	0 0	0 0	0 0	-
	10	E O	0 0	0 0	0 0	0 0	-
	18	E O	0 0	0 0	0 0	0 0	-
	19	E O	0 0	0 0	0 0	0 0	-
	20	E O	0 0	0 0	0 0	0 0	-

E - erythema
O - oedema

+ - positive
- - negative

focal reactions. Positive dermal reactions were demonstrable at first challenge but not at second in animals 2, 4 and 13.

In summary, dermal reactions indicative of sensitivity to nickel sulphate were repeatedly demonstrated in three of thirteen guinea-pigs (23%).

It was considered that the lack of correlation between first and second challenges may be due to:-

1. inability to reproduce identical test conditions;

2. loss of sensitivity or loss of immunological memory in a proportion of animals.

In order to further investigate the first point, the test procedure was repeated in three groups of ten guinea-pigs on three separate occasions. In addition challenge procedures were performed with dual patches, such that two concentrations of nickel sulphate (5% and 1%) could be tested. This slight modification was performed in an attempt to provide more confidence in the challenge results. The severity of reactions in a sensitized animal should be independent, to some extent, of dosage (i.e. an all or none type response). This, therefore, differs from a non-specific irritant response in that animal.

The results of these subsequent investigations are presented in Tables 10 and 11.

The incidence of dermal reactions indicative of sensitivity in each series was as follows:-

TABLE 10

Maximization Test - Dermal Reactions at Challenge of Individual Guinea Pigs - Series 2

Animal No.	Reaction	Reaction after Challenge Application of 5% $NiSO_4$ at				Reaction after Challenge Application of 1% $NiSO_4$ at				Results (+ve or -ve)
		25 Hrs.	48 Hrs.	72 Hrs.	96 Hrs.	25 Hrs.	48 Hrs.	72 Hrs.	96 Hrs.	
21	E O	0 0	0 0	0 0	0 0	0 0	0 0	0 0	0 0	-
22	E O	0 0	0 0	0 0	0 0	0 0	0 0	0 0	0 0	-
23	E O	0 0	0 0	0 0	0 0	0 0	0 0	0 0	0 0	-
24	E O	0 0	0 0	0 0	0 0	0 0	0 0	0 0	0 0	-
25	E O	0 0	0 0	0 0	0 0	0 0	0 0	0 0	0 0	-
26	E O	0 0	0 0	0 0	0 0	0 0	0 0	0 0	0 0	-
27	E O	0 0	0 0	0 0	0 0	0 0	0 0	0 0	0 0	-
28	E O	0 0	0 0	0 0	0 0	0 0	0 0	0 0	0 0	-
29	E O	0 0	2 1	2 1	2 1	0 0	0 0	0 0	0 0	+ (5%)
30	E O	0 0	0 0	0 0	0 0	0 0	0 0	0 0	0 0	-

E - erythema
O - oedema
+ - positive
- - negative

TABLE 11

Maximization Test - Dermal Reactions at Challenge of Indivdual Guinea Pigs - Series 4

Animal No.	Reaction	Reaction after Challenge Application of 5% $NiSO_4$ at				Reaction after Challenge Application of 1% $NiSO_4$ at				Results (+ve or -ve)
		25 Hrs.	48 Hrs.	72 Hrs.	96 Hrs.	25 Hrs.	48 Hrs.	72 Hrs.	96 Hrs.	
41	E	0	1^F	1^F	1^F	0	0	0	0	-
	O	0	0	0	0	0	0	0	0	
42	E	0	0	0	0	0	0	1	1	-
	O	0	0	0	0	0	0	0	0	
43	E	0	2	1	0	0	0	0	0	+ (5%)
	O	0	1	0	0	0	0	0	0	
45	E	0	2	1	1^F	0	2	2	0	+
	O	0	0	0	0	0	0	0	0	
46	E	0	2	2	2	0	0	0	0	+ (5%)
	O	0	0	0	0	0	0	0	0	
47	E	1	2	2	1^F	1	2	2	2	+
	O	0	0	0	0	0	0	0	0	
48	E	1^F	1^F	1^F	0	0	0	1	0	-
	O	0	0	0	0	0	0	0	0	
49	E	0	0	0	0	1	2	2	1	$\pm$
	O	0	0	0	0	0	1	0	0	

E - erythema
O - oedema
F - focal reaction only

\+ - positive
\- - negative
$\pm$ - inconclusive

Guinea pigs 44 and 50 died during the induction phase

Series 2 (Table 10) - 1/10 animals reacted (10%) with 5% nickel sulphate.

Series 3 - No response seen in any animals (0%).

Series 4 (Table 11) - 4/8 animals (50%) showed characteristic responses. Two of these animals responded with both 5% and 1% nickel sulphate. The remaining two with 5% only. One further animal reacted with 1% nickel sulphate only; the results were considered inconclusive in this animal.

The overall results indicated a wide variation of the incidence of positive dermal reactions (0-50%) in guinea-pigs with the standard Maximization test. This irreproducibility may be due to:-

1. inconsistent induction exposure and/or procedure;

2. inconsistent elicitation exposure and/or procedure (demonstration of sensitivity).

These results tend to confirm the interpretations from earlier human and guinea-pig experiments. Nickel is a weak allergen (Vandenberg and Epstein, 1963) and the experimental induction of sensitivity is difficult.

Modified Maximization Test

In order to produce a consistent incidence of positive dermal reactions in the guinea-pig, techniques were developed to increase the exposure to nickel without producing excessive local toxicity or systemic toxicity.

The design of the Optimization test necessitates increasing the concentration of injected nickel sulphate solution, since the number of intradermal injections already occupied a large proportion of dorsal skin (Figure 8). However, higher concentrations of injected nickel sulphate would produce excessive local toxicity as demonstrated in preliminary tests (Table 5). The potential for further development of the Optimization test was thus considered to be limited.

In the Maximization test, induction was performed by both intradermal injection and topical application. Both procedures had the potential for further development as follows:-

1. increasing the intradermal injection volume to 0.1 ml/site (equal to that in the Optimization test);

2. performing a second series of intradermal injections within the interscapular region (Figure 10);

3. using material other than filter paper for the topical applications such that less nickel sulphate solution would be held within the patch at both the topical induction and elicitation phases. Surgical lint was used.

This modified Maximization test was undertaken and the results are presented in Table 12.

A series of 20 animals were used. Two guinea-pigs died following the induction phase. A total of 18 animals were challenged with 5% and 1% w/v aqueous solutions of nickel sulphate.

A further 5 animals received the same induction treatments but without nickel sulphate. These animals were challenged with 5% nickel sulphate and thus served as controls.

Dermal reactions indicative of sensitivity were observed in 16 of the 18 guinea-pigs (88.8%) challenged with nickel sulphate. Five of these animals (27.7% of total) responded to both concentrations of nickel sulphate. The remaining 11 animals reacted to 5% $NiSO_4$ only.

The dermal reactions of animals considered to be representative of this series were photographed and are presented as Figures 11, 12, 13 and 14.

Animal No. 12 showed only slight local dermal reactions to 5% nickel sulphate. The evidence for sensitivity in this animal was inconclusive.

Animal No. 4 showed no dermal response at challenge and was not considered to be sensitive.

No dermal reactions were observed in the control animals.

In order to confirm this high incidence of positive dermal reactions the testing procedure was repeated in three series of 10,

TABLE 12

Modified Maximization Test - Dermal Reactions at Challenge (Day 29) of Individual Guinea Pigs

Treatment	Animal No.	Reaction	Reaction after Challenge Application of 5% $NiSO_4$ at				Reaction after Challenge Application of 1% $NiSO_4$ at				Results (+ve or -ve)
			25 Hrs.	48 Hrs.	72 Hrs.	96 Hrs.	25 Hrs.	48 Hrs.	72 Hrs.	96 Hrs.	
$NiSO_4$	1	E O	1 0	2 1	2 2	2 1	0 0	0 0	0 0	0 0	+ (5%)
	2	E O	1 0	2 1	2 1	2 1	0 0	0 0	0 0	0 0	+ (5%)
	3	E O	2 1	3 2	2 2	2 2	0 0	0 0	0 0	0 0	+ (5%)
	4	E O	0 0	0 0	0 0	0 0	0 0	0 0	0 0	0 0	-
	5	E O	1 1	3 2	2 1	2 1	1 0	2 1	2 1	2 1	+
	6	E O	1 0	2 1	2 1	1 1	0 0	0 0	0 0	0 0	+ (5%)
	7	E O	3 2	2 3	2 2	2 1	1 0	0 0	0 0	0 0	+ (5%)
	8	E O	2 1	3 2	2 2	2 2	0 0	2 1	1 1	1 0	+
	9	E O	2 1	3 2	2 2	2 2	0 0	2 1	0 0	0 0	+
	10	E O	1 1	2 1	1 1	1 1	0 0	0 0	0 0	0 0	+ (5%)
	11	E O	2 1	3 2	1 2	1 2	0 1	2 2	0 0	0 0	+
	12	E O	1[F] 0	1 1	1 0	1 0	0 0	0 0	0 0	0 0	±
	13	E O	1[F] 0	2 2	1 1	1 1	0 0	0 0	0 0	0 0	+ (5%)
	14	E O	1[F] 0	2 2	2 1	2 1	0 0	0 0	0 0	0 0	+ (5%)
	15	E O	2 0	3 2	1 2	2 2	0 0	0 0	0 0	0 0	+ (5%)
	16	E O	2 0	2 1	2 1	2 1	0 0	0 0	0 0	0 0	- (5%)
	17	E O	2 2	2 2	2 1	2 1	1 1	2 1	2 1	1 1	+
	20	E O	1 0	2 2	2 1	2 1	0 0	0 0	0 0	0 0	+ (5%)
Control	21	E O	0 0	0 0	0 0	0 0					
	22	E O	0 0	0 0	0 0	0 0					
	23	E O	0 0	0 0	0 0	0 0					
	24	E O	0 0	0 0	0 0	0 0					
	25	E O	0 0	0 0	0 0	0 0					

E - erythema
O - oedema
F - focal reaction only

\+ - positive
\- - negative
± - inconclusive

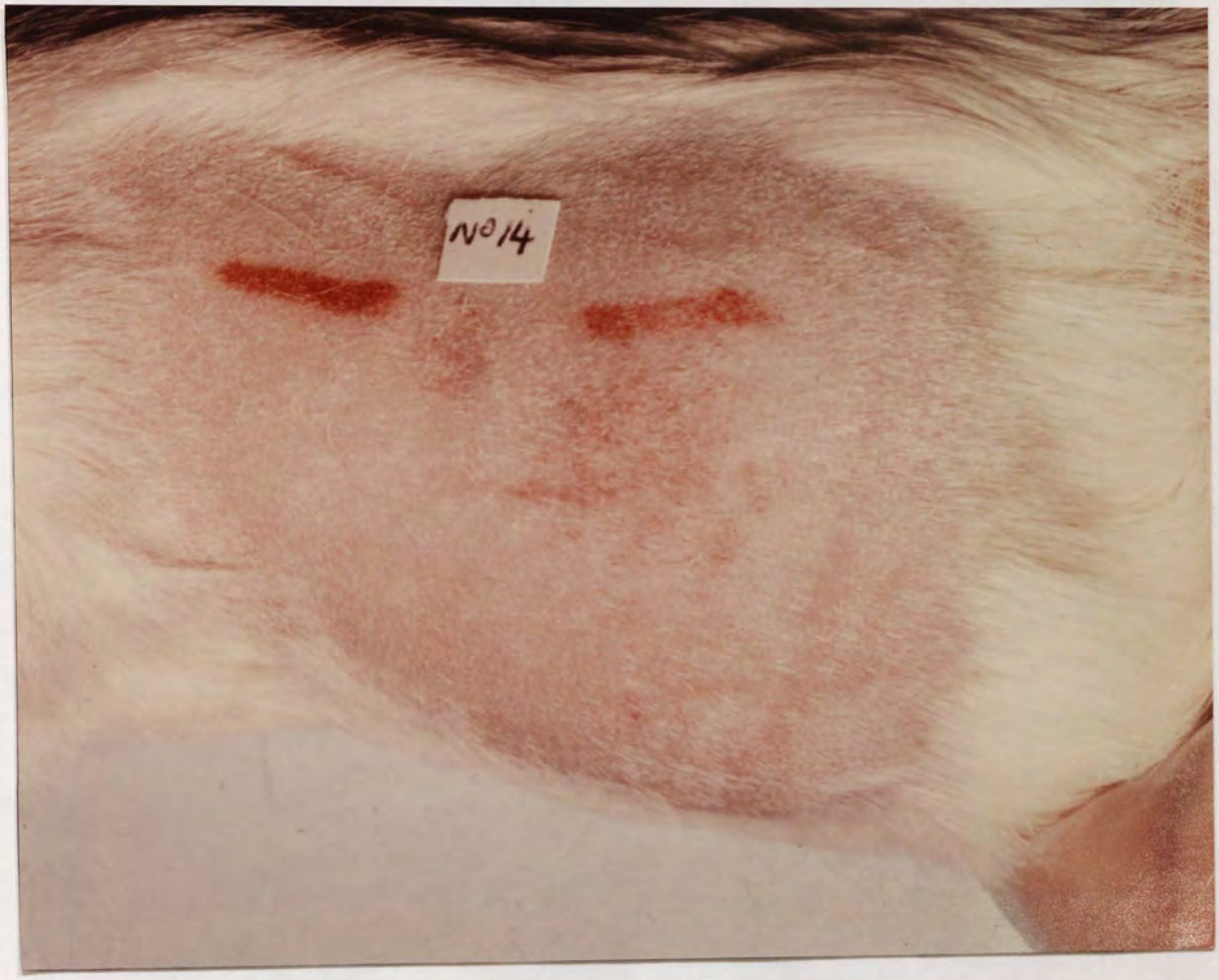


Figure 11

Modified Maximization test. Dermal reactions of guinea-pig 14, at 48 hours following challenge application of 5% $NiSO_4$ (right site) and 1% $NiSO_4$ (left site). These reactions were scored as 2 erythema, 2 oedema (right site) and no reaction (left site). The red lines indicate the position of the test patches when the occlusive dressings were removed.

The reactions at the right site were considered to be indicative of sensitization.

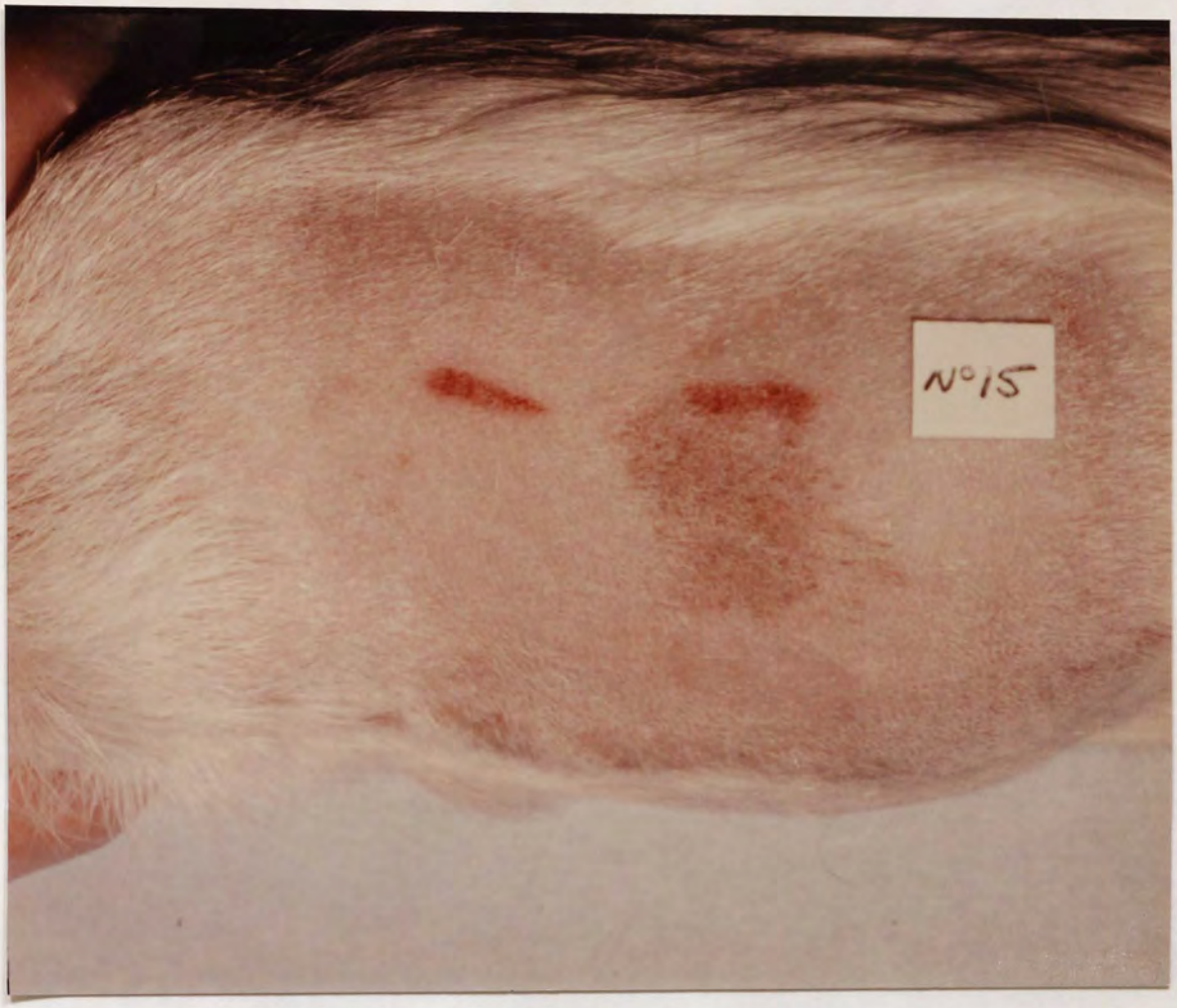


Figure 12

Modified Maximization test. Dermal reactions of guinea-pig 15, at 48 hours following challenge application of 5% $NiSO_4$ (right site) and 1% $NiSO_4$ (left site). These reactions were scored 3 erythema, 2 oedema (right site) and no reaction (left site). The red lines indicate the position of the test patches when the occlusive dressings were removed.

The reactions at the right site were considered to be indicative of sensitization.

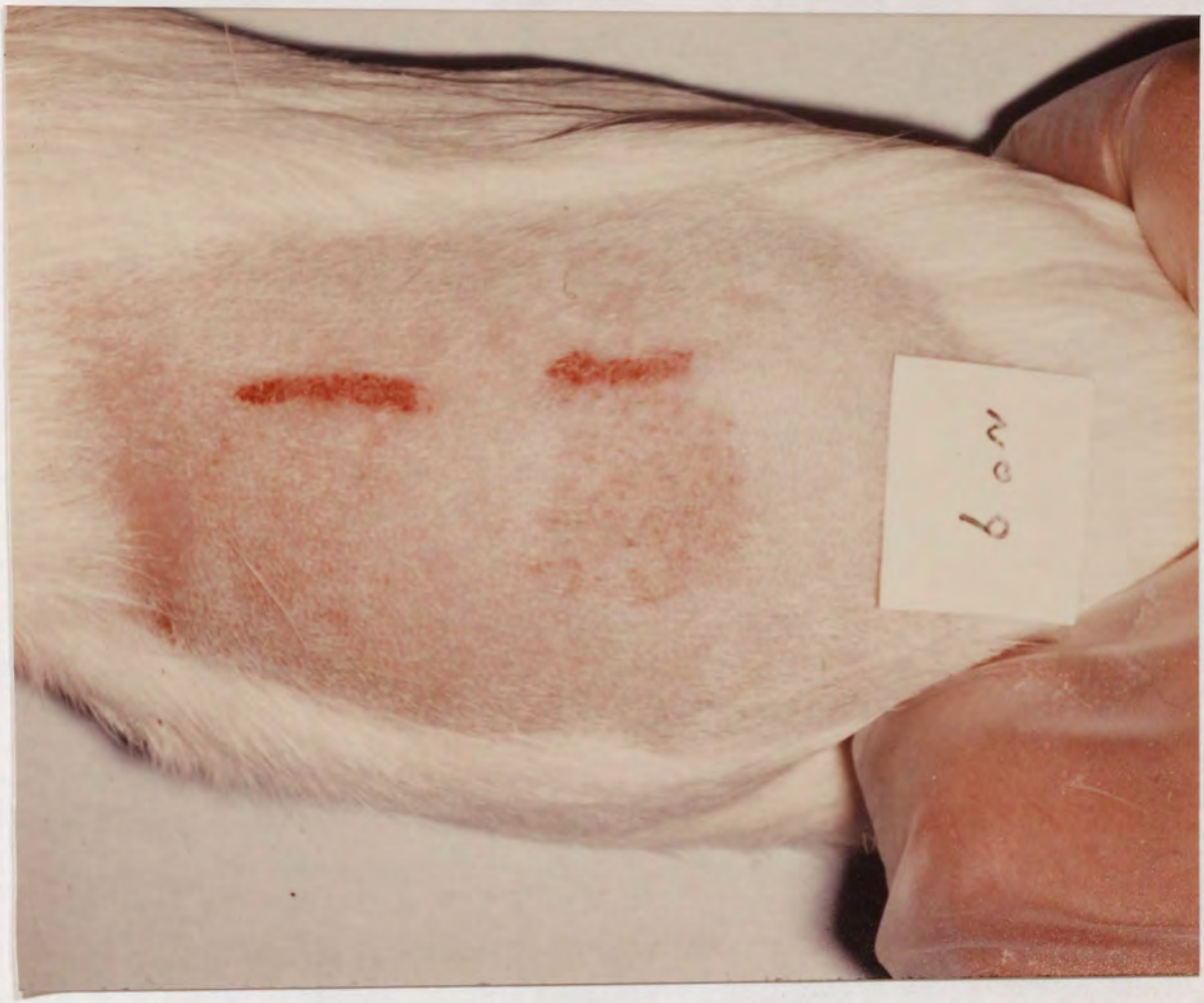


Figure 13

Modified Maximization test. Dermal reactions of guinea-pig 9, at 48 hours following challenge application of 5% $NiSO_4$ (right site) and 1% $NiSO_4$ (left site). These reactions were scored 3 erythema, 2 oedema (right site) and 2 erythema, 1 oedema (left site). The red lines indicate the position of the test patches when the occlusive dressings were removed.

The reactions at both test sites were considered to be indicative of sensitization.

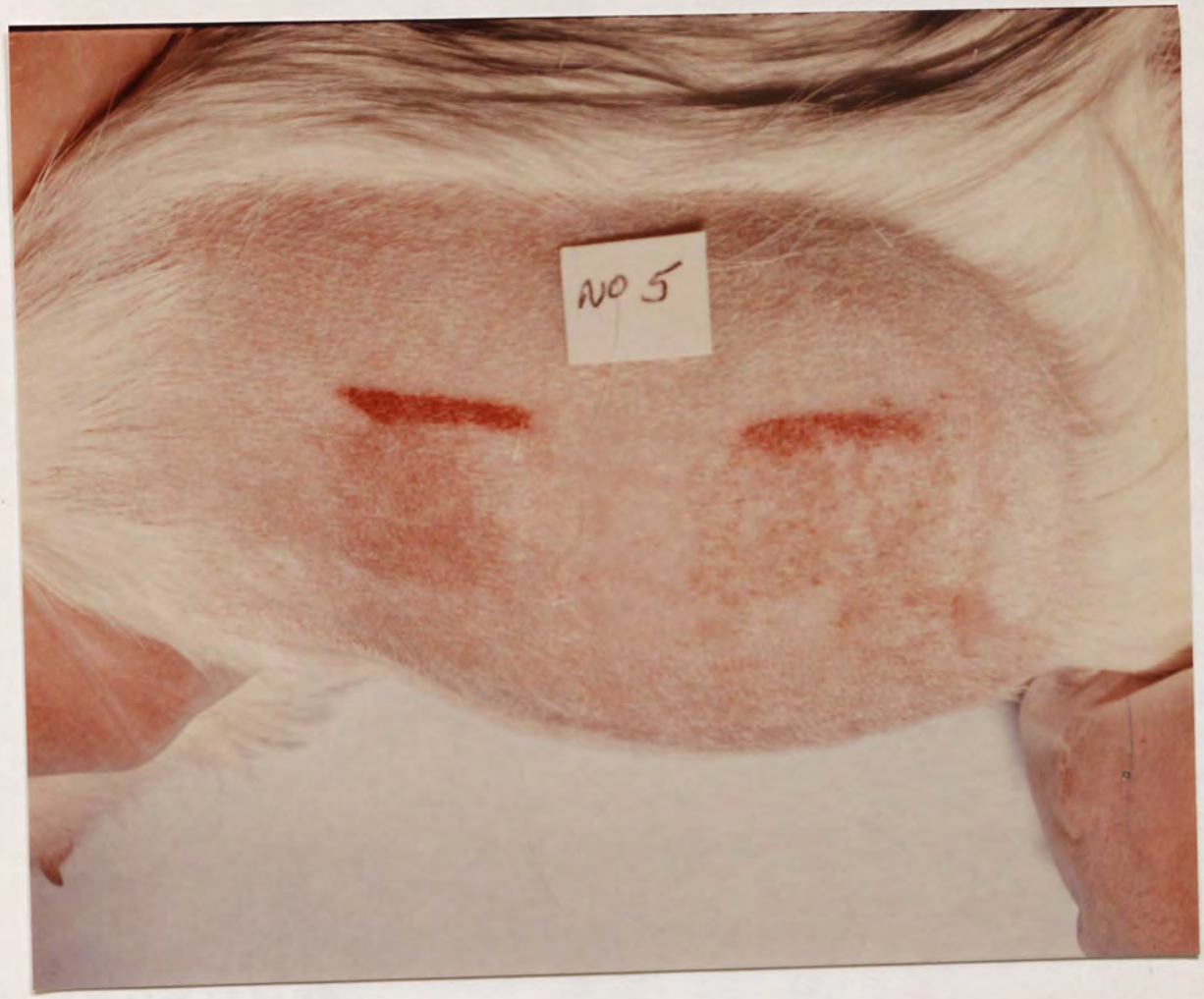


Figure 14

Modified Maximization test. Dermal reactions of guinea-pig 5, at 48 hours following challenge application of 5% $NiSO_4$ (right site) and 1% $NiSO_4$ (left site). These reactions scored 3 erythema, 2 oedema (right site) and 2 erythema, 1 oedema (left site). The red lines indicate the position of the test patches when the occlusive dressings were removed.

The reactions at both test sites were considered to be indicative of sensitization.

15 and 20 guinea-pigs respectively. Similar dual challenges were performed using 5% and 1% w/v aqueous nickel sulphate solutions. In the last series, challenge was also performed with copper sulphate, in order to demonstrate that the positive reactions were due to nickel and were not non-specific reactions to a metal sulphate salt.

The results of initial and subsequent challenge applications in the standard Maximization test indicated a lack of correlation between challenges which may be resultant from a reduction in demonstrable sensitivity in a proportion of animals. In an attempt to check this observation in the Modified Maximization test, second challenges were performed one week following the initial challenge in two series of guinea-pigs.

The results from each series of animals are presented in Tables 13 to 17.

In series 2, dermal reactions indicative of sensitivity were produced in 7 of the 10 animals (70%), four of which (40%) reacted with both 5% and 1% nickel sulphate. On subsequent rechallenge, positive responses were again seen in 5 of the 10 animals (50%), three of which still reacted at both concentrations of nickel sulphate.

One animal initially responding with 5% nickel sulphate did not show any response at second challenge (Animal 32).

One animal initially responding with both concentrations showed no reactions (Animal 34).

TABLE 13

Modified Maximization Test - Dermal Reactions at First Challenge (Day 29) of Individual Guinea Pigs - Series 2

Animal No.	Reaction	Reaction after Challenge Application of 5% $NiSO_4$ at				Reaction after Challenge Application of 1% $NiSO_4$ at				Results (+ve or - ve)
		25 Hrs.	48 Hrs.	72 Hrs.	96 Hrs.	25 Hrs.	48 Hrs.	72 Hrs.	96 Hrs.	
26	E O	0 0	0 0	0 0	0 0	0 0	0 0	0 0	0 0	-
27	E O	1 0	2 1	2 1	1 1	0 0	0 0	0 0	0 0	+ (5%)
28	E O	2 1	2 2	2 3	2 2	1[F] 0	1 2	1 1	0 0	+
29	E O	1 1	3 2	3 2	2 2	1 1	2 1	2 2	2 1	+
30	E O	0 0	0 0	0 0	0 0	0 0	0 0	0 0	0 0	-
31	E O	0 0	0 0	0 0	0 0	0 0	0 0	0 0	0 0	-
32	E O	1 0	2 1	2 2	2 2	1[F] 0	1[F] 0	0 0	0 0	+ (5%)
33	E O	0 0	2 2	3 2	2 2	0 0	0 0	0 0	0 0	+ (5%)
34	E O	1 1	2 2	2 1	1 1	0 0	1 1	2 1	1 1	+
35	E O	2 2	3 3	3 2	2 2	2 1	2 2	3 2	2 2	+

E - erythema
O - oedema
F - focal reaction only

+ - positive
- - negative

TABLE 14

Modified Maximization Test - Dermal Reactions at Second Challenge (Day 36) of Individual Guinea Pigs - Series 2

Animal No.	Reaction	Reaction after Challenge Application of 5% $NiSO_4$ at				Reaction after Challenge Application of 1% $NiSO_4$ at				Results (+ve or -ve)
		25 Hrs.	48 Hrs.	72 Hrs.	96 Hrs.	25 Hrs.	48 Hrs.	72 Hrs.	96 Hrs.	
26	E O	0 0	0 0	0 0	0 0	0 0	0 0	0 0	0 0	-
27	E O	0 0	2 2	2 2	2 1	0 0	0 0	0 0	0 0	+ (5%)
28	E O	1 1	2 1	2 2	2 1	0 0	1 1	2 1	1 0	+
29	E O	1 1	2 2	3 2	2 2	1 0	2 1	2 1	1 1	+
30	E O	0 0	0 0	0 0	0 0	0 0	0 0	0 0	0 0	-
31	E O	1^F 0	1^F 0	0 0	0 0	0 0	0 0	0 0	0 0	-
32	E O	0 0	0 0	0 0	0 0	0 0	0 0	0 0	0 0	-
33	E O	2 1	3 2	2 2	1 1	0 0	0 0	0 0	0 0	+ (5%)
34	E O	1^F 1	1^F 1	1^F 0	1^F 0	0 0	0 0	0 0	0 0	-
35	E O	2 2	3 3	2 2	2 1	1 1	2 2	3 2	2 1	+

E - erythema
O - oedema
F - focal reaction only.

+ - positive
- - negative

TABLE 15

Modified Maximization Test - Dermal Reactions at First Challenge (Day 29) of Individual Guinea Pigs - Series 3

Animal No.	Reaction	Reaction after Challenge Application of 5% $NiSO_4$ at				Reaction after Challenge Application of 1% $NiSO_4$ at				Results (+ve or -ve)
		25 Hrs.	48 Hrs.	72 Hrs.	96 Hrs.	25 Hrs.	48 Hrs.	72 Hrs.	96 Hrs.	
36	E O	2 2	3 3	3 2	2 1	1 2	2 2	3 3	3 2	+
37	E O	2 1	3 2	3 2	2 2	0 0	1 2	2 1	1 1	+
38	E O	2 2	2 2	3 2	2 1	0 0	1 1	2 1	1 0	+
39	E O	2 2	2 2	2 2	1 1	0 0	1[F] 1	1[F] 1	0 0	+ (5%) ± (1%)
40	E O	1 1	2 1	3 2	2 2	0 0	1[F] 1	2 1	2 0	+
41	E O	1 1	3 2	3 3	2 1	0 0	2 1	1 1	0 0	+
42	E O	2 1	3 3	3 2	2 1	1 1	2 3	3 3	2 1	+
43	E O	2 1	3 3	2 2	1 0	2 1	3 2	2 1	1 1	+
44	E O	1 2	2 2	2 2	2 1	0 0	1 1	2 1	0 0	+
45	E O	2 2	3 2	3 2	1 1	0 0	0 0	0 0	0 0	+ (5%)
46	E O	2 2	2 2	3 2	2 1	0 0	1 2	2 2	2 2	+
47	E O	1 1	2 2	2 1	1 0	1 1	2 1	2 1	1 1	+
48	E O	1 0	3 2	2 1	1 1	0 0	0 0	0 0	0 0	+ (5%)
49	E O	2 1	2 2	2 2	2 1	0 0	0 0	0 0	1 0	+ (5%)
50	E O	2 1	2 3	3 3	2 2	0 0	0 0	0 0	0 0	+ (5%)

E - erythema
O - oedema
F - focal reaction only

+ - positive
- - negative
± - inconclusive

TABLE 16

Modified Maximization Test - Dermal Reactions at Second Challenge (Day 36) of Individual Guinea Pigs - Series 3

Animal No.	Reaction	Reaction after Challenge Application of 5% $NiSO_4$ at				Reaction after Challenge Application of 1% $NiSO_4$ at				Results (+ve or -ve)
		25 Hrs.	48 Hrs.	72 Hrs.	96 Hrs.	25 Hrs.	48 Hrs.	72 Hrs.	96 Hrs.	
41	E O	2 1	2 2	2 2	1 1	0 0	0 0	0 0	0 0	+ (5%)
42	E O	2 1	3 2	3 2	2 2	1 0	2 2	2 2	1 1	+
43	E O	1 0	2 1	1 1	1 0	1 1	1 1	2 2	2 2	+
44	E O	0 0	0 0	0 0	0 0	0 0	0 0	0 0	0 0	-
45	E O	1 1	2 2	2 2	2 1	0 0	1 1	1 0	0 0	+
46	E O	2 2	3 2	3 2	2 1	2 1	2 2	3 2	2 2	+
47	E O	1 1	2 1	1 1	1^F 1	0 0	0 0	0 0	0 0	+ (5%)
48	E O	0 0	0 0	0 0	0 0	0 0	0 0	1 0	0 0	-
49	E O	1 0	0 0	0 0	1^F 0	0 0	0 0	0 0	0 0	-
50	E O	2 0	2 0	3 1	2 2	1 1	2 1	2 1	1 1	+

E - erythema
O - oedema
F - focal reaction only

+ - positive
- - negative

TABLE 17

Modified Maximization Test - Dermal Reactions at First Challenge (Day 29) of Individual Guinea-Pigs - Series 4

Treatment	Animal No.	Reaction	Reaction after Challenge Application of 5% $NiSO_4$ at				Reaction after Challenge Application of 1% $NiSO_4$ at				Reaction after Challenge Application of 1% $CuSO_4$ at				Result for $NiSO_4$	Result for $CuSO_4$
			25 Hrs.	48 Hrs.	72 Hrs.	96 Hrs.	25 Hrs.	48 Hrs.	72 Hrs.	96 Hrs.	25 Hrs.	48 Hrs.	72 Hrs.	96 Hrs.		
$NiSO_4$	51	E	1	2	2	2	0	2	2	1	0	0	0	0	+	-
		O	1	2	2	1	0	1	2	1	0	0	0	0		
	52	E	1	2	2	2	1	2	2	1	2	1	0	0	+	-
		O	0	1	2	1	0	1	1	1	1	0	0	0		
	53	E	0	0	0	0	0	1	0	0	0	0	0	0	-	-
		O	0	0	0	0	0	0	0	0	0	0	0	0		
	54	E	2	3	3	2	1	2	2	2	1	0	0	0	+	-
		O	1	2	3	2	1	2	2	1	0	0	0	0		
	55	E	1	3	3	2	0	1	2	0	1	0	1	0	+	-
		O	0	2	2	2	0	1	2	0	0	0	0	0		
	56	E	1	2	2	2	1	1	0	1	0	0	0	0	+ (5%)	-
		O	1	2	2	1	0	0	0	0	0	0	0	0		
	57	E	0	2	2	1	0	0	1	0	0	0	0	0	+ (5%)	-
		O	0	2	1	1	0	0	0	0	0	0	0	0		
	58	E	0	0	0	0	1	0	0	0	0	0	0	0	-	-
		O	0	0	0	0	0	0	0	0	0	0	0	0		
	59	E	1	0	1	0	1	0	0	0	0	0	0	0	-	-
		O	0	0	0	0	0	0	0	0	0	0	0	0		
	60	E	2	2	3	2	1	2	2	1	1	0	0	0	+	-
		O	0	2	2	1	0	2	2	1	0	0	0	0		
	61	E	2	3	2	1	1	2	2	1	0	0	0	0	+	-
		O	1	2	2	1	1	2	2	0	0	0	0	0		
	62	E	1	2	2	2	0	0	0	1	0	0	1	0	+ (5%)	-
		O	1	1	2	1	0	0	0	0	0	0	0	0		
	63	E	2	2	2	2	1	1	0	0	0	0	0	0	+ (5%)	-
		O	0	2	2	2	0	0	0	0	0	0	0	0		
	64	E	1	2	2	1	0	0	0	0	0	0	0	0	+ (5%)	-
		O	0	2	2	1	0	0	0	0	0	0	0	0		
	65	E	2	2	3	2	1	3	2	2	0	0	0	0	+	-
		O	2	2	2	1	1	2	1	1	0	0	0	0		
	66	E	2	2	2	1	0	1	0	0	1	0	0	0	+ (5%)	-
		O	1	2	1	0	0	0	0	0	0	0	0	0		
	67	E	2	2	3	2	1	2	3	2	0	0	0	0	+	-
		O	1	2	3	3	0	2	2	1	0	0	0	0		
	68	E	1	2	2	1	1	0	0	0	1	0	0	0	+ (5%)	-
		O	1	2	2	1	1	0	0	0	0	0	0	0		
	69	E	1	2	2	1	0	0	1	0	0	0	0	0	+ (5%)	-
		O	0	2	1	0	0	0	0	0	0	0	0	0		
	70	E	1	2	2	1	0	0	0	0	0	0	0	0	-	-
		O	0	1	2	0	0	0	0	0	0	0	0	0		
Control	71	E	0	0	0	0	-	-	-	-	0	0	1	0	-	-
		O	0	0	0	0					2	0	0	0		
	72	E	1	0	0	0	-	-	-	-	0	0	0	0	-	-
		O	0	0	0	0					0	0	0	0		
	73	E	2	1	1	0	-	-	-	-	0	0	0	0	-	-
		O	0	0	0	0					0	0	0	0		
	74	E	1	0	0	0	-	-	-	-	0	0	0	0	-	-
		O	0	0	0	0					0	0	0	0		
	75	E	0	0	1	0	-	-	-	-	1	0	0	0	-	-
		O	0	0	0	0					0	0	0	0		
	76	E	0	0	0	0	-	-	-	-	0	0	0	0	-	-
		O	0	0	0	0					0	0	0	0		
	77	E	1	1	0	0	-	-	-	-	0	0	0	0	-	-
		O	1	0	0	0					0	0	0	0		
	78	E	0	1	0	0	-	-	-	-	0	0	0	0	-	-
		O	0	0	0	0					0	0	0	0		
	79	E	0	0	0	1	-	-	-	-	0	0	1	0	-	-
		O	0	0	0	0					0	0	0	0		
	80	E	0	0	0	0	-	-	-	-						
		O	0	0	0	0										

E - erythema
O - oedema

+ - positive
- - negative

In Series 3, all guinea-pigs (100%) showed reactions indicative of sensitivity, ten of which (66%) reacted with both 5% and 1% nickel sulphate. On subsequent rechallenge, positive reactions were again seen in 7 of 10 animals (70%) maintained until this stage, 5 of which (50%) reacted with both concentrations.

Animals 41 and 47 initially responded with 5% and 1% nickel sulphate but on rechallenge reacted to only 5%.

Animal 44 initially responded with both concentrations but subsequently to none.

Animals 45 and 50 initially reacted with 5% but subsequently reacted with both concentrations.

Animals 48 and 49 initially reacted with 5% but subsequently to none.

In series 4, nickel sulphate (5% and 1%) and copper sulphate (1%) were used at the first challenge only. The incidence of reactions indicative of sensitivity to nickel sulphate was similar to the other series; 17 of the 20 guinea-pigs (85%) reacted to 5% nickel sulphate, 8 of which (40%) also reacted to the 1% level.

The reactions of test animals to copper sulphate were similar to the controls. No animals showed significant reaction. This indicates that the reactions of the test animals to nickel sulphate is specific for nickel.

In summary very high incidences of specific positive reactions to nickel sulphate were produced in the Modified Maximization test (up to 100%). The results were reasonably reproducible (range 70-100%) on subsequent testing or rechallenge (50-70%).

DISCUSSION

In order to compare the results of each method used, the relative incidences (expressed as percentages) of dermal reactions indicative of sensitivity are tabulated as follows:-

Test method		Incidence (%) at First Challenge with			Incidence (%) at Second Challenge with	
		5%	1%	0.1%	5%	1%
Optimization (Maurer, et al, 1978)		-	-	20 (intra-dermal)	20	ND
Maximization (Magnusson and Kligman, 1970)	1	46	ND	-	38	ND
	2	10	0	-	ND	
	3	0	0	-	ND	
	4	50	25	-	ND	
Modified Maximization (developed by author)	1	89	27	-	ND	
	2	70	40	-	50	30
	3	100	66	-	70	50
	4	85	40	-	ND	

ND - not performed

The following points may be noted from these figures:-

1. the highest incidence of sensitivity was achieved under the most stringent testing conditions (Modified Maximization test);

2. a greater correlation was achieved between tests using the Modified Maximization test;

3. there is a slight reduction in the demonstrated incidence of sensitivity when one test series is rechallenged.

The cause of the slight reduction in the incidence of sensitivity is unknown but may be indicative of a loss of sensitivity or decrease in the degree of sensitivity in a proportion or all of test animals, or indicative of an inability of the method to reproduce identical test conditions. Individual guinea-pig variability or skin changes between challenges may also be contributory.

These results in general tend also to support the results of earlier experimental induction of sensitivity in humans and guinea-pigs. The experimental induction of sensitivity to nickel is difficult and nickel is a weak allergen (Vandenberg and Epstein, 1963).

Under stringent exposure conditions a high incidence of sensitivity was consistently produced in the guinea-pig. This species was thus considered to be a suitable animal model for further investigation of the underlying mechanisms of allergic contact dermatitis. However, there was a slight indication that the sensitivity in guinea-pigs, unlike that of the human, may be lost easily.

SECTION IV - EXPERIMENTAL STUDIES ON THE MECHANISMS OF THE AFFERENT ARC OF THE SENSITIZATION RESPONSE TO NICKEL

OBJECTIVES

In the preceeding section results are presented which demonstrate specific sensitivity to nickel can be induced in the guinea-pig and that this species is a suitable model for further investigations.

The objectives of this section of the project are to assess the absorption and conjugation of nickel within guinea-pig skin in an attempt to identify in which areas of the skin complete antigen formation could occur, and to isolate the conjugates formed.

INTRODUCTION

Absorption of Nickel in the Skin

The highly keratinized stratum corneum of the skin is the main barrier to external chemical insults and is generally considered to be the rate-limiting step in the absorption of compounds applied to the skin. However, consideration must be given to skin appendages (hair follicles, etc.) and the areas of damaged skin where the stratum corneum barrier is broken.

In 1956, Wells reported results of histological studies on the absorption of nickel in human skin biopsy specimens. Nickel was demonstrated in the skin by dithio-oxamide complexation in formalin fixed paraffin sections. "Stained" nickel was seen predominantly in the stratum corneum and the inner root sheath of hairs. Penetration of nickel appeared to occur only at sweat duct and hair follicle openings. However, in sections taken from the skin damaged by needle scratch, nickel was demonstrated in the deeper layers of the stratum corneum and in the Malpighian layer of the epidermis.

In order to examine for passage of nickel salts through the skin, Kolpakov (1963) used human cadaver skin clamped in a diffusion chamber.

Diffusion through the skin could not be demonstrated and the stratum corneum was considered to be the barrier obstructing penetration. Following the effective removal of this barrier, the hypodermis, dermis and Malpighian layer of the epidermis were readily permeable. The greatest accumulation of nickel was found by dimethylglyoxime staining in the Malpighian layer.

These results were confirmed by Samitz and Katz (1976) using a similar diffusion chamber technique on cadaver skin with radioactive ^{63}Ni. Diffusion of nickel through the epidermis was very slight and was not enhanced by sweat or detergents. During a 48 hour period less than 0.1% of the nickel at the outer surface of the skin diffused through the epidermis.

The absorption of radioactive ^{57}Ni through the skin of normal and nickel-sensitive humans has been studied *in vivo* (Nørgaard, 1955 and 1957). The results were converse to the above *in vitro* studies and indicated that approximately 66% of the applied nickel was absorbed within 24 hours. Most of this was absorbed within the first few hours after application. This high level of absorption was similar for both normal and nickel sensitive persons.

It is not clear from the limited published data whether or not nickel salts are absorbed through the skin. The *in vitro* work of Kolpakov and Samitz and Katz was performed on skin taken from human cadavers, which must be considered an abnormal system. Although skin biopsies can remain viable for several days, they are in a steady decline and blood and lymphatic supplies are obviously absent. These factors will influence absorption of a compound.

The in vivo work of Nørgaard was performed using techniques which would be considered to be open to enormous variation by todays standards although it is appreciated that they may have been the best available in 1955. The short half life of ^{57}Ni (36 hours), the method of radioactivity measurement (Geiger-Müller tube placed on the skin site) and the assumption that disappearance of radioactivity equals absorption are considered to limit the use of these data.

The histological results on the accumulation of nickel in the skin (Wells, 1956 and Kolpakov, 1963) were obtained from sections fixed and processed by routine histological methods. In these processes any soluble nickel conjugates would not be demonstrated in the final section. It is important to use techniques which would not remove soluble nickel conjugates since these could represent the complete antigen. This point is explained earlier in the text in Section II (Absorption and Conjugation of the Hapten).

It is evident that the published data on absorption and accumulation of nickel in the skin are insufficient for full and correct assessment at this time. There does not appear to be any publication of animal data.

Evaluation of the absorption and accumulation of the hapten is essential in the complete understanding of the afferent and efferent arcs of the sensitivity response. Further studies on this aspect were undertaken and are described in detail later in this section.

Binding of Nickel to Biological Substances

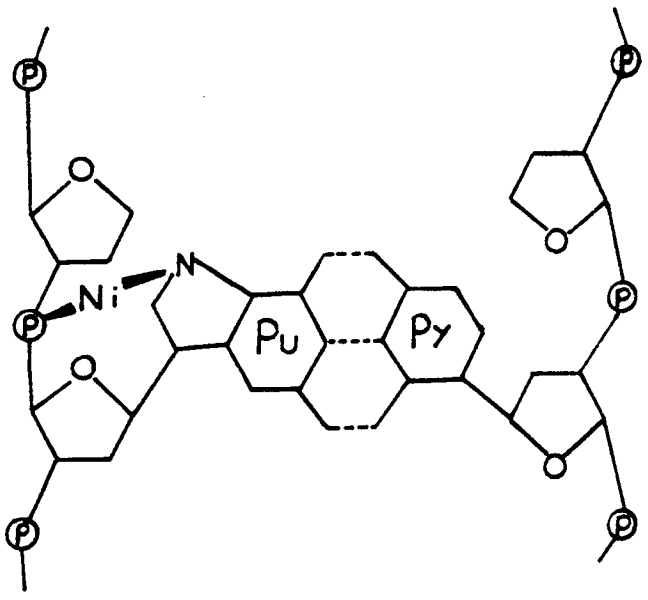
To aid the understanding of the antigenicity of nickel conjugates, knowledge of the nickel binding sites within a tissue or cell and the effects of this binding to a particular site is essential. However, little is known about the sites attacked by nickel within the target tissue or cell and consideration must be given to reactions of nickel ions with isolated

substances. This has been extensively reviewed by the National Research Council (1975).

To Nucleic Acids

Nickel ions have been shown to bind to DNA and RNA (Eichhorn and Shin, 1968) nucleotides (Izatt, et al, 1971; Sissoëff, et al, 1976) and related compounds such as ATP (Glassman, et al, 1971) and thiamine pyrophosphate (White and Drago, 1971). Nickel binds to these materials via the base and phosphate groups (Sissoëff, et al, 1976) and its interaction sites are similar to those of manganese ions and cobalt ions. These sites in DNA and RNA are believed to be between the N_7 of purine bases and the phosphate group of the same strand

e.g.



This binding may have important implications in both the immunologic specificity of allergic contact dermatitis and the carcinogenic effects of nickel and other metal ions. Indeed Sissoëff et al (1976) suggested that a specificity exists in the metal-DNA complexes which is more strict than a chemical recognition of one metal by one base and is dependent on the nature of the metal, the nature of the DNA sequence and on various environmental conditions.

To Proteins, Peptides and Amino Acids

The binding of nickel to proteins has been studied using bovine or human serum albumin (Cotton, 1964; Magnus, 1958; Rao, 1962, Callan and Sunderman, 1973). Imidazole and carboxyl groups have been implicated as the binding site in these studies. Rao (1962) suggested that the imidazole and carboxyl groups compete for the binding of nickel but the carboxyl groups have a much lower affinity.

Callan and Sunderman (1973) studied the binding of nickel to serum albumin of five different species using a technique of equilibrium dialysis. Albumins of dog and pig were found to have a lower affinity for nickel than rat, rabbit or human albumins. The authors postulated that nickel forms a square planar complex involving the terminal amino nitrogen, the first two peptide nitrogens and the imidazole nitrogen of histidine at the third locus from the amino terminus. The dog and pig albumins probably lack the histidine at the third locus thus reducing their affinities.

Nickel binding via carboxyl and amino groups has also been reported for human γ-globulin, casein, gelatine and keratin (Cotton, 1964). However these complexes were considered to be of low stability.

The nickel complexes with simple peptides (diglycines and triglycines) have been studied by X-ray crystallography (Freeman, et al, 1968). In the diglycine complex, nickel was bound to three electron donor sites, the free carboxyl group, the free amino group and the peptide nitrogen. In the triglycine complex, nickel was actively chelated by the terminal amino and the first peptide nitrogen from the N-terminal end of the molecule.

From similar studies (Bryce and Gurd, 1966; Chang and Martin, 1969) it appears that when amino acids containing sulphur or heterocyclic nitrogen atoms are incorporated in a peptide, these electron donor sites also participate in the chelation of nickel. The imidazole nitrogen was implicated in the binding of nickel to glycyl-L-histidine and L-histidyl-L-histidine and cysteine sulphur is involved in the binding to glutathione.

Nickel appears to bind predominantly to amino acids which contain unique functional groups such as those that contain sulphydryl groups (cysteine, cystine, methonine, etc) (Chang and Martin, 1969), those that contain heterocyclic nitrogens (histidine) (Bryce and Gurd, 1966; Morris and Martin, 1970; Barnes and Petit, 1970) and diamino-mono carboxylic acids (lysine) (Hutchinson, et al, 1975).

Amino acids, in isolation, that do not contain unique functional groups chelate nickel through the carboxyl and amino group (Sunderman, et al, 1975).

In this brief review of binding to biological substances it is evident that nickel will chelate with a diversity of materials (DNA, RNA, structural and functional proteins, co-enzymes etc). However, it only indicates that nickel could interfere or participate in biochemical processes in many different ways. In the mechanisms of allergic contact dermatitis, it is important to consider which conjugates are formed in the skin and which could represent the complete antigen.

Possible Nickel Antigens in the Skin

In view of the data suggesting that nickel will bind with a diversity of biological substances, it is not surprising that published information on the identity of nickel allergens in the skin is conflicting.

From the early work of Wells (1956) keratin was considered to be the binding site in the skin, with nickel bound predominantly to the free carboxyl groups. However, the nickel-keratin complex was later considered to be of low stability (Cotton, 1964) and was unlikely to act as a complete antigen. This latter concept was conceived earlier from human experiments with nickel salts (Everall, et al, 1954). These workers, in fact, refuted the hapten concept and suggested that nickel ions alone are the antigens. However, these

interpretations did not take account of the high binding ability of nickel with biological substances or the possible poor absorption of nickel ions.

Attempts to induce sensitization in pigs (Jansen, et al, 1964) and guinea-pigs (Samitz, et al, 1975) using nickel-amino acid conjugates have also proved to be conflicting. Jansen, et al, reported successful sensitization of pigs with Ni-alanine complex, which was considered to be a better sensitizer than nickel sulphate per molecule of applied substance.

These results could not be reproduced in the guinea-pig using nickel conjugated with several amino acids (alanine, tyrosine, glycine, phenylalanine) or with nickel-guinea-pig skin extract (Samitz, et al, 1975). Sensitization was not induced with nickel sulphate either, which possibly indicates that the induction procedure of three separate intradermal injections alone was insufficient.

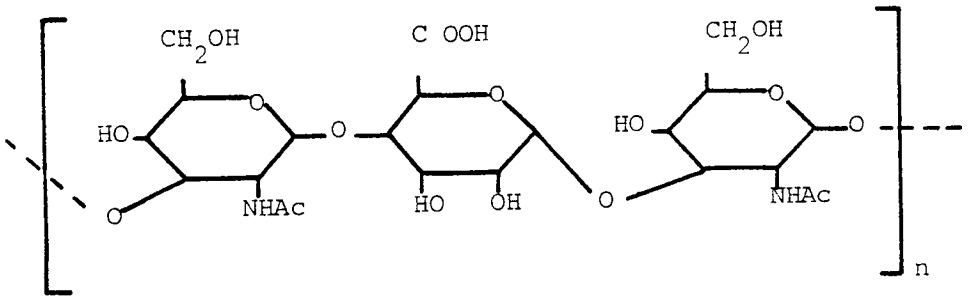
Jansen, et al (1964) and Berrens (1965) proposed that the low molecular weight conjugates of haptens act as messengers for antigenic information to the reticulo-endothelial system. This concept is presented earlier in Section II.

These data for nickel are again limited and conflicting and cannot clearly indicate which conjugate or conjugates can act as the complete antigen. However, results of studies with other haptens may allow, by analogy, a more complete assessment of the underlying mechanisms.

In studies of chromium sensitivity, in both humans (Cohen, 1968) and guinea-pigs (Cohen, 1966), conjugates of chromium III with heparin or hyaluronic acid were shown to be reactive in sensitive humans and able to induce specific sensitivity in guinea-pigs.

Hyaluronic acid is a mucopolysaccharide composed of D-glucuronic acid and N-acetyl-D-glucosamine subunits,

e.g.



It is a widely distributed naturally occurring polymer, of variable M. Wt., and is usually found in association with protein. It exists in human skin as a constituent of the connective tissue ground substance. Cohen (1968) suggested that chromium binds to the free carboxyl of the D-glucuronic acid subunit. This moiety is also likely to bind nickel.

In more recent experiments to isolate hapten-protein complexes in picryl chloride sensitivity (Miyagawa, et al, 1977 and 1978) some success was apparent. In these studies epidermis from picryl chloride sensitive guinea-pigs was homogenised and repeatedly frozen and thawed in order to obtain a soluble extract of the epidermis which was separated by Sephadex gel fractionation. Three main protein containing fractions were separated which were characterised by immunoelectrophoresis against rabbit anti-guinea-pig epidermis and rabbit anti-guinea-pig whole serum. The antigenicity of each fraction was also assessed by macrophage migration inhibition assays (an in vitro test system based on the ability of antigen to inhibit the migration of macrophages obtained from sensitized animals).

Only one fraction demonstrated antigenicity and was shown to consist of at least six epidermal proteins of molecular weight 10,000-200,000. The remaining two fractions (M.Wt. > 200,000 or < 10,000) did not show any antigenicity and were not entirely of epidermal origin.

These data demonstrate that water soluble proteins of picryl chloride treated guinea-pig epidermis appear antigenic in the test systems used. However, it is important to note that the procedures for preparation of epidermal extracts were very stringent and obscure the origin of the proteins within the epidermis. It is possible that carrier proteins might be solubilized from cellular organelles.

This alternative concept is supported by similar experiments in DNCB sensitized guinea-pigs (Parker and Turk, 1970; Nishioka, et al, 1971; Nishioka, et al, 1973). In these studies subcellular components (mitrochondria, lysosomes, microsomes) were fractionated from DNCB painted epidermis of sensitized guinea-pigs by homogenisation and ultra-centrifugation. Results of the earlier studies indicated that specific sensitivity could be induced in guinea-pigs by the DNCB-microsomal fraction. In the later experiments an in vitro assay was used to assess antigenicity. This was evaluated by the ability of each subcellular fraction to stimulate lymphocytes from DNCB sensitive guinea-pig donors to release the lymphokine MIF (macrophage inhibition factor). This was in turn assayed by the macrophage inhibition test. Significant inhibition of macrophages was only obtained by pretreatment of sensitive lymphocytes with the microsomal fraction. This fraction coupled with DNCB was suggested to be the complete antigen.

The ability ~~for~~ of nickel to bind to subcellular organelles has been demonstrated with nickel carbonyl (Sunderman and Selin 1968), nickel acetate (Whanger 1973) and nickel chloride (Oskarsson and Tjälve 1979). The results of these studies indicate nickel binds to nuclei, mitochondria and microsomes of lungs, liver and kidneys after exposure by inhalation, intravenous injection or dietary inclusion.

Processing of haptenic or antigenic information by the metabolic/catabolic organelles of epidermal cells and cells of the immune system (e.g. macrophages, Langerhans cells) could be an important function in the induction phase.

In summary, it is apparent from the published data that several aspects of the afferent arc of sensitization to nickel require further investigation.

1. Most of the data on the absorption and accumulation of nickel has been based on skin biopsies, which may be considered to be an atypical system. The limited in vivo work was performed with techniques and based on assumptions which may not be acceptable by today's scientific standards. Therefore, some further in vivo investigations are necessary.

2. Data on the conjugation of nickel demonstrates that the metal ion binds to a diversity of biologically important substances but the nature of the antigen is not apparent.

3. Is the true antigen formed with soluble protein or sub-cellular fractions and are there several antigens?

EXPERIMENTS ON THE ABSORPTION AND ACCUMULATION OF NICKEL IN GUINEA-PIG SKIN

MATERIALS AND METHODS

The absorption and conjugation of nickel in guinea-pig skin were examined by the use of the radionucleotide ^{63}Ni

The investigations were carried out in five interlinked phases;

1. qualitative assessment of absorption and accumulation
2. quantitative assessment of absorption and accumulation
3. quantitative assessment of sub-cellular distribution
4. isolation of sub-cellular organelle Ni-conjugates
5. isolation of soluble conjugates.

The methods and materials used for each phase are described separately.

In the previous animal studies (Section III) dermal reactions indicative of sensitization peaked within 48 to 72 hours of dosing. It was considered that absorption and conjugation sufficient to elicit a response occurred within 48 hours. This time period was therefore selected as the maximum for these investigations.

Nickel containing test compound

Radioactive nickel chloride ($^{63}NiCl_2$) in 0.1M hydrochloric acid was obtained from the Radiochemical Centre Ltd., Amersham, Buckinghamshire, England. ^{63}Ni is a beta emitting nucleotide of low energy (67 keV) (McKay 1971) and long half-life (100 years) (Barnes *et al* 1971). The

specific activity of the obtained preparations was between 80.6 and 81.0µg Ni (II)/mCi.

Test solutions of ^{63}Ni for the various studies were prepared by quenching in cold aqueous nickel sulphate solution.

Animals

The guinea-pigs used in this study were random-bred albino animals from the Dunkin Hartley strain. Young, mature and healthy animals only were used with the same age and weight criteria described in Section III.

The method of housing, feed and environmental conditions were as previously described in the sensitization studies in Section III.

QUALITATIVE ASSESSMENT OF ABSORPTION AND ACCUMULATION

This phase was studied in guinea-pig skin by the technique of micro-autoradiography.

Administration of Test Compound

Test solutions of ^{63}Ni of approximately 10µCi/ml were prepared with cold nickel sulphate solution (5% w/v aqueous).

A total of 24 guinea-pigs were used which were allocated to six groups, each composed of four animals. Prior to administration of the test material the left flank of each animal was clipped free of hair with electric clippers and shaved with an electric shaver. A volume of 0.2ml of the $^{63}NiCl_2/NiSO_4$ mixture was applied to a 1 x 1cm piece of surgical lint on a strip of occlusive adhesive tape (Blenderm, 3M Company, Minnesota, U.S.A.) which was in turn attached to a length of adhesive bandage (Elastoplast, Smith & Nephew Co. Ltd., Hull, England). The patch was placed on the clipped and shaven

flank of one animal and the bandage wrapped firmly around the trunk. The time period of exposure for each group of animals was as follows:-

Group	Exposure time (hours)	No. of animal
1	0.5	4
2	1	4
3	4	4
4	12	4
5	24	4
6	48	4

Preparation of Skin Samples

Following the respective exposures each animal was killed by pentobarbitone overdosage (Expiral, Abbott Agricultrual Veterinary Products, Kent, England) and the bandage removed. The skin at the treatment sites was excised, dissected free of subcutaneous fat and cut into several strips measuring approximately 0.2 x 1cm. Each strip was folded in half with epidermis on the outside and immersed in isopentane in a liquid nitrogen bath (-160°C).

From this stage onwards all procedures were performed at -20°C and all equipment pre-cooled, in an attempt to maintain to a minimum any localised thawing of the tissue and possible spread or loss of soluble radioactive material.

Preparation of Autoradiographs

The techniques of autoradiography were similar to those described by Rogers (1969) and Pelc (1956). Minor modifications and developmental changes were made where the techniques were not practical for the laboratory facilities or materials used.

All procedures involving the undeveloped autoradiographic film were performed under Kodak Safelight filter No. 1 (red) with a 15w bulb and set at least four feet from the working surfaces.

Fine grain autoradiographic stripping film AR.10 (Kodak, Hemel Hempstead, England) was used. Sections of the film (approximately 3 x 5cms) were stripped from the glass backing plate and floated, emulsion site upwards, on a bath of distilled water at room temperature. Each section was allowed to swell for approximately 2 minutes.

Microscope slides were prepared by wetting one side with dilute gelatin solution (approximately 1% - to aid adhesion of the stripping film) and by placing a coverslip on the other side. The swollen stripping film was then picked up from the water bath, such that the coverslip was sandwiched between the film and microscope slide.

A diagram of the total slide preparation is presented as Figure 15.

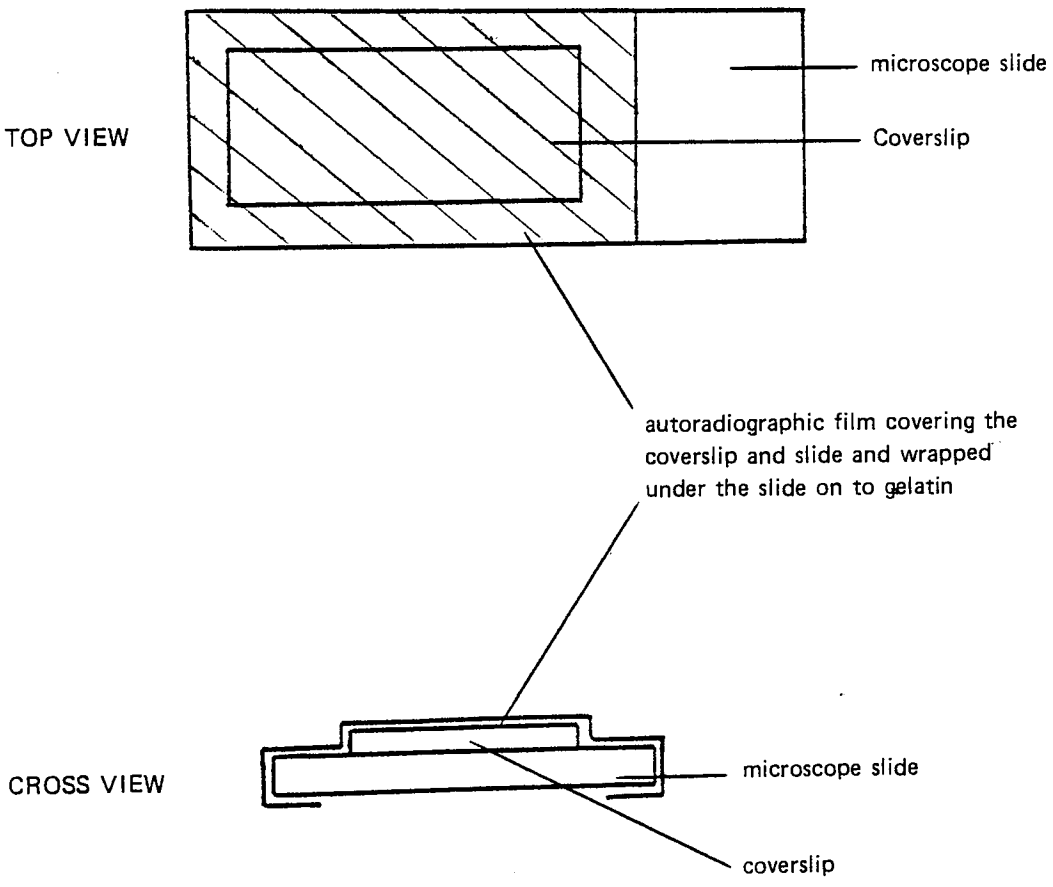
Each slide was air-dried for approximately 4 hours, further dried over silica gel crystals for 48 hours in a lightproof bag, transferred to a lightproof box, and cooled to -20°C.

Serial sections of skin were cut at approximately 10µm thickness using a cryostat microtome (-20°C) and picked up directly on to the precooled autoradiographic film prepared as above. Autoradiographs were then exposed in lightproof boxes for 21 days at a temperature of -20°C.

At the end of this exposure period slides were allowed to thaw to room temperature and dipped in a dilute gelatin solution (~ 1%) to avoid section movement. The tissue sections were fixed in 5% acetic acid in ethanol for 2 minutes and washed in tap water for 4 minutes prior to photographic development.

FIGURE 15

Autoradiographic slide preparation



Each slide was developed in Kodak D-19 developer for 5 minutes, placed in a stopbath of 1% acetic acid for 1 minute, fixed in 30% sodium thiosulphate for 10 minutes and washed in tap water for 4 minutes.

Tissue fixation and film development procedures were performed at 18°C. At higher temperatures the risk of film and section detachment is increased.

All slides were air-dried prior to histological staining. The sections were then stained with Ehrlich's haematoxylin and eosin to show morphological features and then air dried again.

The preparation was removed from the backing slide (by cutting through the film at the edges of the coverslip) and mounted on a fresh slide using DPX medium (Raymond A. Lamb, Middlesex).

Tissue sections were viewed through the autoradiographic film by light microscopy.

The following control slides were prepared with each batch of test sections:-

1. Non-radioactive normal skin section + autoradiographic film - to define any staining and development artefact.

2. Radioactive test skin section without autoradiographic film - to define any staining and development artefacts.

3. Radioactive test skin section + previously light exposed autoradiographic film - to detect any negative chemiography.

QUANTITATIVE ASSESSMENT OF ABSORPTION AND ACCUMULATION

Administration of Test Compound

Test solutions containing 40μCi of ^{63}Ni were prepared with non-radioactive nickel sulphate solution (1% w/v aqueous).

A total of 19 guinea-pigs were used in four groups. Prior to the administration of the test solution both flanks of each animal were clipped free of hair with electric clippers to expose an area of skin approximately 40cm^2 per flank. A volume of 6ml of the test solution was applied over two 4 x 4cm patches of surgical lint (~ 3ml/patch) on a length of occlusive adhesive bandage (Elastoplast backed with Sleek, Smith & Nephew Co. Ltd., Hull, England). The patches were placed on the clipped flanks of one animal and the bandage wrapped firmly around the trunk.

The time periods of exposure for each group of guinea-pigs were as follows:-

Group	Exposure time (hours)	No. of animal
1	4	4
2	12	3
3	24	7
4	48	5

Each animal was housed in a metabolism cage for the collection of urine throughout the respective exposure period.

The minimum time period of 4 hours was selected on the basis of the results of microautoradiography. At this time accummulation in the epidermis had commenced.

The dose preparation containers were retained for the estimation of residual dose for inclusion in the calculation of total radioactivity recoveries.

Samples for analysis

At the end of each exposure period for each animal, samples of plasma, urine, epidermis, bandage and skin washings were collected for analysis in the following manner.

A 10ml blood sample was withdrawn by cardiac puncture under light ether anaesthesia. Each sample was placed in a heparinised container, centrifuged at approximately 3,000 r.p.m. and the plasma pipetted in a separate container.

The total volume of urine excreted by each animal was measured and an aliquot decanted into a separate container.

Each animal was killed after blood sampling and the bandage removed. Care was taken to minimise the spread and loss of residual radioactivity. The exposed skin sites and surrounding areas of each animal were thoroughly washed with distilled water ($\sim$ 200ml) and the washings collected. The final volume of the washings was measured and an aliquot decanted into a separate container. The bandage from each animal was cut into small pieces and placed in distilled water and shaken for 3 hours. An aliquot of the resultant liquor was decanted into a separate container.

Samples of plasma, urine, skin washings and bandage extractions were stored at -20°C prior to further analysis.

The epidermis of each flank of each animal was removed by surgical keratome (Silvers knife, Downs Surgical Ltd., Mitcham, Surrey, England) across the whole area of exposure. The epidermal sample was weighed and immediately processed for further analysis and isolation of nickel conjugates.

The epidermis only was selected for examination on the basis of the time-coursed autoradiographic investigations. These qualitative results, which are presented later, indicated accumulation in the epidermis, hair follicles and highly keratinized areas of the skin only. Radioactivity was not observed in the dermis.

Preparation of Epidermal Samples

The methods used to prepare epidermal homogenates were performed either on ice or at +4°C. All solutions were maintained at +4°C.

The epidermal slices from each guinea-pig were minced finely with scissors on a glass plate on ice and homogenised in sucrose (5% w/w) in a glass/teflon Potter-Elvehjem homogeniser. Two to three strokes with the teflon plunger rotating at approximately 150 r.p.m. were used to disrupt the epidermal samples.

Several methods for homogenization of the epidermis were attempted during the development of techniques and the above method was selected as providing the greatest yield for the least disruption of subcellular

organelles. This method was also preferred to enzymic solubilization which could release nickel from protein conjugates or denature the conjugates.

The epidermal homogenate was then filtered through nylon mesh to remove non-homogenisable debris and hair shafts. The filtrate was centrifuged for 2 minutes at 1500 r.p.m. (~600g) to remove unfiltered non-homogenisable debris and 20ml of the supernatant was immediately processed for isolation of nickel conjugates.

The non-homogenisable debris obtained by filtration and light centrifugation was pooled. A sample of the debris (200-300mg wet weight) was placed into 1ml of a tissue solubilizer (Solune 350, Packard Instrument Ltd., Caversham, Berks., England) and incubated at 60°C for 48 to 72 hours. The digest was then diluted with 5ml distilled water prior to estimation of radioactivity.

Ultracentrifugation of Epidermal Homogenates

The technique of rate zonal ultracentrifugation across a linear sucrose gradient was used to examine the subcellular distribution of nickel and to isolate nickel conjugates.

This technique was preferred to differential rate centrifugation or density gradient centrifugation in conventional rotors because:-

a. the technique allows relatively large volumes of whole tissue homogenate to be centrifuged in one run. This is important when the homogenate is composed of organelles which are largely of unknown size (Hinton and Dobrota 1976).

b. developmental studies using differential rate centrifugation did not yield organelle fractions of sufficient purity to allow an interpretation of nickel distribution.

Centrifugation

A B-XIV titanium zonal rotor was used for these studies fitted to a Superspeed 65 ultracentrifuge (MSE Scientific Instruments Ltd., Crawley, Sussex, England). The sucrose gradient was made using an MSE gradient maker and the rotor was loaded and unloaded by peristaltic pumps. The experimental set-up is presented diagramatically as Figure 17.

The rotor, sucrose and test samples were precooled to +4°C.

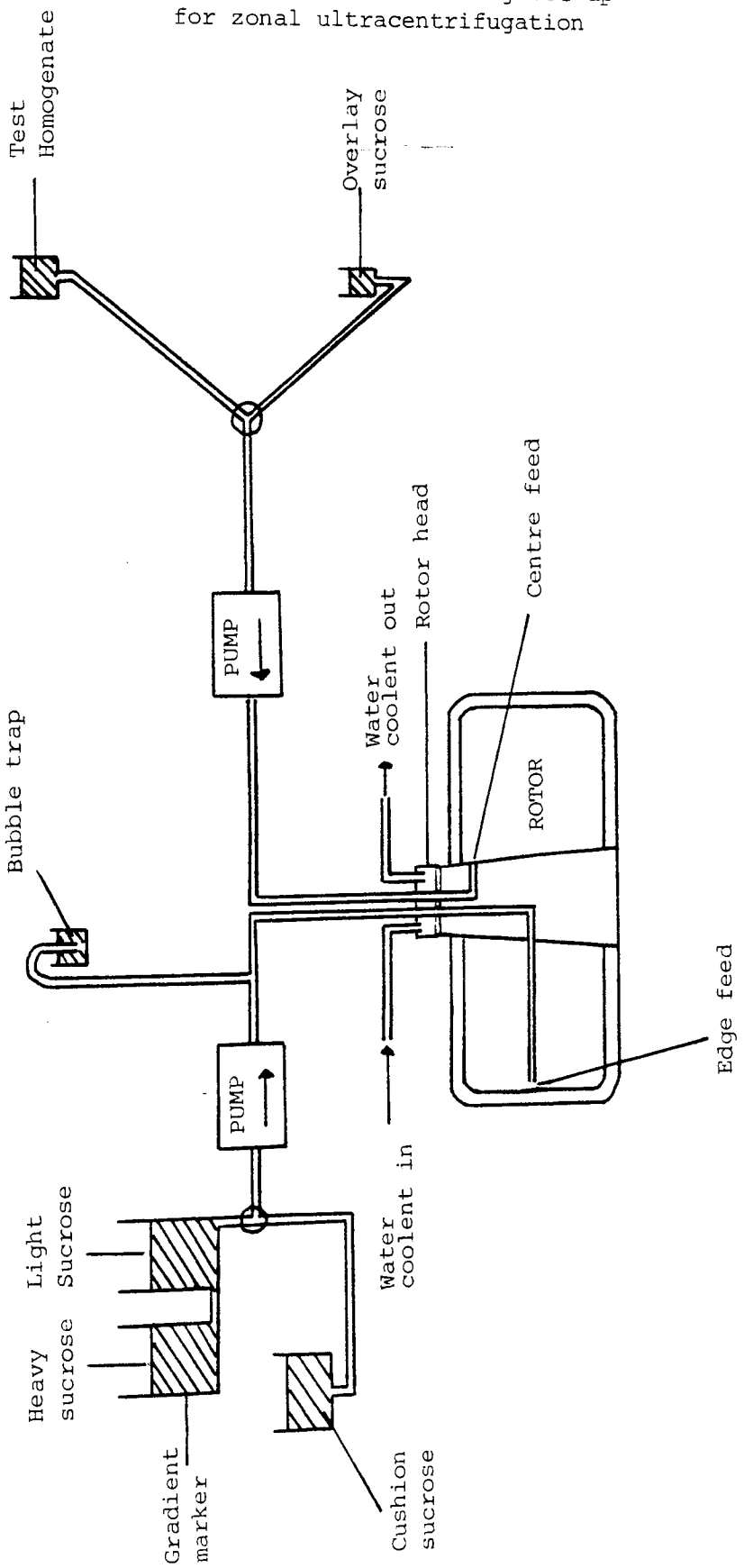
A 400ml gradient of 10 to 40% w/w sucrose was formed by dynamically loading the rotor via the edge feed, with the rotor spinning at 2,000 r.p.m. The remaining capacity of the rotor was filled with 55% w/w sucrose as a cushion.

The flow rates during loading were reduced (30 to 10ml/minute) to suit the viscosity of the sucrose and prevent excessive back pressure and resultant cross leakage at the loading head.

The test homogenate (20ml) followed by 2% w/w sucrose as overlay (25ml) were loaded via the central feed. A flow rate of 2.75ml/minute was used in an attempt to minimise zone broadening

Figure 16

Diagramatic view of loading set-up
for zonal ultracentrifugation



After loading, the centrifuge was closed, a vacuum developed (100 torr) and the rotor brought to 16,000 r.p.m. for 45 minutes at +4°C.

The rotor was unloaded at 2,000 r.p.m. by displacing the gradient with cushion sucrose (55% w/w) via the edge feed. Twenty fractions of 20ml each were collected from the central feed.

Assay of fractions

Each zonal fraction and the original homogenate were assayed for the following parameters:

1. ^{63}Ni content by scintillation counting
2. sucrose density by gravimetric measurements
3. total protein by the method of Lowry et al (1951)
   This colorimetric assay is based on the reduction of phosphomolybdate-phosphotungstate reagent (Folins) by tyrosine and tryptophan residues of protein. Optical densities were measured at 750nm
4. marker enzymes;
   a. Succinate dehydrogenase as a mitochondrial marker using the method of Prospero (1974). The assay is based on the reduction of 2-(p-iodophenyl)-3-(p-nitrophenyl) -5-phenyl tetrazolium chloride (INT). Sodium succinate is the substrate. Reduced INT is measured colourimetrically at 490nm.

b. acid phosphatase as a lysosomal marker using the method of Robinson (1972) which is based on the fluoride sensitive hydrolysis of ∝-naphthyl phosphate. Estimation of released ∝-naphthyl is by colour reaction with diazotized Fast Red ITR and measurement at 550nm.

c. catalase as a peroxisomal marker using the method of Baudhuin (1964), which is based on the disappearance of hydrogen peroxide from imidazole buffer. The reaction is stopped with acidified titanium sulphate and the yellow 'peroxy' titanium sulphate measured colorimetrically at 410nm.

d. glucose-6-phosphatase as microsomal marker using a two stage assay; firstly the fluoride insensitive hydrolysis of glucose-6-phosphate described by Connock et al (1971) and secondly estimation of released inorganic phosphate by the colorimetric assay of Baginski et al (1967) at 700nm.

Control centrifugations were performed using epidermal homogenates from six non-exposed normal guinea-pigs. Epidermal samples were taken and the homogenates prepared in an identical manner to that described for the test animals. Homogenates from three animals served as controls for the centrifugation procedures by adding $^{63}NiCl_2$ to the homogenate. Homogenates from the remaining three animals served as controls for the combined homogenization and centrifugation procedures by adding $^{63}NiCl_2$ to the sucrose solution used for homogenization. In each case 82µl of a standard solution of $^{63}NiCl_2$ was added (1.87×10^6 d.p.m. total).

Equilibrium Dialysis of Epidermal Fractions

The technique of equilibrium dialysis was performed using the Dianorm system (MSE Scientific Instrument Ltd.) to quantitatively distinguish between bound and unbound nickel in the main radioactivity containing zonal fractions. The soluble (fraction 2) and microsomal (fraction 4) samples were examined in this way for each centrifuged epidermal homogenate, including the controls.

A series of teflon cells were constructed with presoaked benzoylated dialysis membrane (Sigma Chemical Co., Poole, Dorset, England). The molecular weight cut off for this membrane is approximately 2000 daltons. Epidermal fractions were dialysed against 5% w/w sucrose and approximately 1.4ml was loaded in each respective half cell. The dialysing volume and area under this system are 1.0ml and 4.52cm^2 respectively.

The constructed and loaded cells were rotated at 12 r.p.m. (to ensure mixing) for 3½ hours at room temperature. The time was predetermined from developmental experiments using free $^{63}NiCl_2$ in 5% w/w sucrose.

After the required dialysing time 0.5ml of the fractions and dialysate were unloaded from the respective half cells and the ^{63}Ni content assessed by scintillation counting.

The level of bound and unbound ^{63}Ni were calculated as follows:

$$CL_{(b)} = CL_{(T)} - 2 \times CL_{(f)}$$

where $CL_{(f)}$ = the concentration of free ligand (^{63}Ni) in the dialysate (cell B). Since equilibrium exists in the system $CL_{(f)}$ is the same on both sides of the membrane.

$CL_{(T)}$ = total concentration of ^{63}Ni in both the dialysate (cell B) and the test fraction (cell A)

$CL_{(b)}$ = concentration of bound ^{63}Ni in the test fraction.

Levels of bound and free ^{63}Ni were expressed as absolute values and as a percentage of the total concentration.

Analytical recoveries of total radioactivity were calculated as percentages using $CL_{(T)}$ and the total starting concentration of ^{63}Ni in tested fractions ($CL_{(o)}$).

Liquid Scintillation Counting

Radioactivity was measured by a Phillips PW 4510/01 Automatic Liquid Scintillation Analyser or an Intertechnique SL 4000 Analyser using an external standard quench correction method (Kyobashi and Maudsley 1969). The analysers were calibrated using chemically quenched standard preparations of $^{63}NiCl$. The quenching agent was chloroform and volumes of 10 to 800µl were added to a known quantity of $^{63}NiCl$ in scintillation fluid. Packard MI-31, (Packard Instrument Ltd., Caversham, Berks., England) was the scintillation fluid. This is a pseudo-cumene (1,2,4-trimethylbenzene) based cocktail containing 2,5-diphenyloxazole (PPO) and p-bis-(o-methylstyryl)-benzene (bis-MSB) as scintillators.

Test sample volumes of 0.5 or 1ml were prepared for counting in 15ml of scintillator in glass vials. Distilled water (0.5ml) was also added to the preparations of zonal centrifugation fractions to keep the sucrose in solution.

Standard $^{63}NiCl_2$ containing preparations and background count vials were included with each batch of test samples counted. The background values were subtracted from each test sample result.

The counting time was 4 or 10 minutes and counting efficiency was approximately 63% for the Phillips machine and 71% for the Intertechnique analyser.

RESULTS

QUALITATIVE ASSESSMENT OF ABSORPTION AND ACCUMULATION

The results of the micro-autoradiographic studies on guinea-pig skin are presented as Figures 17 to 30 These photomicrographs are considered to be examples representative of the whole study and in each case are presented with a legend describing any apparent labelling by radioactivity.

It is important to note that each autoradiograph consists of an approximately 10μ thick tissue section which is viewed through the film of approximately 15μ thickness. Silver grains, formed in the autoradiographic film as a result of ionising radiation, will be apparent throughout the film. Any evidence of radioactivity is, therefore, at a higher plane of focus than the tissue section and the two images cannot be presented together in total definition. An attempt has been made in most examples to pictorially present the results in terms of labelling in a general area of the skin, at the expense of greater structural resolution.

The overall results of control sections were as follows:-

1. Staining and film development artefacts:
   Dark areas were noted at the edges of hair shafts and outer edges of hair follicles and could be mistaken for radioactive labelling at lower power magnification. This was particularly evident where hair shafts were cut obliquely. This problem was overcome by checking at

higher magnification for the grains indicative of radioactivity.

Pale uneven histological staining was evident in some sections and was considered to be due to an uneven gelatin layer applied prior to development.

2. Negative chemiography:

This artefact was not detected in any of the previously light exposed control slides.

In the test sections, little evidence was produced to suggest that nickel is absorbed across the dermis or accumulates within the dermis during the 48 hour period. However, passage of radioactive nickel was apparent down hair shafts and into the root sheath of hair follicles within a one hour exposure period and absorption of nickel could be seen into the epidermis following a four hour exposure. These observations suggest that passage down skin appendages could be an early route of entry through the skin. Absorption across the stratum corneum may be a route into the epidermis although occurring slightly later.

Following exposure of guinea-pig skin for periods of 4 hours and above, accumulation of nickel was apparent in the stratum corneum and at lower levels of the epidermis. In many sections, marked labelling at the openings of hair canals was noticed and was considered to be a physical artefact produced by the method of application of nickel solution.

Marked labelling of the stratum corneum, labelling within certain areas of the epidermis and of individual cells were apparent in most sections from guinea-pigs exposed for 4 hours to 48 hours. However, variation in the intensity of labelling was noted in individual animals. This variability

may be due to one or more of the following factors:-

1. the pressure of patch application and bandaging;
2. variation in the efficiency of hair clipping and shaving;
3. individual animal variation in stratum corneum thickness;
4. low stability of nickel to keratin binding (Cotton, 1964).

The general picture of stratum corneum labelling was similar at each exposure period above one hour.

Of particular interest in three of the four animals exposed for 4 hours, was a fine line of radioactive labelling along the middle of the epidermis at the stratum lucidum/stratum granulosm level. This specific line of labelling was not as clearly evident in skin sections taken from animals exposed for greater than 4 hours. It may be noted that this general area is where epidermal Langerhans cells have been demonstrated by other techniques (Juhlin and Shelly, 1977; Stingl, et al, 1978a). In the reports of light microscopy demonstration of Langerhans cells (Juhlin and Shelley, 1977), nickel was employed as an active agent. The autoradiographic observations may be related to this apparent Langerhans cell/nickel affinity but similar labelling was not seen in skin sections of animals exposed for greater than 4 hours. The biological relevance of these autoradiographic observations remains uncertain at this stage.

At the higher magnifications of the micro-autoradiographs prepared from skin exposed for 4 to 48 hours, radioactive labelling of individual basal and supra-basal epidermal cells was discernible. The general pattern of labelling was similar at each exposure period of 4 hours and above, and in many appeared to form silhouettes of the cells. It is not possible using

this technique to draw definite conclusions of whether nickel binds to the cellular membranes or accumulates in the extracellular spaces. However, cellular involvement in the induction of sensitivity is an important consideration in the design of further studies as indicated by the work of Nishioka, et al (1971 and 1973).

The results of these qualitative assessments are further discussed in conjunction with quantitative analyses at the end of this section of the thesis.

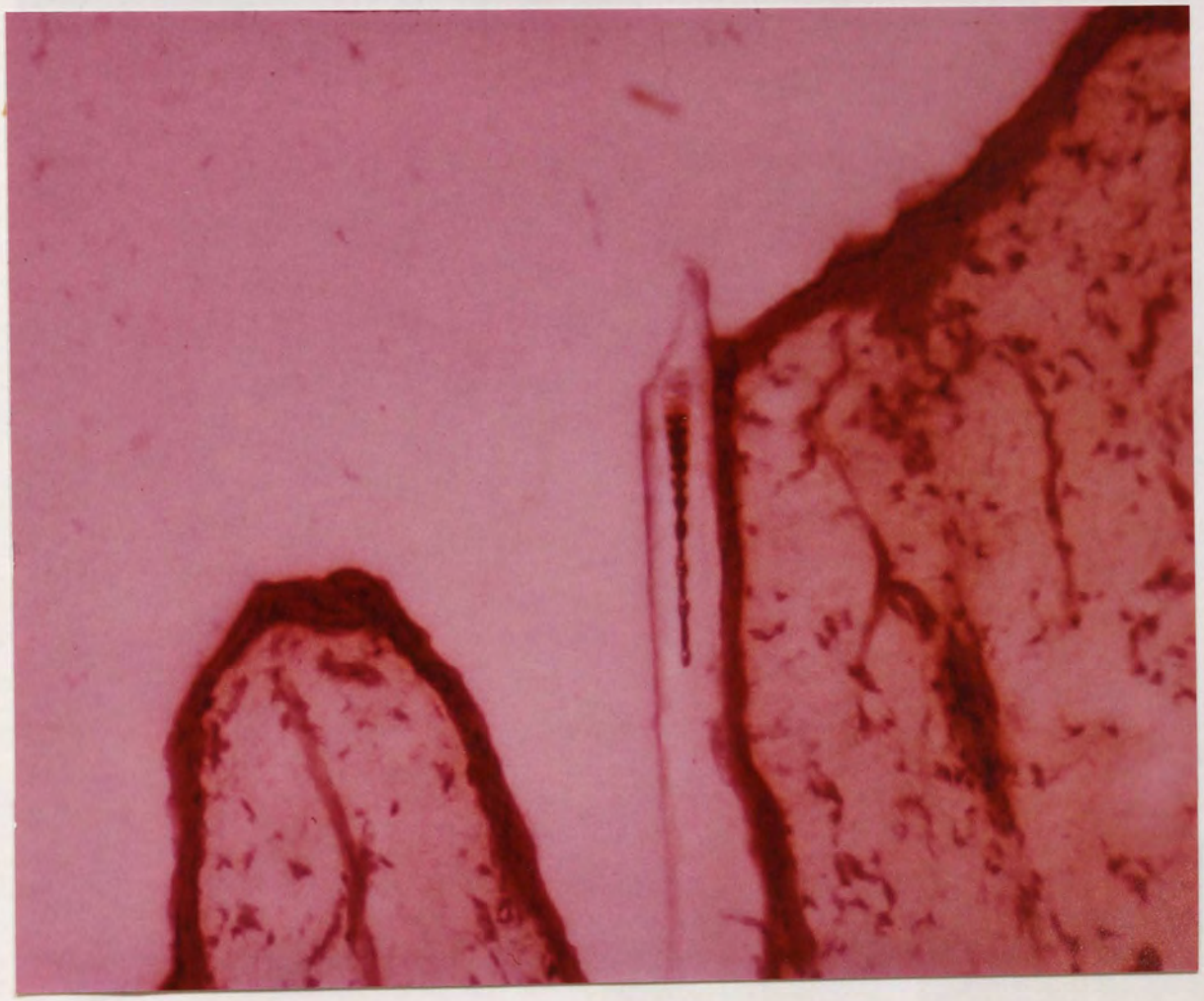


Figure 17

Guinea-pig skin following 0.5 hour exposure to ^{63}Ni. There is little evidence to suggest accumulation of ^{63}Ni in the epidermis, dermis or hair shaft. (H & E X67)

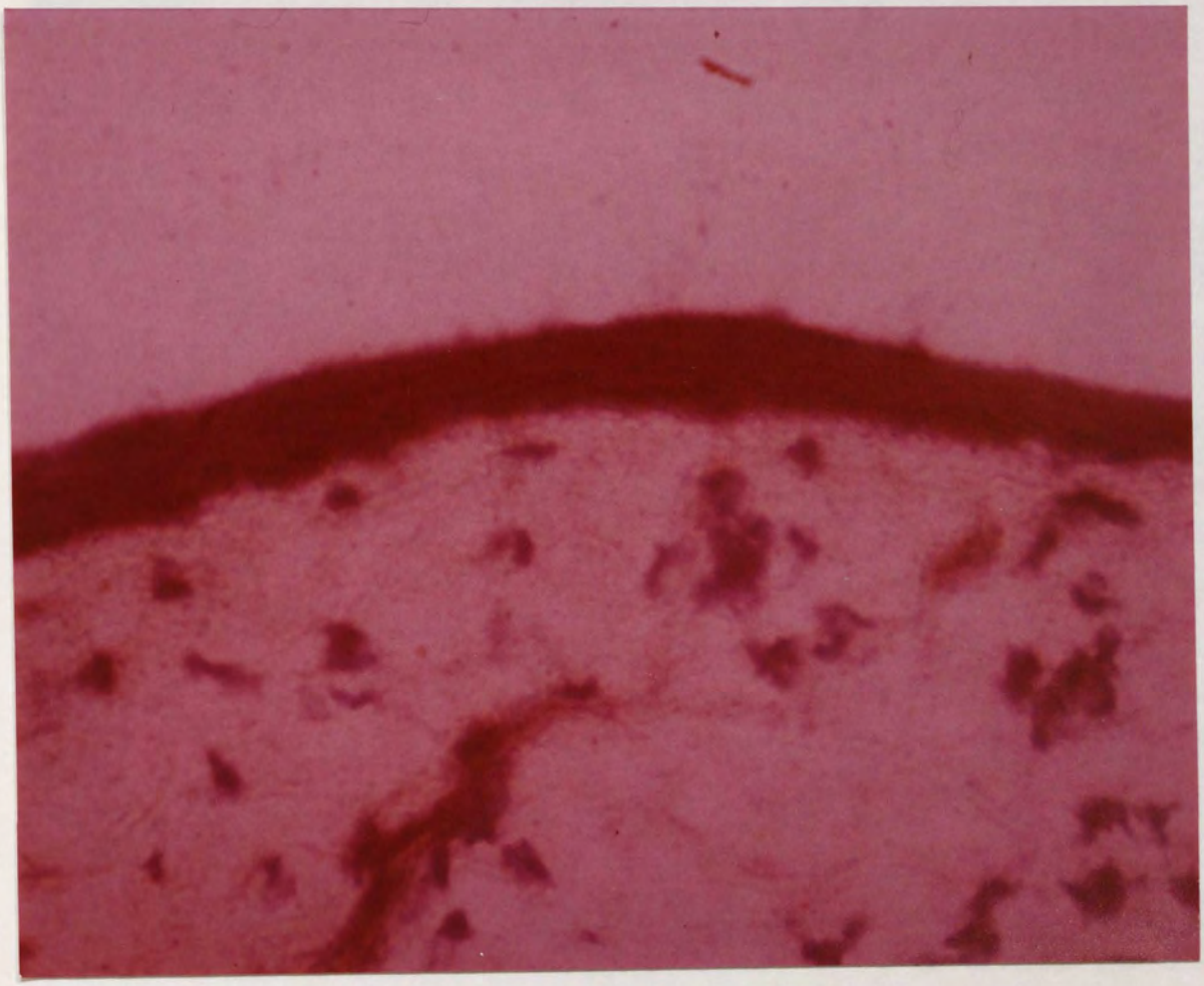


Figure 18

Guinea-pig skin following 0.5 hour exposure to ^{63}Ni. No accumulation of radioactivity is apparent in the epidermis or dermis. The stratum corneum of this animal is thin, but there is no apparent nickel binding. (H & E X208)

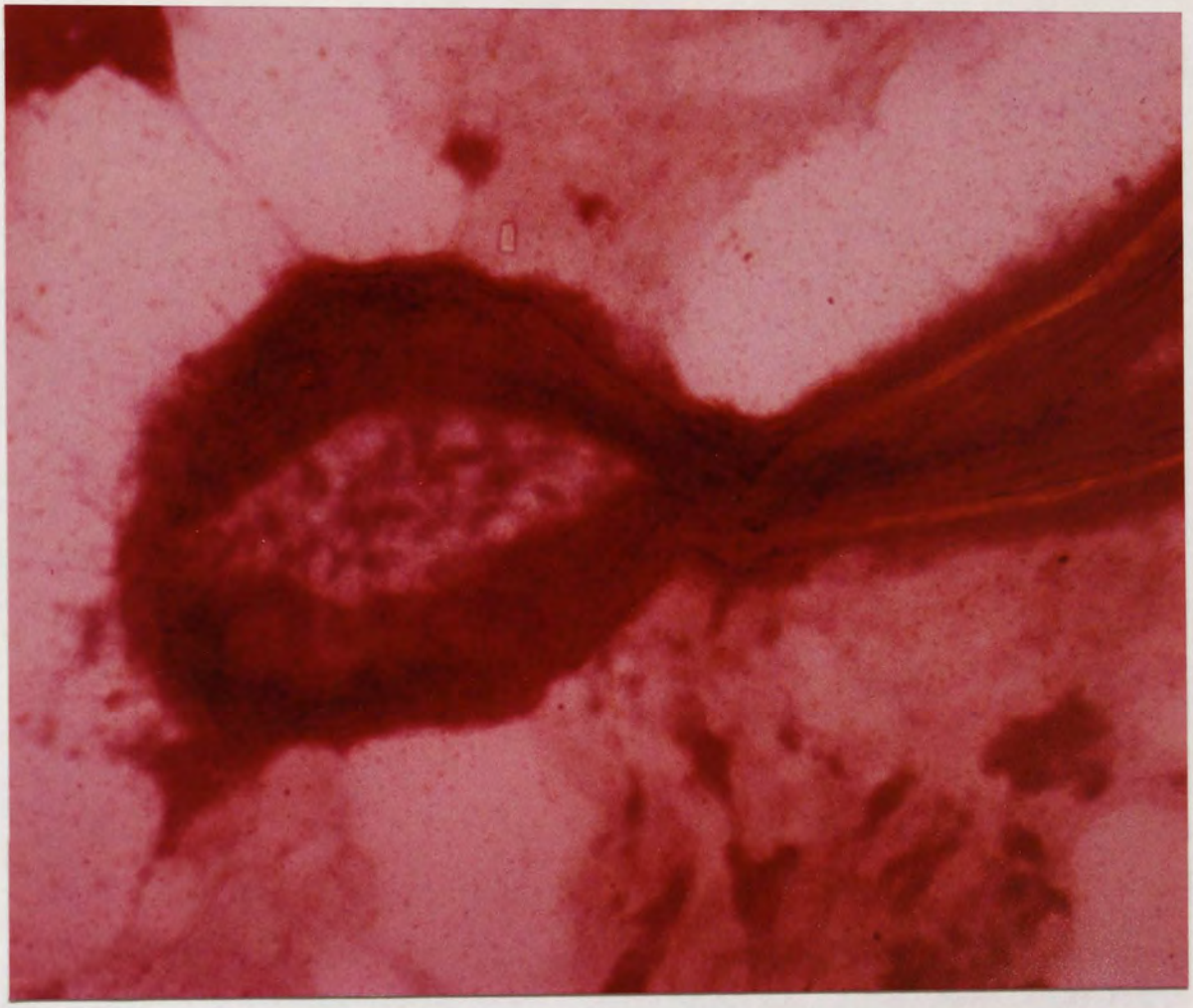


Figure 19

Hair follicle of guinea-pig skin following 1 hour exposure to ^{63}Ni. Labelling can be seen predominantly in the outer root sheath of the bulb and to a lesser degree in the inner root sheath. This appears to extend to the upper areas of the hair follicle. Although the cortex of the follicle is not continuous in this section, there does not appear to be any radioactivity in either the bulb or upper cortical areas. (H & E X130)

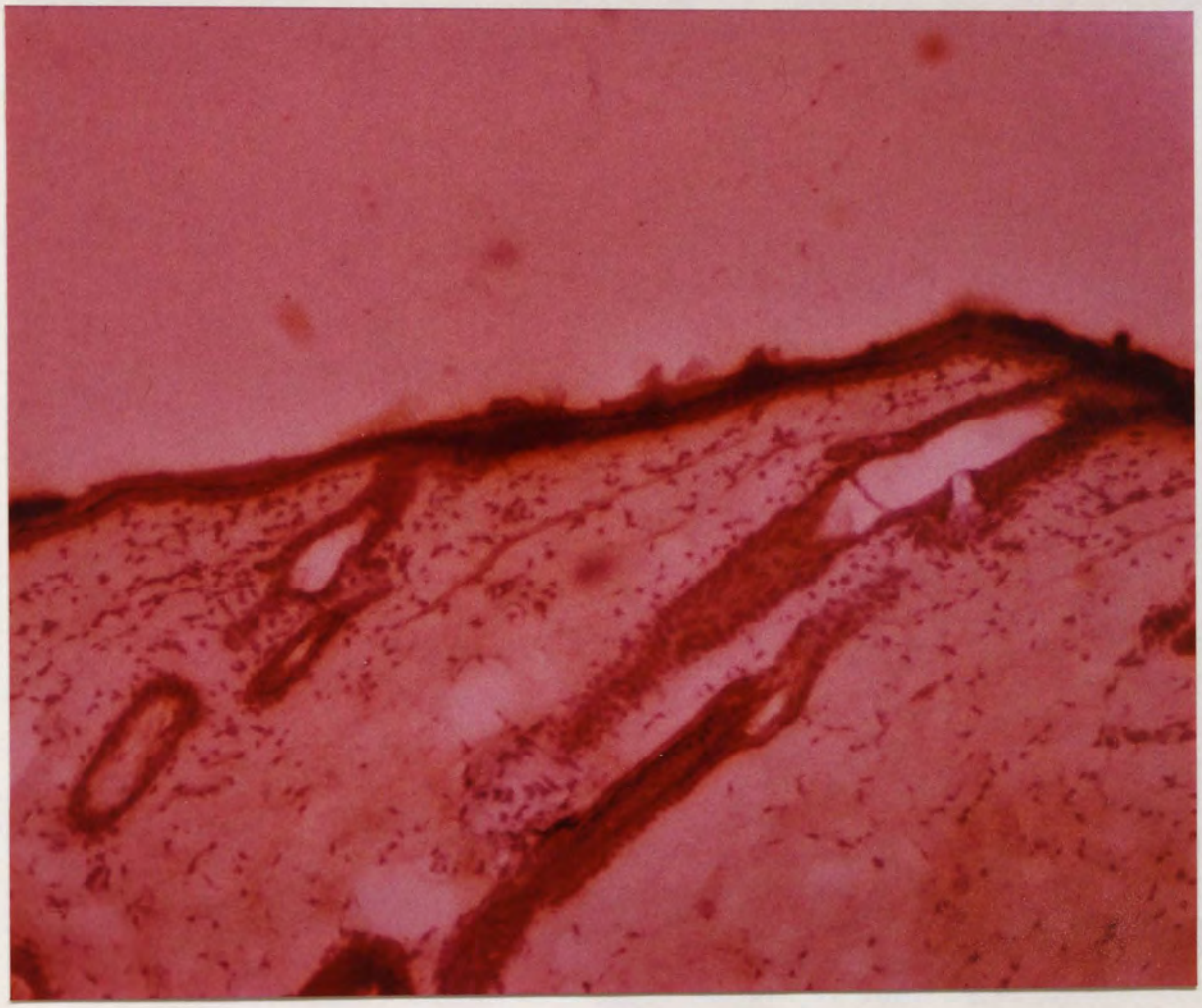


Figure 20

Low power view of guinea-pig skin following 4 hour exposure to ^{63}Ni. Accumulation of radioactivity can be seen in the epidermis, but not in the dermis. Heavy labelling is apparent at hair canal openings. This is considered to be a physical effect of the method of application. (H & E X33)

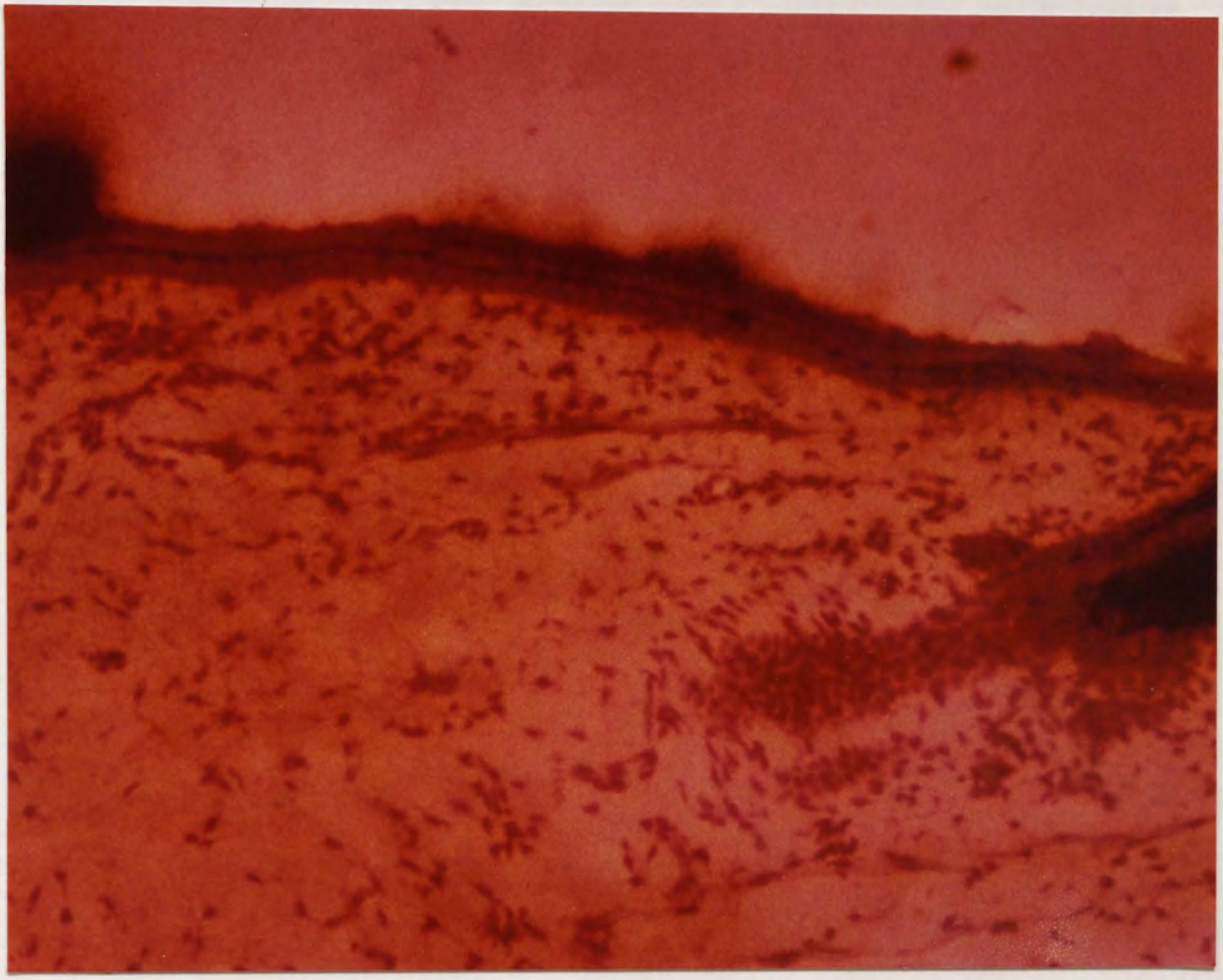


Figure 21

Guinea-pig skin following 4 hour exposure to ^{63}Ni. Labelling is apparent within the epidermis, particularly the stratum corneum. Heavy accumulation (left top corner and right middle edge) can be seen at the top and partly extending down hair canals. Of particular interest in this section is the almost continuous fine line of radioactivity along the middle of the epidermis.
(H & E X52)

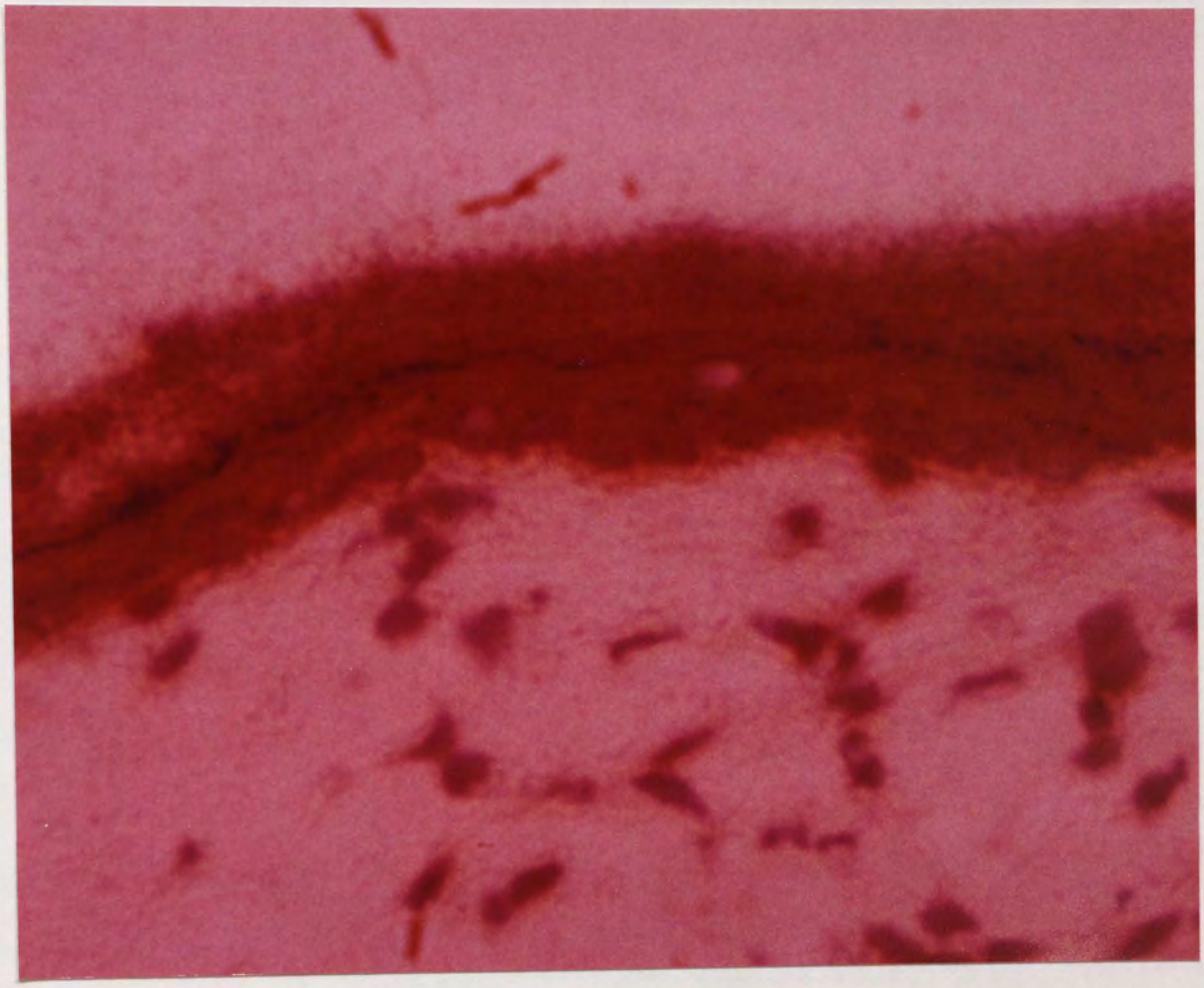


Figure 22

High power view of guinea-pig epidermis following 4 hour exposure to ^{63}Ni. Diffuse labelling can be seen in the stratum corneum. In comparison to the previous photomicrograph, a distinct but broken line of radioactivity is apparent along the middle of the epidermis at the stratum lucidum region. Also of particular interest is light labelling of basal and suprabasal epidermal cells, shown as cell silhouettes. (H & E X215)

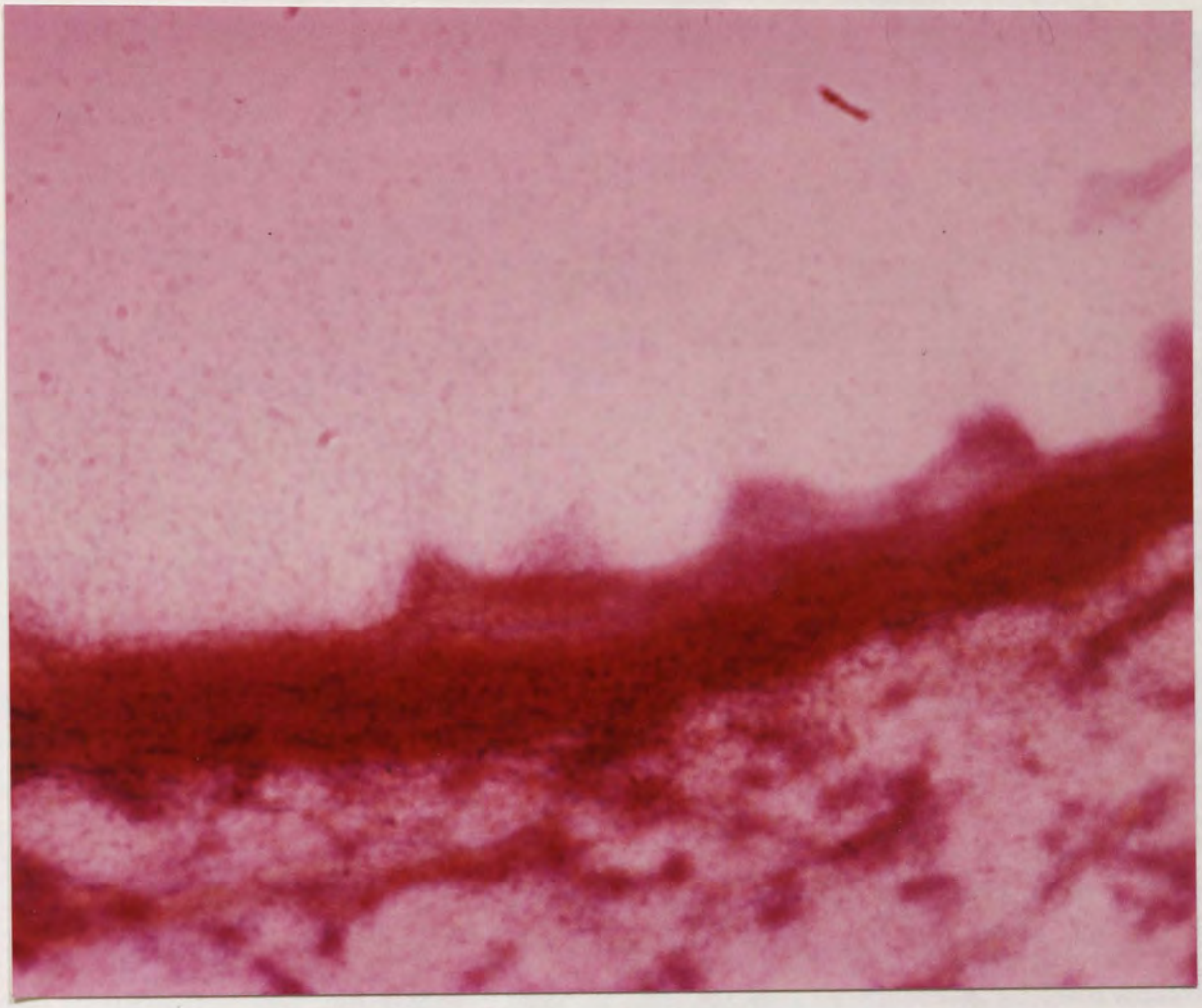


Figure 23

High power view of guinea-pig epidermis following 4 hour exposure to ^{63}Ni. In contrast to the two previous photomicrographs the fine line of radioactivity is not clearly apparent at the stratum lucidum. However, distinct labelling of individual epidermal cells can be seen, particularly in the basal and suprabasal regions.

Some background spread of radioactivity has occurred on each side of the epidermis. The labelling within the dermis was not considered to be indicative of dermal absorption. (H & E X270)

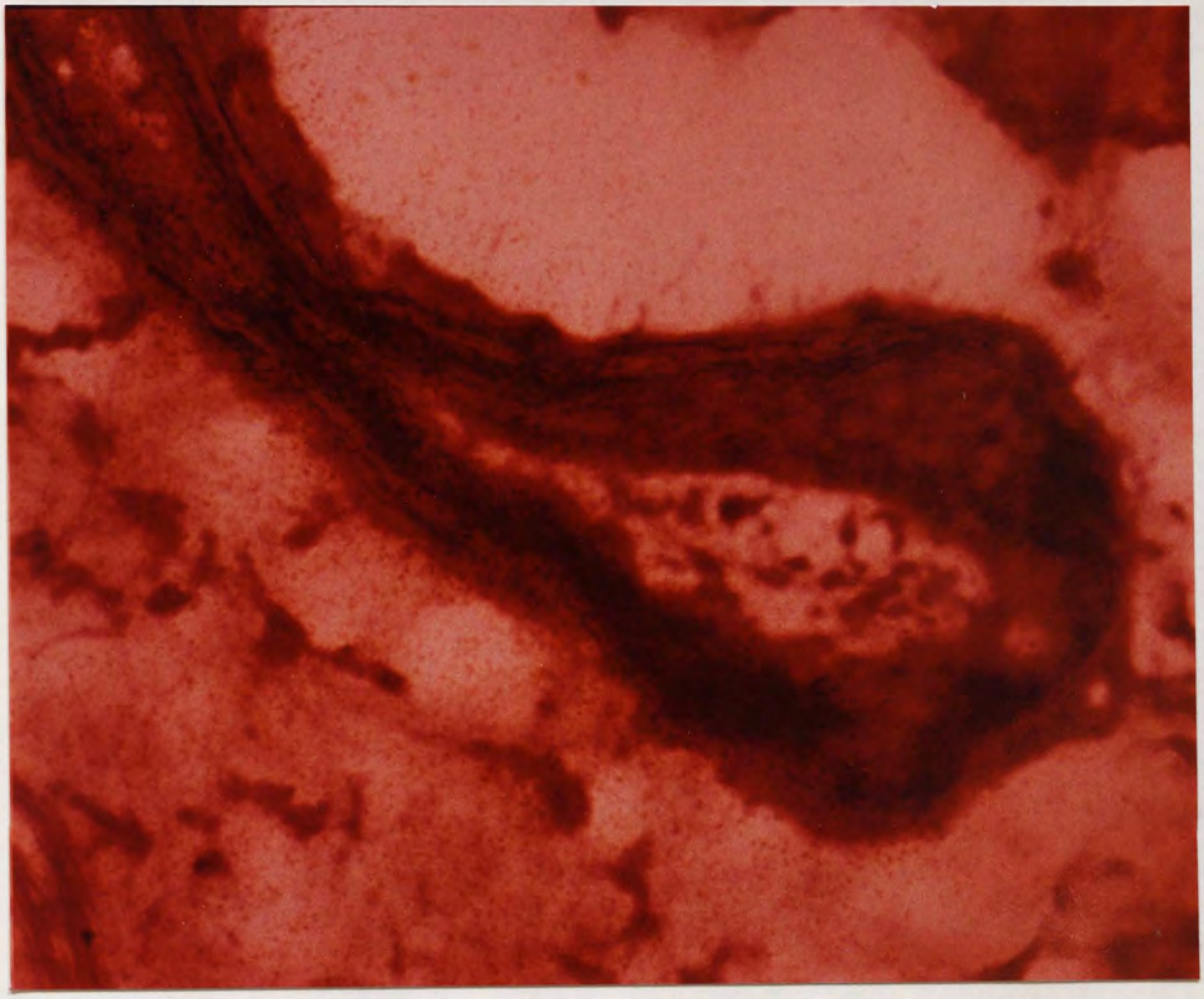


Figure 24

Guinea-pig skin following 4 hour exposure to ^{63}Ni. Marked accumulation can be seen in the bulb and upper hair follicle, particularly in the outer root sheath. The cortex of the follicle appears unlabelled. In comparison with the hair follicle after 1 hour exposure (Figure 19) the degree of labelling has greatly increased. (H & E X130)

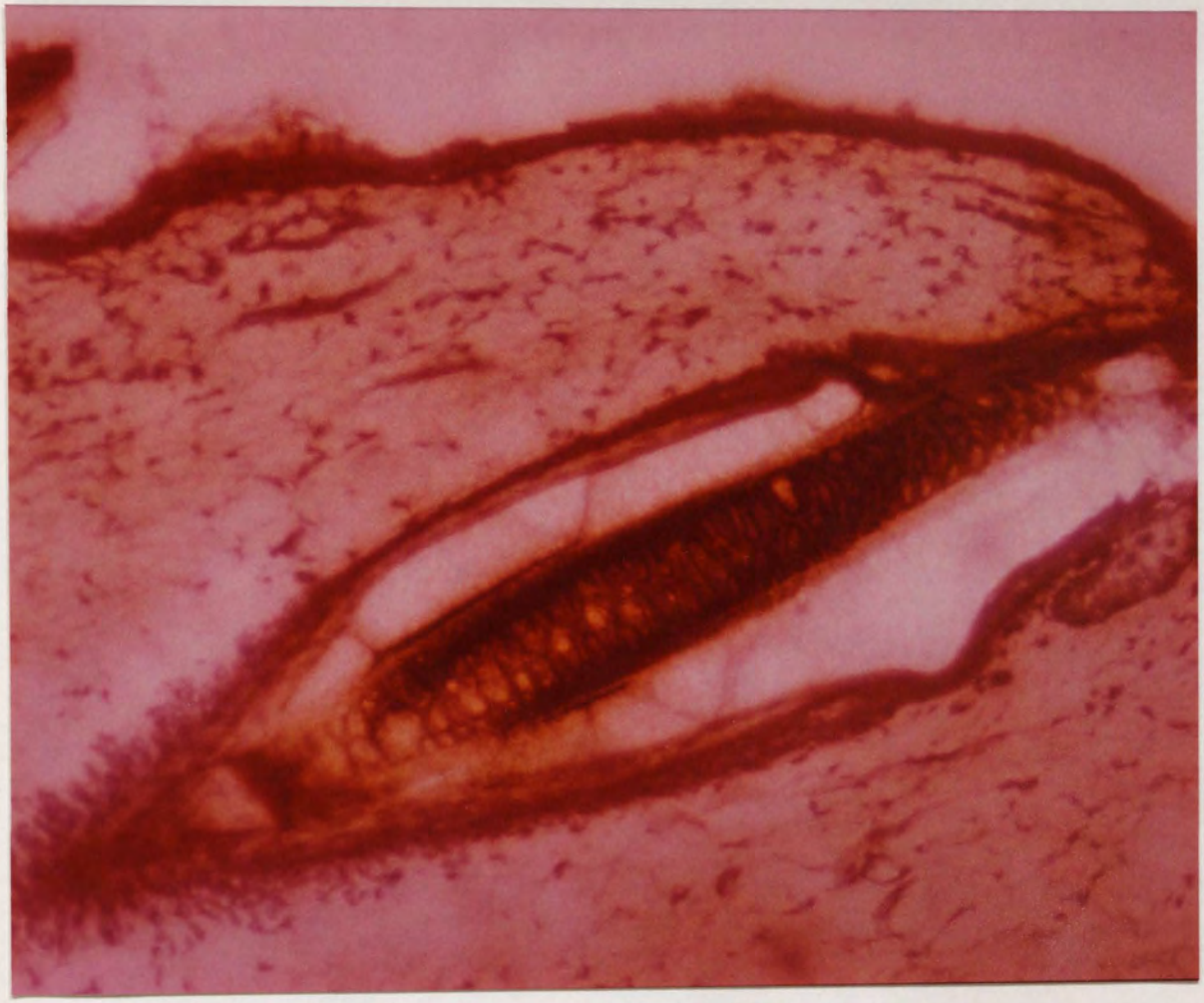


Figure 25

Guinea-pig skin following 12 hour exposure to ^{63}Ni. Marked radioactive labelling can be seen on the hair and diffuse labelling is apparent in the epidermis. (H & E X52)

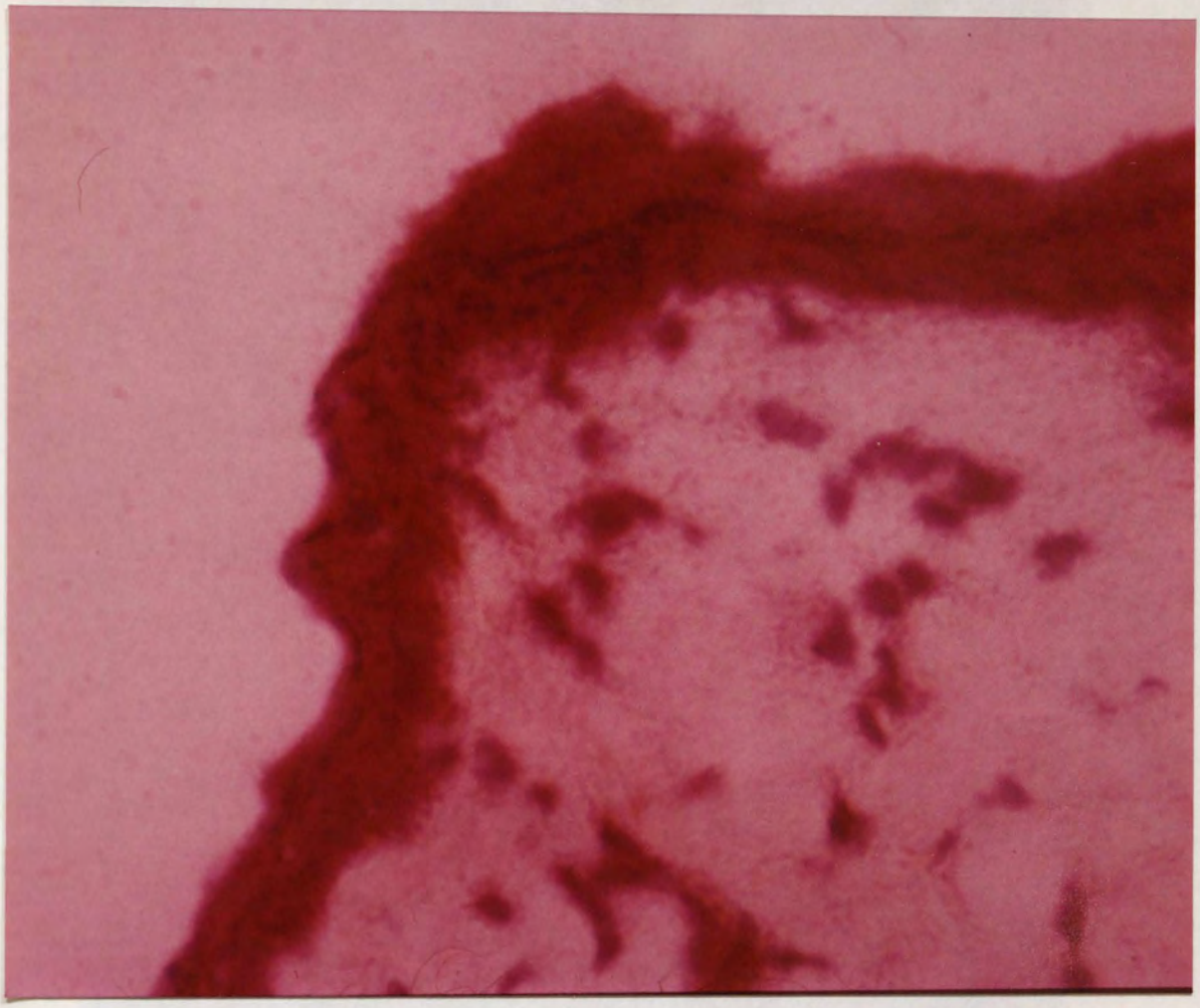


Figure 26

Guinea-pig skin following 12 hour exposure to ^{63}Ni. Diffuse radioactivity is apparent in the stratum corneum and individual basal and suprabasal epidermal cells are labelled. (H & E X208)

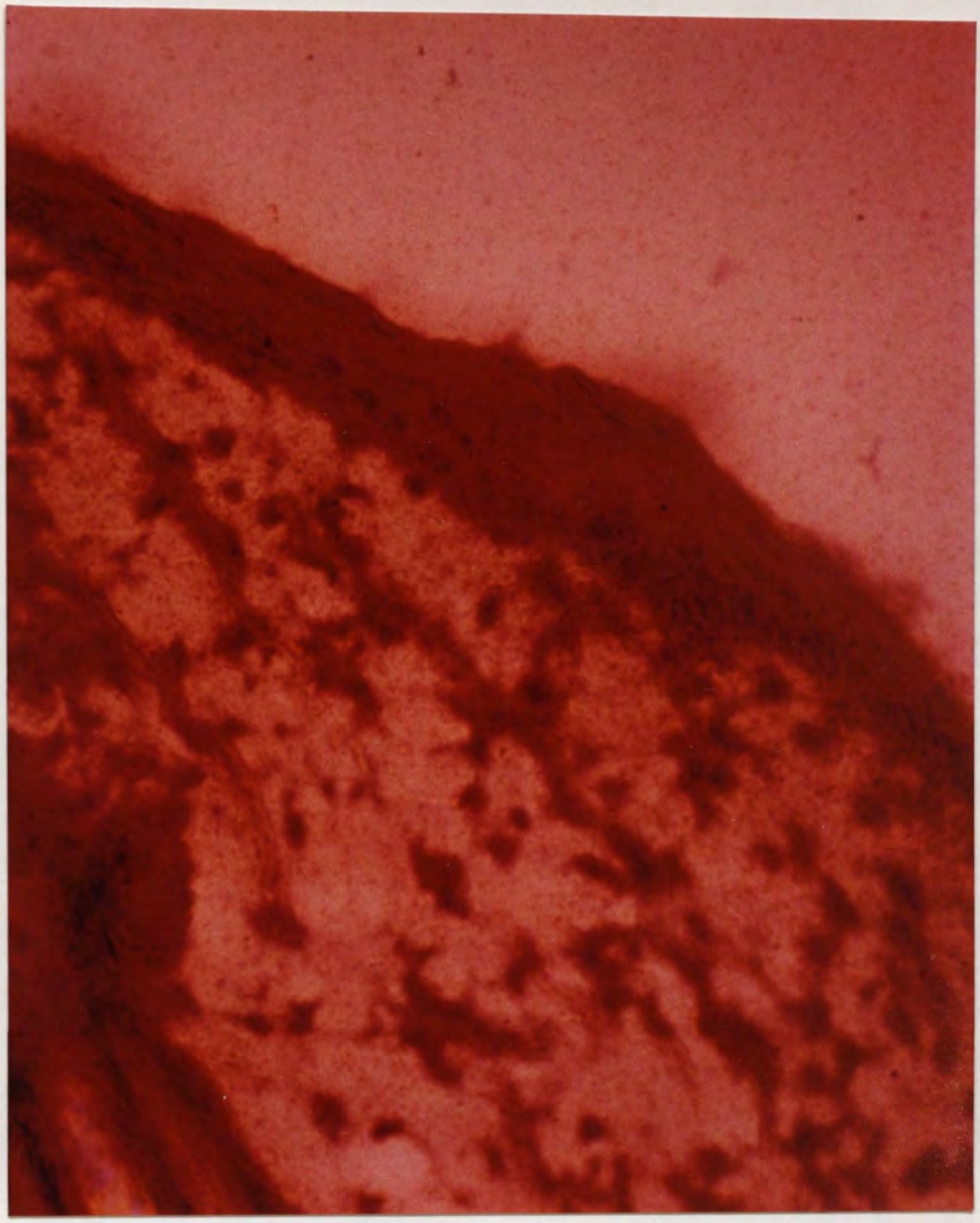


Figure 27

Guinea-pig skin following 24 hour exposure to ^{63}Ni. Labelling can be seen in both the epidermis and dermis. Distinct labelling of basal and suprabasal epidermal cells is apparent. Radioactivity has spread into the upper dermis and some dermal cells appear to be labelled. This dermal radioactivity was not observed in the sections of any other animal and the importance in this case remains uncertain.

Accumulation of ^{63}Ni is also apparent in the hair canal (bottom left). (H & E X130)

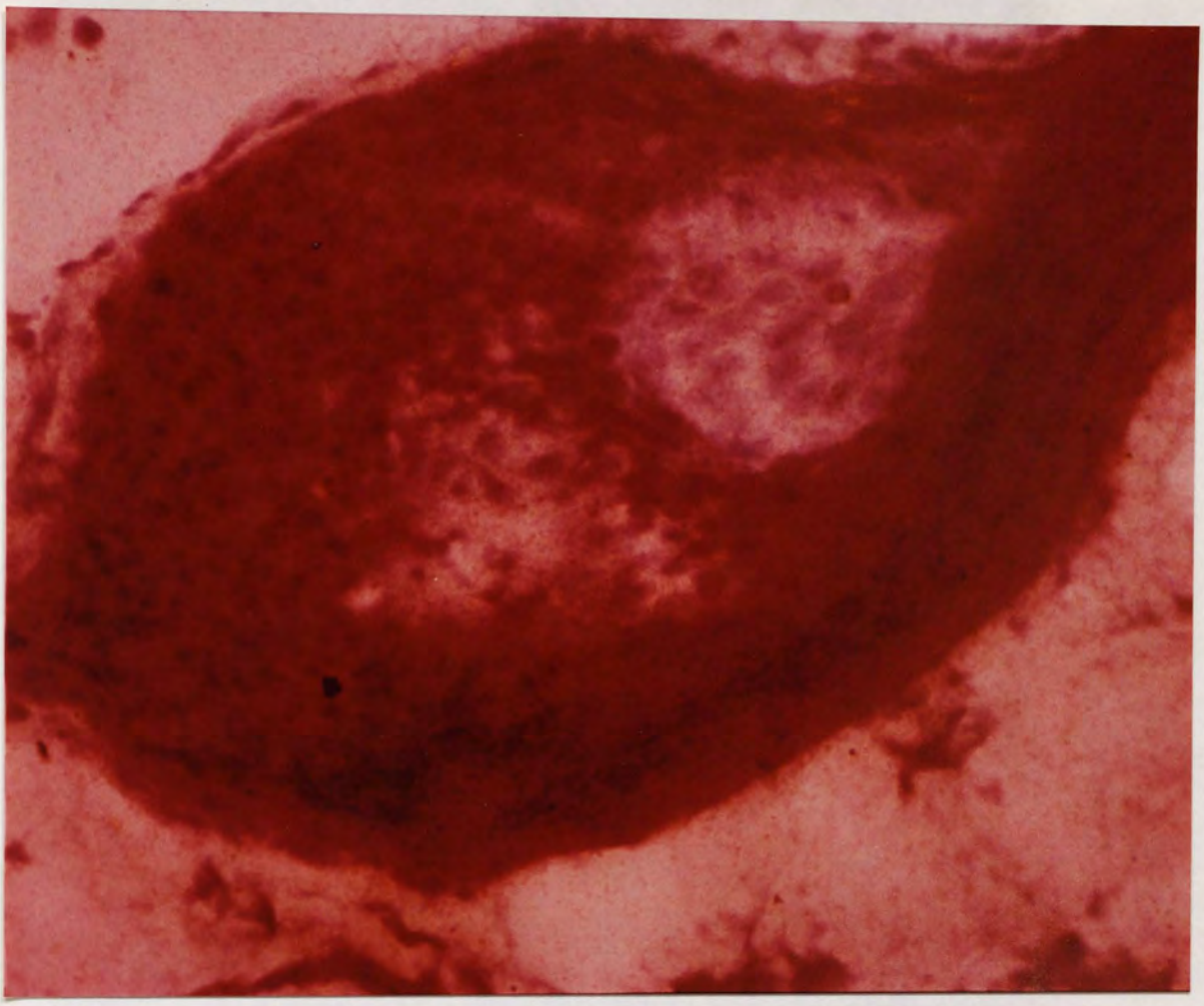


Figure 28

Guinea-pig skin following 24 hour exposure to ^{63}Ni. Marked accumulation of ^{63}Ni in the bulb of a hair follicle. This section is cut obliquely and the detail of the sheath layers of the top half of the bulb is not clear. In comparison to the photomicrograph of the hair follicle after 4 hours exposure (Figure 24) the amount of labelling has not greatly increased. (H&E x 130).

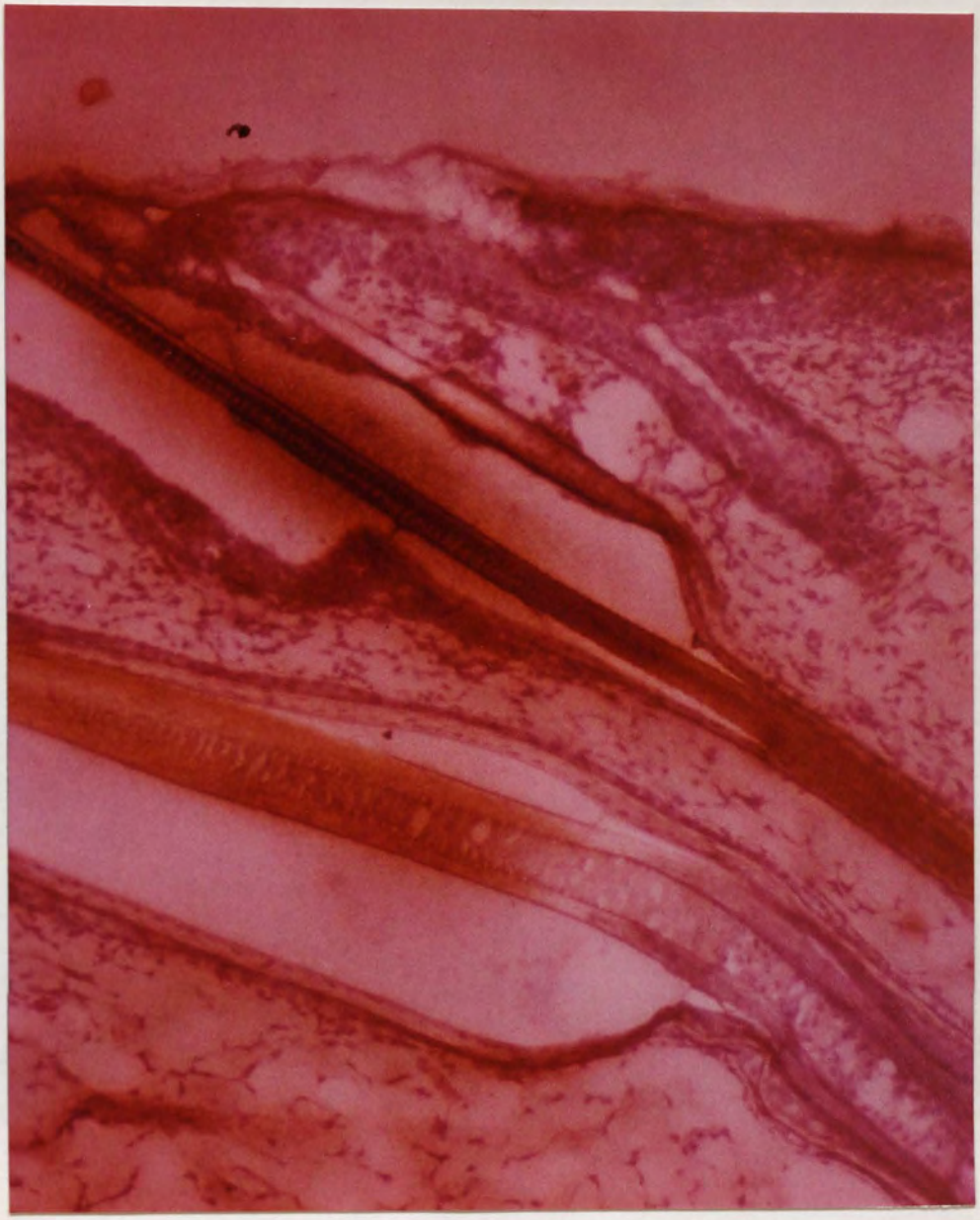


Figure 29

Guinea-pig skin following 48 hour exposure to ^{63}Ni. Marked hair binding and epidermal labelling is apparent. (H&E x 33).

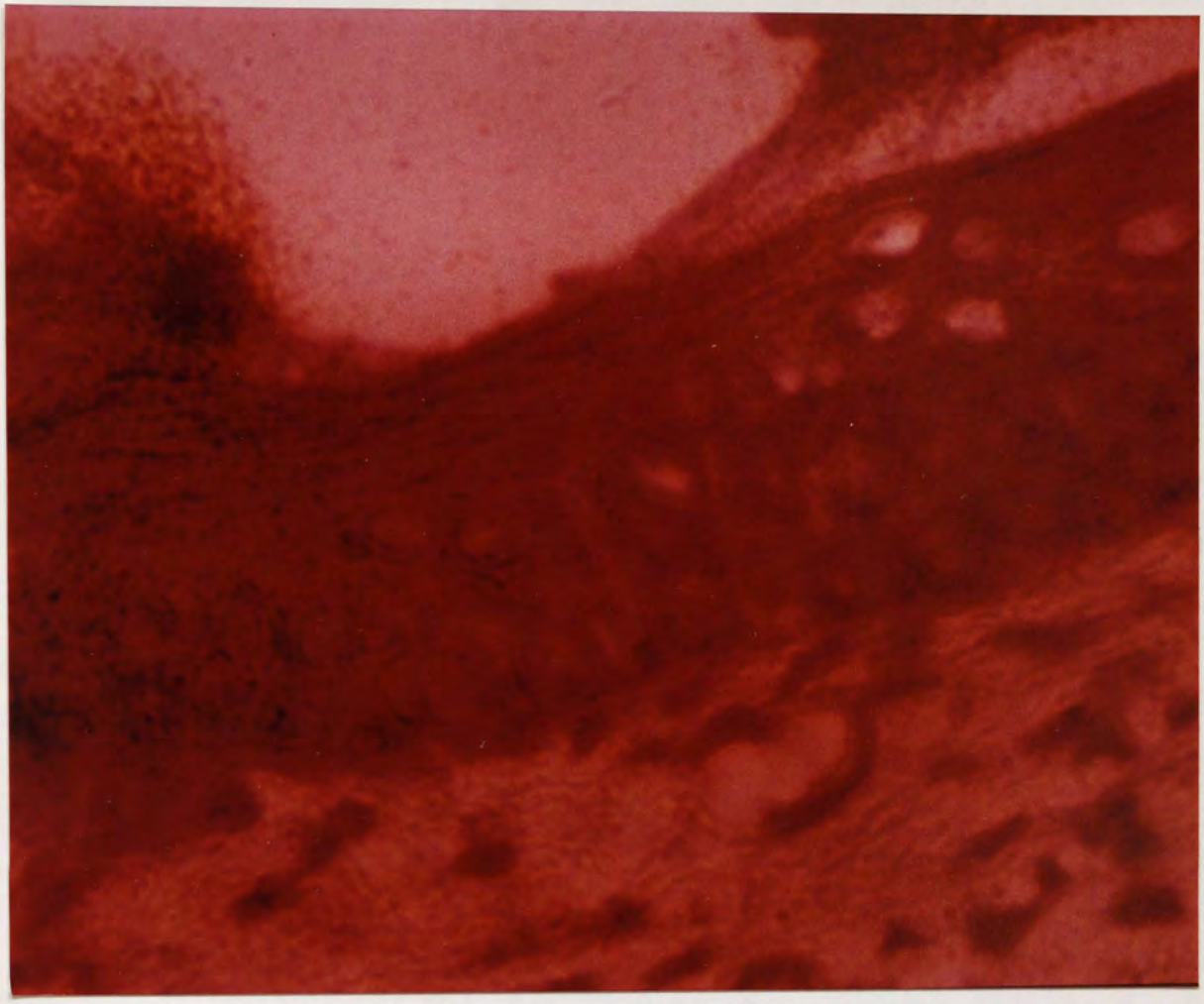


Figure 30

Guinea-pig skin following 48 hour exposure to ^{63}Ni. High power view of epidermis. Some hyperplasia and the beginnings of spongiosis are apparent in the epidermis. This reaction is considered to be due to the irritant properties of the Ni solution under occlusion for 48 hours.

^{63}Ni labelling can be seen in the stratum corneum part of which (top right) has lifted. Labelling of basal and suprabasal epidermal cells can be seen. In some cases this appears to form silhouettes of the cells. (H&E x 268).

QUANTITATIVE ASSESSMENT OF ABSORPTION AND ACCUMULATION

General Performance of Studies

In any study using radioactive tracers the overall performance and accuracy of techniques is reflected by the total recovery of radioactivity in collected samples. It is important to consider this general aspect before commencing the interpretation for individual test samples.

These data for each exposed animal are presented in Tables 18 and 21. In each case test samples containing 9.126×10^7 d.p.m. were prepared. Percentage recoveries for each animal are calculated using the radioactivity of the prepared dose.

In the interpretation of results from the test samples in subsequent sections the applied dose (prepared dose - residual) is the basis of calculations. Although the loss of radioactivity during dosing procedures was relatively small, it is considered to be an experimental variation which should be taken into account.

The mean total recovery of ^{63}Ni for all animals exposed was 85.9% /standard deviation (S.D.) 7.36/. The greatest proportion of radioactivity was residual in the bandage (mean 76.4% of total prepared dose, S.D. 6.47). A mean of 2.5% (S.D. 0.78) was recovered in the treated skin washings and 2.4% (S.D. 0.86) was residual dose.

These figures indicate that on average approximately 14% of the prepared dose was not recovered. Several limiting factors such as incomplete retrieval of radioactivity from bandages, slight loss of dose during bandage applications, seepage of dose into hair surrounding the treatment sites, samples of faeces not collected, cage washings not collected, are all considered to contribute to the slightly reduced total recoveries.

The mean applied dose was 8.85×10^7 d.p.m. equivalent to 22.07mg Ni^{II}.

TABLE 18

Recovery of Radioactivity

(Exposure Time 4 hours)

Results expressed as % prepared dose

Sample	Guinea-pig			
	1	2	3	4
Test samples*	0.01+	1.96	1.98	1.89
Skin washings	3.70	3.07	2.53	2.45
Bandage	64.16	69.71	78.17	73.93
Residual dose	3.14	3.42	3.35	3.09
Total Recovered	71.04	78.16	86.03	81.36
Applied dose (dpm x 10^7)	8.84	8.81	8.82	8.84
Applied dose (mg Ni^{II})	22.05	21.98	22.00	22.06

* includes samples from skin, plasma and urine

+ skin samples not analysed

TABLE 19

Recovery of Radioactivity

(Exposure time 12 hours)

Results expressed as % prepared dose

Sample	Animal No.		
	5	6	7
Test samples*	6.46	8.91	7.56
Skin washings	3.08	1.82	3.14
Bandage	80.91	69.07	70.06
Residual dose	3.02	1.87	2.46
Total Recovered	93.47	81.67	83.22
Applied dose (dpm x 10^7)	8.85	8.96	8.90
Applied dose (mg Ni^{II})	22.07	22.34	22.20

* includes samples taken from skin, plasma and urine

TABLE 20

Recovery of Radioactivity

(Exposure time 24 hours)

Results expressed as % prepared dose

Sample	Guinea-pig						
	8	9	10	11	12	13	14
Test Samples*	8.19	6.54	5.19	6.95	5.05	0.39+	0.36+
Skin washings	3.34	2.24	2.29	4.08	2.55	2.10	3.42
Bandage	79.27	78.39	81.04	73.47	86.75	81.65	63.32
Residual dose	2.36	1.72	2.08	1.34	2.26	4.07	1.12
Total Recovered	93.16	88.89	90.60	85.84	96.61	88.21	68.22
Applied dose (dpm x 10^7)	8.91	8.97	8.94	9.00	8.92	7.68	9.02
Applied dose (mg Ni^{II})	22.22	22.37	22.29	22.45	22.24	19.15	22.51

* includes samples from skin, plasma and urine

+ skin samples not assayed

TABLE 21

Recovery of Radioactivity

(Exposure time 48 hours)

Results expressed as % prepared dose

Sample	Guinea-pig				
	15	16	17	18	19
Test samples*	4.97	4.97	6.37	7.56	0.71+
Skin washings	1.55	1.83	1.83	1.85	1.35
Bandage	78.00	82.88	79.55	79.40	81.27
Residual dose	1.85	1.04	1.77	3.15	3.20
Total recovered	86.37	90.72	89.52	91.96	86.53
Applied dose (dpm x 10^7)	8.96	9.03	8.97	8.84	8.83
Applied dose (mg Ni^{II})	22.34	22.52	22.36	22.04	22.03

* includes samples from skin, plasma and urine

+ skin samples not assayed

Skin

In an attempt to quantify the degree of accumulation in the exposed guinea-pig epidermis measurements of Ni content were taken for both the homogenizable and non-homogenizable portions of the test sample. The non-homogenizable residue of the skin was considered to be comprised of the highly keratinized areas (stratum corneum), the hairs and some hair follicles and large cell debris. The combination of measurements for both portions was considered to indicate the degree of accumulation in the epidermis.

The measurements for individual animals at each time period are presented as Table 22, the groups mean figures are presented as Table 23 and Figure 31.

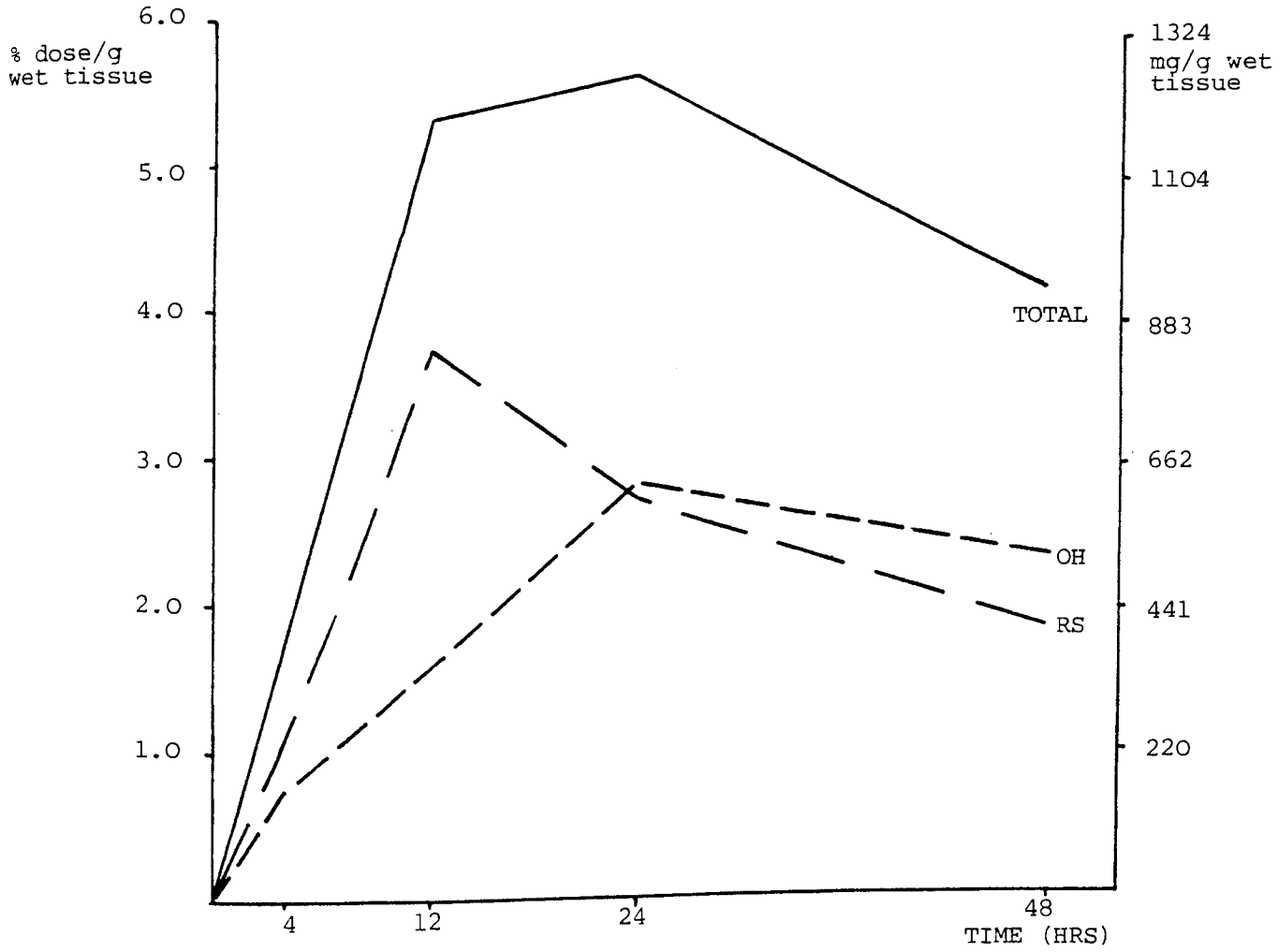
After 4 hours of exposure accumulation of radioactivity was apparent at a small percentage of the applied dose (mean 1.8% - 396µg Ni^{II}/g wet tissue). The levels within the epidermis increased markedly after 12 hours of exposure (mean 5.30% of applied dose - 1168µg Ni^{II}/g wet tissue) and appeared to plateau after 24 hours of exposure (mean 5.71% - 1208µg Ni^{II}/g wet tissue).

Comparison of the levels of radioactivity in the epidermal homogenates and the non-homogenizable debris indicates that the proportion of accumulation are similar in both the keratinised areas and the remainder of the epidermis.

The exceptions to this indication were for two of the three sampled animals exposed for 12 hours. The greatest proportion of radioactivity was found in the non-homogenizable debris.

Figure 31

CONCENTRATION OF RADIOACTIVITY IN TREATED EPIDERMIS



OH - homogenate

RS - residual sample

TABLE 22

Concentration of Radioactivity in Treated Epidermis - Individual Values

Exposure Time	G-Pig	Weight Skin (g)	Total Dpm Found		% Applied Dose/g Wet Tissue			Ni^{II} Content µg/g wet tissue			Ni Content as % Total Found	
			OH	RS	TOTAL	OH	RS	TOTAL	OH	RS	OH	RS
4 Hrs	2	1.100	750100	1023960	1.83	0.77	1.06	403	169	233	41.9	58.1
	3	1.134	634200	1034670	1.66	0.63	1.03	366	139	227	37.9	62.1
	4	1.058	754930	1017440	1.90	0.81	1.09	418	178	240	42.6	57.4
12 Hrs	5	1.740	1069430	4315690	3.49	0.69	2.80	700	152	618	21.7	78.3
	6	1.502	1359210	6515690	5.85	1.01	4.84	1307	226	1081	17.3	82.7
	7	1.107	3051070	3593720	6.75	3.10	3.65	1498	688	810	45.9	54.1
24 Hrs	8	1.042	3087675	3928120	7.56	3.33	4.23	1680	740	940	44.0	56.0
	9	1.110	3560800	2049720	5.63	3.58	2.05	1260	801	459	63.6	36.4
	10	1.090	2423959	1987650	4.53	2.49	2.04	1010	555	455	55.0	45.0
	11	1.168	2185790	3209360	5.13	2.08	3.05	1152	467	685	40.5	59.5
	12	1.180	1844730	2584560	4.21	1.75	2.46	936	389	547	41.6	58.4
48 Hrs	15	1.087	2299860	1422520	3.82	2.36	1.46	853	527	326	61.8	38.2
	16	1.440	2542301	1511560	3.11	1.95	1.16	700	439	261	62.7	37.3
	17	1.330	2550200	2386020	4.14	2.14	2.00	926	479	447	51.7	48.3
	18	1.270	3396800	3222100	5.90	3.03	2.87	1301	668	633	51.3	48.7

OH - homogenate
RS - residual sample

TABLE 23

Concentration of Radioactivity in Treated Epidermis - Group Values

Exposure Time Hrs.	% Applied Dose/g Wet Tissue			Ni^{II} Content µg/g Wet Tissue			Ni Content as % Total Found	
	TOTAL	OH	RS	TOTAL	OH	RS	OH	RS
4	1.80 (0.123)	0.74 (0.095)	1.06 (0.030)	396 (26.8)	162 (20.4)	233 (6.5)	40.8 (2.54)	59.2 (2.54)
12	5.36 (1.684)	1.60 (1.309)	3.76 (1.025)	1168 (416.7)	355 (290.5)	836 (232.6)	28.3 (15.40)	71.7 (15.40)
24	5.71 (1.311)	2.87 (0.703)	2.77 (0.916)	1208 (292.3)	590 (175.9)	617 (203.1)	48.9 (10.01)	51.1 (10.01)
48	4.24 (1.186)	2.37 0.471)	1.87 (0.750)	945 (255.3)	528 (99.9)	417 (163.5)	56.9 (6.22)	43.1 (6.22)

figures in parenthesis are standard deviations of the mean values

OH - homogenate
RS - residual sample

Plasma

The results of plasma radioactivity measurements for individual animals are presented in Table 24 and the group mean figures in Table 25 and Figure 32.

These results indicate that a small percentage of dose applied to the skin is absorbed during the exposure periods. After four hours of exposure a very low concentration was apparent in the plasma (0.74% applied dose/litre ≡ 162ng Ni^{II}/ml). Peak concentrations were observed after the 12 hour exposure period (3.24% applied dose/litre ≡ 716ng Ni^{II}/ml) and a reduction in concentrations was apparent after 24 hours (2.61% applied dose /litre ≡ 549ng Ni^{II}/ml) and 48 hours (0.96% applied dose/litre ≡ 214ng Ni^{II}/ml).

The specific reason for a reduction in plasma concentration during the continuous 24-48 hour exposure is not apparent. However there are at least two possible explanations:

a. The greater proportion of dose could be trapped within the bandage material and after approximately 24 hours of exposure the available portion is exhausted.

b. The accumulation of nickel in the epidermis becomes a limiting factor on the rate of absorption.

An alternate way of expressing the plasma concentrations is to relate the finding to the total plasma volume for each animal. This assumes that total plasma volume is 4% of the bodyweight (Wagner and Manning 1976).

Using this method a maximum of 0.062% of the applied dose (12 hour exposure) was absorbed across the skin to appear in the total plasma volume. These figures were also used in the calculation of total radioactivity recoveries.

Figure 32

CONCENTRATION OF RADIOACTIVITY IN PLASMA

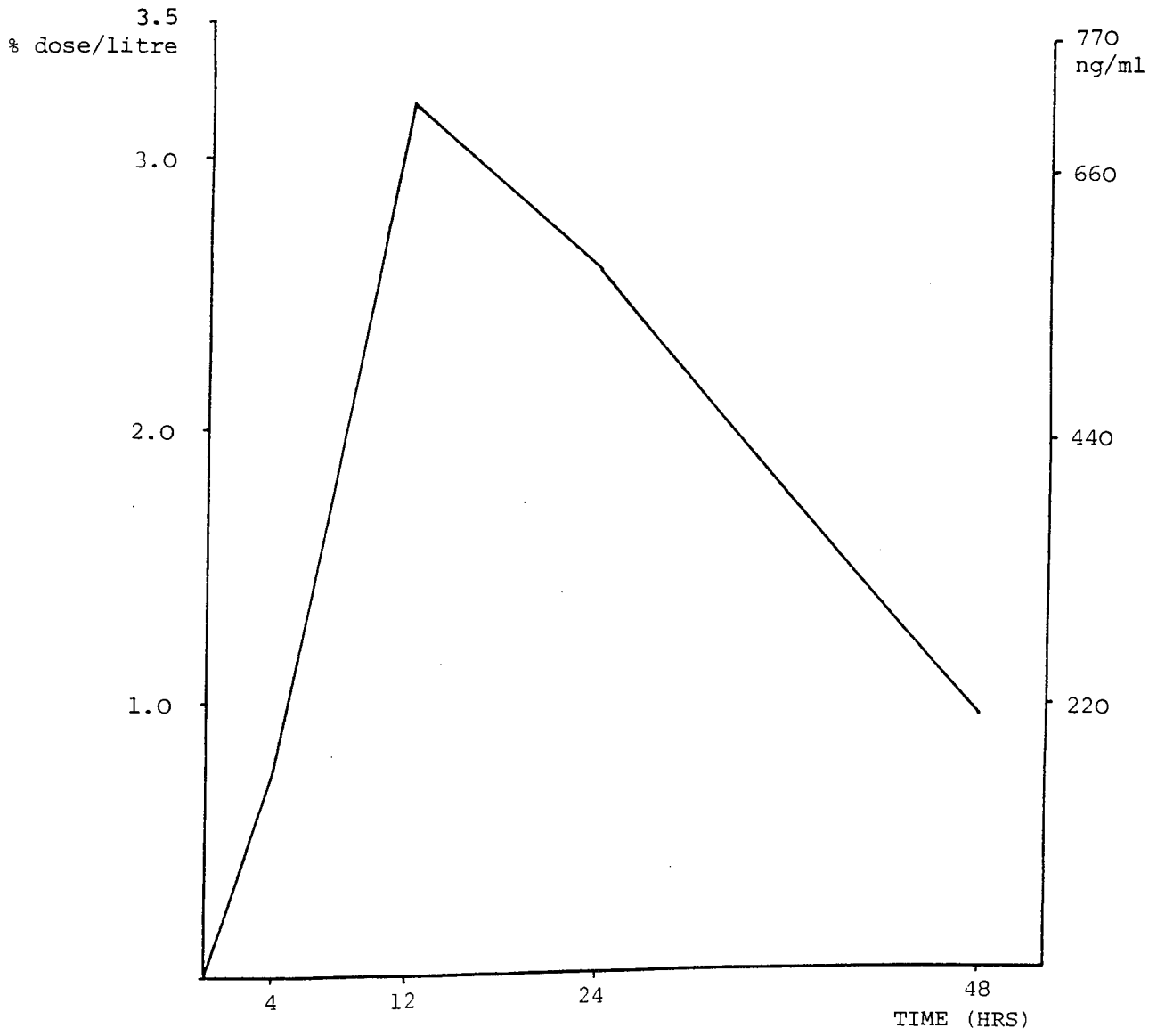


TABLE 24

Concentration of Radioactivity in Plasma

- Individual values

Exposure Time (hours)	G-Pig	Dpm/ml	% Applied dose/litre	ng Ni^{II}/ml	% applied dose/total plasma volume*
4	1	254	0.29	63	0.005
	2	684	0.78	171	0.02
	3	1454	1.65	363	0.02
	4	210	0.24	52	0.005
12	5	2898	3.27	723	0.07
	6	2454	2.74	612	0.05
	7	3264	3.67	814	0.07
24	8	2420	3.15	604	0.06
	9	866	0.97	216	0.03
	10	926	1.04	231	0.03
	11	3290	3.66	821	0.07
	12	1698	1.90	423	0.04
	13	3436	4.47	857	0.09
	14	2784	3.09	694	0.06
48	15	964	1.08	240	0.02
	16	728	0.81	182	0.02
	17	504	0.56	126	0.02
	18	304	0.34	76	0.005
	19	1794	2.03	447	0.04

* assuming total plasma volume approximately 4% of bodyweight

TABLE 25

Concentration of Radioactivity in Plasma

- Group values

Exposure Time (Hours)	Dpm/ml	% applied dose/litre	ng Ni^{II}/ml	% applied dose/total plasma volume*
4	651 (577)	0.74 (0.65)	162 (144)	0.013 (0.009)
12	2872 (406)	3.23 (0.47)	716 (101)	0.062 (0.007)
24	2203 (1061)	2.61 (1.34)	549 (265)	0.051 (0.022)
48	859 (578)	0.96 (0.66)	214 (144)	0.020 (0.012)

* assuming total plasma volume approximately 4% of bodyweight

figures in parentheses are standard deviations of the mean values

Urine

The results of urine radioactivity measurements for individual animals are presented as Table 26 and the group mean values in Table 27 and Figure 33.

Nickel was apparent in the urine after each exposure period, and the concentration increased with length of exposure. After an initial lag-phase at 4 hours the urinary nickel concentration increased sharply to 12 hours. The rate of increase was less, however, during the 24 and 48 hour exposures.

This general pattern is considered to reflect the plasma concentration of nickel, which reached a peak at 12 hours and subsequently reduced.

Figure 33

CONCENTRATION OF RADIOACTIVITY IN URINE

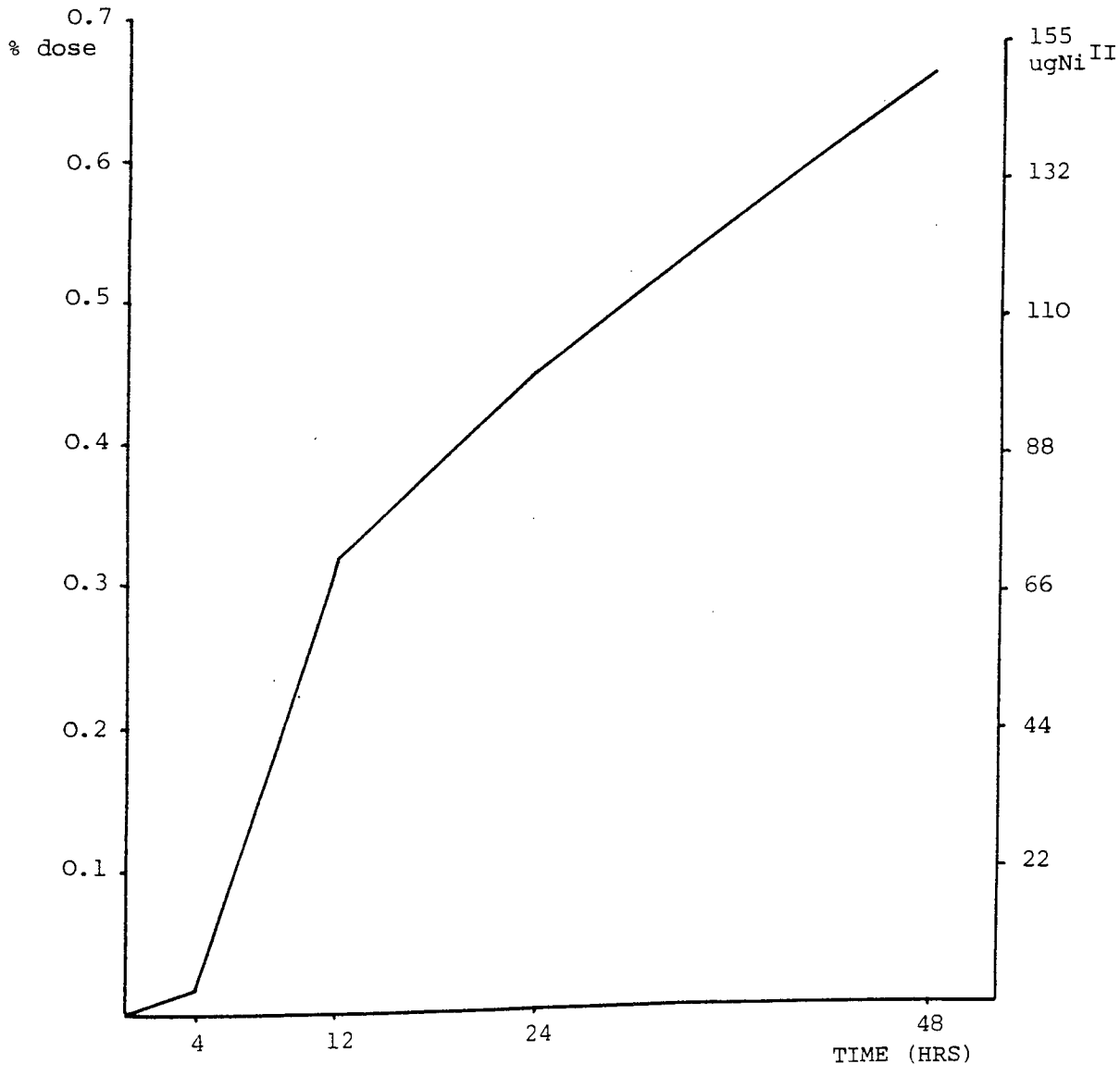


TABLE 26

Concentrations of Radioactivity in Urine

- Individual values

Exposure Time (Hours)	G-Pig	Volume Collected (ml)	Dpm/ml	Total Dpm	% applied dose	µg Ni^{II} total
4	1	6	954	5724	0.006	1.4
	2	27	562	15174	0.017	3.8
	3	21	1512	31752	0.036	7.9
	4	4.5	1820	8190	0.009	2.0
12	5	29	15694	455126	0.514	113.5
	6	12	17068	204816	0.229	51.1
	7	39	4968	193752	0.218	48.3
24	8	42	9834	413028	0.464	103.0
	9	44	7672	337568	0.376	84.2
	10	43	6938	298334	0.334	74.4
	11	40	22228	889120	0.988	221.7
	12	40	7442	297680	0.334	74.2
	13	42	6844	287448	0.374	71.7
	14	16	16914	270624	0.300	67.5
48	15	77	10306	793562	0.886	197.9
	16	70	6682	467740	0.518	116.7
	17	72	12008	864576	0.964	215.6
	18	88	3120	274560	0.311	68.5
	19	72	8472	609984	0.691	152.1

TABLE 27

Concentration of Radioactivity in Urine

- Group values

Exposure Time (Hours)	Volume (ml)	Dpm/ml	Total Dpm	% applied dose	µg Ni^{II} total
4	14.6 (11.1)	1212 (562)	15210 (11732)	0.017 (0.013)	3.8 (2.93)
12	26.7 (13.6)	12577 (6625)	284565 (147814)	0.320 (0.168)	71.0 (36.86)
24	38.1 (9.9)	11125 (6053)	399115 (221176)	0.453 (0.242)	99.5 (55.15)
48	75.8 (7.3)	8118 (3431)	602084 (240280)	0.674 (0.267)	150.2 (59.90)

figures in parentheses are standard deviations of the mean values.

Results of Zonal Ultracentrifugation

Epidermal homogenates from a total of 21 animals were examined by rate zonal ultracentrifugation on linear sucrose gradients. Of these animals 15 were exposed to $^{63}Ni^{II}$ for periods of 4 to 48 hours and the remaining 6 animals served as controls.

The group values for each exposure period and each control group are presented as Tables 28 to 32. The values are also presented graphically as Figures 34 to 39 for ease of comparison. The results of marker enzyme assays and scintillation counting are presented relative to the gradient as a ratio of the concentration of activity in each fraction to the total activity if it were equally distributed across the whole gradient ($^{CF}/CI$). This is considered to be a better indication of the purity of each fraction (De Duve 1967). Calculation of specific activity (activity relative to protein concentration per fraction) could be slightly misleading when the protein concentration varies considerably between some fractions.

Sucrose density and total protein results are presented as absolute values.

Marker Enzymes and Total Protein

Each fraction collected was analysed for the following sub-cellular marker enzymes in addition to total proteins:

acid phosphatase	-	lysosomes, plasma membranes
succinate dehydrogenase	-	mitochondria membrane
catalase	-	peroxisomes
glucose-6-phosphatase	-	microsomal membrane

Protein distribution across the gradient indicated that 48-59% of the total protein was in the soluble fraction. The remaining protein was distributed across the gradient with minor increases above background levels in certain fractions.

Mitochondrial distribution for all animals as indicated by succinate dehydrogenase activity was found at the heavy end of the gradient (approximately 1.181g/ml, sucrose density). A small proportion of activity was evident in the soluble fraction (approximately 1.033g/ml, sucrose density) and was considered to indicate disruption of a small proportion of the organelles. Additionally, in intact viable epidermis some mitochondria will be in a disrupted state in those cells transforming into the squamous cells of the stratum corneum (Gray and Yardley 1975). In this report Gray and Yardley found similar 'heavy' mitochondria and soluble succinate dehydrogenase activity in porcine and human epidermis samples.

Acid phosphatase activity was more widely spread across the whole gradient. Four easily discernible peaks of activity were observed; two mid gradient (sucrose density 1.069 and 1.091) and two towards the heavy end (sucrose density 1.137 and 1.154). In comparison with published reports of the epidermal distribution of this enzyme, the general picture seems expected. Gray et al (1980), using pig epidermis, found similar peaks of activity at densities of 1.09, 1.16 and 1.30. The peaks in the lower densities also coincided with plasma membrane marker enzymes and may be associated with the membranes (Robinson 1969). Gray et al (1980) also reported the major proportion of pig epidermal acid phosphatase activity associated with the soluble fraction. This was not observed in the experiments for this thesis, but a small proportion of the enzyme activity

was detected in the soluble fraction although it did not constitute the 82% of total activity reported by Gray et al. This may be due to a species difference in activity distribution, different methods of epidermal homogenate preparation or different methods of enzyme determination.

Catalase activity was almost totally soluble but a small proportion of the activity appeared at the very edge of the gradient (density 1.047).

Glucose-6-phosphatase activity (microsomal) was maximal in the light end of the gradient (density 1.057) but a large proportion was apparent in the soluble fraction.

Gray and Yardly (1975) were unable to conclusively detect glucose-6-phosphatase in porcine and human epidermis due to the incomplete fluoride inhibition of acid phosphatase during assay procedures and the high levels of epidermal acid phosphatase. However, in the guinea-pig the large 'light' acid phosphatase activity was absent and this possible masking effect was not apparent. The distribution of glucose-6-phosphatase activity (NaF inhibited PO_4-release) was resolved in quite different positions of the gradient to acid phosphatase.

The pattern of marker enzyme distribution was consistent for each animal exposed to ^{63}Ni at each exposure period and also comparable with distribution patterns for epidermal homogenate from guinea-pigs not exposed in vivo to ^{63}Ni (controls groups 1 and 2).

The analytical recoveries for each assay varied between 90 and 120% of the total homogenate activities but were considered to be acceptable and comparable with similar published studies.

Nickel

All the radioactivity found after centrifugation was located at the light end of the gradient. The recovery of ^{63}Ni from the homogenate averaged approximately 97%.

The majority of the radioactivity was associated with the soluble fractions but in each case extended into the early part of the gradient (fractions 4-8). This movement onto the gradient did not occur in control centrifugation runs and thus does not appear to be an artifact of centrifugation (e.g. diffusion).

When a known amount of ^{63}Ni was added to the epidermal homogenate from non-exposed guinea-pigs the radioactivity was totally limited to the soluble fraction. This pattern was similar when the ^{63}Ni was added to the minced epidermis prior to homogenisation or to the prepared homogenate. In these control groups the spread of radioactivity into the zonal fractions 1 and 3 were considered to reflect artefacts of diffusion and rotor deceleration but on no occasion was radioactivity apparent in the light gradient. In comparison with this control distribution, the presence of ^{63}Ni in the light gradient was therefore considered to be a genuine observation for the exposed animals.

The extent of movement of radioactivity into the light end of the gradient coincided with glucose-6-phosphatase distribution in this area. This can be illustrated by plotting the relative radioactivity against sucrose density (Fig. 40).

Thus, two localizations of nickel were apparent following zonal centrifugation of epidermal homogenates from exposed guinea-pigs. One area associated with the soluble portion of the homogenate (representing 91% of the found nickel) and the second associated with the "microsomal" portion (representing 9% of the found nickel).

The proportions of nickel in each area were similar for each exposure period.

TABLE 28

Results of Zonal Centrifugation - Group values

Exposure Time, 4 Hours.

Number of Animals, 3.

Fraction	Sucrose Density (g/ml)	Total Protein µg x 10^2	^{63}Ni CF/CI	Acid Phosphatase CF/CI	Catalase CF/CI	Glucose-6-Phosphatase CF/CI	Succinate Dehydrogenase CF/CI
1	1.020	35.9	5.7	1.3	3.8	2.0	1.0
2	1.033	68.1	10.9	1.9	12.0	4.9	4.3
3	1.048	21.0	4.1	1.7	6.2	4.5	0.1
4	1.061	6.9	1.6	2.0	0.2	7.0	0
5	1.064	3.8	0.5	2.6	0.3	5.5	0
6	1.068	2.1	0.1	6.6	0	0.2	0
7	1.072	1.7	0.1	3.5	0	0.1	0
8	1.077	1.7	0	5.2	0	0	0
9	1.083	3.8	0	5.3	0	0	0
10	1.090	4.0	0	7.5	0	0	0
11	1.098	3.7	0	3.5	0	0	0
12	1.105	3.0	0	3.9	0	0	0
13	1.114	1.8	0	7.3	0	0	0
14	1.123	3.5	0	9.5	0	0	0
15	1.132	7.0	0	10.1	0	0	0
16	1.143	6.9	0	2.8	0	0	0.1
17	1.155	3.2	0	9.8	0	0	1.8
18	1.172	3.2	0	6.6	0	0.8	10.5
19	1.194	3.6	0	1.3	0	1.9	8.5
20	1.212	3.5	0	1.4	0	2.5	4.5
Recovery from Homogenate (%)	-	102	98	106	96	97	90

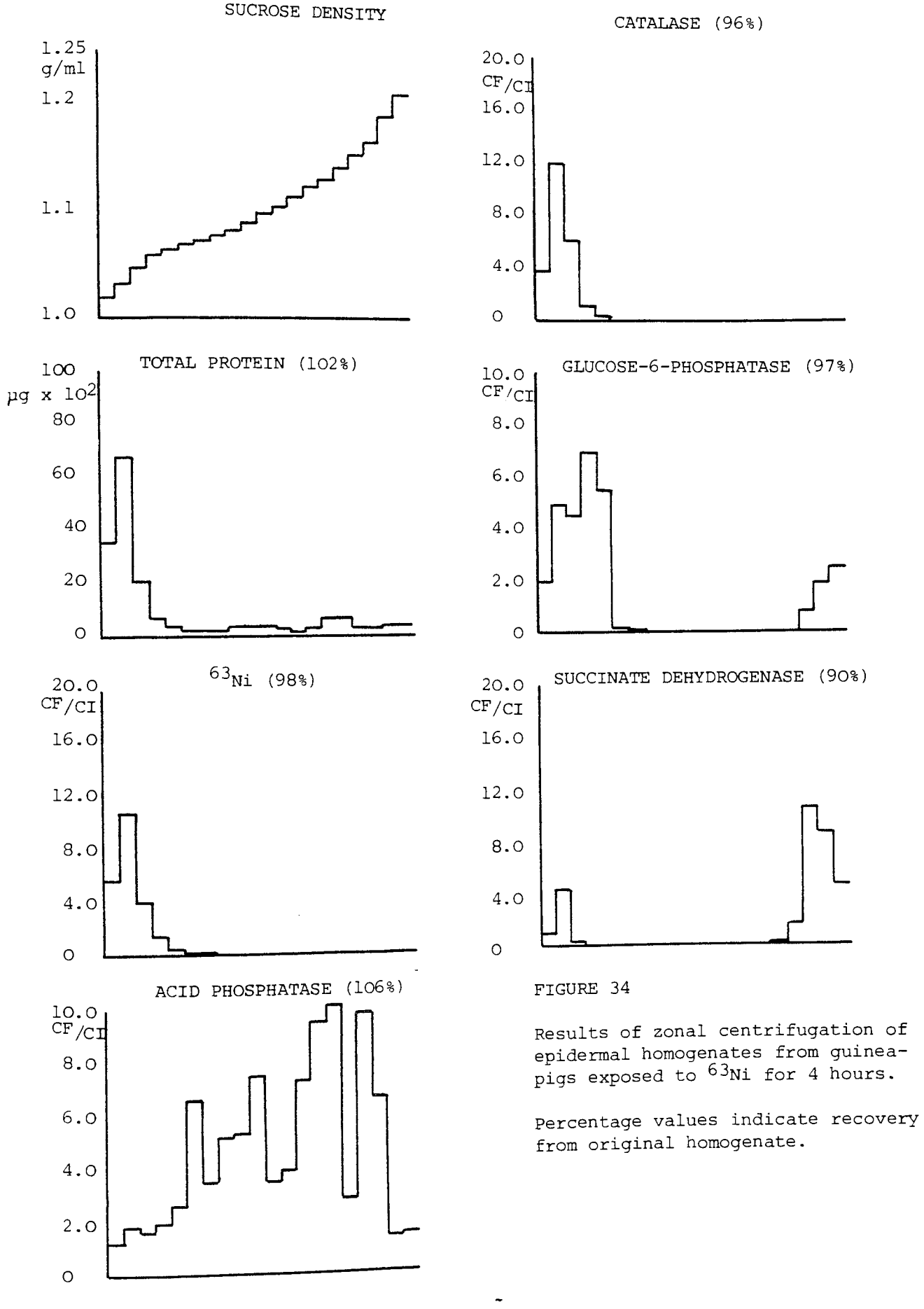


FIGURE 34

Results of zonal centrifugation of epidermal homogenates from guinea-pigs exposed to ^{63}Ni for 4 hours.

Percentage values indicate recovery from original homogenate.

TABLE 29

Results of Zonal Centrifugation - Group values

Exposure Time, 12 hours.
Number of Animals, 3.

Fraction	Sucrose Density (g/ml)	Total Protein µg x 10^2	^{63}Ni CF/CI	Acid Phosphatase CF/CI	Catalase CF/CI	Glucose-6-Phosphatase CF/CI	Succinate Dehydrogenase CF/CI
1	1.010	31.7	6.4	2.2	5.5	1.1	1.5
2	1.030	51.3	9.9	3.2	18.4	4.2	5.9
3	1.050	18.8	1.5	1.5	2.6	5.1	0
4	1.057	8.5	1.2	1.3	0.2	7.4	0
5	1.060	2.5	0.1	0.5	0	4.1	0
6	1.063	2.3	0	3.0	0	0.1	0
7	1.065	1.7	0	6.5	0	0	0
8	1.067	1.9	0	1.5	0	0	0
9	1.076	4.9	0	0.5	0	0	0
10	1.086	4.1	0	7.3	0	0	0
11	1.092	2.3	0	6.1	0	0	0
12	1.102	2.2	0	4.8	0	0	0
13	1.108	2.5	0	7.3	0	0	0
14	1.120	3.4	0	5.9	0	0	0
15	1.130	4.3	0	3.3	0	0	0
16	1.139	2.8	0	1.4	0	0.5	0
17	1.147	3.0	0	6.3	0	2.5	2.3
18	1.164	4.1	0	3.3	0	0.8	10.0
19	1.181	6.3	0	3.1	0	0	10.1
20	1.202	5.6	0	1.9	0	0	5.2
Recovery from Homogenate (%)	-	97	97	100	92	106	89

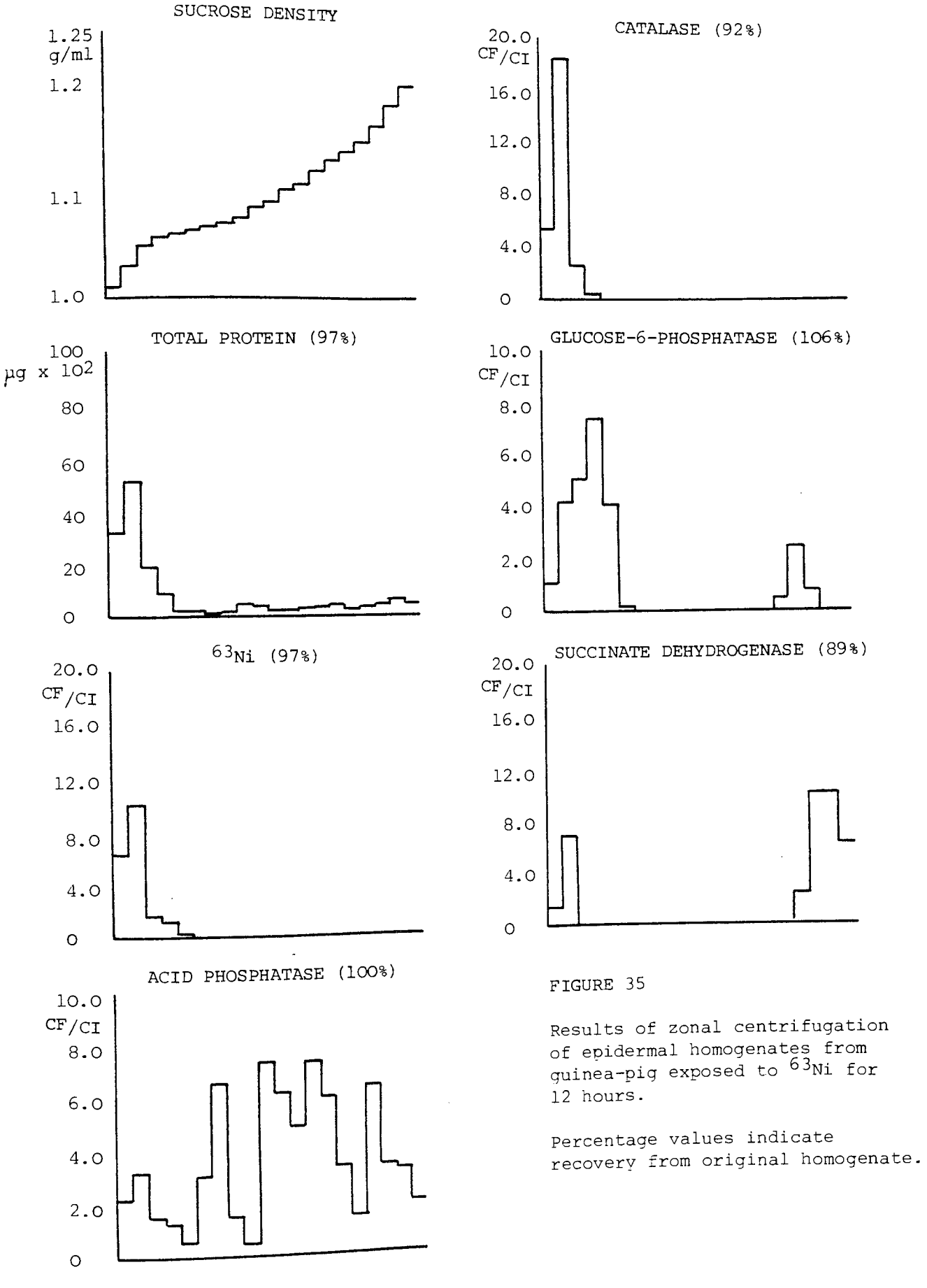


FIGURE 35

Results of zonal centrifugation of epidermal homogenates from guinea-pig exposed to ^{63}Ni for 12 hours.

Percentage values indicate recovery from original homogenate.

TABLE 30

Results of Zonal Centrifugation - Group values

Exposure Time, 24 Hours.
Number of Animals, 5.

Fraction	Sucrose Density (g/ml)	Total Protein µg x 10^2	^{63}Ni CF/CI	Acid Phosphatase CF/CI	Catalase CF/CI	Glucose-6-Phosphatase CF/CI	Succinate Dehydrogenase CF/CI
1	1.018	35.9	8.7	1.4	8.2	2.0	1.5
2	1.036	67.2	9.6	2.7	15.3	3.7	4.1
3	1.053	21.1	2.1	3.1	1.1	5.2	0.1
4	1.061	7.7	0.8	1.4	0.4	7.3	0
5	1.065	3.9	0.3	2.1	0.2	3.1	0
6	1.071	1.7	0.1	5.7	0.1	0.1	0
7	1.075	1.8	0.1	6.2	0.1	0	0
8	1.079	1.6	0.1	1.3	0.1	0	0
9	1.083	1.4	0	1.6	0	0.5	0
10	1.091	2.2	0	8.2	0	0	0
11	1.100	1.9	0	7.1	0	0	0
12	1.111	3.4	0	9.0	0	0	0
13	1.120	2.1	0	4.7	0	0	0
14	1.129	2.9	0	7.2	0	0	0
15	1.139	4.4	0	6.1	0	0	0
16	1.148	4.4	0	3.8	0	0	0
17	1.162	5.2	0	3.5	0	1.0	3.1
18	1.176	6.3	0	1.5	0	0	6.5
19	1.199	7.7	0	0.8	0	1.3	10.4
20	1.223	7.4	0	1.0	0	0	5.1
Recovery from Homogenate (%)	-	97	94	91	91	94	90

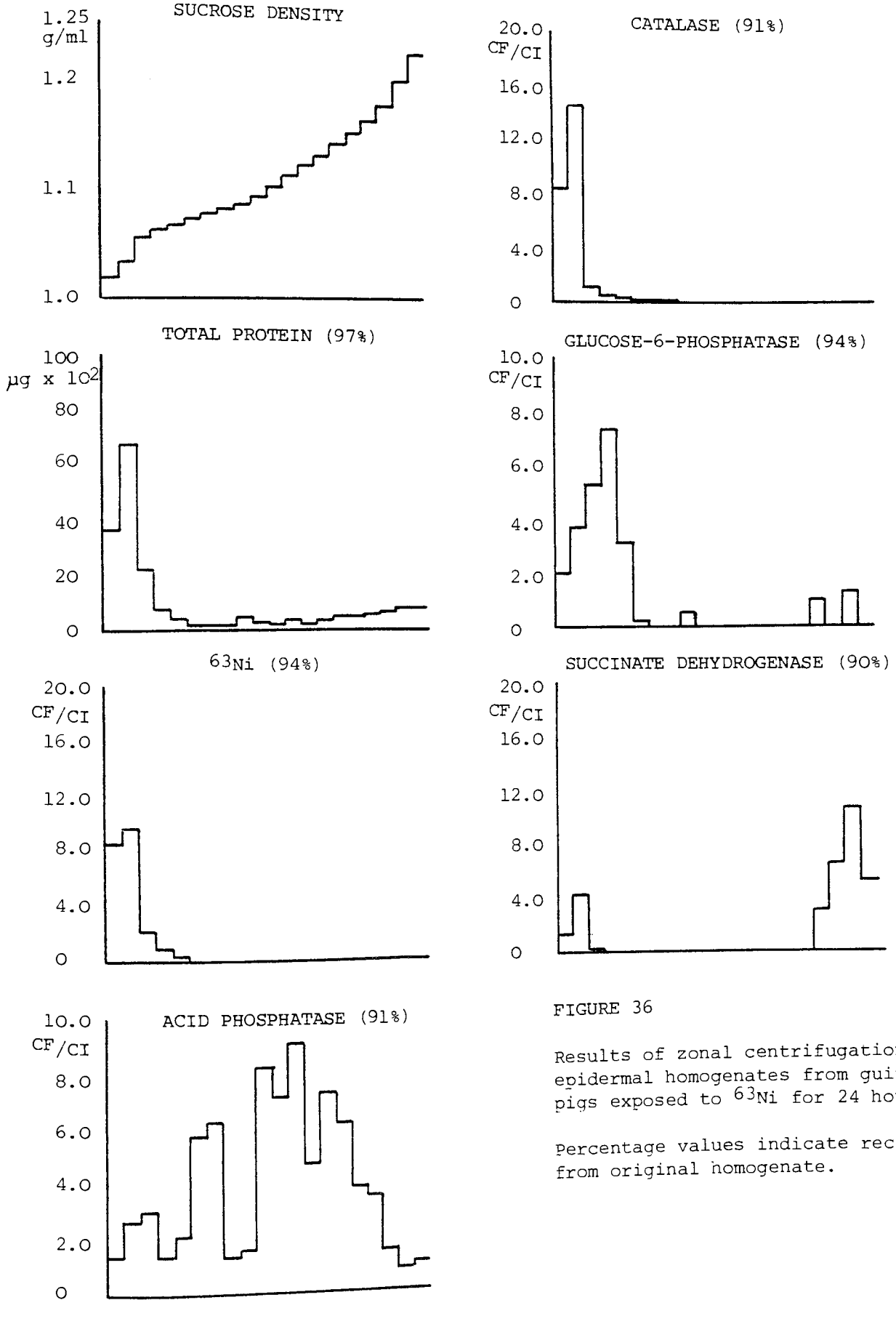


FIGURE 36

Results of zonal centrifugation of epidermal homogenates from guinea-pigs exposed to ^{63}Ni for 24 hours.

Percentage values indicate recovery from original homogenate.

TABLE 31

Results of Zonal Centrifugation - Group values

Exposure Time, 48 Hours.
Number of Animals, 4.

Fraction	Sucrose Density (g/ml)	Total Protein µg x 10^2	^{63}Ni CF/CI	Acid Phosphatase CF/CI	Catalase CF/CI	Glucose-6-Phosphatase CF/CI	Succinate Dehydrogenase CF/CI
1	1.020	40.8	5.2	1.4	8.9	1.0	1.6
2	1.033	93.9	11.1	2.3	16.7	2.7	3.4
3	1.039	18.8	5.3	2.8	1.6	4.4	0
4	1.051	8.2	2.1	0.6	0.1	7.5	0
5	1.058	3.9	0.7	1.0	0	4.9	0
6	1.064	1.4	0.2	6.2	0	1.4	0
7	1.070	1.0	0.1	4.3	0	0.2	0
8	1.078	0.5	0.1	1.5	0	0.3	0
9	1.083	3.6	0	2.4	0	0	0
10	1.089	4.5	0	7.3	0	0	0
11	1.096	3.7	0	9.1	0	0	0
12	1.106	7.4	0	8.0	0	0	0
13	1.117	5.2	0	7.6	0	0	0
14	1.130	1.7	0	5.3	0	0	0
15	1.138	2.3	0	6.8	0	0.4	0
16	1.149	2.3	0	4.5	0	1.4	0.1
17	1.161	3.0	0	4.7	0	0	1.3
18	1.175	6.8	0	1.8	0	0.6	9.6
19	1.202	8.6	0	1.3	0	0	9.4
20	1.221	8.9	0	0.6	0	0	3.8
Recovery from Homogenate (%)	-	98	98	96	90	102	95

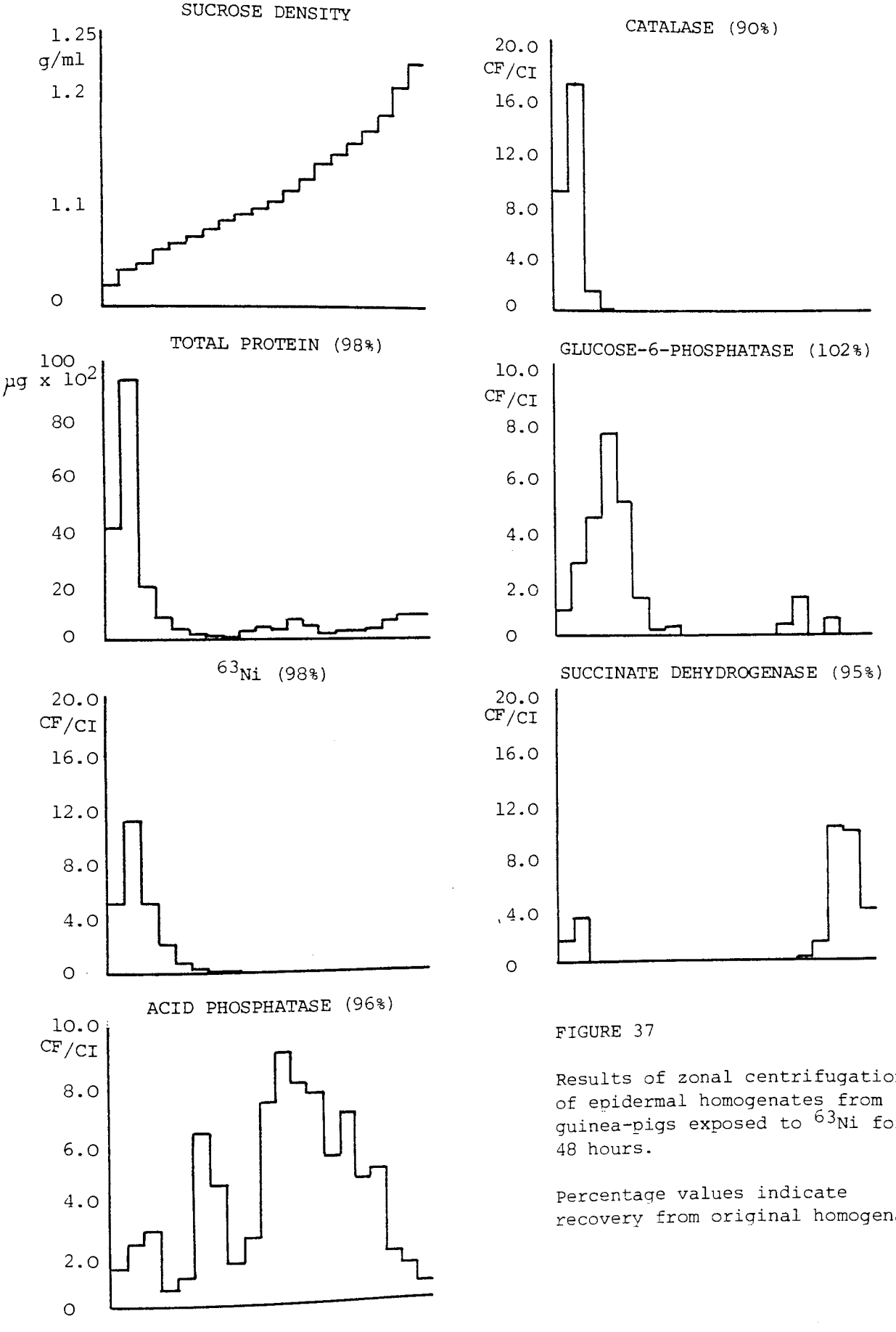


FIGURE 37

Results of zonal centrifugation of epidermal homogenates from guinea-pigs exposed to ^{63}Ni for 48 hours.

Percentage values indicate recovery from original homogenate.

TABLE 32

Results of Zonal Centrifugation Control - Group values

Control 1 - ^{63}Ni Added Post-Homogenization. Number of Animals, 3.

Control 2 - ^{63}Ni Added Pre-Homogenization. Number of Animals, 3.

Fraction	Sucrose Density (g/ml)		Total Protein µg x 10^2		^{63}Ni CF/CI		Acid Phosphatase CF/CI		Catalase CF/CI		Glucose-6-Phosphatase CF/CI		Succinate Dehydrogenase CF/CI	
	1	2	1	2	1	2	1	2	1	2	1	2	1	2
1	1.02	1.02	32.2	31.9	5.9	5.9	1.5	2.3	7.0	5.1	0.6	1.8	2.3	0.9
2	1.036	1.033	64.1	60.2	18.4	20.3	2.3	2.7	15.0	17.9	4.7	3.6	4.5	3.4
3	1.045	1.045	19.6	17.0	1.0	0.6	2.0	2.7	4.1	2.4	5.9	5.1	1.1	0.4
4	1.054	1.059	8.9	6.5	0	0	1.2	1.3	0.8	0.7	7.6	7.2	0.1	0
5	1.062	1.064	5.2	3.5	0	0	2.8	0.9	0.1	0	4.3	1.8	0	0
6	1.067	1.073	2.1	2.7	0	0	7.0	4.1	0	0	0.1	0.1	0	0
7	1.072	1.080	2.2	2.6	0	0	3.6	5.1	0	0	0	0	0	0
8	1.078	1.087	2.4	2.0	0	0	1.1	1.4	0	0	0	0	0	0
9	1.081	1.092	5.4	4.3	0	0	3.7	7.7	0	0	0	0	0	0
10	1.093	1.104	4.1	6.8	0	0	9.1	8.1	0	0	0	0	0	0
11	1.102	1.111	2.6	4.6	0	0	6.0	3.6	0	0	0	0	0	0
12	1.115	1.121	4.5	4.7	0	0	1.8	2.1	0	0	0	0	0	0
13	1.122	1.131	2.9	2.5	0	0	3.7	8.2	0	0	1.4	0	0	0
14	1.132	1.139	5.0	1.9	0	0	7.2	8.6	0	0	0	0	0	0
15	1.146	1.149	5.6	3.1	0	0	8.4	8.1	0	0	0	0	0	0.1
16	1.159	1.158	5.5	4.2	0	0	5.8	4.2	0	0	0	0	0.1	1.6
17	1.173	1.172	6.9	4.4	0	0	4.2	1.4	0	0	0	0.5	7.7	5.9
18	1.183	1.184	6.5	3.7	0	0	3.3	2.5	0	0	1.1	1.4	10.2	11.4
19	1.200	1.206	8.2	6.6	0	0	1.4	2.1	0	0	0	0	9.6	8.8
20	1.221	1.226	6.3	6.2	0	0	1.7	2.2	0	0	0	0	5.3	4.4
Recovery from Homogenate (%)	-	-	96	98	97	97	105	93	100	104	96	118	91	93

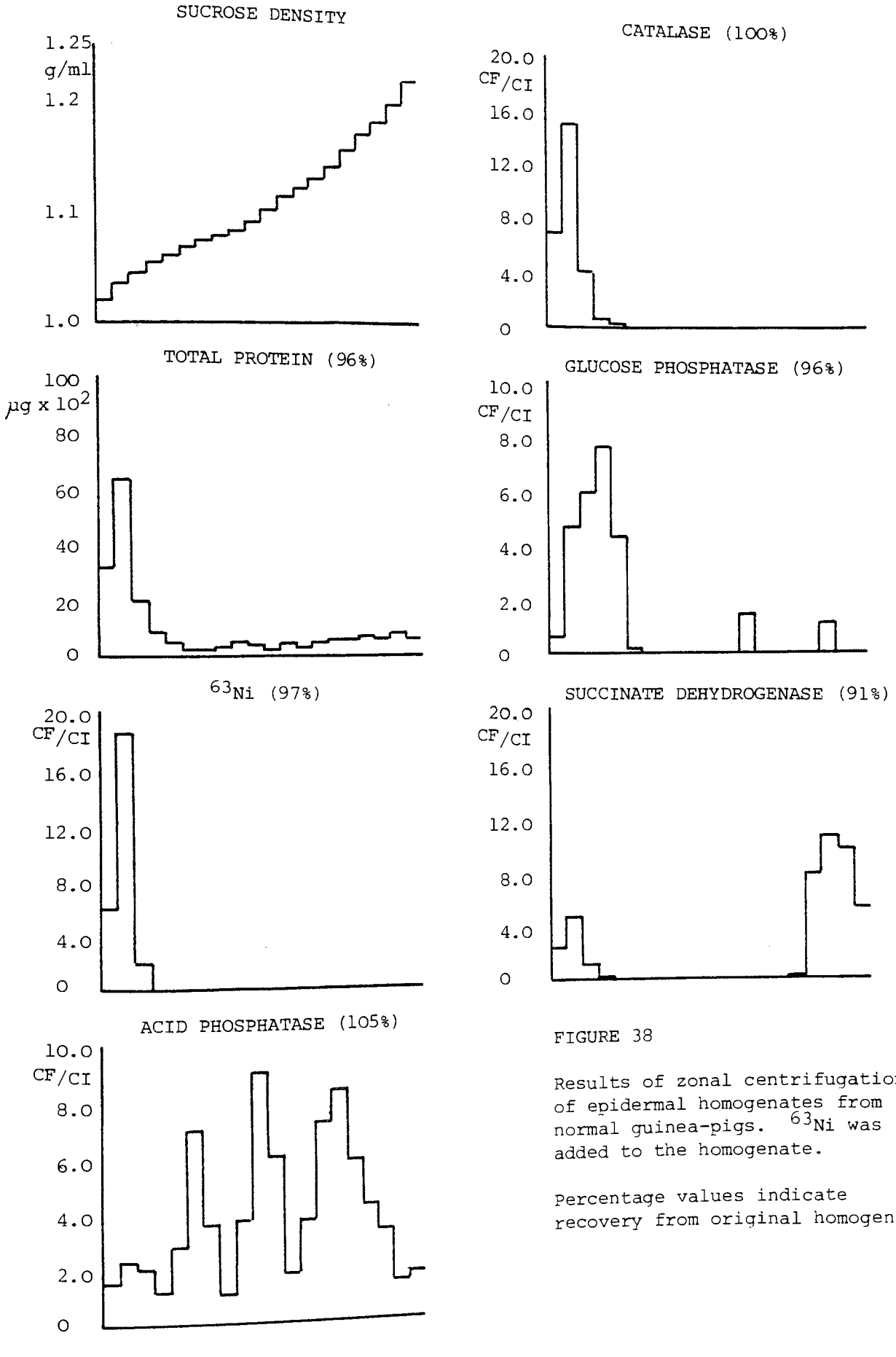


FIGURE 38

Results of zonal centrifugation of epidermal homogenates from normal guinea-pigs. ^{63}Ni was added to the homogenate.

Percentage values indicate recovery from original homogenate.

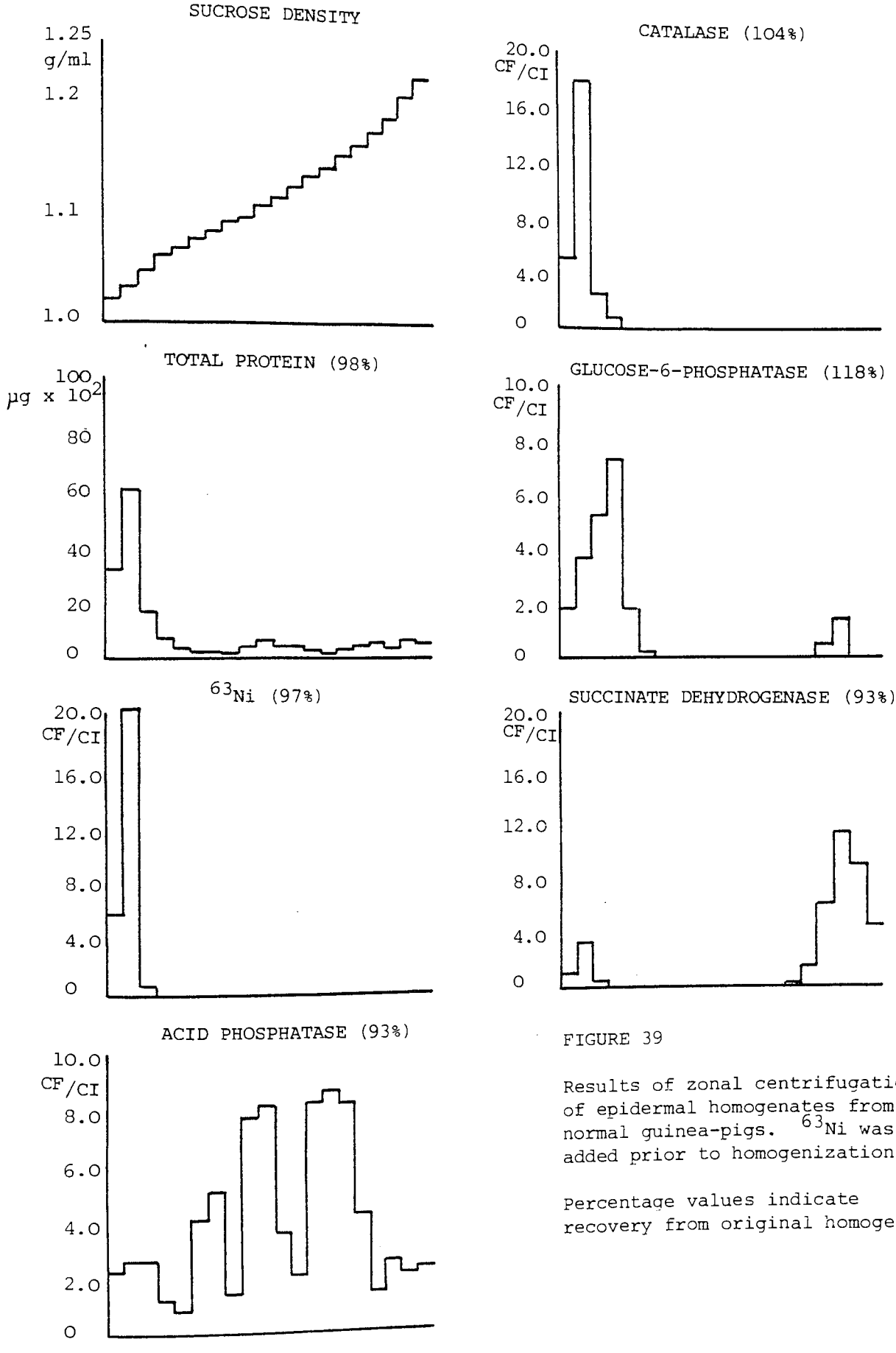


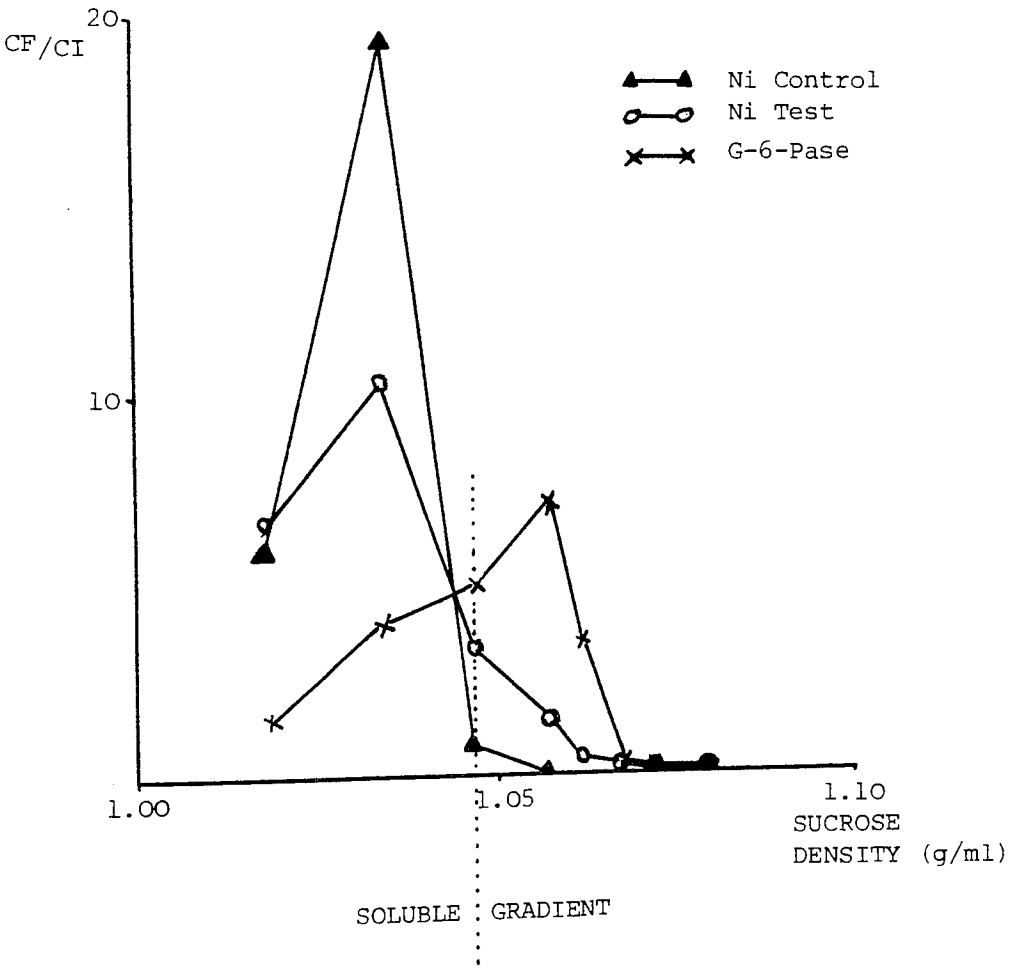
FIGURE 39

Results of zonal centrifugation of epidermal homogenates from normal guinea-pigs. ^{63}Ni was added prior to homogenization.

Percentage values indicate recovery from original homogenate.

FIGURE 40

Density distribution of nickel-63 in zonal fractions 1 to 9



Equilibrium Dialysis of Zonal Fractions

The technique of equilibrium dialysis using teflon half cells separated by dialysis membrane was performed to quantitatively distinguish between bound (non-dialysable) and unbound (dialysable) ^{63}Ni in selected zonal-centrifugation fractions.

The initial parameter to be decided during the development of this technique was the time for nickel ions to reach equilibrium under the conditions of dialysis. The results of these developmental assays are presented as Figure 41.

In 5% w/w sucrose $^{63}NiCl$ reached equilibrium within 2½ hours and was stable thereafter. The concentrations of $^{63}NiCl$ in each cell were equal at equilibrium and indicated that any binding to the dialysis membrane was negligable and need not be accounted for in the calculations for the test samples.

A dialysis time of 3½ hours was chosen for the zonal fractions.

The molecular weight exclusion limit of the dialysis membrane was 2000 daltons.

Equilibrium dialysis was performed on zonal fractions 2 (soluble) and 4 (microsomal) for each guinea-pig. These fractions were selected as the two main localizations of nickel in the epidermal homogenates.

The results for individual animals are presented as Tables 33 and 34 and group values in Tables 35 and 36.

The microsomal fractions for control animals were not assayed because no radioactivity could be detected during the anlaysis of these fractions after centrifugation.

In the soluble fractions for test animals the largest proportion of ^{63}Ni was found to be dialysable (82-86%) and therefore of a molecular weight of less than 2000 daltons. The remainder (14-18%) was excluded by the membrane and therefore bound in conjugates of molecular weight greater than 2000 daltons.

The proportions of bound and unbound nickel remained similar for each period of exposure, but the actual concentrations of nickel in each fraction reflected the changes seen with exposure time for total homogenate nickel concentrations.

When the soluble zonal fractions from control epidermal homogenates were assayed a proportion (7.5-10%) of the nickel content was also bound. However, the values obtained were slightly less than for the test guinea-pigs. This observation suggests that binding of nickel to the soluble proteins of epidermis occurs *in vitro*.

In the microsomal fractions for test animals, the largest proportion (89-95%) of Ni was not dialysable and therefore bound in conjugates of molecular weight greater than 2000 daltons.

Again, the proportion of bound and unbound nickel remained similar for each period of exposure, but the actual concentrations reflected the changes seen with exposure time for total homogenate nickel concentrations.

No radioactivity could be detected in the microsomal fractions of the control homogenates which suggests that binding of nickel in the microsomal fractions of test animals occurs *in vivo*.

The analytical recoveries for these investigations averaged 99.6% for soluble fractions and 99.4% for microsomal fractions.

FIGURE 41

EQUILIBRIUM DIALYSIS OF $^{63}NiCl_2$ IN SUCROSE (5% w/w)
(mean of triplicate assays)

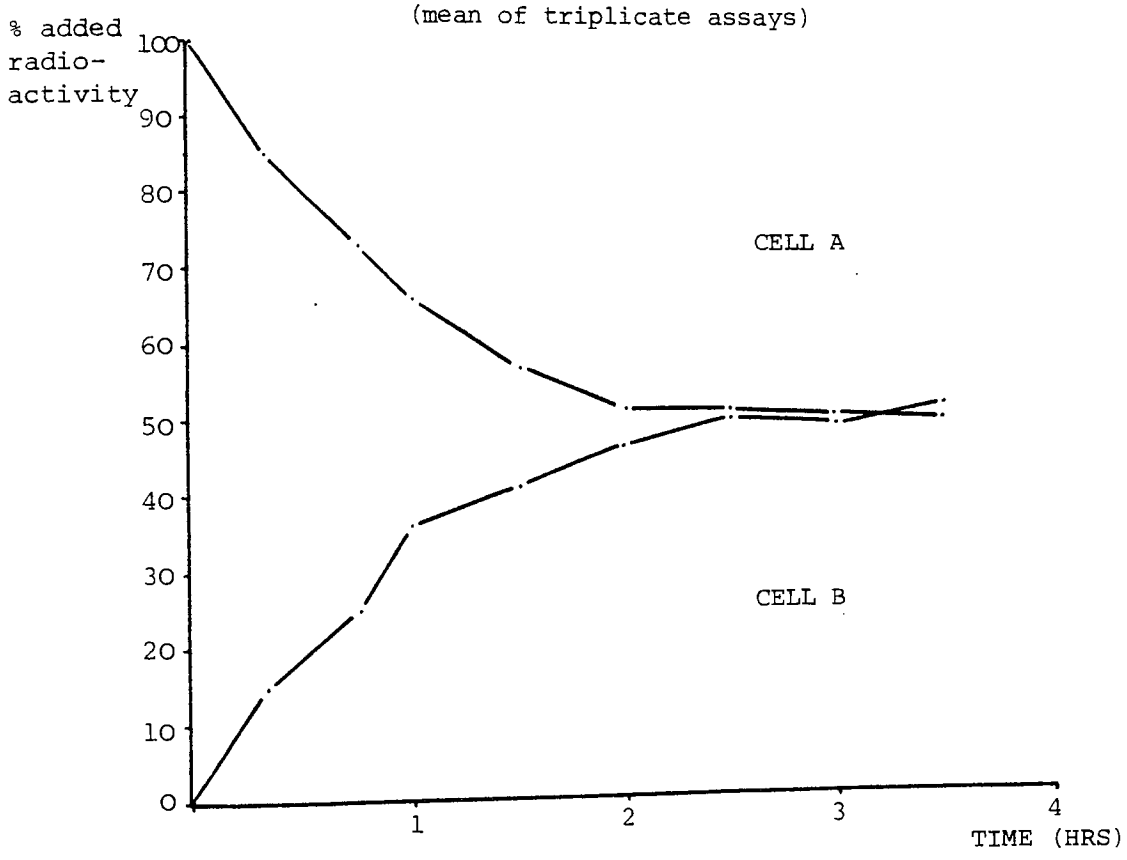


TABLE 33

Equilibrium Dialysis of Zonal Fractions 2 (Soluble) - Individual values

Exposure Time (Hours)	Guinea Pig	Cell A (Dpm/ml) $CL_{(b)} + CL_{(f)}$	Cell B (Dpm/ml) $CL_{(f)}$	Free fraction % f	Bound fraction % b	Starting concentration (Dpm/ml) $CL_{(o)}$	Recovery %
4	2	7170	5309	85.1	14.9	12476	100.0
	3	6726	4932	84.6	15.4	11748	99.2
	4	7209	5149	83.3	16.7	12408	99.6
12	5	12154	8784	83.9	16.1	21128	99.1
	6	21870	15519	83.0	17.0	37390	100.0
	7	29525	19238	78.9	21.1	48818	99.9
24	8	38732	26365	81.0	19.0	63570	102.4
	9	34065	26657	87.8	12.2	61960	98.0
	10	35631	27937	87.9	12.1	64212	99.0
	11	22682	17009	85.7	14.3	29492	100.5
	12	27286	21438	88.0	12.0	48774	99.9
48	15	28930	19381	80.2	19.8	49111	98.4
	16	27665	22637	90.0	10.0	50300	100.0
	17	28589	21874	86.7	13.3	50720	99.5
	18	31822	23858	85.7	14.3	56241	99.0
Control 1	20	26511	23057	93.0	7.0	49585	99.9
	21	26190	21982	91.1	8.9	49172	98.2
	22	27294	23953	93.5	6.5	50549	101.4
Control 2	23	30219	24561	89.7	10.3	54769	100.0
	24	27906	21836	87.8	12.2	50745	98.0
	25	27432	23660	92.6	7.4	51228	99.7

TABLE 34

Equilibrium Dialysis of Zonal Fractions 4 (Microsomal) - Individual values

Exposure Time (Hours)	Guinea Pig	Cell A (Dpm/ml) $CL_{(b)} + CL_{(f)}$	Cell B (Dpm/ml) $CL_{(f)}$	Free fraction % f	Bound fraction % b	Starting concentration (Dpm/ml) $CL_{(o)}$	Recovery %
4	2	1490	27	3.6	96.4	1498	101.3
	3	1857	29	3.1	96.9	1886	100.0
	4	1586	63	7.6	92.4	1756	93.9
12	5	1125	58	9.8	90.2	1282	92.3
	6	4372	90	4.0	96.0	4490	99.4
	7	7190	530	14.7	85.3	7624	101.3
24	8	5620	570	18.4	81.6	6180	100.2
	9	1968	142	13.6	86.4	2040	103.4
	10	6074	437	13.4	86.6	6496	100.2
	11	2034	83	7.8	92.2	2086	101.5
	12	1510	29	3.8	96.2	1526	100.9
48	15	5958	314	10.0	90.0	6372	98.4
	16	5770	236	7.9	92.1	6106	98.4
	17	8697	393	8.6	91.4	9060	100.3
	18	8308	408	9.4	90.6	8746	99.7
Controls 1 and 2	NOT ASSAYED						

TABLE 35

Equilibrium Dialysis of Zonal Fractions 2 (Soluble)

- Group values

Exposure Time (Hrs)	$CL_b + CL_f$ (Dpm/ml)	$CL_{(f)}$ (Dpm/ml)	f %	b %	CL_o (Dmp/ml)	Recovery %
4	7635	5130	84.3	15.6	12211	99.6
12	21183	14514	81.9	18.1	35779	99.7
24	31679	23881	86.1	13.9	55602	100.0
48	29252	21938	85.7	14.4	51593	99.2
Control 1	26665	22997	92.5	7.5	49769	99.3
Control 2	28519	23352	90.0	10.0	52247	99.3

TABLE 36

Equilibrium Dialysis of Zonal Fractions 4 (Microsomal)

- Group values

Exposure Time (Hrs)	$CL_b + CL_f$ (Dpm/ml)	CL_f (Dpm/ml)	f %	b %	CL_o (Dmp/ml)	Recovery %
4	1644	40	4.8	95.2	1713	98.4
12	4229	226	9.5	90.5	4465	97.7
24	3441	252	11.4	88.6	3666	101.2
48	7183	338	9.0	91.0	7571	99.2
Controls 1 and 2	NOT ASSAYED					

DISCUSSION

The objective of the time-course studies were firstly to qualitatively and quantitatively assess the absorption and accumulation of nickel both within and across the skin and secondly to isolate nickel conjugates which could represent the complete antigen(s).

The results of the micro-autoradiography studies indicated that nickel is poorly absorbed across the skin and does not penetrate the tissue further than the epidermis. Accumulation in the highly keratinized areas (stratum corneum and hair shafts) was also apparent. These observations are in agreement with the in vitro work of Wells (1956), Kolpakov (1963) and Samitz and Katz (1976) on cadaver skin.

There was some evidence to suggest that passage down hair shafts and into the follicles may be the route of absorption across the skin although the quantities would be expected to be small.

The immunological importance of the hair shaft route of entry remains uncertain. For nickel to contact cells of the reticulo-endothelial system (e.g. macrophages, T-lymphocytes) it may have to escape from the hair follicles. This could be achieved by;

a) passage of nickel with hair growth, in which case it would be lost from the skin;
b) diffusion of nickel from the hair into the dermis. This was not apparent in autoradiographs taken over the 48 hour exposure period;

c) diffusion of nickel from the follicle into the hair blood capillary network. From the work of Frey and Wenk (1957) the blood system, unlike the lymphatics, does not appear to play an important role in the induction of sensitivity (see Section II).

The accumulation of nickel in the epidermis at levels lower than the stratum corneum and apparent labelling of individual epidermal cells may be of more importance in the induction of sensitivity. Contact of bound nickel by cells of the lymphoid system may be possible once nickel is past the stratum corneum. Alternatively drainage in extra-cellular fluids and then into the lymphatic system may be possible.

In the quantitative experiments the poor absorption of nickel across the skin was confirmed by examination of the appearance of nickel in the plasma. Maximum plasma levels occurred after 12 hours exposure but constituted only approximately 3% of the applied dose per litre plasma, which was equivalent to approximately 0.06% of the applied dose in the total plasma volume. This latter figure and the results of Samitz and Katz (1976) in vitro work appear to be comparable and less than 0.1% of the dose is absorbed across the epidermis.

These low plasma levels, together with the micro-autoradiography of follicular absorption suggest that the passage down skin appendages may be the only route of entry of nickel across the skin.

If skin appendages are the major or only portals of entry across the skin, the heavy accumulation to the hair and the keratinized hair shaft canals could limit the rate at which absorption can continue (i.e. the greater the accumulation with time the lesser the passage of nickel into hair follicles and blood). This could explain why plasma and urine concentrations of nickel reached a peak after 12 hours exposure but reduced at 24 and 48 hours.

This, of course, assumes that absorption of nickel is simple diffusion and does not take account of the slightly reduced total nickel content of skin observed after 48 hours of exposure. The possibility that the dose in the bandage is no longer available could still hold true as an explanation of reduced plasma concentrations.

The results of radioactivity counting of the homogenisable and residual (non-homogenisable stratum corneum, hairs, etc.) portions of the epidermis indicate that nickel accumulates in both the highly keratinized areas and the remaining areas. Generally the different areas of the epidermis contained similar amounts of radioactivity. However, the results for the keratinized areas after 12 hours of exposure were higher for 2 of the 3 exposed guinea-pigs (approximately 80% of total epidermal radioactivity). There is no apparent explanation for the deviation of these two animals from the general picture.

The subcellular distribution of epidermal nickel was examined by rate zonal ultracentrifugation of the epidermal homogenates. Data from these experiments indicate two localizations of nickel. The major proportion of nickel was associated with the soluble fraction of the homogenate and did not enter the sucrose gradient. This represented approximately 91% of the homogenate nickel content.

The second localization of nickel was associated with the light sucrose end of the gradient and coincided with the peak of microsomal marker enzyme activity (glucose-6-phosphatase). The nickel content represented 9% of the total homogenate concentration. In contrast to the soluble fraction, this 'microsomal' localization did not occur in control epidermal samples which had nickel added before or after homogenization. The slight, but definite movement into the sucrose gradient was, therefore, not considered to be an artefact of the homogenization or centrifugation techniques.

Microsomal localization of nickel following exposure to nickel carbonyl and nickel acetate has been reported for lung, liver and kidney (Sunderman and Selin 1968, Whanger 1973). In these reports binding to nuclei and mitochondria was also noted, but this was not observed in these present studies on skin. However, in comparison with studies using DNCB as the hapten (Nishioka et al 1971 and 1973), microsomal conjugates were antigenic in in vitro assays. It is therefore possible that the nickel-microsomal fraction could represent the possible antigen in nickel allergic dermatitis.

The soluble fraction of the epidermis could also represent the possible antigen. However at this stage of isolation it must still be considered as a complex mixture of at least free nickel, low molecular weight conjugates and high molecular weight conjugates. Further characterization and isolation was necessary before any attempts at biological assay of antigenicity.

Equilibrium dialysis of soluble and microsomal fractions containing nickel was performed to assess the degree of binding within each fraction. In the soluble fractions approximately 16% was excluded by the membrane and therefore bound in conjugates of a molecular weight greater than 2000 daltons. Bound nickel in soluble fractions from control homogenates represented approximately 9% of the total concentration and suggests that at least some of the conjugation occurs in vitro.

By contrast, the majority ($\sim$91%) of nickel in the microsomal fractions was bound. No radioactivity could be detected in control microsomal fractions.

The proportions of nickel found bound in the microsomal, soluble or non homogenisable fractions of the epidermis were similar for each fraction from each animal at each of the exposure times. However, the actual nickel concentrations reflected the changes in total epidermal nickel concentration seen with time.

In summary therefore, the results of experiments in this section indicate the following points of behaviour of nickel in the skin of the guinea-pig:

a) soluble nickel salts are poorly absorbed across the skin and trans-appendageal passage may be the major route and possibly the only route;

b) a small proportion of topically applied nickel salts appears in the plasma and is excreted in the urine. Peak plasma levels were seen at 12 hours exposure.

c) after 4 hours of exposure distinctive labelling along the middle of the epidermis was noted. This area is consistent with Langerhans' cell localization.

d) nickel accumulates in the highly keratinized areas of the skin, binds to individual cells at levels of the epidermis lower than the keratinized layers, but does not appear in the dermis.

e) the accumulation in the keratinized areas may be rate limiting on the trans-appendageal absorption.

f) two areas were isolated from epidermal homogenates which could represent or contain the true antigen(s); the soluble and microsomal fractions.

g) the potential antigen formation occurred at each exposure time used in the quantitative experiments and the proportion of nickel concentration represented by each fraction was similar for each time. The actual concentrations reflected the changes in total epidermal nickel seen with increasing exposure time.

In view of the above findings, the soluble and microsomal fractions from each animal were selected for further examination. A summary of the group mean bound nickel content of each fraction for each time period is as follows:

Fraction	Exposure Time (Hrs)	% Bound	dmp/ml	ng Ni^{II}/ml	% applied dose
Soluble	4	15.6	1905	475	0.002
	12	18.1	6476	1470	0.007
	24	13.9	7729	1927	0.009
	48	14.4	7429	1853	0.008
Microsomal	4	95.2	1631	407	0.002
	12	90.5	3987	905	0.004
	24	88.6	3248	810	0.004
	48	91.0	6890	1718	0.008

Both fractions of the epidermis could contain antigenic constituents but the soluble fraction requires additional characterization.

SECTION V - ISOLATION OF SOLUBLE NICKEL CONJUGATES

OBJECTIVES

The results of rate zonal untracentrifugation described in the previous section indicated two areas of nickel localization in epidermal homogenates; the microsomal fraction and soluble fraction. Further isolation of nickel conjugates from the soluble fraction was considered essential before assessment of antigenic activities.

The objectives of this section of the project are to survey, isolate and characterize soluble nickel conjugates formed in the guinea-pig skin.

INTRODUCTION

In the previous section (IV) it has been stated that published data concerning nickel absorption and conjugation in the skin are limited. Similarly, there are no apparent publications of data concerning soluble nickel conjugates in the skin. There are, however, a few reports of the distribution of nickel in the "cytosol" of cells from other tissues and in plasma or serum. It is worth considering these data for comparison with the skin.

Studies on the nickel distribution within plasma or serum from several mammalian species have generally indicated three locations: a) albumin bound nickel b) low molecular weight ultrafiltrable nickel, and c) a nickel containing α_2-macroglobulin, subsequently called 'nickeloplasmin' (Nomoto et al 1971, Soestbergen and Sunderman 1972, Callan and Sunderman 1973, Nomoto et al 1971, Lucassen and Sarkar 1979, Oskarsson and Tjälve 1979, Nomoto 1980, Sarkar 1980).

In almost all the above reports, by far the greatest proportion of total serum nickel (77 - 96%) was found associated with albumin. In *in vitro* equilibrium dialysis studies (Callan and Sunderman 1973), the degree of albumin binding was shown to be species dependant. Lower levels of binding were observed for dog and pig albumins (55% and 77% of total Ni II respectively) than for human, rabbit and rat albumin (87 - 89%). This difference was suggested to result from the replacement of histidine by other amino acids at the primary nickel binding site in albumin.

The existence of low molecular weight ultrafiltrable nickel has been consistently identified. The proportion of total serum nickel that this represents is reported to be between 1% and 10% and probably represents nickel-amino acid conjugates. It is suggested that this fraction of serum nickel plays an important role in nickel homeostasis by serving as diffusable conjugates for extracellular transport and excretion of nickel (Soestbergen and Sunderman 1972, Lucassen and Sarkar 1979).

The presence of the α_2-macroglobulin Ni^{II} fraction has been reported by several investigaters although the data for the proportion of total serum nickel that this represents are conflicting. Nomoto *et al* (1971) found "nickeloplasmin" to represent 44% of total serum nickel but Lucasson and Sarkar (1979) found less than 0.1%.

The physiologic role of "nickeloplasmin" remains uncertain, although it may be involved in the hypernickelemia observed in some human diseases (Nomoto 1980, McNeely *et al* 1971).

Nickel distribution within soluble fractions of cells is being studied by several groups. However data are restricted to target organs other than the skin (e.g. lung, liver, kidney) and literature searches for data on the skin have been unrewarding.

Webb (1972) and Sabbioni and Marafante (1975) used gel filtration of rat liver "cytosol" to examine binding of $^{63}Ni^{II}$ to the metalothionein protein associated with cadmium toxicity. Neither studies produced evidence of Ni binding to this protein but did elute nickel in peaks around the void volume of Sephadex G-75 (M. Wt. $\geq$ 50,000). In similar studies Oskarsson and Tjälve (1979) examined nickel distribution in the "cytosol" of lung, liver and kidney from mice treated with $^{63}NiCl_2$ or $^{63}Ni(CO)_4$. Two nickel containing protein peaks were eluted of Sephadex G-75 from each tissue; one at the void volume (M. Wt. > 50,000) and the second at the bed volume (M. Wt. < 2,000). A third peak (M. Wt. ~30,000) was eluted from lung and kidney.

Sarkar (1980) used gel filtration on Sephadex G-50 to examine the distribution of nickel in rat kidney cytosol and eluted one peak of molecular weight between 5,000 to 10,000. After further characterization techniques it is suggested that the nickel may be associated with a metalothionein-like-protein.

Sunderman et al (1981) also examined rat kidney cytosol on Sephadex G-200. On this gel matrix six nickel containing constituents were eluted. Peak 1 eluted at the void volume (M. Wt. > 130,000); Peak 2 (M. Wt. ~70,000) was possibly albumin; Peak 3 (M. Wt. ~55,000); Peak 4 (M. Wt. 30,000) was similar to that described by Oskarsson and Tjälve (1979); Peak 5 (M. Wt. ~10,000) was suggested to correspond with a

metalothionein-like-protein; Peak 6 (m. Wt. < 2,000) eluted at the bed volume and contained the major portion of nickel (52 - 76%).

All the current published data are, of course, useful to the interpretation of nickel-tissue distribution. However, it is impossible to extrapolate to the skin, other than to predict that several nickel-protein conjugates of various molecular weights could be eluted from the soluble fraction of the skin.

MATERIALS AND METHODS

The distribution of nickel in soluble epidermal fractions was examined by preparative gel filtration chromotography.

Test Samples

The soluble fractions (zonal fraction 2) from the epidermal homogenates of ^{63}Ni exposed guinea-pigs were pooled for each time period. A total of four skin derived samples were therefore investigated.

In addition, plasma samples from four ^{63}Ni exposed guinea-pigs (one from each exposure time) were investigated. These samples were examined primarily to check that the plasma nickel of this animal model was distributed in a comparable manner to previous reports.

Preparation of skin soluble fractions

In the previous section, the results of equilibrum dialysis (Tables 33 and 35) indicated the zonal soluble fractions from each animal contained a large proportion ($\sim$84%) of dialysable nickel (M. Wt. < 2,000). At this stage of the investigation it was considered advantageous to first

remove this fraction in order to avoid possible masking of chromatography elution profiles.

This was acheived by gel chromatography down a short desalting column. Commercially prepared PD-10 disposable columns prepared with medium grade Sephadex G-25 (bed volume 9.1ml) were used (Pharmacia Ltd., Hounslow, Middlesex, England). The molecular weight exclusion limit of these columns was approximately 5,000 daltons. This preparation procedure served to remove the low molecular weight nickel and exchange sucrose for the buffer used in the main chromatography (phosphate buffered saline). The method was preferred to dialysis which can take several hours and use large volumes of buffer solutions.

PD-10 columns were equilibrated with four bed volumes ($\sim$ 36ml) of phosphate buffer saline (P.B.S.) of pH 7.45. Samples of each pooled soluble zonal fraction were loaded (load volume 2.5ml/run) and separated in P.B.S.. Fractions of 1ml were collected and the protein and ^{63}Ni contents assayed. Total protein was monitored by the method of Lowry et al (1951) and ^{63}Ni was estimated by scintillation counting as described in the previous section.

The first resolved peak of radioactivity and protein found in fractions 2 to 5 was used in the main experiments. The second peak in fractions 6 to 10 was not analysed further.

Chromatography of skin soluble fractions

Ascending gel filtration chromatography was performed on Sephacryl S-200 Superfine gel (Pharmacia Ltd., Hounslow, Middlesex, England) in phosphate buffered saline (P.B.S.). Sephacryl is an N,N-methylene bisacrylamide crosslinked dextran gel with a molecular weight fractionation range for proteins of 5,000 - 250,000.

The experimental set-up for the gel filtration chromatography is shown diagrammatically in figure 42. A borosilicate glass chromatography column (K16 type - Pharmacia Ltd.) of 16mm internal diameter and 100cm height was used throughout these procedures. The column was packed with Sephacryl S-200 gel and equilibrated in degassed P.B.S. at a flow rate of 1.3ml/minute. At least two bed volumes (approximately 400ml) were pumped through the column in both the downward and upward directions.

The homogeneity of the gel bed was checked by passing through a 1ml sample of Blue Dextran 2,000 (2mg/ml in P.B.S.) (Pharmacia Ltd.) at a flow rate of 1.3ml/minute.

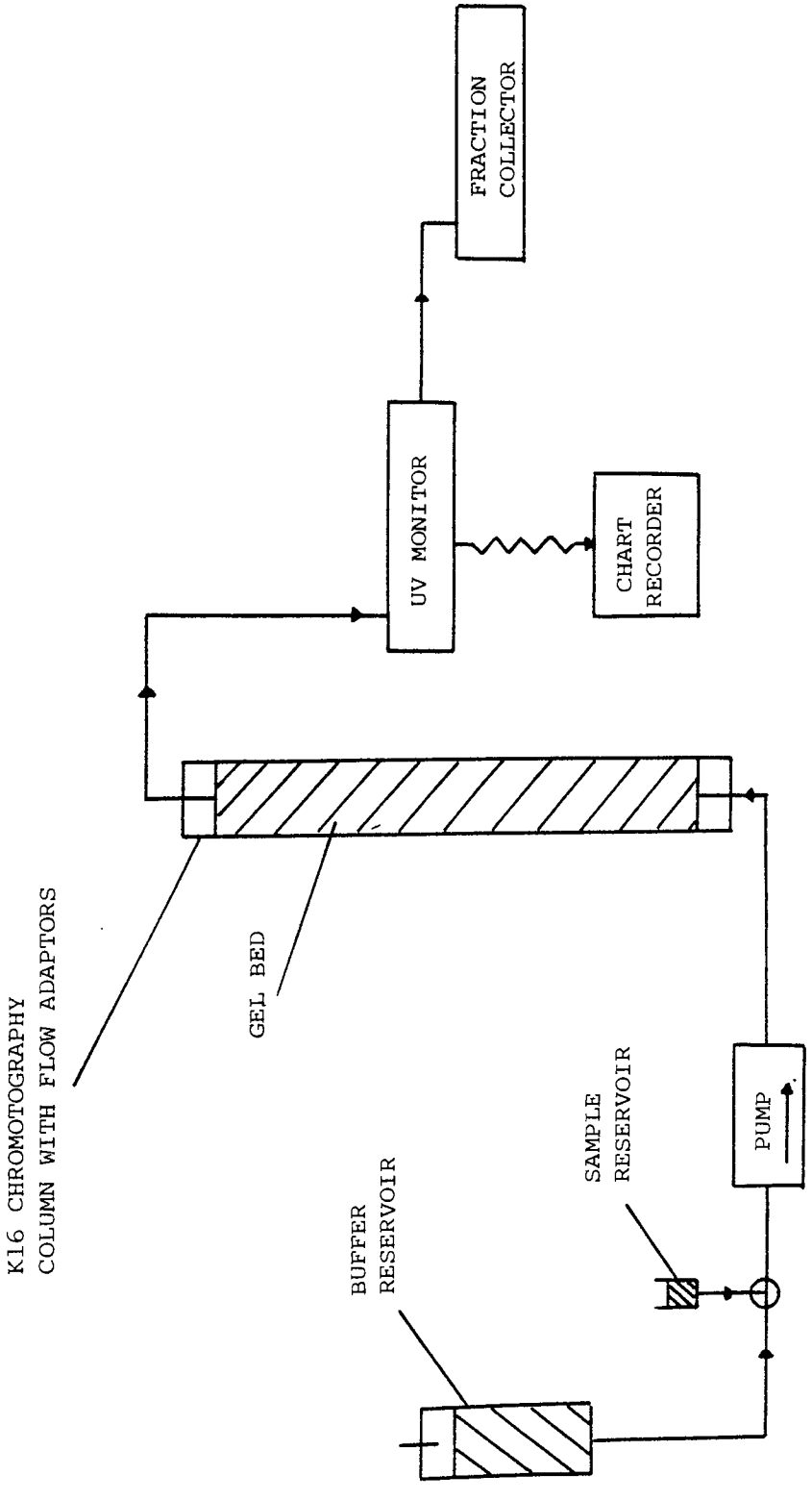
Once the column was packed and equilibrated, the parameters which vary with the packed bed size were assessed and the column was calibrated for molecular weight determinations.

The total bed volume (V_t) was assessed by direct measurement of the bed height and subsequent calculation.

FIGURE 42

EXPERIMENTAL SET-UP FOR

GEL FILTRATION CHROMATOGRAPHY



The void volume (V_o) was determined by passing the Blue Dextran 2,000 (2mg/ml in P.B.S.) through the column at the flow rate used for the test samples (0.16ml/minute).

Molecular weight calibration was carried out by passing protein standards of known molecular weight (Pharmacia Ltd.) through the column at a flow rate of 0.16ml/minute. The following standards were used:

albumin (bovine serum) 5mg/ml P.B.S. M. Wt. 67,000

ovalbumin (hen egg) 5mg/ml P.B.S. M. Wt. 43,000

chymotrypsinogen A (bovine pancreas) 5mg/ml P.B.S. M. Wt. 25,000

ribonuclease A (bovine pancreas) 5mg/ml P.B.S. M. Wt. 13,700

The loaded volume of each standard was 1ml.

After calibration the following test samples were run in P.B.S. at a flow rate of 0.16ml/min:

soluble zonal fractions from exposed guinea-pigs, pooled for each time period and prepared as stated above. A 4ml volume was loaded in each case.

plasma samples from one guinea-pig from each time period diluted 1 in 5 with P.B.S. to avoid viscosity dragging problems associated with high protein concentrations. A 1ml volume was loaded in each case

The elution curve for each chromatography run was monitored continuously at 280nm. The column effluent was passed through a silica-glass flow cell (path length 1cm, internal volume 80µl) fitted in a U.V.

spectrophotometer (Cecil Instruments Ltd., Cambridge, England). Absorbancies were recorded on a chart recorder.

Effluent fractions of 5ml were collected during the chromatography of the test samples using a drop-counting fraction collector (LKB-Ultrarac, LKB Instruments Ltd., Croydon, England).

Nickel-63 content of each fraction was measured by scintillation counting as previously described in Section IV.

Calculation of Chromatographic Behaviour

In order to characterize and interpret the elution patterns of test samples it is important to account for variations in the gel bed (e.g. bed colume, degree of packing) (Fischer 1980). The following parameters were therefore calculated:

Vo - void volume. The elution volume of molecules which are only distributed in the mobile phase because they are too large to enter the largest pores of the gel. This was determined by Blue Dextran 2,000 elution as described above.

Vt - total bed volume measured as described above.

Ve - elution volume. The average volume of eluant required to carry the separated substance through the column. This was measured from the inflexion point of each peak of absorbancy at 280nm

Kav - partition coefficient between the stationary phase and mobile phase of the column bed

$$Kav = \frac{Ve - Vo}{Vt - Vo}$$

In this calculation the total bed volume minus the mobile (void) volume is considered to represent the stationary phase, although it does not account for the volume of unavailable gel matrix.

RESULTS

General Performance of Studies

As in the previously described radiotracer studies (Section IV), total recovery of radioactivity and protein were used as indicators of the overall performance of the techniques. Recovery values for each gel filtration run are shown with the individual elution profiles.

In general ^{63}Ni and protein recoveries for soluble skin samples averaged 90% and 94% respectively. ^{63}Ni recoveries from plasma samples averaged 97%. Protein recovery was not examined for plasma samples because it was considered that the gel filtration experiments were a brief examination of nickel distribution in plasma and not protein distribution which has been extensively studied by many researchers.

Preparation of Soluble Skin Samples

Group separation of the soluble fractions from zonal centrifugation allowed the low molecular weight nickel ($\leq$ 5,000) to be removed from the high molecular weight ($\geq$5,000) conjugates and also served as a comparative test for the equilibrium dialysis in Section IV.

Group separation on PD-10 columns resulted in two peaks of radioactivity; the first eluted in fractions 2 to 5 and corresponding to the high molecular weight conjugates ($\geq$5,000), the second eluted over fractions 6 to 10 and corresponding to the low molecular weight nickel ($\leq$ 5,000).

The proportions of radioactivity recovered in each peak are shown in Table 37. These results show that the greatest proportion (87%) of radioactivity in soluble fractions from zonal centrifugation is of molecular weight less than 5,000. The results of equilibrium dialysis similarly indicated that approximately 85% was less than 2,000.

Distribution of Nickel in Plasma

The chromatography elution profiles of plasma samples from exposed guinea-pigs are presented as figures 43 to 46. Partition coefficients (Kav) and nickel contents are shown in Table 38.

In general three locations of nickel and six protein peaks were resolved. The nickel was found associated with protein peaks 2 (Kav 0.03), 4 (Kav 0.19) and 6 (Kav 1.00) which are considered to correspond with the elution behaviour of α_2-macroglobulin, albumin and low molecular weight ($\leq$ 5,000) fractions respectively (Hudson and Hay 1976, Fischer 1980, Mahler and Cordes 1971). Radioactivity could not be detected in the

TABLE 37

Group Separation of Radioactivity from

Soluble Zonal Fractions

Exposure Time (hrs.)	Percentage of Recovered Radioactivity in Peak		Overall Recoveries of Radioactivity (%)
	1*	2*	
4	11.9	88.1	95.6
12	16.6	83.4	93.9
24	12.6	87.4	97.0
48	12.2	87.8	92.0

* Peak 1 - M. Wt. $\geq$ 5,000

Peak 2 - M. Wt. $\leq$ 5,000

FIGURE 43

ELUTION PROFILE OF PLASMA FROM GUINEA-PIG NO. 3 EXPOSED TO ^{63}Ni FOR 4 HOURS

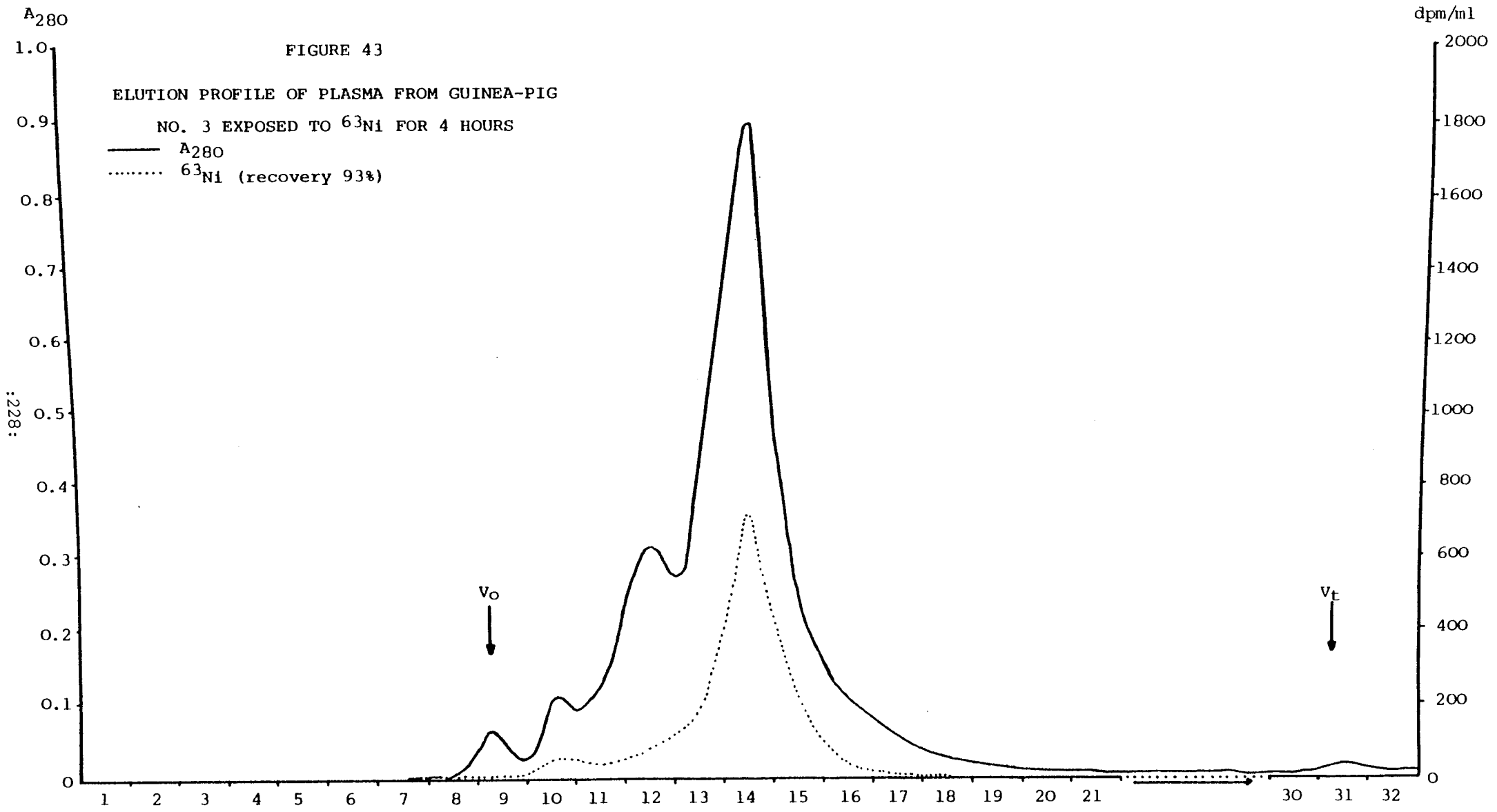


FIGURE 44

ELUTION PROFILE OF PLASMA FROM GUINEA-PIG NO. 7 EXPOSED TO ^{63}Ni FOR 12 HOURS

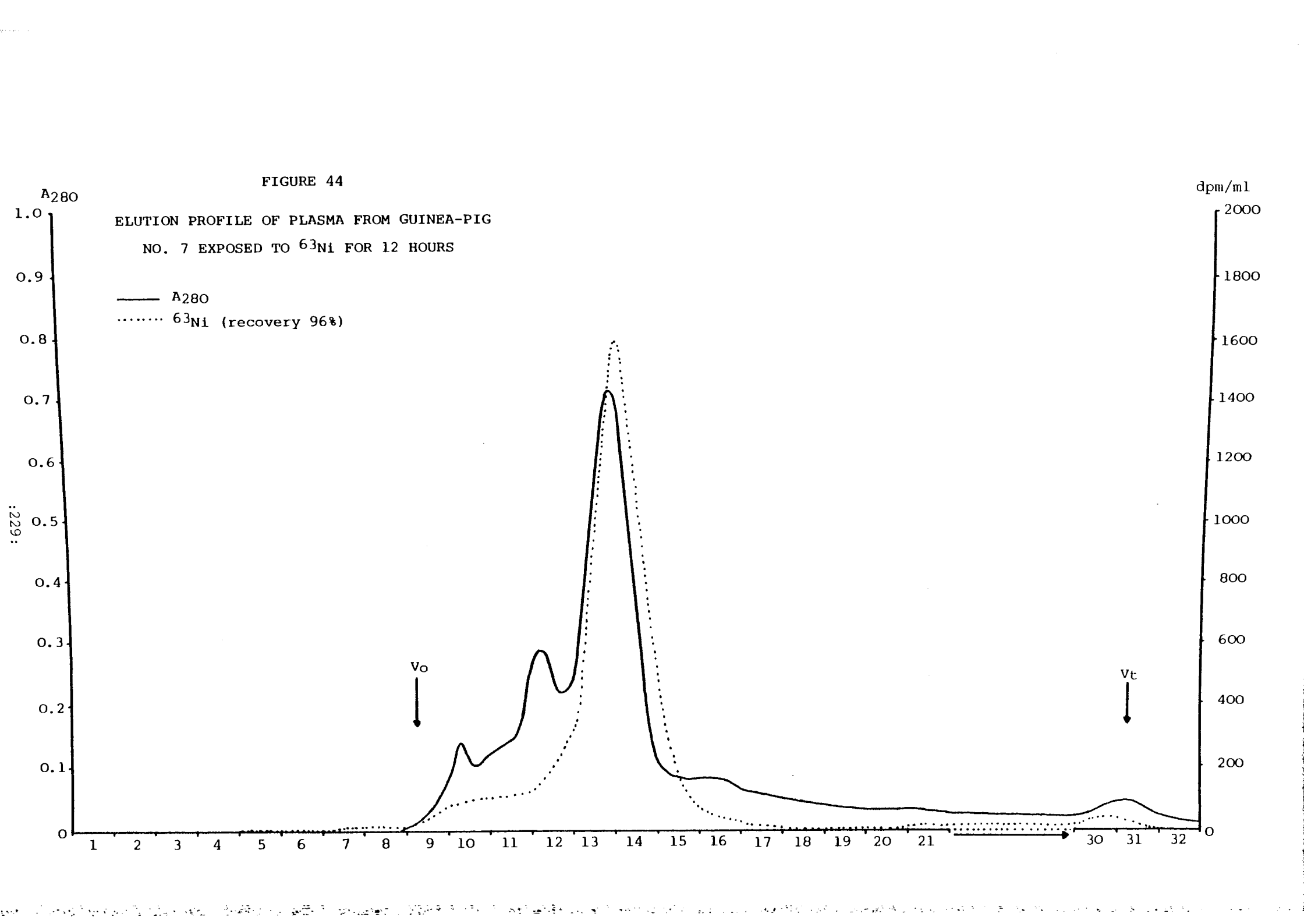


FIGURE 45

ELUTION PROFILE OF PLASMA FROM GUINEA-PIG NO. 11 EXPOSED TO ^{63}Ni FOR 24 HOURS

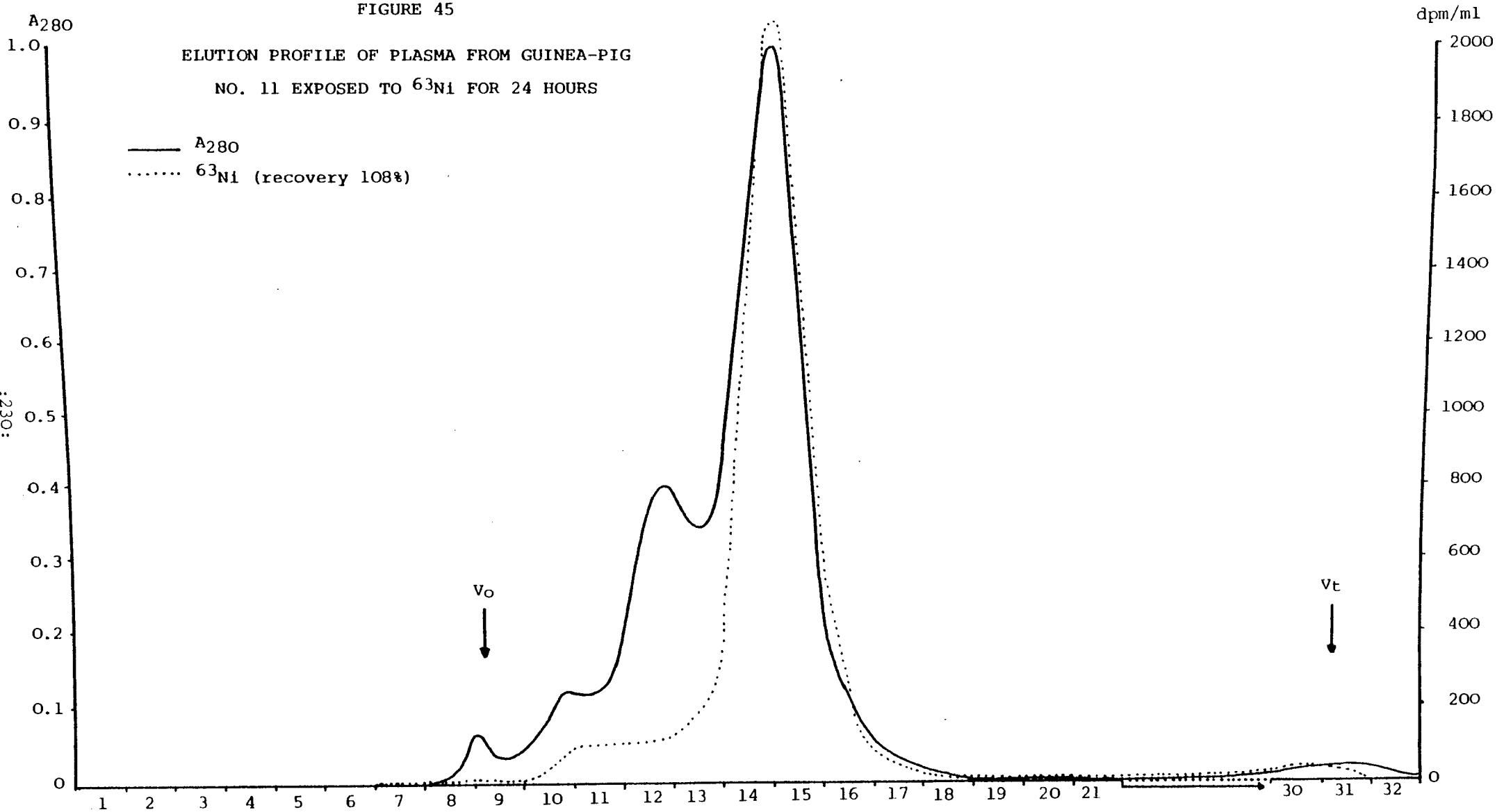


FIGURE 46

ELUTION PROFILE OF PLASMA FROM GUINEA-PIG NO. 15 EXPOSED TO ^{63}Ni FOR 48 HOURS

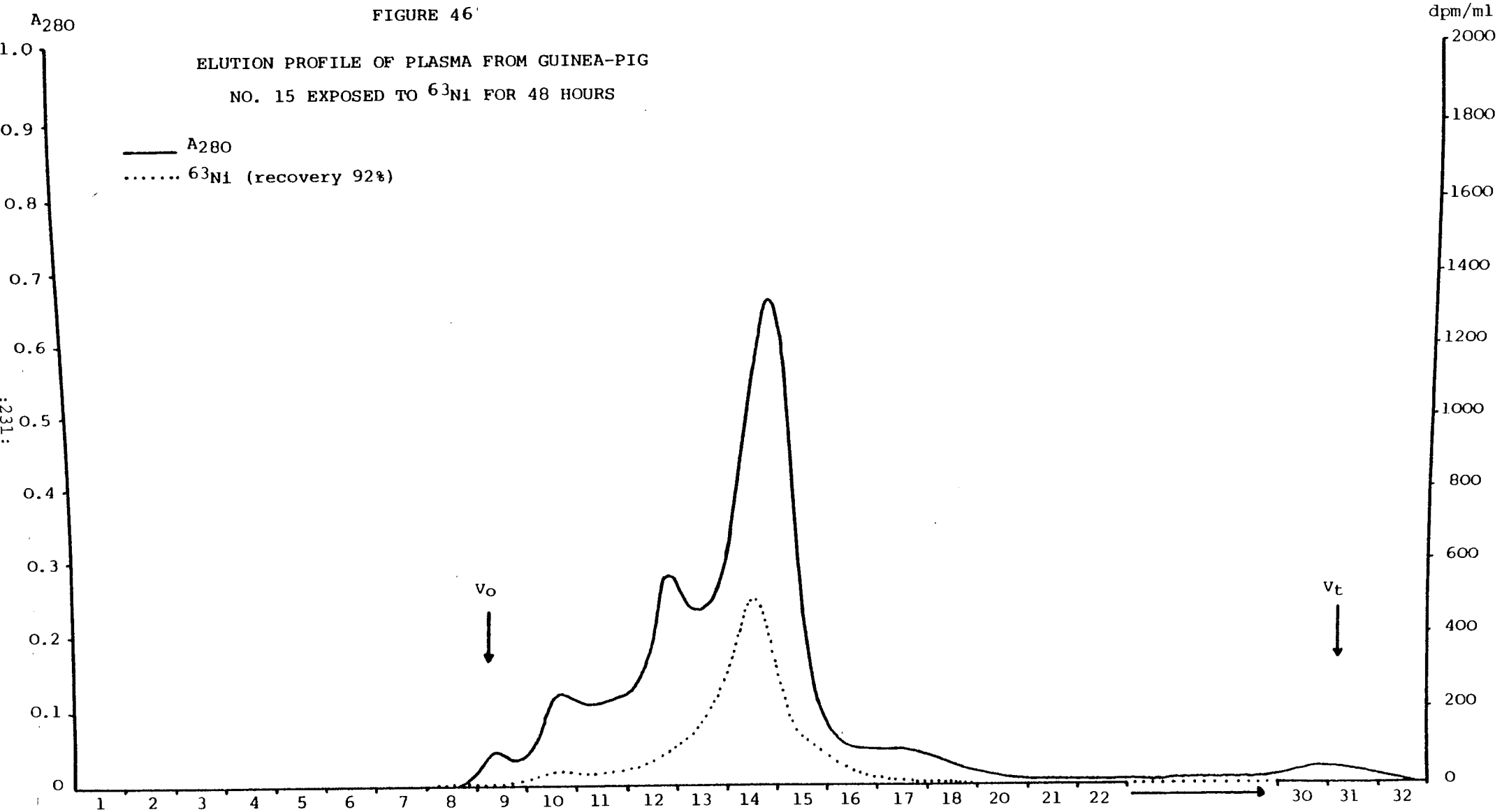


TABLE 38

Results of Gel Filtration Chromatography of Plasma Samples

Animal no. / Exposure Time (hours)	Peak	Ve (ml)	Kav	Resolved Ni content		
				dpm	ng	% of total
3 / 4	1	40.7	-0.05	-	-	-
	2	47.1	0.04	52	13	3.8
	3	56.4	0.09	-	-	-
	4	66.2	0.19	987	246	72.7
	5	NR	-	-	-	-
	6	146.0	0.97	-	-	-
7 / 12	1	45.6	-0.01	-	-	-
	2	50.1	0.03	131	33	4.2
	3	54.9	0.08	-	-	-
	4	63.3	0.16	2226	555	71.1
	5	75.1	0.28	-	-	-
	6	149.0	1.00	37	9	1.2
11 / 24	1	39.8	-0.06	-	-	-
	2	48.6	0.02	102	25	2.9
	3	57.9	0.10	-	-	-
	4	68.7	0.21	3182	794	89.0
	5	NR	-	-	-	-
	6	149.0	1.00	35	8	0.9
15 / 48	1	41.2	-0.04	-	-	-
	2	47.1	0.04	48	12	4.8
	3	58.4	0.11	-	-	-
	4	68.2	0.20	842	210	84.4
	5	81.4	0.34	-	-	-
	6	147.0	0.98	-	-	-

NR = not resolved

remaining protein peaks 1 (Ig m, α_1-macroglobulim), 3 (Ig G, IgA) and 5 (haemoglobin). This latter peak was resolved in two of the four animals examined and probably reflects slight haemolysis of the blood samples in these guinea-pigs.

The low molecular weight nickel fraction (peak 6) was not detected from the plasma samples of guinea-pigs exposed for 4 or 48 hours. This probably reflects the low total plasma nickel concentrations in these animals.

The proportion of nickel within the three identified areas were reasonably constant between the different animal exposure times, although actual concentrations obviously varied with time (see section IV). The greatest proportion of plasma nickel was associated with the albumin fraction (80%). The α_2-macroglobulin fraction accounted for 4% of the recovered nickel and the low molecular weight fraction for only 1%.

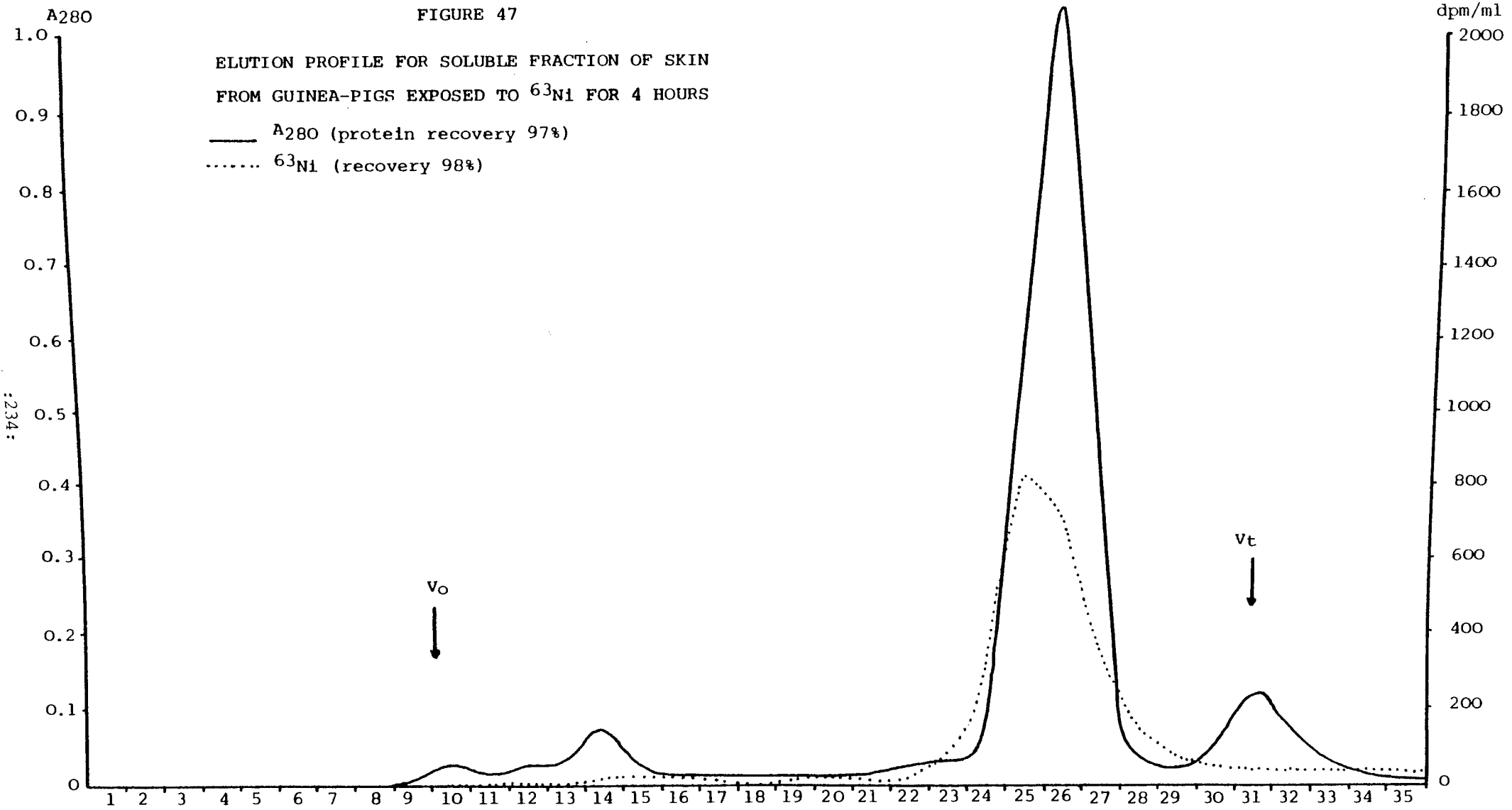
Distribution of nickel in skin soluble fractions

The chromatography elution profiles of pooled soluble skin fractions from each group of exposed guinea-pigs are presented as figures 47 to 50. Partition coefficients (Kav) and nickel contents are shown in Table 39.

In general six protein peaks were resolved. Comparison of partition coefficients with the plasma elution profiles indicates that the first three resolved peaks from soluble skin fractions are of plasma origin. Peak 1, eluted at the void volume (Kav 0.002) corresponds with the high molecular weight plasma proteins, peak 2 (kav 0.1) corresponds with plasma peak 3 (IgG, IgA) and peak 3 (Kav 0.19) corresponds with plasma peak 4 (albumin).

FIGURE 47

ELUTION PROFILE FOR SOLUBLE FRACTION OF SKIN FROM GUINEA-PIGS EXPOSED TO ^{63}Ni FOR 4 HOURS



A280 (protein recovery 97%)

^{63}Ni (recovery 98%)

FIGURE 48

ELUTION PROFILE OF SOLUBLE FRACTION OF SKIN FROM GUINEA-PIGS EXPOSED TO ^{63}Ni FOR 12 HOURS

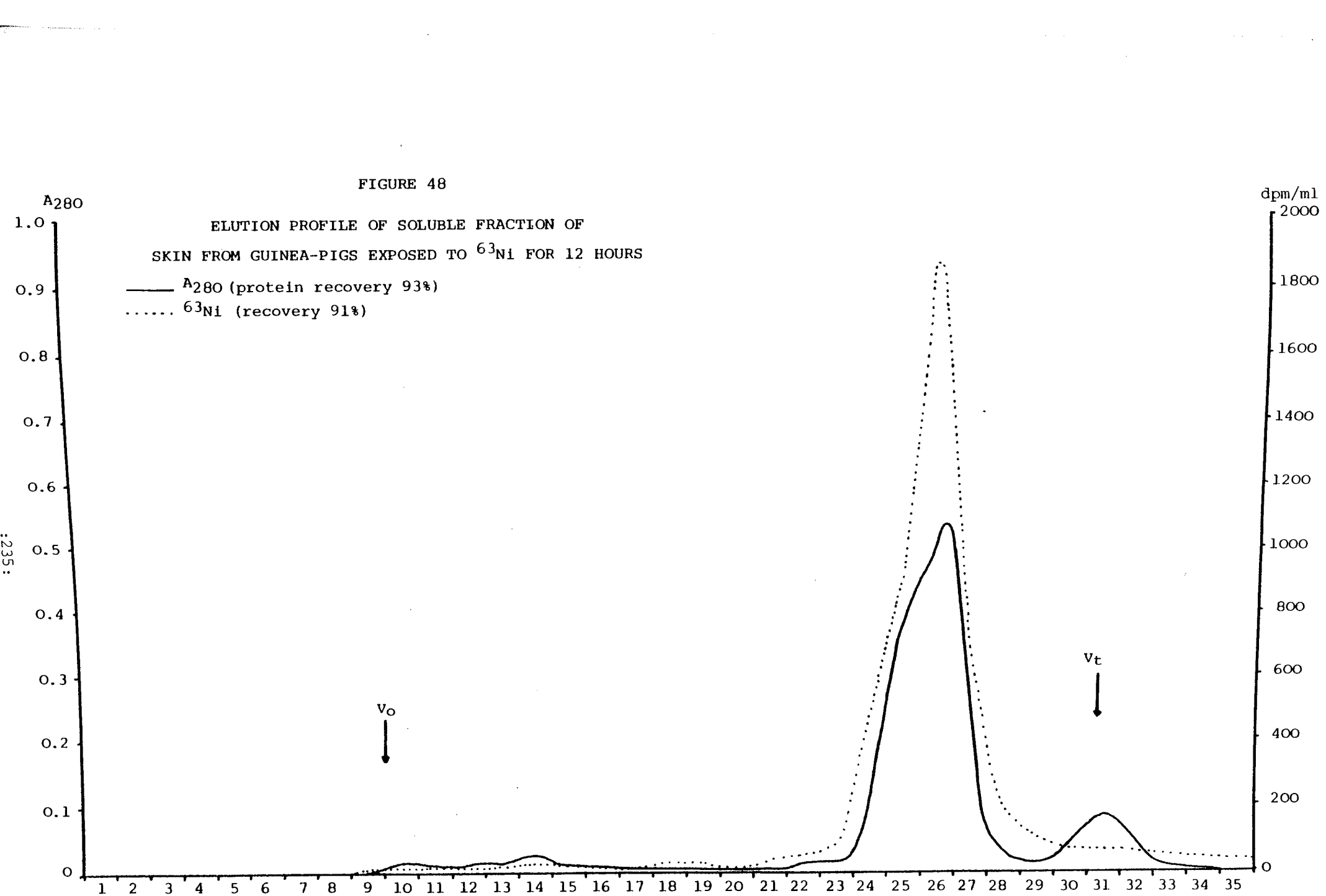


FIGURE 49

ELUTION PROFILE OF SOLUBLE FRACTION OF SKIN FROM GUINEA-PIGS EXPOSED TO ^{63}Ni FOR 24 HOURS

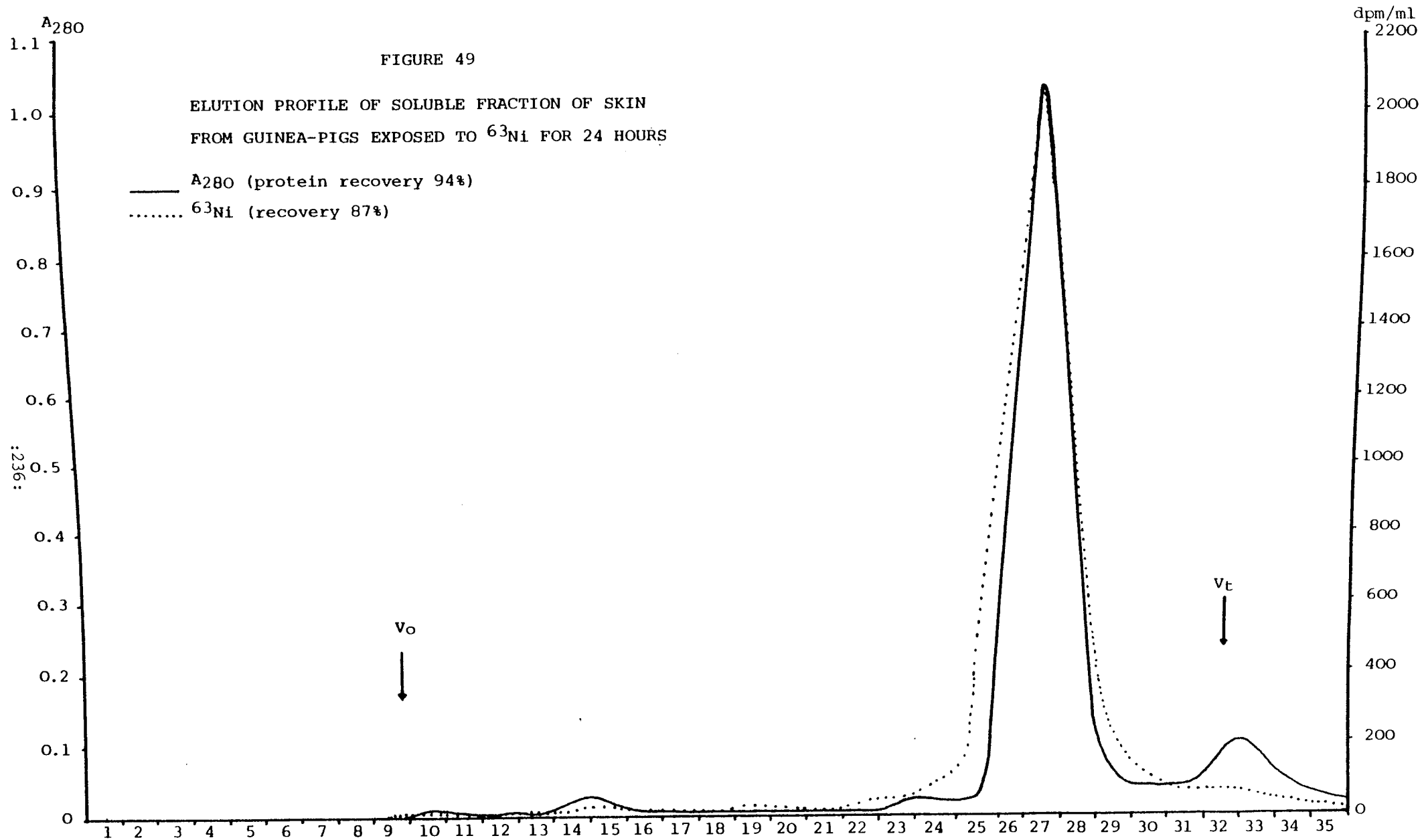
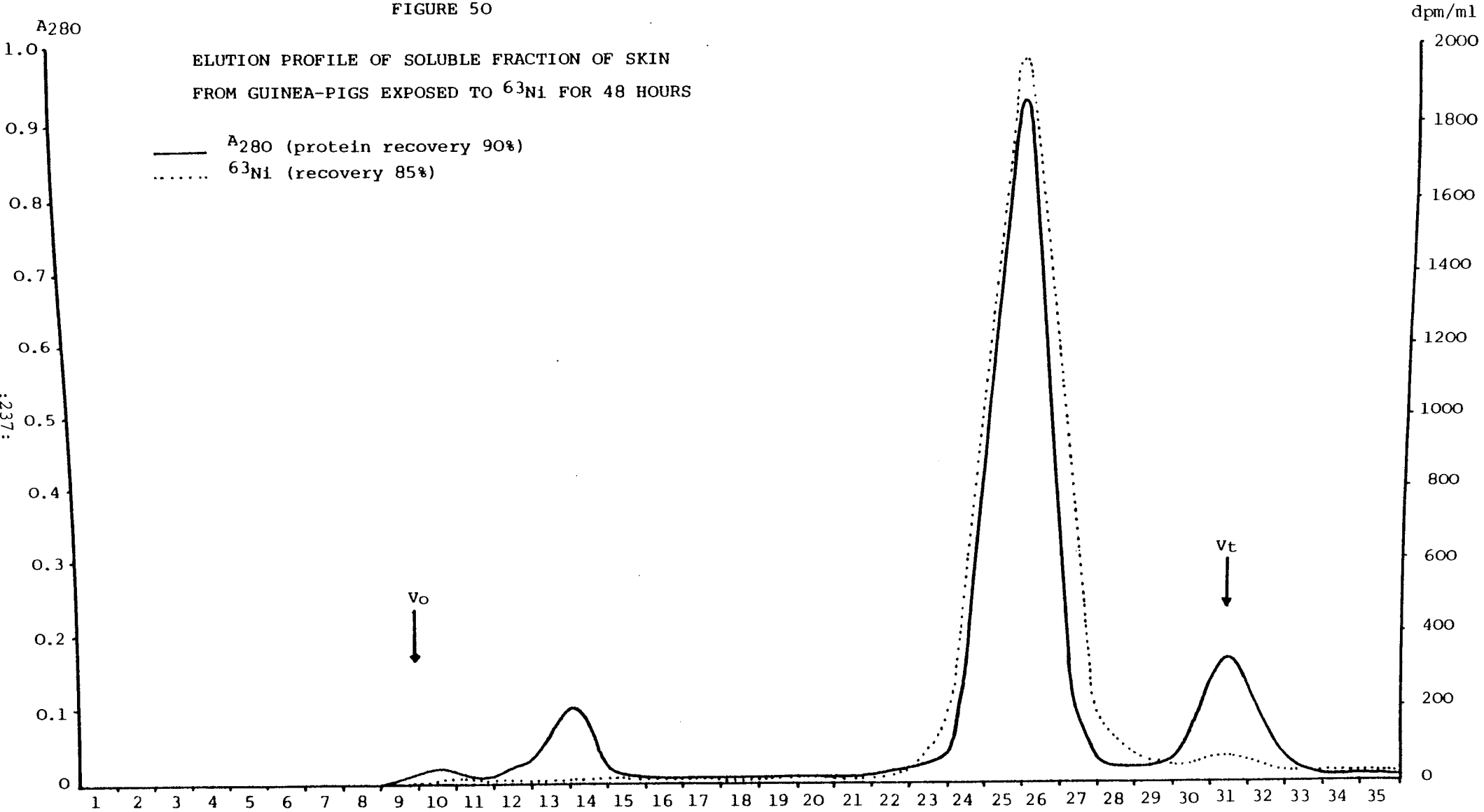


FIGURE 50

ELUTION PROFILE OF SOLUBLE FRACTION OF SKIN FROM GUINEA-PIGS EXPOSED TO ^{63}Ni FOR 48 HOURS



A280 (protein recovery 90%)

^{63}Ni (recovery 85%)

TABLE 39

Results of Gel Filtration Chromatography of Skin Soluble Fractions

Exposure Time (hrs)	Peak	Ve (ml)	Kav	Resolved Ni content		
				dpm	ng	% of total
4	1	46.4	-0.003	-	-	-
	2	56.2	0.09	-	-	-
	3	65.6	0.18	164	41	1.1
	4	108.0	0.60	-	-	-
	5	124.3	0.76	11129	2776	74.6
	6	150.0	1.01	795	198	1.3
12	1	47.4	0.007	-	-	-
	2	57.9	0.11	-	-	-
	3	66.4	0.19	166	41	0.7
	4	108.0	0.60	-	-	-
	5	126.3	0.78	20157	5027	85.8
	6	149.6	1.00	849	212	3.6
24	1	47.4	0.007	-	-	-
	2	58.2	0.11	57	14	0.2
	3	68.7	0.21	123	31	0.5
	4	112.9	0.65	-	-	-
	5	129.8	0.81	21816	5441	81.0
	6	157.0	1.07	940	234	3.5
48	1	46.5	-0.002	-	-	-
	2	56.3	0.09	-	-	-
	3	64.8	0.18	112	28	0.4
	4	NR	-	-	-	-
	5	122.7	0.74	20682	5158	81.7
	6	149.5	1.00	691	172	2.7

NR = Not resolved

Peaks 4 and 5 resolved from skin fractions were not resolved in the gel filtration of plasma and are therefore, of skin origin. Peak 4 was not resolved from the soluble skin fractions of guinea-pigs of the 48 hour exposure period. In this profile the eluted protein probably existed as a leading shoulder to peak 5, but the resolution was not sufficient to measure the partition coefficient. Peak 6, eluted at the column bed volume (Kav 1.02) would be low molecular weight ($\leqslant$5000) proteins, probably originating from both skin and plasma.

Radioactivity was found to be associated with three of the resolved protein peaks; the albumin fraction (peak 3 Kav 0.19) the major skin protein peak (peak 5 Kav 0.77) and the low molecular weight fraction (peak 6 Kav 1.02). In the sample from guinea-pig skin at the 24 hour exposure period a low level of radioactivity was also associated with peak 2 (Kav 0.1) and may represent the α_2-macroglobulin associated nickel seen in the plasma elution profiles.

The proportions of nickel within the three identified areas were reasonably constant between the different animal exposure times, although the actual concentrations again varied with time (see section IV). The greatest proportion of nickel was associated with the major skin protein peak (81%). The albumin fraction accounted for 0.7% and the low molecular weight fraction for 3%.

In addition to the above protein associated nickel distribution, two very small peaks of radioactivity were observed which were not associated with resolved protein peaks. One of these peaks was apparent in all soluble skin samples examined and had a Kav value of 0.44.

The second peak (Kav 0.57) was only resolved in skin samples from guinea-pigs of the 12 and 24 hour exposure periods. The proportion of nickel within these peaks accounted of 0.6% and 0.9% of the recovered radioactivity respectively and their relevance without apparent association with protein is uncertain.

Molecular weight determination of nickel skin proteins

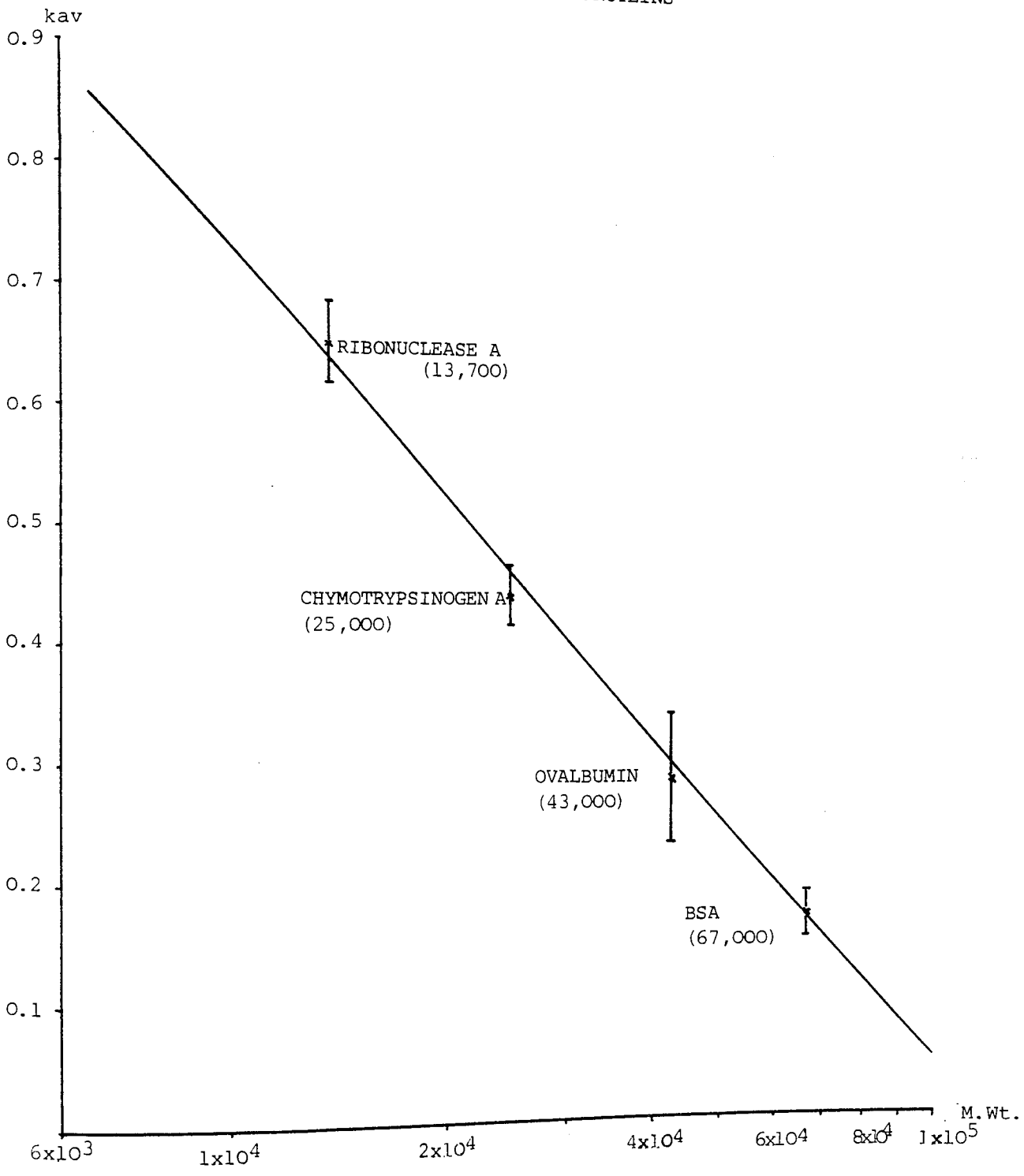
The column calibration curve using commercial protein standards is shown in figure 51. By comparison of the experimental partition coefficients with this curve an approximation of the molecular weights of resolved protein peaks can be achieved within the fractionation range of the chromatography gel. However, it is noted that measurement by gel filtration can be affected by the tertiary and quaternary structure of proteins (Fischer 1980). Gel filtration does allow a reasonably accurate assessment without denaturation of the protein.

The approximate molecular weights of protein resolved from the soluble skin fractions were as follows:

Peak 1 (Kav 0.002, void volume)	- M.Wt. $\geqslant$ 250,000	
Peak 2 (Kav 0.1)	- M.Wt. $\sim$83,000	
Peak 3 (Kav 0.19) albumin	- M.Wt. $\sim$62,000	(Ni-albumin)
Peak 4 (Kav 0.62)	- M.Wt. $\sim$14,500	
Peak 5 (Kav 0.77)	- M.Wt. $\sim$ 8,700	(Ni-skin protein)
Peak 6 (Kav 1.02, bed volume)	- M.Wt. $\sim$ 5,000	(Ni-low molecular weight residue)

FIGURE 51

MOLECULAR WEIGHT CALIBRATION OF CHROMATOGRAPHY COLUMNS - MEAN kav VALUES FOR STANDARD PROTEINS



DISCUSSION

The objectives of the experimentation in this section were to survey, isolate and characterize nickel-protein conjugates within the soluble fraction of guinea-pig skin. Nickel distribution in plasma from exposed guinea-pigs was also examined for comparison of the animal model with previous publications.

Gel filtration of plasma samples from one animal of each exposure period revealed, in general, three areas of nickel localization. The greatest proportions of nickel were associated with the albumin fraction. Much smaller proportions were eluted with the macroglobulin fraction and at the bed volume. In comparison with previous published studies of nickel plasma distributions, the pattern seen in this study was considered to be similar. This tends to confirm that the animal model was not atypical for absorbed nickel.

Group separation chromatography of the initial soluble skin fractions on small Sephadex G-25 columns indicated that a proportion of the nickel content ($\sim$ 13%) was macromolecular (M. Wt $\geq$ 5,000). The remaining radioactivity ($\sim$ 87%) was associated with the second eluted peak (M. Wt $\leq$ 5,000). These fractions were similar to the portions of dialysable and non-dialysable nickel seen in the equilibrium dialysis of the previous section.

Gel filtration on Sephacryl S-200 of the macromolecular portion of soluble skin fractions showed three main areas of nickel localization. The major proportion of nickel was associated with a protein peak of skin origin and molecular weight of approximately 8,700. The two

other areas of nickel localization, which represented much smaller proportions of the nickel content, were eluted with the albumin peak and at the bed volume respectively. The latter peak (M. Wt $\leq$ 5,000) was considered to be low molecular weight material carried over from the group separation columns.

The combination of gel filtration on both types of columns used, leads to an assessment of the distribution of nickel within the total soluble fraction of skin homogenates derived by zonal centrifugations. The greatest proportion of nickel was found to be associated with low molecular material and accounted for approximately 88% of the total nickel. Approximately 11% was associated with the protein peak of skin origin (M. Wt 8,700) and less than 1% was associated with albumin.

The association of nickel with low molecular weight material and albumin could be expected from the results of previous reports with different tissues (Sunderman et al 1981). The elution of the protein peak of skin origin tends to coincide with the metalothionein-like-proteins studied by Sarkar (1980) and Sunderman et al (1981), but further characterization of the protein by other techniques is required before any such tenuous links could be proposed.

It is not known from the gel filtration experiments, whether or not the isolated nickel conjugates are formed in vivo or in vitro (i.e. by active or passive binding). This would be useful information for future evaluation, but is not considered to detract from the primary objective at isolating nickel-conjugates as possible antigens.

In summary gel filtration of soluble skin fractions revealed three areas of nickel localization i) Ni-albumin ii) Ni-skin protein iii) Ni-low molecular weight residues. Each area could represent or contain possible antigens and each was selected for assessment of antigenicity.

SECTION VI - IN VITRO ASSESSMENT OF THE ANTIGENICITY OF ISOLATED NICKEL CONJUGATES

OBJECTIVES

Zonal centrifugation (Section IV) of epidermal homogenates isolated two areas of nickel localization, a microsomal fraction and a soluble fraction. Further separation of the latter fraction by gel filtration chromatography (Section V) isolated three areas, Ni-albumin, Ni-skin protein and Ni-low molecular weight residues.

The objective of this section of the project is to quantitatively assess the potential stimulus that isolated nickel-conjugates could present to the immune system.

INTRODUCTION

There are essentially three ways of assessing the stimulus that an antigen presents to the immune system in allergic contact dermatitis conditions. These are (i) skin testing (usually patch testing) of sensitive subjects (ii) passive transfer of sensitivity (iii) *in vitro* techniques with cells from sensitive subjects.

Patch testing of sensitive subjects relies upon the absorption of the antigen into the skin, subsequent recognition by 'sensitive' cells, clinical manifestation of this recognition and subjective assessment of the reactions (macroscopic and microscopic). It is considered that the problems of macromolecular absorption and subjective assessments reduce the applicability of patch testing for this quantitative phase of the project.

Passive transfer of sensitivity is based upon the transfer of 'sensitive' cells from reactive donors to normal recipients and manifestation of the sensitivity in the recipients. The technique relies upon histocompatibility between the donor and recipient and a method of demonstrating successful transfer. The latter aspect is usually skin

testing, particularly patch testing, which is open to the same problems as stated above.

The technique of passive transfer, although an extremely useful tool in the demonstration of cell mediated immunity, is not considered appropriate for quantitative assessment of antigenicity.

Of the many *in vitro* tests used in the study of the immune system, two are frequently used as indicators of type IV allergic manifestations. These are the lymphocyte transformation test and the macrophage-migration inhibition assay (Urbaniak et al 1978, Polak 1980, Turk 1980).

The lymphocyte transformation test is based upon the ability of lymphocytes to respond to a stimulus in culture by the production of blast-like cells (large, pyroninophilic cells) which synthesize DNA *de novo*. The major responding cells in this assay are T-cells (Urbaniak et al, 1978) and the response is considered to be analogous to the *in vivo* proliferative phase in the development of delayed hypersensitivity (see Section II) and is macrophage dependant (Oppenheim and Rosenstreich 1976, Rosenstreich et al 1976). The transformation can be monitored morphologically or quantitatively by the measurement of radiolabelled DNA precursor uptake (e.g. tritiated thymidine).

The macrophage-migration inhibition assay is a measure of lymphokine production by sensitized lymphocytes following stimulation in culture. The assay is based specifically on the production of macrophage inhibition factor (MIF) and is considered to be analogous to the elicitation phase of delayed hypersensitivity (see Section II).

Inhibition in culture of the migration of macrophages from capillary tubes can be demonstrated;

(a) directly by the culture of peritoneal exudate cells (lymphocytes and macrophages) from sensitized animals with the antigen

(b) indirectly by first incubating sensitized lymphocytes with the antigen and then assessing the inhibitory effect of the cell-free supernatant on the migration of normal peritoneal exudate cells.

In either case the end result is a planimetric measure of the extent of migration.

These *in vitro* techniques have been used to examine allergic contact dermatitis to nickel. However, their use has been limited to human cell cultures with an objective of devising reliable tests to aid the diagnosis of nickel sensitivity. The success of the *in vitro* tests has been variable.

Successful stimulation of cultured lymphocytes from nickel sensitive humans was first reported by Macleod *et al* (1970). Significantly increased carbon-14 labelled thymidine uptake was seen for 7 of 12 subjects at a Ni^{2+} concentration of 10^{-4} mEq/ml culture. The failure of the remaining five subjects was attributed to the cytotoxicity of nickel. A later report from the same group (Hutchinson *et al* 1972) indicated a similar success with 6 of 8 nickel sensitized subjects examined. No lymphocytic response was detected in a nickel non-sensitive control group. In these later experiments, nickel sulphate and acetate were used at the antigens but there was no difference in response to the two salts. It was suggested that nickel ions may bind with amino acids in the culture medium, particularly alanine and these conjugates are stimulatory.

Similarly successful lymphocyte transformation assays have been reported by several groups (Forman and Alexander 1972, Millikan et al 1973, Gimenez-Camarasa et al 1975, Silvennoinen-Kassinen 1981). Good correlations with nickel patch test reactions were reported for each study. The successful lymphocyte transformation reported by Svejgaard et al (1978) did not show a good correlation with the patch test results. The lymphocytes from 3 of 9 control subjects diagnosed as non-sensitive by negative patch test, showed a significant response in culture. The authors suggested that nickel may present a weak non-specific stimulus (mitogenic potential) to lymphocytes, but it is also possible that patch testing did not elicit clinically manifest reactions.

The possibility that nickel may possess non-specific mitogenic properties was previously suggested by Pappas et al (1970). In this study, the lymphocytes from 8 normal humans and 3 nickel sensitive humans gave approximately the same response to nickel acetate.

Unsuccessful lymphocyte tests were reported by Aspegren and Rorsman (1962) and Grosfeld et al (1966). One suggested explanation for these negative results was that the nickel conjugates formed in culture were not sufficiently similar to the original sensitizing conjugate for the response to be initiated.

Migration inhibition assays have been used much less than lymphocyte transformation tests in the examination of nickel sensitivity and the results are conflicting.

Forman and Alexander (1972) used both the indirect macrophage migration inhibition test and the lymphocyte transformation test as

in vitro indicators of nickel sensitivity. Significant macrophage inhibition was seen for only 3 of 15 nickel sensitive patients although 100% correlation was observed with the lymphocyte transformation assay.

The direct migration inhibition assay using peripheral blood cells from nickel sensitive patients was similarly ineffective in demonstrating a response to nickel salts (Macleod et al 1976). The migration indices of cells from nickel sensitive or non-sensitive humans were not significantly different, at the two concentrations of $NiSO_4$ added to culture.

Successful migration inhibition assays using peripheral human blood cells have been reported by Mirza et al (1975) and Thulin (1976). In both studies nickel salts were ineffective but success was reported for protein conjugates of nickel such as nickel-albumin (human or bovine) and nickel-human epidermal proteins. The latter preparation was a 'crude' soluble fraction obtained from homogenized epidermis after centrifugation at 1000g, and conjugated with nickel under in vitro laboratory conditions.

Both studies, however, indicated a protein carrier requisite for the in vitro demonstration of nickel sensitivity.

In summary, it appears that early attempts to use the lymphocyte transformation test as a diagnostic tool in nickel sensitivity were unsuccessful. However, several later studies incorporating developmental modifications to the test have proved successful and show good correlation with patch test diagnosis.

Migration inhibition assays have been used to a very limited degree in nickel sensitivity and the results are conflicting.

On the basis of these background data the lymphocyte transformation test was selected for assessment of antigenicity in this currect project, although it is noted that both assays may be equally quantitative. A dual system of lymphocyte transformation coupled to indirect migration inhibition would be ideal but in the context of the current objectives the much greater workload was considered to outweigh the benefits.

MATERIALS AND METHODS

Lymphocyte transformation assays were performed using cells prepared from nickel-sensitive and control guinea-pigs. The animals were sensitized to nickel using the modified maximization procedure described in Section III and sensitivity was confirmed by patch testing. Non-sensitized cells were prepared from guinea-pigs on which the modified maximization procedures had been performed but without nickel.

Both sets of guinea-pigs had, therefore, received intradermal injections of Freunds Complete Adjuvant containing killed M. tuberculosis. All animals were considered to be sensitized to the bacterial proteins, although this was not confirmed by in vivo challenge. This dual sensitivity was, however, used as a positive control in the lymphocyte transformation assays, by culturing the cells with purified protein derivative of tuberculin (PPD). This control procedure served to demonstrate the ability of cultured cells to respond to a "recall" antigen in both nickel sensitive and non-sensitive cultures.

The procedures used for the lymphocyte transformation assays were based on those of Urbaniak et al (1979), Hudson and Hay (1976), Waithe and Hirschhorn (1979) and those previously described for nickel (see introduction).

The culture medium used in the preparation and culture of cells was bicarbonate buffered RPMl 1640 (Flow Laboratories, Ayrshire, Scotland) containing gentamicin (50 µg/ml) and fungizone (2.5 µg/ml) and supplemented with 5% normal guinea-pig serum.

Tritiated thymidine (1mCi/ml) was obtained from Amersham International (Bucks., England) and diluted in culture medium to 0.1 mCi/ml.

Test and Control Antigens

The experimental antigens examined by the lymphocyte transformation assays were those described and isolated in Sections IV and V. Two positive control materials were also used to monitor the procedures, and cell cultures without any antigen served as negative controls.

Phytohaemagglutinin (PHA purified grade HA 16/17 Wellcome Reagents Ltd., Kent, England) is a non-specific mitogen and was used as a positive control to show the ability of T-cells to transform in culture.

Purified protein derivative of tuberculin (PPD of human tuberculin, MAFF, Surrey, England) was used as a specific "recall" antigen and served as a positive control to demonstrate the ability of cells in culture to specifically recognise an antigen and then respond by transformation.

Experimental antigens from the previous studies were selected to contain the greatest observed nickel concentrations as follows:

(a) Ni-microsomal fraction pooled from zonal samples of guinea-pigs exposed for 48 hours and containing 1720 ng Ni^{II}/ml.

(b) Ni-total soluble fraction pooled from zonal samples of guinea-pigs exposed for 24 and 48 hours and containing 13,370 ng Ni^{II}/ml.

(c) Ni-skin serum fraction pooled from column chromatography samples 14 to 19 of guinea-pigs exposed for 24 hours and containing 350 ng Ni^{II}/ml.

(d) Ni-skin protein fraction pooled from column chromatography samples 24 to 28 of guinea-pigs exposed for 24 hours and containing 1,090 ng Ni^{II}/ml.

(e) Ni-skin low molecular weight residues pooled from group separation chromatography samples of guinea-pigs exposed for 24 hours and containing 11,600 ng Ni^{II}/ml.

(f) $NiSO_4.7H_2O$ - analar grade (Fisons Scientific Apparatus Ltd., Leics., England).

The experimental nickel-protein conjugates were used at three concentrations (as isolated, 1/10 and 1/50) to examine any dose-response relationships.

PHA, PPD and $NiSO_4$ were used at concentrations which were determined in preliminary experiments and are described later in the text.

Preparation of Cells

Nickel sensitive guinea-pigs and control guinea-pigs were killed by cervical dislocation within one hour of the final challenge reaction observations (i.e. 97 hours after challenge application). The anterior

axillary lymph nodes (4 from each animal) were dissected out under aseptic conditions and placed in culture medium at room temperature.

Cell suspensions were prepared as follows using pre-sterilized equipment and aseptic techniques in lamina-flow hoods at room temperature:

(i) The lymph nodes from each animal were dissected free of fat and finely minced with scissors in fresh culture medium.

(ii) The minced tissue was then gently rubbed through a sterile nylon mesh to obtain a coarse cell suspension. The remaining tissue was discarded. The cell suspensions were allowed to stand for 5 minutes to permit large tissue particles and aggregated cells to sediment. The sediment was discarded.

(iii) Cell suspensions were centrifuged at approximately 1000 rpm for 5 minutes and washed three times in fresh culture medium. Cells were resuspended in 10 ml of medium for viability counting.

(iv) Viability counts for cell suspensions were performed by Trypan blue exclusion on improved Neubauer haemocytometers. Cell suspensions (20 µl) were diluted 1 in 50 in Trypan blue (0.2% in culture medium) and counted within 5 minutes. Red blood cells, large macrophage-like cells and blue stained (dead) cells were disregarded. The concentrations of viable cells (unstained lymphocytes) per millilitre of suspension were calculated.

(v) The cell suspensions were then diluted with culture medium to give a final concentration of 10^6 viable cells/ml for culture. This final dilution was dispensed into several sterile universal pots prior to addition of the respective antigens.

It should be noted here that the final cell suspensions were mixtures of cells from the lymph nodes and would contain primarily lymphocytes, although other cells including macrophages would be present. In the initial development of these tests for the project, lymphocyte suspensions were used after purification on Ficoll-Hypaque or Plasmogel gradients. These suspensions give little or no response to PHA in culture. The response was only produced when macrophages from peritoneal exudates were added to the culture. The cruder cell suspensions from lymph nodes gave good responses to PHA and were selected for the further experimentation on the basis that they represented a mixed population of cells involved in the recognition and response to antigen.

Culture of Cells

Before the main culture series using the experimental antigens were undertaken, several smaller cultures were performed in order to define the optimum conditions of culture and stimulation. The specific objectives of the experiments reported within the context of this thesis are as follows:

(a) to assess the optimal dose of PHA in culture of cells from 3 naive guinea-pigs

(b) to assess the optimal culture time for PHA stimulation of cells from 6 naive guinea-pigs

(c) to assess the optimal culture time and dose for PPD response in cells from 5 Freund's Complete Adjuvant treated guinea-pigs and using 3 naive guinea-pigs as controls

(d) to assess the cytotoxicity and non-specific mitogenicity of Ni^{++} in culture with cells from 4 naive guinea-pigs. Cytotoxicity was assessed by Trypan blue exclusion counting after culture.

(e) to assess the optimum culture time for response to Ni^{++} in cells from 6 sensitized guinea-pigs.

(f) to assess the antigenicity of experimental antigens (main study series) using cells from 8 sensitized guinea-pigs and 4 non-sensitized control guinea-pigs.

Each objective relates to a separate series of cultures. The general techniques used in these series were similar and are described in general.

The appropriate antigens or mitogens were added to the cell suspensions in sterile universal pots to give the required final concentrations. The "dosed" suspensions were dispensed into multiwell tissue culture plates (Flow Laboratories, Ayrshire, Scotland) using 0.2 ml of the suspension (2×10^5 cells) into respective wells. All

suspensions from each animal used were cultured in triplicate for each dosage concentration and culture time. An untreated cell suspension from each animal was similarly dispensed and served as individual controls for background thymidine uptake.

Medium alone was also dispensed into six wells of each plate, and served as procedural control.

The prepared plates were then placed in "wet boxes" for humid incubation at 37°C in an atmosphere of 5% CO_2 in air.

Twenty-four hours before the end of the required culture time the contents of each well were pulsed with tritiated thymidine 1.0 µCi/well. The plates were then returned to the incubator for the required period before harvesting.

Cell Harvesting and Measurement of Radioactivity Uptake

After the required culture and pulsing times, cells were harvested onto glass fibre mats using the semi-automatic Titretek Cell Harvester (Flow Laboratories, Ayrshire, Scotland). This is a vacuum operated system whereby cultures were sucked from the culture plates, the cells were trapped on glass fibre discs and precipitated with 10% trichloroacetic acid. Unbound radioactivity was removed by washing with water and methanol. These discs were then dried overnight at ambient temperature.

Tritiated thymidine uptake was measured by liquid scintillation counting, using a samples channel ratio method on an Intertechnique SL 4000 Analyser. Dried discs were placed into 10 ml of Scintillation Cocktail T (Fisons, Loughborough, Leics.). This is a toluene based

cocktail, containing 2,5-diphenyloxazole (PPO) and p-bis/-2-(5-phenyloxazolyl)/-benzene (POPOP) as scintillaters.

The counting time for each sample was 4 minutes and results were automatically calculated as disintergrations per minute (dpm).

Treatment of Results

The culture wells containing medium alone were 'harvested' and used as background controls in the scintillation counting. These background control figures were subtracted from the radioactivity counts for all other samples from the respective culture plates. The mean count of triplicate cultures was then calculated.

The radioactivity uptake of the test cultures was expressed as a stimulation index using the cell culture controls for the respective animals as follows:

$$\text{Stimulation index} = \frac{\text{mean dpm of test culture 1 for animal x}}{\text{mean dpm of cell control for animal x}}$$

Radioactivity uptake in the test culture, at least twice that of the respective control culture (i.e. stimulation index $\leq$ 2.0) was considered to be a positive response. Where dosage relationships were also examined the magnitude of the stimulation index versus antigen or mitogen concentration was an equally important factor in evidence of a positive response.

Statistical comparisons of the stimulation indices obtained in the main studies were performed by analysis of variance. These analyses were carried out in collaboration with Dr. D.O. Chanter and Mr. A. McAllister of Statistic Unit, HRC.

Two questions were of interest

(a) for each test anitgen, at each time of culture, was there a dosage-related difference between cells from nickel sensitive and control animals?

(b) was any test antigen presenting a greater stimulus to the cells?

In order to examine the first question a two-way analysis of variance was performed for each test antigen at each time of culture using sensitivity (i.e. control animals vs Ni-sensitive animals) and antigen concentration as factors. Any interactions between these two factors were also taken into account. Where there were no significant interactions between the factors (i.e. the dose-response curves for control and Ni-sensitive animals were parallel) the group mean stimulation indices were averaged over concentration and vice versa. Where there were significant interactions (i.e. the dose response curves were not parallel) analyses were performed separately for each concentration against sensitivity.

The stimulation indices for PHA and PPD were submitted to a one-way analysis of variance because antigen concentration was not a factor for these cultures.

In order to examine the second question the mathematical differences between antigens were submitted to a two-way analysis of variance using the antigen (i.e. antigen 1 vs antigen 2, etc.) and concentration as factors. These analyses were separately performed for Ni-sensitive animals and control animals and for each culture time. The responses to PHA and PPD were not included in the comparisons.

RESULTS

The results of preliminary experiments to define optimum conditions of lymphocyte transformation are described briefly before the main study series.

Phytohaemagglutinin stimulation

The mean stimulation indices for the dose-response and time-response experiments with naive guinea-pigs are presented as Table 40.

The greatest stimulation by PHA was seen at concentrations of 1.25 and 0.625 µg/ml. A concentration of 1.0 µg/ml was selected for further studies. The optimum time for the response at this selected concentration was 48 hours (including 24 hours pulsing time).

Purified protein derivative of tuberculin stimulation

The mean stimulation indices for the cells from tuberculin-sensitive (Freund's Complete Adjuvant treated) animals and non-sensitive animals are shown in Table 41.

In the sensitized animals the greatest response for each PPD concentration occurred at 96 hours of culture. A PPD concentration of 1.0 µg/ml culture gave the largest mean stimulation index (5.7) at this time. The response was specific for sensitized animals as indicated by low indices for non-sensitized animals.

In view of these results, 96 hours of culture with PPD 1.0 µg/ml were considered to be the optimum conditions for use in the main studies.

The responses to PHA (positive control) in both PPD sensitive and non-sensitive cells were as expected from the previous results (see above).

TABLE 40

Optimum Phytohaemagglutinin Stimulation

Response vs Dose

	PHA concentration (µg/ml culture)					
	10	5	2.5	1.25	0.625	0.0
SI	8.5	7.1	14.8	22.0	22.7	1.4
SD	3.51	2.98	6.72	5.32	8.90	0.41
n	9	9	9	9	9	9

Response vs Time (PHA concentration 1 µg/ml culture)

	Culture time (hours)		
	48	96	144
SI	24.9	4.8	2.6
SD	8.10	2.64	2.31
n	18	18	18

SI = mean stimulation index
SD = standard deviation
n = number of observations

TABLE 41

Optimum Stimulation by Purified Protein Derivative of Tuberculin

Freund's Complete Adjuvant Treated Guinea Pigs

Culture Time (Hours)		PPD concentration (µg/ml culture)			PHA Control
		10	1.0	0.1	
48	SI SD	2.6 0.76	2.1 0.81	1.2 0.23	28.0 5.70
96	SI SD	3.0 0.82	5.7 0.91	2.2 0.53	5.4 2.15
144	SI SD	1.1 0.22	1.6 0.29	0.8 0.35	1.3 0.31
	n/SI	15	15	15	15

Naive Guinea Pigs

Culture Time (Hours)		PPD concentration (µg/ml culture)			PHA Control
		10	1.0	0.1	
48	SI SD	0.8 0.32	0.8 0.36	0.5 0.11	17.1 4.11
96	SI SD	0.8 0.16	0.8 0.24	1.2 0.69	4.0 1.89
144	SI SD	1.2 0.29	1.0 0.24	0.7 0.16	3.1 0.63
	n/SI	9	9	9	9

SI = mean stimulation index
SD = standard deviation
n/SI = number of observations in each mean index

Cytotoxicity and mitogenicity of $NiSO_4$

Mean stimulation indices and viabilities for cells from naive guinea-pigs are shown in Table 42.

The cytotoxicity of $NiSO_4$ was assessed by examining the viability (trypan blue exclusion) of cells after 48 hours of culture. The percentage of viable cells remaining was compared with the mean viability of cells cultured without $NiSO_4$. The results indicated that concentrations of 100, 50 and 10 µg/ml in culture were markedly toxic. Concentrations of 1.0 and 0.1 µg/ml produced viability counts similar to cells cultured without nickel, and were selected for further study. A third level of 0.01 µg/ml was also selected to examine possible dose-response relationships.

The radioactivity uptake of nickel-treated cultures was approximately the same as non-treated cells and stimulation indices approached unity. These results were considered to indicate that $NiSO_4$ in culture does not possess mitogenic activity under the conditions of these assays.

Nickel sulphate stimulation

The mean stimulation indices for this preliminary examination are presented in Table 43.

It is evident from these low indices that transformation of sensitive lymphocytes did not occur in response to $NiSO_4$ in culture. However, the responses to PHA were considered normal for the assay and indicated that the cultures were viable.

TABLE 42

Cytotoxicity and Mitogenicity of
$NiSO_4$ in Culture with Naive Cells for 48 Hours

	$NiSO_4$ Concentration (µg/ml) culture						PHA Control
	100	50	10	1.0	0.1	0.0	
SI SD n	0.3 0.24 12	0.4 0.21 12	1.0 0.52 12	1.3 0.33 12	1.3 0.29 12	1.1 0.42 12	21.2 6.71 12
Viable Cells Remaining (%)	7	11	56	87	87	84	89

SI = mean stimulation index
SD = standard deviation
n = number of observations

TABLE 43

Optimum Stimulation of $NiSO_4$
in Culture with Sensitized Cells

Culture Time (Hours)		$NiSO_4$ Concentration (µg/ml culture)			PHA Control
		1.0	0.1	0.01	
48	SI SD	1.2 0.69	1.0 0.29	1.0 0.42	26.4 6.66
96	SI SD	1.1 0.38	1.0 0.23	1.0 0.15	5.0 2.5
144	SI SD	1.2 0.43	1.0 0.89	1.0 0.29	2.1 0.85
	n/SI	18	18	18	18

SI = mean stimulation index
SD = standard deviation
n/SI = number of observations in each mean index

The lack of response of sensitive cells to $NiSO_4$ in culture was a recurring theme and will be discussed in detail later in the text. However, the objective of these preliminary tests were to select the optimum culture time for a response to nickel and, since this was not achieved, three culture times of 48, 96 and 144 hours were continued in the main study.

Main study series

The mean stimulation indices are shown in Table 44.

The results for the two positive controls were as expected from the preliminary investigations. The response to PHA occurred at 48 hours of culture and at 96 hours for PPD. The degrees of response were similar to previous experiments, and there were no statistical differences between nickel-sensitive and control cells for either material.

Evidence of positive transformation in nickel-sensitive cells was seen for three test antigens. The Ni-microsomal fraction, Ni-total soluble fraction and Ni-skin protein fraction each resulted in higher stimulation indices for the nickel-sensitive cells than in the control cells.

For the Ni-microsomal fraction, cells from seven of the 8 nickel-sensitive animals gave higher indices than the controls at 48 hours of culture. This group difference was statistically significant at the 1% level and was concentration related in that the indices decreased with reduced concentration. The indices at the lowest concentration (1 in 50) were similar to the control. Indices at longer culture times of 96 and 144 hours, were also similar to the controls.

TABLE 4-1

Lymphocyte transformation assays (main studies) - group mean stimulation indices

Culture Time (hours)	Cells	Test antigens																		Positive controls	
		Antigen	Ni - microsomal			Ni - total soluble			Ni - skin serum			Ni - skin protein			Ni - skin low M.wt residues			$NiSO_4$			PHA	PPD
		ng Ni/ml	1720	172	34	13,370	1337	267	350	35	7	1090	109	22	11,600	1160	232	1000	100	10	1.0µg PHA/ml	1.0µg PPD/ml
48	Control	Mean S.I.	1.5	1.1	1.3	1.3	1.3	1.4	1.4	1.3	1.4	1.1	1.1	1.1	1.2	1.1	1.0	0.9	1.2	1.1	20.4	1.4
		SD	0.55	0.38	0.57	0.54	0.44	0.36	0.61	0.40	0.31	0.27	0.42	0.24	0.08	0.21	0.15	0.17	0.45	0.47	7.39	0.53
		No. of responders	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	4	0
	Ni - sensitive	Mean S.I.	3.2**	2.2	1.5	3.3+++	2.6+++	1.9	1.0*	1.0	1.1	3.5+++	2.4++	1.7	1.9	1.6	1.2	1.2	1.2	1.1	25.6	1.3
		SD	1.72	0.60	0.50	0.93	0.40	0.40	0.27	0.44	0.43	0.94	0.60	0.46	1.41	0.83	0.45	1.01	0.56	0.50	7.07	0.78
		No. of responders	7	7	1	8	7	2	0	0	0	8	6	3	1	1	1	0	0	0	8	1
96	Control	Mean S.I.	1.0	1.0	1.0	1.0	1.0	0.9	1.0	1.0	0.8	1.0	0.9	1.2	1.0	1.0	0.8	1.1	1.1	1.1	4.2	5.3
		SD	0.57	0.37	0.57	0.57	0.49	0.13	0.43	0.29	0.32	0.56	0.42	0.34	0.36	0.42	0.17	0.40	0.54	0.32	2.67	1.03
		No. of responders	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	4	4
	Ni - sensitive	Mean S.I.	1.0	1.1	1.2	1.8**	1.3	1.5	1.1	1.1	1.1	1.6*	1.4	1.1	1.1	1.1	1.1	1.0	1.0	1.1	9.5	6.0
		SD	0.51	0.49	0.54	0.88	0.47	0.37	0.36	0.31	0.40	0.71	0.57	0.37	0.48	0.39	0.24	0.30	0.30	0.43	10.88	2.76
		No. of responders	1	1	0	2	0	0	0	0	0	2	1	0	0	0	0	0	0	0	8	8
144	Control	Mean S.I.	0.6	0.7	0.6	0.8	0.9	0.8	0.8	0.6	0.9	0.9	0.9	0.8	1.0	0.9	0.9	0.7	0.8	0.7	3.0	0.7
		SD	0.36	0.17	0.35	0.22	0.38	0.19	0.21	0.82	0.61	0.31	0.25	0.42	0.18	0.28	0.34	0.25	0.26	0.30	1.02	0.18
		No. of responders	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	3	0
	Ni - sensitive	Mean S.I.	0.7	0.9	0.8	0.6	0.8	0.9	0.9	0.9	0.9	1.0	1.0	0.8	0.9	0.8	1.0	0.9	0.9	0.9	6.5	1.2
		SD	0.24	0.22	0.37	0.23	0.40	0.37	0.26	0.19	0.30	0.23	0.21	0.21	0.18	0.20	0.20	0.25	0.27	0.29	7.66	0.62
		No. of responders	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	6	1

Group size - Control 4 animals
Ni - sensitive 8 animals

Responder - Defined as an animal from which cells gave a stimulation index greater than 2.0 and which showed concentration dependence

Number of observations/S.I.: Control 12
Ni - sens 24

Mean S.I. = Mean stimulation indices for triplicate cultures for each animal.

SD = Standard deviation

Level of significance: blank - not significant
* - $P<0.05$ analysis performed using sensitivity and concentration factors combined
** - $P<0.01$
++ - $P<0.01$ analysis performed separately for each concentration. (significant interactions between sensitivity and concentration factors)
+++ - $P<0.001$

A similar response was apparent after culture with the Ni-total soluble fraction. The group response was significantly higher than the controls at the 0.1% level after 48 hours of culture and at the 1% level after 96 hours. Cells from all sensitive animals showed positive transformation with this antigen and the response was concentration dependant. This 'dose' relationship was seen in both the degree of transformation and the incidence of positive responses amongst the sensitive group of animals.

After 144 hours of culture, the indices for nickel-sensitive cells had diminished to levels which were comparable with the controls.

For the nickel-skin protein fraction, cells from all sensitive animals showed positive transformation. After 48 and 96 hours of culture the group responses were significantly higher than the controls at the 0.1% and 1% levels respectively. The response had diminished after 144 hours of culture. At the peak transformation time (48 hours) the degree and incidence of response were concentration dependant.

The general pattern of lymphocyte transformation response to these three antigens was considered to be similar. After 48 hours of culture the majority of cells from nickel-sensitive animals showed positive transformation, but at longer culture times the degree and incidence of response diminished. At the peak time the degree and incidence of response were concentration dependant, in that they decreased with decreasing concentrations of antigen.

The group stimulation indices for the remaining test antigens (Ni-skin serum, Ni-skin low molecular weight residues and $NiSO_4$) were similar for both sensitive cells and control cells. Statistical analyses of the results did not reveal any significantly higher differences for the sensitive cells.

The stimulation indices for cells from the majority of sensitive animals were clearly similar to the controls for these antigens. However, at the 48 hour cultures two animals did show responses to the Ni-skin residues and one of these also reacted to $NiSO_4$. In the one animal that responded to both, the indices were not strictly concentration dependant. The indices were 3.0, 3.2 and 2.1 with decreased concentrations of Ni-residues and 3.6, 1.9, 2.1 with decreasing $NiSO_4$. In the second animal that responded to Ni-residues the indices were concentration related (4.9, 2.5, 1.0).

The results in these individuals, were exceptional for the Ni-sensitive group, and may be an indication of weak antigenicity of Ni-residues and $NiSO_4$. Alternatively, they may be artifactual, but indices of a similar magnitude were not seen in any of the controls.

The second question that was of interest in the main study results was whether or not any one antigen presented a greater stimulus to the sensitive cells. As described in the methods section, this was examined by subtracting the indices for each antigen and comparing the difference. An example of the pairwise comparison is given in Table 45.

TABLE 45

Pairwise statistical comparison of antigens - Differences between group mean stimulation indices for Ni-sensitive cells (48 hour culture).

A \ B		Ni-Total Soluble	Ni-Skin Serum	Ni-Skin Protein	Ni-Skin Low M. Wt. Residues	$NiSO_4$
Ni-Microsomal	Mean	-0.25	1.30***	-0.21	0.76***	1.54***
	Neat	-0.02	2.27	-0.22	1.35	2.05
	1:10	-0.41	1.20	-0.19	0.58	1.03
	1:50	-0.32	0.43	-0.21	0.35	-
Ni-Total Soluble	Mean		1.55***	0.04	1.01***	1.76***
	Neat		2.29	-0.20	1.37	2.07
	1:10		1.61	0.22	0.99	1.44
	1:50		0.74	0.11	0.67	-
Ni-Skin Serum	Mean			-1.50***	-0.54**	-0.20
	Neat			-2.49	-0.92	-0.23
	1:10			-1.39	-0.62	-0.16
	1:50			-0.63	-0.07	-
Ni-Skin Protein	Mean				0.97***	1.75***
	Neat				1.57	2.27
	1:10				0.77	1.22
	1:50				0.56	-
Ni-Skin Low M. Wt. Residues	Mean					0.58*
	Neat					0.70
	1:10					0.46
	1:50					-

Difference for stimulation indices calculated as A-B
Negative values indicate A<B
Positive values indicate A>B
Mean value calculated over concentrations

Significantly different from zero: * $p<0.05$
** $p<0.01$
*** $p<0.001$

As expected from these comparisons the antigens which gave positive responses in sensitive cells when compared with controls, are also significantly higher (0.1% level) than those antigens which did not produce transformation, i.e. Ni-microsomal, Ni-total soluble and Ni-skin protein fractions were better antigens than Ni-skin serum, Ni-residues and $NiSO_4$. The following results were also of interest.

(a) At the peak culture time for response (48 hours) the indices for the Ni-microsomal, Ni-total soluble and Ni-skin protein fraction were similar i.e. each antigen presented a similar stimulus.

(b) At the 96 hour culture time the Ni-total soluble and Ni-skin protein fractions produced slightly higher indices (0.1% and 5% significance respectively) than the Ni-microsomal fraction, i.e. the response was more prolonged for these antigens.

(c) At the 48 hour culture time, the stimulation indices for the Ni-residues fraction were higher than Ni-skin serum (1% significance) and $NiSO_4$ (5% significance).

In all the cases described, the difference between antigens was concentration related, in that the difference decreased as the concentration of antigen reduced.

When the same pairwise comparisons were performed for each antigen with non-sensitive cells or for the 144 hour culture of sensitive cells no real differences were apparent.

DISCUSSION

In summary of the results presented in Table 44 the peak responses of the tested nickel antigens were as follows:-

NICKEL ANTIGEN	Peak S.I. in		Overall antigenicity
	control cells	sensitive cells	
Ni-microsomal fraction	1.5	3.2	+
Ni-total soluble fraction	1.3	3.3	+
Ni-skin serum	1.4	1.0	-
Ni-skin protein	1.1	3.5	+
Ni- low M. Wt.	1.2	1.9	-
$NiSO_4$	0.9	1.2	-

The results of these in vitro assays indicate that 3 of the 6 nickel antigens used, produced positive transformation of sensitized cells. The three positive antigens were nickel-microsomal fraction, nickel-total soluble fraction and nickel-skin protein. The stimulation indices produced by these antigens were statistically significantly greater than the indices both for non-sensitized cells and for the remaining 3 antigens which did not produce positive responses (nickel-skin serum, nickel-skin low molecular weight residues and $NiSO_4$). Peak responses to the 3 positive antigens occurred after 48 hours of culture and the stimulation indices were similar for each antigen.

The assays with $NiSO_4$ and nickel-skin low molecular weight residues were not entirely negative. The cells from 1 and 2 animals respectively showed increased stimulation indices at 48 hours of culture, although the dose-response relationships were slightly atypical.

The difference between the stimuli presented by the 3 positive antigens and $NiSO_4$ indicates that there is a protein-carrier requirement for the recognition of antigen. Additionally, the results of culture of sensitized cells with nickel-skin serum were totally negative. This tends to indicate that there is a degree of protein-carrier specificity in the reactions. It may be possible that the protein-carriers are antigenic or mitogenic. However, no responses were seen with non-sensitized cells cultured with the antigens, suggesting that the response to nickel in sensitive cells is produced by specific nickel-protein conjugates.

The positive responses of cells from two animals in culture with nickel-skin low molecular weight residues may indicate weak antigenic properties for this fraction. This could also explain the response of one animal to $NiSO_4$. Soluble nickel salts will ionize in the culture medium and the nickel ions will probably bind to amino acids, thus representing low molecular weight conjugates.

The presence or absence of a positive response to the test antigens may be explained by the concentration of nickel in the preparation, since this was not equalized prior to testing. It is important, therefore, to examine the responses with nickel concentration.

Of all the antigens, the nickel-skin serum fraction contained the least nickel (350 ng Ni^{II}/ml as isolated) and may therefore present the least stimulus. However, distinct positive responses were obtained with lower concentrations of nickel-microsomal (172 ng Ni^{II}/ml, 1/10 dilution) and nickel-skin protein (109 ng Ni^{II}/ml, 1/10 dilution). The negative response to nickel-skin serum does not appear to be a false result related to nickel concentration.

Similarly the nickel-skin low molecular weight residues and $NiSO_4$, represented a potential stimulus, at least equal to or greater than the positive antigens (1000 ng $NiSO_4$/ml and 11,600 ng Ni^{II}/ml for the residues compared with 1090 ng Ni^{II}/ml for nickel-skin protein). The markedly reduced responses to these antigens were, thus, considered to be real.

The nickel-total soluble fraction represented the greatest potential stimulus (13,370 ng Ni^{II}/ml as isolated) and was included in the series as a check for loss of antigenic material during column

chromatography experiments. The response, however, was similar to that of the constituent, nickel-skin protein, even though the nickel concentration was approximately 12 times greater. The responses to the other isolated constituents were either negative or very weak.

The nickel-skin protein portion of the total soluble fraction was considered to be responsible for the positive response seen with the total fraction.

The nickel content of the microsomal fraction was slightly higher than the nickel-skin protein fraction (1720 ng Ni^{II}/ml compared with 1090 ng Ni^{II}/ml as isolated) but the responses to the nickel-skin protein were very slightly greater. The differences in responses between these two antigens were not statistically different and may be accounted for by biological variation. It is not possible, on this basis, to conclude that either antigen presents a greater stimulus to the sensitized cells.

It is important to emphasize, at this point, that the techniques currently available for assessing in vitro sensitivity are all based on the use of pre-sensitized cells. The systems, therefore, simulate the proliferative and elicitation phases of allergic contact dermatitis and not the induction phase. It is possible that the same antigens are both inducing and elicitating but it is also possible that elicitation may be slightly less specific. The lymphocytes, primed by one specific antigen, may recognise a group of hapten-carrier conjugates.

The results, however, indicate that two of the isolated nickel conjugates are antigenic and that low molecular weight residues may represent a weak stimulus. The allergic contact dermatitis to nickel appears to be a complex sensitivity reaction to more than one antigen

formed in the skin, each possessing similar antigenicities. There is a protein-carrier requirement in the reactions and this is semi-specific in that not all nickel-protein conjugates are stimulatory.

These findings confirm and expand upon previous publications of *in vitro* assays for nickel sensitivity. The variability seen in early reports using nickel salts could be explained by the protein-carrier requisite in the recognition of antigen. Later research (Mirza *et al*, 1975, Thulin, 1976) involving nickel-protein conjugates gave more successful results with nickel-albumin or nickel-epidermal fraction. This latter antigenic material may be considered to be equivalent to the nickel-total soluble fraction used in the present studies of this thesis. The nickel-skin protein (M.Wt. 8,700) has been identified as the major antigenic component of total soluble fraction.

Hutchinson *et al* (1972) suggested that nickel ions are stimulating after conjugation with amino acids in the culture medium, but earlier Grosfeld *et al* (1966) indicated that these low molecular weight conjugates are not sufficiently similar to the sensitizing conjugate for the response to be initiated. The results in this thesis with nickel-low molecular weight conjugates from the skin compromise both previous observations. These conjugates produced only a limited response in cells from two animals, suggesting that they have some antigenic properties but they are not the primary antigens.

Nickel-skin protein (M. Wt. 8,700) and nickel-microsomal fractions were found to be the primary antigens in the systems studied. Further examination of these conjugates is necessary to elucidate the exact composition and epidermal origin of these conjugates.

SECTION VII - GENERAL DISCUSSION AND CONCLUSIONS

In the preceding sections, the experimentation has been discussed within the context of the specific objectives. It may now be useful to consolidate these discussions to summarise the project and draw conclusions.

Allergic contact dermatitis is a specific cutaneous inflammatory response to an external agent and is produced as a consequence of an immunological process, the end result of which is adverse to the host. The allergy may be induced by repeated dermal contact over several days or several years, but once acquired it can be easily maintained and the dermatitis readily triggered by very low exposures.

The aetiology for cases of allergic contact dermatitis is often unknown and may be responsible for recurrent exacerbation of the disorder. The prevention of subtle re-exposure to contact allergens is often difficult especially when the causal agent is unknown or of domestic origin.

In the face of the increasing number of compounds to which we are exposed and poor knowledge of the molecular mechanisms of the immunological processes, the dermatological problem is potentially serious.

It is the overall objective of this project to examine the molecular processes of the induction of allergic contact dermatitis by using nickel as an example of a contact allergen, in the search for a better understanding of this dermatological problem.

The incidence of allergic contact dermatitis to nickel in the general population is unknown but has been reported to be approximately 4.5% to 5.8% amongst "normal" volunteers who may or may not have been attending dermatology clinics (Peltonen, 1979, Keiffer, 1979, Prystowsky, 1979). The incidence amongst dermatology clinic patients is around 11%. However, the majority of epidemiological surveys are based on populations of patients attending dermatological clinics for therapy and therefore do not reflect the true incidence in the general population.

These surveys, together with many individual case reports, describe the dermatitis as a papular or papulovesicular erythematous eruption within the area of direct contact to nickel but often with secondary spreading within localised areas. Pompholyx (a deep seated vesicular reaction on the palms and fingers) is a frequently reported associated eruption.

In the early epidemiological surveys, the exposure responsible for nickel sensitivity was considered to be of occupational origin, but preventative measures and technologic improvements have largely reduced the incidence in the major nickel using industries. Exposure of non-occupational origin is now considered to be the major cause of the sensitivity and responsible for more than 80% of the cases. The incidence of nickel sensitivity in females is strikingly higher than in males. This difference may be attributed to a greater exposure of domestic origin, with cosmetic jewellery, clothing fasteners/appliances, stainless steel kitchens and nickel containing detergent powders, being the main sources of contact.

Published data on the duration of nickel sensitivity suggest that the allergy, once acquired is seldom lost. However, the ubiquitous nature of nickel in the environment may dictate that complete avoidance of contact is impossible and subtle re-exposure may be responsible for the persistence and exacerbation of the dermatitis.

Thus, allergic contact dermatitis to nickel stands as a dermatological problem in its own right and nickel was selected as a prime example of a contact allergen.

The concepts of the immunological processes involved in allergic contact dermatitis have been presented as Section II of this report. This section by no means describes all the contributing immunological processes and mechanisms, since a complete account is outside the scope of this project. However, the review attempts to define and highlight the mechanisms which are largely unknown.

Allergic contact dermatitis has been classified as a type IV (delayed, cell-mediated) immune response. The contactant or hapten is not considered to be the complete antigen. Complete antigenicity may only be produced by absorption of the hapten into the skin and conjugation with proteins or cells. Contact sensitivity may be a complex response to numerous conjugates formed in situ. The number and nature of these conjugates are unknown.

The sensitivity is induced following contact by circulating antigen inexperienced lymphocytes (thymus-dependent) with the complete antigen(s). This encounter results in a "triggering" of the T-cells. Alternatively hapten or antigen may be taken up by macrophages or epidermal Langerhans cells and antigenic information relayed to inexperienced T-cells.

The specifically triggered T-cells drain from the skin site via the lymphatics into the regional lymph node where proliferation takes place to form a population of sensitized lymphocytes (effector cells).

These cells are capable of response against the antigen but are incapable of further proliferation and differentiation.

On subsequent re-exposure to the hapten the same process of absorption and conjugation may take place. Contact of the effector cells with the antigen can elicit the dermatitis, by release of soluble mediators (lymphokines). These mediators may be responsible for the recruitment of non-sensitized cells (macrophages, lymphocytes) for increased vascular permeability and for tissue damage.

If the first contact with the hapten is sufficient to trigger the immune mechanism, then the whole process to the sensitized condition can take 5-7 days. The majority of this time is required for cell proliferation. The dermatitis, once elicited, usuallly peaks within 24-48 hours of exposure and ameliorates under experimental conditions within a few days.

This project was designed to assess the induction processes of nickel sensitivity by examining the early phases of hapten absorption and antigen formation. The specific objectives were:-

1. to select an animal model and develop experimental techniques for the consistent induction and demonstration of allergic contact dermatitis to nickel;

2. to qualitatively and quantitatively assess, in vivo, the absorption and accumulation of nickel in the skin;

3. to survey and isolate the soluble and sub-cellular organelle conjugates of nickel formed in the epidermis;

4. to assess the antigenicity of isolated conjugates.

The albino guinea-pig was selected as a suitable species, in which two standard methods of inducing sensitivity, the Optimization test (Maurer et al, 1978) and the Maximization Test (Magnusson and Kligman, 1970) were initially assessed. Both methods have been reported to produce a high incidence of sensitivity to nickel in the guinea-pig. The results presented in this report do not confirm these previous publications. The incidences of dermal reactions indicative of sensitivity amongst the tested groups were 20% in the Optimization test and 0 to 50% in the Maximization test. Reproducibility was poor.

In order to produce a high, consistent incidence of positive dermal reactions, the Maximization test was modified to increase the exposure to nickel without producing excessive local or systemic toxicity. Under these stringent conditions, an acceptably consistent high incidence of sensitivity (70-100% of the test groups) was produced. There was, however, a slight indication that the sensitivity in the guinea-pig, unlike that in the human, may be readily lost. These findings support the suggestion that nickel is a weak allergen (Vandenberg and Epstein, 1963).

Time coursed in vivo exposure studies, employing $^{63}NiCl_2$ as a radiotracer, were undertaken to qualitatively and quantitatively assess the absorption and accumulation of nickel.

These studies indicated that soluble nickel salts are poorly absorbed across the skin. Maximum plasma levels were seen after 12 hours of continuous skin exposure, when only 0.06% of the applied dose was found in the plasma (716 ng Ni^{II}/ml). Microautoradiography of exposed skin suggested that nickel did not penetrate further than the epidermis, but that it did accumulate on hair shafts and in hair follicles. This observation, together with the low plasma levels suggest that trans-appendageal

passage may be the major route, if not the only route of entry into the body. These findings from *in vivo* exposures expand upon and confirm the limited number of previous reports of nickel absorption in *in vitro* systems (Wells, 1956; Kolpakov, 1963; Samitz and Katz, 1976).

Absorbed nickel was excreted in the urine and urinary concentrations reflected the changes in plasma levels seen with duration and exposure.

In the skin, nickel accumulated in the keratinized areas and penetrated the stratum corneum within 4 hours of exposure. Labelling of individual cells at levels of the epidermis lower than the stratum corneum was noted after exposure for 4 or more hours. This detail of nickel accumulation has not been reported from *in vitro* systems (Wells, 1956; Kolpakov, 1963).

The subcellular distribution of epidermal nickel was examined by rate zonal ultracentrifugation of epidermal homogenates on sucrose gradients. These experiments resulted in two localizations of nickel. The majority ($\sim$ 91% of the total homogenate) was associated with the soluble fraction, in which only 16% of the nickel was bound in conjugates of molecular weight greater than 2,000. The use of control experiments indicated that at least some of macromolecular conjugation occurred *in vitro*.

The second area of nickel localization was in the microsomal fraction and represented approximately 9% of the total homogenate nickel content. In contrast to the soluble fraction, the microsomal fraction contained a major proportion of non-dialysable nickel (M.Wt. $>$ 2,000) and this did not appear to be formed *in vitro*.

The conjugation of nickel within each area occurred at each examined exposure time and the proportions of the nickel concentration represented by each fraction were similar at each time. The actual concentrations reflected the changes in total epidermal nickel content observed with duration of exposure.

Microsomal binding of nickel has also been reported from other tissues after inhalation, intravenous injection or dietary inclusion of nickel salts (Sunderman and Selin, 1968; Whanger, 1973; Oskarrsson and Tjälve, 1979). Binding to nuclei and mitochandria in lungs, liver and kidneys was also reported, but the skin appears to be different from these organs in that nuclear and mitochandrial bound nickel could not be detected in the present studies.

Microsomal involvement in allergic contact dermatitis has previously been reported using the strong allergen, dinitrochlorobenzene (Parker and Turk, 1970; Nishioka et al, 1971 and 1973).

Further characterization of the soluble zonal fractions was carried out by gel filtration chromatography. This technique generally resolved three nickel containing protein peaks. The major portion of nickel was found to be associated with low molecular weight residues (M.Wt. $<5,000$) which confirmed previous dialysis results. The second main area was associated with a protein peak of epidermal origin. This eluted with an approximate molecular weight of 8,700. A minor proportion of the nickel in the soluble fraction was found to be eluted with albumin (M.Wt. 62,000). Similar nickel containing peaks were eluted from kidney cytosol (Sunderman et al 1981) and one protein peak (M.Wt. $\sim$10,000) was suggested to correspond with a metalothionein-like-protein.

Each nickel containing area of the skin soluble fraction elution profile was considered to represent potential antigenic material. Thus, the antigenicity of each fraction, together with the microsomal and total soluble fractions were assessed by *in vitro* lymphocyte transformation assays.

In this system, increased uptake of tritiated thymidine by lymph node cell suspensions from sensitized guinea-pigs was used to indicate positive transformation in response to culture with test antigens. Nickel-skin protein (M.Wt. 8,700) and nickel-microsomal fractions were found to be the primary antigens. The positive transformation produced by these antigens were consistant amongst the sensitized cell cultures and were concentration dependant. The stimulation indices were statistically greater than those of control (non-sensitized) cell cultures and greater than those of the other tested antigens with sensitized cells.

Nishioka *et al* (1973) found similar antigenicity in *in vitro* assays using DNCB coupled microsomal fractions. A fractionated group of picryl chloride coupled epidermal proteins (M.Wt. 10,000 - 200,000) was also found to be antigenic in *in vitro* systems by Miyagawa *et al* (1977 and 1978). However, the molecular weight distribution of these soluble proteins tend to suggest that they are not the same protein as found for nickel in the present studies.

The nickel low-molecular weight residues also appeared to possess some very weak antigenic properties. Nickel sulphate did not stimulate transformation of the sensitized cells in the majority of cultures. There was a slight suggestion, however, that nickel sulphate may possess some antigenic properties, possibly by ionisation in the culture medium and binding to amino acids (i.e. low molecular weight residues). This was also indicated from the research of Hutchinson *et al* (1972) which conflicted

with earlier publications (Grosfeld et al, 1966). The results in this thesis with nickel sulphate and nickel-low molecular weight residues from the skin tend to compromise both previous observations. These materials produced only a limited response, suggesting that they have some antigenic properties but they are not the primary antigens. The protein-carrier requisite for primary antigenicity could account for the failure of early attempts to use lymphocyte transformation assays as diagnostic tools.

In summary, the results of the presented studies indicated that allergic contact dermatitis to nickel and by analogy to other haptens, is a complex sensitivity reaction to more than one antigen formed in the skin. The recognition of antigen is protein-carrier dependant and only epidermal protein (M.Wt. 8,700) and microsomal conjugates with nickel satisfy this requirement. Other conjugates with albumin or small peptides and amino acids do not possess the antigenic properties.

Microsomal involvement appears to be a common theme, at least between the haptens nickel and DNCB. Processing of haptenic or antigenic information by the metabolic/catabolic organelles of epidermal cells and reticuloendothelial cells (e.g. macrophage, Langerhans' cells) could be an important function in the induction phase of allergic contact dermatitis.

The requirement for a soluble protein conjugate is also a common theme, between nickel and picryl chloride, but on the basis of the presented results it appears that the carrier protein for each hapten may be different. This difference in carrier protein may dictate the specificity of allergic contact dermatitis.

It is essential, however, to consider that both primary antigens found with nickel may still comprise mixtures of material. The microsomal fraction will, almost certainly, contain ribosomes and portions of Golgi bodies and endoplasmic reticulum. The skin protein fraction, although

resolved in one peak, may contain several proteins with similar elution properties under the conditions used. Further characterization would be necessary to elucidate the exact composition of these antigenic fractions.

Finally, the antigenicity of the nickel-microsomal fraction strongly implies a cellular involvement in sensitivity reactions. It would be of interest to examine the "information processing" role of epidermal cells in allergic contact dermatitis.

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